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Toxikologická sekce České společnosti pro experimentální a klinickou farmakologii a toxikologii ČLS J. E. Purkyně

Edice příspěvků, které odezněly v červnu 2007 na 12. mezioborové česko-slovenské toxikologické konferenci v Praze, je příležitostí pro připomenutí jubilea jiné významné pražské toxikologické akce. Právě před čtyřiceti lety (v roce 1967) se v našem hlavním městě uskutečnil jeden z prvních evropských toxikologických kongresů vůbec. Garantovala ho Evropská společnost pro studium toxicity léčiv, ustavená v roce 1963. Mezi její zakládající a zvlášť aktivní členy patřila Československá toxikologická sekce. Nezapomenutelnou zásluhu na tom měla nestorka světové farmakologie prof. MUDr. Helena Rašková, DrSc., která patřila mezi iniciátory evropských toxikologických společenství. Na jednom z toxikologických shromáždění minulého desetiletí zazněla otázka, proč se mezinárodní toxikologie ve svých začátcích organizovala především z podnětů farmakologů, ačkoliv toxikologický akční rádius zasahuje daleko mimo rámec farmakologických problematik. Za dominantní provokující příčinu byla označena tzv. thalidomidová (Conterganová) teratogenní aféra ze začátku šedesátých let minulého století, která nastartovala sérii mezinárodních snah po revizi předklinického experimentálního ověřování potenciálních léčiv, především z hlediska bezpečnostních záruk. Původní Evropská společnost pro studium toxicity léčiv se ještě během šedesátých let transformovala do podoby současného EUROTOXu (Sdružení evropských toxikologických společností). Z českých farmakologů-toxikologů byl v letech 1969–1975 členem výboru EUROTOXu nedávno zesnulý doc. RNDr. Ivo Janků, DrSc. Jeho zásluhou se konala v roce 1974 jedna z dalších Evropských toxikologických konferencí v Karlových Varech. V devadesátých letech převzal naši štafetu ve výboru EUROTOXu prof. MUDr. Vladislav Eybl, DrSc. a byla to především jeho iniciativa, že se kongres „EUROTOX 1995“ konal opět v České republice, tentokrát v Praze.

V rámci „jubileem“ vyprovokovaného vzpomínání je snad na místě stručné shrnutí dosavadních aktivit tuzemského toxikologického sdružování, vázaného na EUROTOX. Československá toxikologická sekce vznikla v roce 1963 jako součást Čs. farmakologické společnosti. Kromě mezinárodní angažovanosti se koncentrovala na organizování každoročních pražských toxikologických symposií s vytypovanými diskusními okruhy (například: problematika přenosu dat z pokusných systémů na člověka, hierarchie experimentálního modelování v toxikologii, predikce nežádoucích účinků xenobiotik, etická pravidla pro animální toxikologické pokusy, celulární toxikologie, toxikologické diagnostické testy a jiné). Iniciátory byli nejprve prof. MUDr. Olga Benešová, DrSc., později prof. MUDr. Vladislav Eybl, DrSc. a prof. RNDr. Jaroslav Květina, DrSc. Po rozpadu Československa se toxikologové obou států dohodli na vytvoření tradice každoročních společných mezinárodních toxikologických konferencí, uskutečňovaných střídavě v České republice a na Slovensku. První z nich se konala (pod patronátem osvědčené organizátorské dvojice: doc. RNDr. Eduard Ujházy, PhD. a ing. Jana Navarová PhD.) v Piešťanech v roce 1996. Nejvýznamnějším předsevzetím bylo otevřít tato diskusní fóra mezioborově co nejširší toxikologické veřejnosti. Užitečnost tohoto záměru dokládá i soudobá akce „REACH“ iniciovaná Evropským společenstvím. V tomto smyslu se stala zlomovým startem konference konaná v roce 2004, na její intence pak navázaly další konference, včetně té letošní s rekordní účastí více jak 150 účastníků. Její úroveň se stala odrazovým můstkem i závazkem pro čtyřletou perspektivu nově zvoleného výboru České toxikologické sekce ve složení: Jaroslav Květina, Věra Štětinová, Vladislav Eybl, Miloň Tichý, Pavel Anzenbacher.

Jaroslav Květina
předseda České toxikologické sekce

Analytická chemie a toxikologie

Není sporu o tom, že existuje celá řada styčných ploch mezi toxikologií a analytickou chemií. Kolegové pracující v oblasti toxikologie jistě budou souhlasit s mým tvrzením, že bez analytické chemie by nemohli získat řadu zásadních poznatků nutných k úspěšnému rozvoji jejich oboru. A za analytické chemiky mohu prohlásit, že jsou rádi, když jimi vyvíjené metody a principy nekončí v šuplíku, ale slouží k ochraně lidského zdraví, což v případě jejich využití v toxikologii jistě platí.

Proto jsem velmi rád, že se v rámci České společnosti chemické úspěšně rozvíjí široká a mnohostranná spolupráce mezi odbornou skupinou analytické toxikologie a odbornou skupinou analytické chemie. Jejím výsledkem je i spoluúčast katedry analytické chemie Přírodovědecké fakulty Univerzity Karlovy v Praze na 12. mezioborové česko-slovenské toxikologické konferenci. Z té vzešly následující příspěvky v tomto čísle Chemických Listů.

Chtěl bych při této příležitosti poděkovat organizátorům této konference, zejména doc. RNDr. Miloňovi Tichému, DrSc. a jeho kolektivu ze Státního zdravotního ústavu za příkladnou organizaci této konference a přípravu následujících příspěvků a paní Ing. Radmile Řápkové za jejich pečlivé technické zpracování. A vyslovit přání, aby bylo co nejvíce takovýchto akcí důstojně reprezentujících úspěšnou práci českých a slovenských vědců v nejrůznějších chemických i lékařských oborech.

Jiří Barek
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Ochrana veřejného zdraví a toxikologie

Výhodou úvodních slov, předmluv a doslovů je, že vážený čtenář mnoho nezíská, když je přečte a většinou nic neztratí, když je nepřečte. Nevím proč jsem byl požádán o „několik slov úvodem“. Jestli pro jistou etapu svého života, kdy i já se snažil poodhrnout tajemství toxikologie a zejména uchránit lidstvo před negativním působením chemických látek a přípravků nebo proto, že se mi dostalo cti být ředitelem a řídit Státní zdravotní ústav.

Lidstvo a jeho touha po poznání. Když jsem se probíral obsahem tohoto čísla, tak jsem byl příjemně překvapen tím, že stále se mezi námi nalézá spousta aktivních vědkyň a vědců, aktivních kolegů a kolegyň, kteří nelení se podělit o své znalosti a zpřístupnit toxikologii a s ní související obory odborné veřejnosti. Té, skoro anonymní skupině čtenářů, která hledá poučení. Někdy mezi autory hledám jména těch, které jsem znal a kteří vytrvali. I to je informace milá a povzbudivá.

Toxikologie je nádherná věda, která jistě začínala jako touha ochránit vznikající lidstvo před smrtí otravou, přes dlouho trvající a jistě na některých pracovištích ještě praktikovanou touhu zjistit, jak nejlépe otrávit nepřítele, přes krásnou touhu nalézt léky na naše neduhy, které by více léčily než škodily až po současnou aktivitu, podobající se prapočátkům toxikologie a vedoucí k lepším znalostem. A tak ke zlepšení chemické bezpečnosti lidstva.

Důležitá, potřebná a krásná věda je toxikologie. Přeji všem, kteří se podíleli na vzniku tohoto čísla i všem ostatním toxikologům hodně vědeckých radostí a osobního uspokojení a štěstí. Dobrá mysl není jen půl zdraví, ale je i nezbytným předpokladem k přežití všech úskalí a ústrků, kterým jsme ve vědeckém světě vystavováni. Tak se nedejte otrávit!

Jaroslav Volf
ředitel Státního zdravotního ústavu, Praha

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L01
HEALTH RISKS OF HARMFULL COMPOUNDS FIXED ON PARTICULATE MATTER ($PM_{2.5}$) IN URBAN AIR

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Keywords: particulate matter, polyaromatic hydrocarbons, urban air, health risks, traffic

Introduction

Amount of gases and solid pollutants emitted from anthropogenic activities to the atmosphere continually increase and demonstrably have adverse effects on human health and environmental ecosystems. These unfavourable conditions are evident especially in large cities where transport becomes the dominant source of pollution. 347,900 European died in the year 2000 in accordance with the newest EU study[1] due to air pollution. These results thus testified former epidemiological studies that indicate an early death of hundreds of thousands people in Europe[2] exposed to these pollutants and life shortening[3] of a couple of years because of cardiovascular[4] and respiratory[5] diseases. Last studies actually advert to cancer origin[6]. Particulate matter (PM) and some non limited pollutants such as polyaromatic hydrocarbons (PAH), benzene, 1,3-butadiene become an actual part of research not only due to increasing concentrations in the ambient air and primarily because of their negative effects on human health (respiratory and cardiovascular diseases). Possible human health effects of PM are associated with particle diameter and of course with chemical composition because even particle diameter is the dominant factor determining whether it can be inhaled and in which part of the respiratory system it can be deposited. PM smaller than 2.5 μm and pollutants adsorbed on their surface can penetrate into the bronchioles. Particles with diameter les than 1 μm penetrate into pulmonary alveoli where can be accumulated or ultrafine particles enter the cardiovascular system and are transported to different organism tissues[3]. The most life-threatening group of people are those living near the streets with intensive road transport and first of all children[7].

Described facts confirming recency of solving of this issue there through was realized air quality monitoring with respect to NV 350/2002 Collection of Law as amended by later documents in period from 2005 till 2006. The goal was to clarify long term trends in $PM_{2.5}$ concentrations and composition on selected localities including assessment of possible negative impacts on population health state.

Experimental Part

Localities in Brno city with high traffic intensity and different morphology of surroundings were chosen for taking air samples. Locality Arboretum representing wide opened site with a lot of trees is situated near the I. transport city circular near the crossroads Drobného, Lesnické, Provazníkové and tř. generála Píky streets. Locality Kotlářská is situated also near the I. transport city circular but in contrast to the first locality in the surroundings stand five floor residential buildings and this street could be considered "street canyon".

Middle volume samplers LECKEL MVS6 (Sven Leckel Ingenierbüro, Germany) were used for taking ambient air samples, two on each locality – one equipped with nitrocellulose filter for $PM_{2.5}$ concentrations determination, second with quartz filter for analyses of PAH adsorbed on the surface of this PM fraction. Gravimetric analysis on Mettler-Toledo MX5/A balance (Mettler-Toledo GmbH, Switzerland) of each exposed filter was used for $PM_{2.5}$ concentrations determination. 112 samples from both localities were taken and analysed during seven days campaigns in 24 hours intervals. Vertical distribution and continual measurements of $PM_{1.0}$, $PM_{2.5}$ a PM_{10} fractions were performed by using EnvironCheck 107 (Grimm Aerosol Technik GmbH, Germany).

PAH extraction from quartz filters (IKA, IKA – Werke GmbH, Germany) and disturbing compounds separation on silica gel column were done before proper analysis. Gas chromatograph with mass detector SHIMADZU QP 2010 (SHIMADZU, Japan) equipped with autosampler was used for PAH concentration analysis. Helium was the carrier gas in the analysis. Internal standard analysis (*p*-terfenyl) with calibration with standard mixture of 16 PAH from US EPA MIX 63 and coronen (Dr. Ehrenstorfer, Germany) was used for PAH concentrations determination. Chromatograms evaluation was done by peak manual integration and data were expressed as concentration of PAH fixed to $PM_{2.5}$ for unit air volume.

On the basis of experimentally determined annual mean concentration of pollutants that were count over using toxic equivalent factors (TEF) to benzo(*a*)pyrene concentration[8] was assessed long term (chronic) inhalation heath risk of people living near the chosen localities. Health risk assessment was done for simplicity as a risk for ideal average population it means for individual with body weight 70 kg without exposition data splitting between men and woman. Basic exposition scenario considering 3 hours exposition to the ambient air, age 70 years, exposition duration 30 years, exposition frequentation 350 days per year and inhalation rate 0.83 $m^3\ h^{-1}$. The same scenario was used for health risk assessment for children with modification of age to 14 years, body weight 15 kg,

exposition duration 14 years and inhalation rate 3.2 m^3 h^{-1}.

Results and Discussion

The highest $PM_{2.5}$ concentrations were determined in autumn (turn of November and December) whereas the lowest in summer (turn of June and July) (fig. 1). Monitoring results indicate strong correlation of $PM_{2.5}$ and temperature. By the lowest measured temperatures were the highest $PM_{2.5}$ concentrations, by the high temperatures the concentrations were lower. This effect could be either due to loss of volatile PM compounds (such as ammonium salt) in warmer period that coagulate in low temperatures and can be captured on filters or it could have connection to vertical stability of the atmosphere. Better ventilation in warmer season (convection) amuses pollutants further from the sources whereas in colder season ventilation is limited (inversion) and PM cumulate in the baser parts of the atmosphere near the source. Local furnace that heat surrounding houses could be another important source of these particles in colder period. Comparison of the PM concentrations in the range of diameter 2.5–10 μm, 1–2.5 μm a 0–1 μm measured in different seasons shows the significant dependence of separate PM fractions on the season. The share of fine fraction $PM_{2.5}$ on total PM_{10} concentration is higher in colder period when it is 92.9 % from PM_{10} and in warmer period the same share is 59 % respectively. This fact confirm the higher burden of peoples respiratory system in colder seasons.

Very important group of harmful compounds often fixed to PM are PAH originating especially during the incomplete combustion of fossil fuels. Obtained results show that concentrations of selected PAH indicate significant seasonal correlation such as $PM_{2.5}$ and exist some specific context with temperature. Two PAH groups were identified on the basis of multidimensional analysis, "volatile PAH" containing naftalene, acenaftylene, fluorene, fenanthrene a antracene, "non volatile PAH" containing fluoranthene, pyrene, benz(*a*)anthracene, chrysene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*a*)

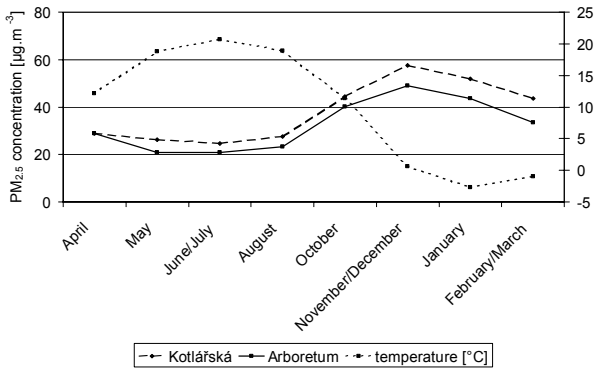

Fig. 1. **$PM_{2.5}$ concentration and temperature progress during the observed period**

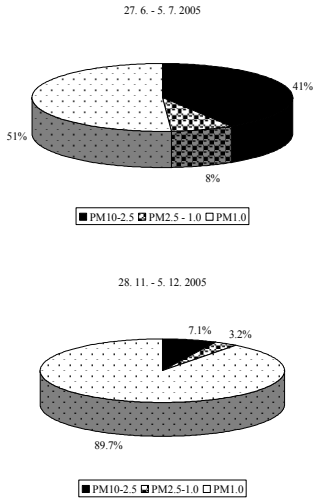

Fig. 2. **PM_{10}, $PM_{2.5}$ and $PM_{1.0}$ distribution comparison**

pyrene, indeno(1,2,3-*cd*)pyrene, benzo(*ghi*)perylene, coronene. PAH concentrations fixed to $PM_{2.5}$ have different presence during the warm season from April till August and in colder season from October till April. Principal component analysis Varimax for two factors was used to possible sources identification. This spend 89 % of total variability of results that can be considered sufficient for describing problem. According to factor loadings values benzo(ghi)perylene and coronene represent traffic sources and benzo(*a*)pyreneme other sources than traffic. The other PAH are significantly produced by both sources. Comparing the ratios of coronene – benzo(*a*)pyrene and benzo(*ghi*)perylene – benzo(*a*)pyrene show traffic as the dominant source of air pollution in the warmer season (May – August) with better ventilation conditions. Other sources than traffic become important in March, January and February and become dominant in turn of November and December.

PAH concentrations were and number of people living near the chosen localities were used as input data for carcinogenic risk assessment due to inhalation of these compounds. Possibility of tumor diseases occurrence due to PAH inhalation for adult individual is acceptable on both localities, this risk for children individual is on the edge of acceptance (Table I). Inclusion the population living near the chosen localities to the assessment show that the possibility of tumor diseases is for both adults population and especially children population higher than the barrier of socially assumed point of risk for tumor diseases occurrence $1 \cdot 10^{-6}$.

Conclusion

Measured annual mean $PM_{2.5}$ concentrations confirm the higher burden of people on locality Kotlářská (38.1 μg m^{-3}) than on locality Arboretum (32.5 μg m^{-3}). These values are in both cases higher than limits (25 μg m^{-3})

Table I
Individual and population risk due to PAH inhalation

Locality	ILCR – individual lifetime cancer risk		APCR - population lifetime cancer risk	
	adult	children	adult	children
Kotlářská	1.38E-07	5.78E-06	1.30E-04	2.72E-03
Arboretum	1.24E-07	5.21E-06	9.02E-05	1.90E-03

that are being prepared in the new Europen Union dirrective (Directive of the European Parliament and council on ambient air quality and cleaner air for Europe). Obtained results thus confirm conclusions with dependence of PM and PAH concentrations[9] on temperature when the highest concentrations were also by the lowest measured temperatures. We can also hypothesize that the source of air pollution can be also other sources than traffic especially by lower temperatures near zero. Limit for benzo(*a*)pyrene settled in NV 350/2002 Collection of Law was not exceeded on both localities. Measured PM and PAH concentrations stand with the concentrations measured on localities with mean traffic intensity[10,11] and industry[12]. Possibility of tumor diseases occurrence due to PAH inhalation for adult individual is acceptable on both localities, but not for children individual (Table I). Possibility of tumor diseases is not acceptable for both adults population and especially children population. Results unambiguously show that already PAH inhalation is potentially harmful for exposed population living near both localities.

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L02
POSSIBILITY OF PHARMACOLOGICAL PROPHYLAXIS AGAINST HIGHLY TOXIC ORGANOPHOSPHATES

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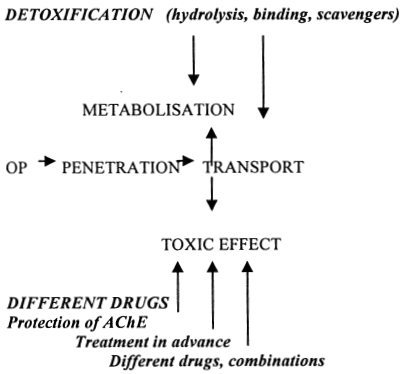
Key words: neve agents, prophylaxis, detoxification, protection, acetylcholinesterase inhibition

Inhibitors of cholinesterases are very important chemicals in the group of organophosphates (OP). These compounds are used in industry, in veterinary or human medicine and, last but not least, these compounds are, unfortunately, usable (and used) for military purposes as chemical warfare agents (nerve agents) and as poisons used by terrorists as it was documented in the Tokyo subway. The broadest spectrum of these compounds is used as pesticides (insecticides), acaricides etc. According to the World Health Organization, more than one million serious accidental and two million suicidal poisonings with insecticides occur worldwide every year, and of these approximately 200,000 die, mostly in developing countries[1-3]. The mechanism of their action, prophylaxis and treatment of intoxications with OP is a very hot topic at present. Basic mechanism of action of OP is based on their ability to inhibit the enzyme acetylcholinesterase (EC, 3.1.1.7) at cholinergic peripheral and central synapses[2]. The term prophylaxis used in this article is limited to medical countermeasures applied relatively shortly before penetration of a toxic agent into the organism with the aim of protecting the organism against the toxic drug. In general, the prophylaxis can be focused on protection of AChE against the inhibition using reversible cholinesterase inhibitors. The diminishing the level of OP using enzymes hydrolyzing these agents or enzymes binding the agents (to specific proteins or to antibodies) and thus reducing the OP level and inhibition of cholinesterases – AChE and butyrylcholinesterase (BuChE, EC 3.1.1.8) (scavenger effect) in the organism can be described as detoxification. Another approach to prophylaxis is based on using present antidotes and other drugs[2,4] (Scheme 1).



Scheme 1. **Four basic reactions of OP in the organism (in CAPITALS) and *possible targets for prophylaxis (in italics, bold)***

Protection of AChE against inhibition

Keeping AChE intact is a basic requirement for effective prophylaxis. This can be achieved by using reversible inhibitors, which are able to inhibit AChE reversibly and after spontaneous recovery of the activity, normal AChE serves as a source of the active enzyme. The ability of some carbamates to protect an organism poisoned with OP has been known for many years. Physostigmine and neostigmine have been used to protect animals against DFP. Nevertheless, the protective effect of physostigmine, aminostigmine, pyridostigmine and others against AChE inhibition caused by different OP (mostly soman) has been demonstrated[2,4]. From the results published (and unpublished), it appears that pyridostigmine is the most promising prophylactic drug especially against soman poisoning. On the basis of these results, pyridostigmine was introduced into some armies as a prophylactic antidote against nerve agents. Its prophylactic effect (like the effects of other carbamates) is limited by its dose. With a higher dose, a higher efficacy was observed, but the side effects were more expressed, too (for review, see[2,4]). This problem can be solved by the adding of pyridostigmine antagonizing drugs – anticholinergics. The prophylactic combination of pyridostigmine with trihexyphenidyle and benactyzine called PANPAL was introduced into the Czech Army. The presence of these two anticholinergics allowed us to increase the pyridostigmine dose and to increase its prophylactic efficacy. This combination (including follow-up therapy) is not limited to soman, sarin and VX poisoning but its high efficacy against tabun, GV and cyclosarin[5,6] intoxications was observed. The prophylactic antidotal combination PANPAL has not side effects as it has been demonstrated on volunteers: no statistically different changes in the actual psychic state as well as no negative changes in the dysfunction time and heart function and blood pressure were observed[7]. On the basis of the results with the prophylactic efficacy of other different carbamates, aminostigmine was chosen as the most effective. All the results dealing with reversible AChE inhibitors as prophylactics against nerve agents including initial historical papers were summarized[2,4].

Other carbamates also have a good prophylactic effi-

cacy, especially physostigmine (due to its central effect on the contrary to pyridostigmine)[8,9]. Human study with transdermal physostigmine suggests a serious interest in the prophylactic use of this drug[10]. Structurally different inhibitors from the carbamate and OP groups were also studied. From these compounds (preferably binding to the AChE anionic site), tacrine, 7-methoxytacrine (7-MEOTA) and huperzine A were considered and experimentally studied with respect to prophylaxis in vitro and in vivo[4,11]. The most interesting results were obtained with huperzine A. Huperzine A was tested as a potential candidate against OP for its long-lasting efficacy and relatively low toxicity[11,12]. However, the results obtained do not support replacement of pyridostigmine by these drugs.

Detoxification

This principle involves two different approaches: administration of enzymes splitting the OP or specific enzymes which bind the OP (cholinesterases). OP is bound to the exogenously administered enzyme and thus the OP level in the organism is decreased (it acts as "scavenger"). The use of scavengers as prophylactics either as enzymes binding[13,14] or hydrolysing[15,16]. OP was tested many years ago. A lot of studies have been made with cholinesterases as scavengers. BuChE and AChE were observed to be very effective in protection against OP intoxication[17-22]. The administration of enzymes as scavengers seems to be very promising: the enzyme is acting at the very beginning of the toxic action, without interaction with the target tissues and without side effects[17]. All of these features are of great interest and they are yielding practical results – isolation of the enzyme, examination for lack of and auto immune response and establishment of pharmacokinetic and pharmacodynamic properties[17,20]. Moreover, BuChE pretreatment also showed protective effects on AChE inhibition in the brain parts following low level sarin inhalation exposure[21,22]. Given our increasing knowledge in bioengineering and biotechnology, the connection between these two enzymes will be possible with the aim of obtaining a modified enzyme splitting OP and simultaneously reacting with AChE as a scavenger[23]. Antibodies against OP are in the stage of research and they are more focused on the detection of OP[24].

Standard antidotes as prophylactics

The antidotes currently used for the treatment of OP poisoning can be tested as prophylactics. The aim of this approach is very simple – to achieve sufficient level of antidotes in the blood vessel before intoxication. Standard antidotes were studied in this respect, i.e. anticholinergics, reactivators, anticonvulsants and others[2,4]. The problem with their use is the timing and duration and achievement of sufficient levels of these antidotes after administration. However, the prophylactic efficacy is good as it has been demonstrated in treatment studies but administration of these antidotes mostly takes place very shortly (minutes) after the intoxication. The prolongation of the duration of the antidote effects by achievement of their sufficient level in the blood by oral administration is not possible (especially reactivators) and therefore it is excluded. It was a reason for searching for other routes of administration. Transdermal administration of one of the most effective reactivators (HI-6) was shown to be the most realistic approach[2,4,25]. The final result was the new prophylactic transdermal antidote called TRANSANT. This preparation was clinically tested (including dermal sensitivity) without any harmful effects and field testing was also successful[25]. The final reports were finished and TRANSANT has been introduced into the Czech Army. The prophylactic efficacy of other drugs was studied. As anticonvulsant drugs, benzodiazepines (diazepam, midazolam, alprazolam, triazolam, clonazepam) were studied, but isolated prophylactic administration had not very good effects[2,4,26].

Prophylaxis with other drugs

Prophylactic administration of different drugs (alone or in combination) against intoxication with OP were studied. Calcium antagonists (nimodipine), neuromuscular blockers (tubocurarine), adamantanes (memantine), and the opiate antagonist meptazinol[27,28] were also tested with different results but they were not very useful for practical use. On the other hand, a positive prophylactic effect has been demonstrated with procyclidine (antimuscarinic, antinicotinic and the anti-NMDA receptor drug)[29]. However, all these studies are experimental ones and they have not reached the practical output stage. The combinations of various drugs as prophylactics can be of very different character. They can be used simultaneously (a combination of different drugs) or as pre-treatment and following treatment with different antidotes. Administration of pyridostigmine (or other inhibitors) prior to intoxication and treatment with different drugs is a typical example[8,30-32]. There are other combinations such as the administration of triesterase[32,33], procyclidine[8,29], clonidine[34], sustained release of physostigmine and scopolamine[35]. The results are very dependent on experimental conditions but this approach – administration of different drugs - has yielded some good results though up to now they have been on an experimental level. Only one prophylactic mixture has been introduced into the army – PANPAL composed of pyridostigmine, benactyzine and trihexyphenidyle. When PANPAL[7] and TRANSANT[25] are administered simultaneously, their combination represents the best prophylaxis against nerve agents at present.

Conclusions

There are many drugs tested for their prophylactic efficacy against nerve agent intoxication. However, only three prophylactics (pyridostigmine alone, PANPAL and TRANSANT) were introduced into the military medical practice. Perspective approach seems to be the use of purified enzymes, especially butyrylcholinesterase or acetyl-

cholinesterase produced by biotechnology.

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L03
HAIR ANALYSES FOR DRUG ABUSE AND FORENSIC APPLICATIONS

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Key words: hair analysis, drugs of abuse

Introduction

Many years hair analysis for drugs has been gaining increased attention in toxicology and several comprehensive reviews were published on this topics[1−6]. Hair differs from other materials analyzed in toxicological laboratories due to its relative stability and due to its ability to store foreign substances for long time (months to years) in relation to their temporal appearance in blood or urine. Toxicological hair analyses can be useful above all to confirm or exclude chronic intentional or unintentional poisonings or alcohol or drug abuse in various contexts. The development of analytical technologies, especially mass spectrometry, enabling to improve method sensitivity and selectivity, will expand the field of hair analyses for additional drugs and poisons in trace concentrations and will expand the boundaries of rational interpretation with potential serious impacts to individual.

Hair analyses have potential applications to human performance toxicology as preemploee or employee drug screening, or driving ability examination. Hair analyses are used to gestational drug exposure detection, diagnosis of chronic intoxication or pollutant exposition. The results from hair analyses can elucidate the backgrounds of investigated drug-facilitated crimes. Hair analyses are used in divorce proceedings to assess the parents' reliability for children custody in context of parents' potential alcohol or drug addiction. In postmortem toxicology, they can contribute to explanation of pathological autopsy findings. There is only limited relevance of hair in therapy compliance control. Hair can not substitute blood in therapeutic blood monitoring in quantitative sense.

Hair analysis for drugs is not a simple routine procedure. It is necessary to follow substantial scientific guidelines[7,8] starting from sample collection with respect to the individual dignity, case history and aim of investigation.

Hair grows in cycles: the anagen (active growing stage), the catagen (transitional stage) and telogen (resting stage). The individual length of hair depends on the mutual duration of these stages and on the growth rate. Average values for the anagen stage in human are 4–8 years, the catagen a few weeks, and the telogen stage 4–6 months. The scalp hair growth rate is reported to be in the range 0.6–1.4 cm per month in general[1,3]. There are significant differences both in the proportions anagen/telogen hair and both in the growth rate between hairs from various anatomical part of the body[1,3]. The both parameters are dependent on race, sex, age, health conditions. On the scalp of an adult, the approximately 85 % of the hair is in the growing phase (anagen) and the remaining 15 % is in a resting phase (telogen)[1,3]. The consequence of the cyclic hair growth is the nonhomogenity of the hair bunch at the horizontal level, at a certain distance from the skin.

Incorporation and elimination of drugs in hair

The ideal model assumes that drugs or chemicals enter hair by passive diffusion from blood capillaries into the growing cells at the base of hair follicle. However, experimental data indicate that drugs may enter hair in different locations and in different times from different sources by various mechanisms. The drugs can be transported from blood and also from deep skin compartments not only into hair growing cells but with some time delay also into keratogenous zone during hair shaft formation. The other mechanisms are diffusion from sweat or sebum secretions. A contamination of the hair surface from external environment needs to be diminished by washing. The scheme of possible sources of drug incorporation and elimination is demonstrated in fig. 1.

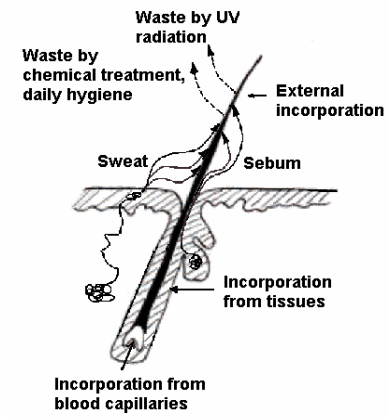

Fig. 1. **The scheme of incorporation of drugs in hair and their potential elimination**

The three key factors which influence the drug incorporation into hair are melanin content in a hair, lipophilicity and basicity of a drug substance. The physicochemical

properties of drugs, lipophilicity and basicity related to molecular structure clearly affect the drug incorporation into hair and on the other side, hair structure and its color plays a very important role too. The pH of melanocytes is between 3 and 5 and significant melanin affinity for basic drugs has been demonstrated in several experimental studies[9]. It was confirmed that drug concentration in pigmented hair was much higher than in blond or grey hair after the same dosage[10,11].

The second important factor is the polarity of a drug or its metabolite. It has been many times documented that more polar metabolites as benzoylecgonine or amphetamine enter the hair in a lesser extent than their more lipophilic precursors cocaine or methamphetamine. The acidity or basicity of a drug is the third important factor. The matrix of hair is more acidic than blood, therefore the resulting pH gradient is more convenient to transfer basic compounds. For example the acidic carboxy metabolite of delta-9-tetra-hydrocannabinol enters the hair only in tiny traces[12].

The retention and stability of drugs in hair is considered to be good, nevertheless it can be affected by cosmetic treatment as bleaching or dyeing and permanent wave application. The long term effects of wheather (sunshine, rain, wind) may cause the damage of hair shaft with impacts to changes of concentration in hair. In case of long hair, above all the structure of distal part could be damaged and its analysis should be avoided.

The practical steps in hair analysis

The meaningful hair analysis needs the complex attitude from sampling to interpretation including the adequate information about the aim of investigation. The main steps of hair analysis include: *1*) Information about the case and aim of investigation; *2*) Hair sampling and documentation; *3*) Washing the external surface; *4*) Segmentation if appropriate; *5*) Opening the inner hair space by grinding or cutting to small strips; *6*) Digestion of hair matrix; *7*) Clean up the hair digest; *8*) Sensitive and specific hair extract analysis (GC-MS or LC/MS); *9*) Interpretation of results, conclusions.

The interpretation of results related to time may be complicated from various reasons:

- Incorrect sampling, mutual shift of individual hair fibers in a sampled bunch
- Nonhomogenous hair fibers growth, individual ratio anagen/telogen, variation in hair growth rate
- Incorporation from other sources than blood (sweat, sebum, delayed incorporation of deposits from tissues)
- Potential waste by environmental effects, daily hygiene, chemical treatment
- Potential longitudinal diffusion

The schemes of various models of drug distribution along the hair tuft after a month consumption a year ago are outlined in the fig. 2.

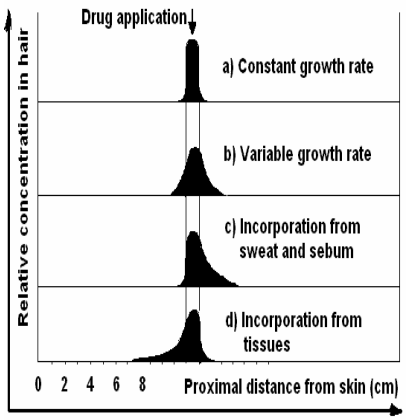

Fig 2. **Models of drug distribution along the hair tuft after continuous one month consumption a year ago**

The information about the assumed period of previous drug consumption in a specific case is very important. When testing for previous drug use about the certain date, the approximate localization of a drug in hair must be estimated. The analysis must be performed at least in 3 segments with one covering the date of the event and two other adjacent segments. Nevertheless, the temporal information gained may be uncertain or insufficient. The width of a segment and their total number selected to cover the time scale of interest are often the compromise between the number of segments and the cost of examination. However, the broader width of a segment can cause the lower concentration of a drug in hair or the concentration bellow the limit of detection, see fig. 3.

Cases of application

Postmortem toxicology

Segmental hair analysis can provide a retrospective calendar of individual's drug use, period of abstinence, or

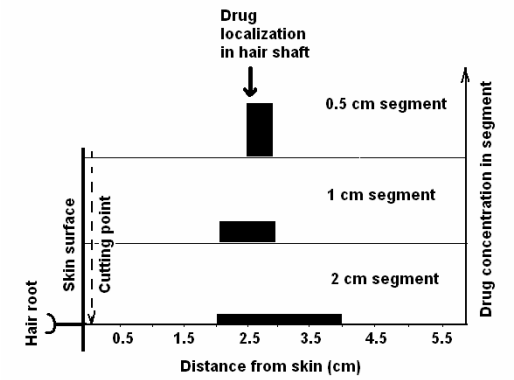

Fig 3. **Drug position in hair and various concentrations in sample segments of various width prepared for analysis**

the evidence of switching from one drug to another or of mixing various drugs e. g. heroin, dihydrocodeine, hydrocodone[13]. A significant factor in interpretation postmortem blood concentration of opioids is chronic or single consumption and the degree of tolerance at the time of death. Only hair results can indicate whether the deceased subject was long term opioid addict and the determined periferic blood concentration was fatal or not, as we have presented earlier in case of a young heroin addict[5].

The extensive pathological autopsy findings in a man, 38 years old, were also in compliance with the toxicological evidence of the drug mixture in hair (methamphetamine, amphetamine, ephedrine, norephedrine, 6-acetylmorphine, morphine, buprenorphine, norbuprenorphine) confirming the chronic and variable drug addiction.

Long term drug abuse or chronic poisoning can gradually induce certain harmful effects on the human organism and can exacerbate some preexisting diseases. For example chronic abuse of methamphetamine is known to be associated with cardiovascular diseases. During autopsy certain types of morphological alterations are found in the hearts of stimulant addicts. The rapid increase in blood pressure after an intravenous methamphetamine dose can be risky for addicts with arteriosclerosis which can develop after long term abuse. However, the information on the life style of a deceased person need not be available to explain the pathological cardiovascular alterations and to classify the cause of death correctly. The findings in hair segments may be useful in this respect. The results in four 2 cm segments and in the fifth distal 7 cm segment were used to explain pathomorphological observation during autopsy of a methamphetamine addict with bleeding into cerebellum. The results provided clear evidence that the man was methamphetamine addict for more than 8 months[14].

Drug facilitated crimes

A case of 13 years old boy belongs to this category. The boy´s tutor was under the suspicion of child sexual abuse and of prohibited methamphetamine administration. In December 2005 the boy was transferred to another children institute. The hairs were sampled in the middle of January 2006. After washing, five 1 cm segments and the resting 2−3 cm distal segment were prepared for GC-MS targeted analysis. Methamfetamine presence was confirmed in all segments except for the proximal segment. The absence of methamphetamine in the proximal segment corresponded to the period after cutting off the contact with the tutor. By hair analyses it was proved that the prohibited drug administration to the infant covered the time window July to November 2005, with the maximum in the third segment roughly corresponding to October at supposed hair rate growth 1 cm month^{-1}. The results of this hair analysis are demonstrated in fig. 4.

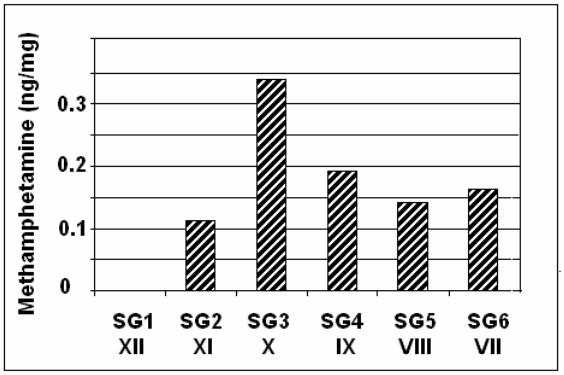

Fig. 4. **Methamphetamine in hair of 13 years old boy. The drug absence in proximal segment SG1 correspon-ded to the time after removing the infant from his tutor**

Human performance toxicology

Many companies have established their own policy for drug testing which is based usually on urine testing where the detection is restricted only to recent dose. In Germany and Italy, the hair testing is allowed in monitoring chronic drug or alcohol abuse in problematic drivers in regranting driver´s licence. In majority of European countries the hair tests for drugs are limited to criminal investigations. As an example from our practice, it is a case of a driver (man 32 years old) who caused a fatal traffic accident. After positive urine drug results for cannabis and amphetamines, the driver admitted the chronic consumption of marijuana and admitted only one dose of methamphetamine after the accident. The hair was sampled 2.5 months after the crash. The hair length was 3.5 cm (covering the approximate period of 4 months of hair growth). Two equal segments were prepared and analyzed. Methamphetamine was confirmed only in the distal segment (0.15 ng mg^{-1}), which corresponded to longer term drug consumption before the accident or to several high doses around the accident time.

Children custody

Hair testing for drugs can be ordered by a judge in divorce proceedings involving parents mutually accused for alcohol or drug addiction. There is the problem to decide whom to charge as a more reliable person for child custody in this situation. The results in hair of a mother or father or even the child can serve as objective evidence supporting the correct decision of the court. We have had the opportunity to provide such evidence in several cases.

Conclusion

The properties of basic drugs as cocaine, amphetamines, opiates are convenient for their efficient incorporation into hair from blood. The transport of acidic or neutral compounds as cannabinoids from blood into hair is less

effective. Therefore to prove their low amount in hair, very sensitive methods are needed. Hair is a unique and stable material for retrospective investigation of a person's life style, to prove or exclude abstinence from alcohol or drugs in a specific time window, to ascertain decreasing or increasing trends in drug abuse. There is no direct relationship between drug concentration in hair and the dose. The relationship between drug concentration in blood and hair is strictly individual and can not be generalized.

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L04

IMMUNOSUPPRESSIVE AND ANTI-INFLAMMATORY EFFECT OF DERMAL EXPOSURE TO COAL TAR AND UV-RADIATION

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Key words: coal tar, polycyclic aromatic hydrocarbons, UV-radiation, Goeckerman regimen, immunosuppressive effect

Introduction

Goeckerman regimen (GR) combines dermal application of therapeutic coal tar (containing polycyclic aromatic hydrocarbons – PAHs)[1] and UV-radiation[2]. GR is often used as the first option for topical treatment of psoriasis[3–8]. PAHs and UV-radiation are carcinogenic (mutagenic) agents[1] with suspected effect to immune system[9–15]. The aim of presented study was to evaluate the changes of selected cellular (lymphocyte subpopulations CD3, CD4, CD8) and humoral immunological parameters (immunoglobulin IgG, IgA, IgM, IgE, β_2-microglobulin, α_2-macroglobulin, transferrin, C3 complement, orosomucoid, prealbumin, haptoglobin, neopterin) in a group of 56 patients with psoriasis, treated by GR.

Research design and methods

Subjects, therapy and sampling

A group of 56 patients (average age 37 years, range 18–77 years), diagnosed with psoriasis and undergoing GR were selected. The group consisted of 34 males and 22 females, 38 % of the patients were smokers. A questionnaire was administered to each subject to determine previous exposures to coal tar (or to other mixtures containing PAHs) and UV-radiation. Patients with positive exposure history were excluded from this study. The efficiency of GR was assessed with respect to erythema, infiltration and desquamation by means of a Psoriasis Area and Severity Index (PASI) score[16].

The GR was applied individually according to disease activity (duration from 8 to 30 days; average time of GR 17 days). Coal tar (CT) ointment (containing 5 % of CT-Pix Lithantracis) was applied daily to affected areas (32–77 % of the patient's body surface). Concurrently, the patients were irradiated daily by ultraviolet radiation-A (UV-A) and ultraviolet radiation-B (UV-B). Radiation was applied individually in relation to the disease activity in intervals from 1 to 20 min. Density of UV-B radiation (134.45 μW cm^{-2}) and UV-A radiation (245.60 mW cm^{-2}) was controlled by a spectroradiometer Sola-Scope 2000 (Solatell Ltd., United Kingdom).

Immunological findings

Blood samples were collected before and after GR. Selected parameters of cell-mediated immunity (lymphocyte subpopulations CD3, CD4, CD8) were determined in heparin-treated blood and the immunophenotyping analysis was performed on a flow cytometer Coulter Epics XL (Coulter-Beckmen, USA). Blood serum was used to estimate the parameters of humoral immunity (immunoglobulin IgG, IgA, IgM, IgE, β_2-microglobulin, α_2-macroglobulin, transferrin, C3 complement, orosomucoid, prealbumin, haptoglobin, neopterin). The levels of serum proteins α_2-macroglobulin, IgG, IgA, IgM, C3 complement, orosomucoid, prealbumin, haptoglobin and transferrin were determined using rate nephelometry (Beckman, USA). IgE and β_2-microglobulin were measured by chemiluminiscent immunoassay (DPC, USA). Levels of neopterin were evaluated using ELISA technique (Brahms, Germany).

Statistical analysis

The data were statistically processed in software "Statistica", version 6.1 (USA). Since the Shapiro-Wilk W test for normality rejected hypothesis of normal distribution, for all followed values we used the nonparametric tests. To assess the effect of treatment, dependent data before and after the GR were compared by the Wilcoxon matched pairs test. The associations between selected parameters were evaluated by Pearson's correlation coefficient. The statistic significance was determined on a probability level less than 0.05 in all calculations.

Results

The levels of IgG, IgM, IgE, transferrin and neopterin were significantly decreased after GR (Table I). The changes of other immunological findings were not significant (lymphocyte subpopulations CD3, CD4, CD8, IgA, β_2-microglobulin, α_2-macroglobulin, C3 complement, orosomucoid, prealbumin, haptoglobin). The levels of PASI score were significantly decreased after GR (22.2 ± 6.9; 6.3 ± 3.7; $P < 0.001$).

Table I
Significantly changed immunological parameters in serum

Units	Before GR					After GR					
	Parameters	n	MED	LQ	UQ	Parameters	n	MED	LQ	UQ	P
[g l^{-1}]	**IgE**	56	**48.5**	30.0	200.0	**IgE**	56	**41.3**	25.1	170.0	**<0.01**
[g l^{-1}]	**IgM**	56	**1.2**	0.9	1.6	**IgM**	56	**1.1**	0.8	1.5	**<0.001**
[g l^{-1}]	**IgG**	56	**11.4**	9.0	13.6	**IgG**	56	**10.5**	9.1	12.8	**<0.001**
[nmol l^{-1}]	**Neo**	56	**6.2**	4.4	8.5	**Neo**	56	**5.3**	4.0	7.1	**<0.01**
[g l^{-1}]	**Trf**	56	**2.6**	3.60	5.20	**Trf**	56	**2.5**	2.1	2.8	**<0.01**

IgE, IgM, IgG = Immunoglobulins, Neo = Neopterin, Trf = Transferrin, MED = median, LQ = lower quartile, UQ = upper quartile, n = number of patients

Discussion

The skin, as an interface between internal and external "environment" plays an important role in protecting and supporting of the organism[7]. Deregulation of skin's immune system, however, frequently occurs and can result into undesirable inflammatory skin processes. A typical example of an undesirable inflammation skin process is a chronic inflammatory skin disease – psoriasis[17].

Psoriasis is a T-cell driven immunologically mediated systemic skin disease (with altered Th1/Th2 cytokine balance)[18], afflicting up to 2.5 % of the world's population[19,20]. Pathogenesis of psoriasis is associated with variable provoking factors of both environmental[21] and endogenous origin in genetically predisposed individuals, e.g. certain HLA (Human Leukocyte Antigens) haplotype (HLA-Cw*0602)[18]. In a case of psoriasis, the complex of interaction between T-lymphocytes, antigen-presenting cells, keratinocytes, pro-inflammatory cytokines and chemokines is disturbed[17,20,22].

Many biological and chemical agents used in the therapy of psoriasis are able to alter the immune functions in human. For example, in one of our previous work we concluded that chemokine pathway of IL-8 and TNF-alpha could be significantly modulated by GR (mainly PAHs)[4]. In another work we found that GR significantly alleviated angiogenic potential, which is in patients with psoriasis usually abnormally increased[1].

In a good agreement with our previous results[5], serum concentrations of IgM and IgG, which are recognized as positive reactants of inflammation, were significantly decreased after GR. It is very likely associated with diminished activity of immunopathological inflammation in treated patients. Also production of IgE class of immunoglobulins significantly decreased in psoriatic patients after GR.

In spite of the fact, that transferrin is a typical negative marker of inflammation, its serum level was significantly decreased after GR. At this moment we are not able seriously explain to this fact, nevertheless, given reality is in accordance with our previous findings[5].

Neopterin is a non-specific marker of activation of cell-mediated immunity[23]. In presented study the serum level of neopterin was significantly decreased, immediately after GR. In agreement with other authors, these findings confirmed that serum neopterin concentration reflects disease activity in psoriasis[20,23,24].

According to the PASI score, our study confirms effectiveness of GR for treatment of chronic psoriasis. The level of PASI was significantly decreased after GR ($P < 0.001$). It was in a good compliance with our previous results[5] and with the results of other authors[6–8].

Conclusions

The results of the study indicate that combine dermal exposure to therapeutic coal tar and UV-radiation (GR) induced significant immunosuppressive and significant anti-inflammatory effects.

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L05
SKIN SENSITISATION AND APPROACHES FOR THE DEVELOPMENT OF CELL-BASED TESTING METHODS

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Key words: skin sensitization, dendritic cells, Langerhans cells

Introduction – mechanisms

This paper is focused specifically on the impact of chemical exposure on immune target cells and the potential application of derived information in the development of cell-based assay for the assessment of skin sensitization potential of chemicals *in vitro*. The possibilities of discrimination between irritating and sensitizing action of chemicals on *in vitro* level are also discussed.

The development of skin sensitization is associated with, and requires, the activation and clonal expansion of allergen responsive T lymphocytes (T-cells). These cells orchestrate the cutaneous allergic reaction. In recent years, much has been learned of the characteristics of immune responses to skin sensitizing chemicals and of the roles played by dendritic cells (DC), cytokines and chemokines. Langerhans cells (LC), the principal DC, are settled in the epidermis. The LC form a network designed to „trap" foreign antigens that have entered the skin, including chemical allergens[1].

One of the characteristics of a chemical allergen, which has been used for the assessment of skin sensitization potential is its ability to react with proteins prior to the induction of skin sensitization[2]. In order to determine if reactivity correlates with sensitization potential, 38 chemicals representing allergens and nonsensitizers were evaluated for their ability to react with glutathione or three synthetic peptides containing either cysteine, lysine, or histidine. UV detection was used to monitor the depletion of glutathione or the peptide following reaction. The results show that a significant correlation exists between allergenic potency and the depletion of glutathione, lysine, and cysteine[3].

Methods for the detection of skin sensitization

A variety of animal test methods are available for the identification of chemicals that have the potential to cause skin sensitization and allergic contact dermatitis. Originally, guinea pigs represented the species of choice for skin sensitization predictive tests and two methods using this species, the guinea pig maximization test[4] and the occluded patch test of Buehler[5], have found wide application. In the early 1980s the mouse ear swelling test (MEST) was developed to provide a lower cost, shorter and objectively graded alternative to the existing guinea pig tests for assessment of skin sensitization. Initially the MEST was employed to evaluate sensitization potential for industrial chemicals only[6]. It has since found much wider utility, particularly in those areas where a cost effective screen for strong irritants and sensitizers is required (such as for finished fabrics and medical devices)[7]. Further method using mouse, the popliteal lymph node assay (PLNA), has been proposed as a screening test for detecting chemicals with potential of inducing allergic reactions in humans[8]. More recently, similar method in the mouse has been developed, the murine local lymph node assay (LLNA)[9,10], having been endorsed by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) as a stand-alone method for the evaluation of skin sensitizing potential[11]. However, even with the significant animal welfare benefits provided by the LLNA, there is still interest in the development of nonanimal test methods for skin sensitization testing using *in vitro* approaches.

Discrimination of irritant and sensitizing agents

Cutaneous toxicity may have several forms – those of greatest prevalence being allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD)[12]. Although the skin elicited reactions are usually indistinguishable with respect to macroscopic or histopathological appearance, the mechanistic bases for ACD and ICD are clearly very different, with the former, but not the latter, being dependent upon the initiation of a primary cutaneous immune response and skin sensitization[13]. It is well established that antigen presentation by antigen presenting cells to the T-cells are essential in the mechanism of ACD. In contrast ICD is believed to activate the immune cascade independent of the antigen presentation pathway, by stimulating release of proinflammatory mediators and cytokines that directly recruit and activate T cells. The precise mechanism of skin irritancy is still unclear[1].

Increases in lymph node cellularity were observed in both allergen- and irritant-treated mice compared to naive or vehicle-treated animals. Mice treated with allergens

showed a preferential increase in the percentage of $B220^+$ (specific cell marker) B cells compared with irritant-treated mice. Treatment with allergens, but not irritants, resulted in a selective increase in the percentages of $CD4^+$ (marker of T-cell help) and $CD8^+$ (marker of cytotoxic T-cell) cells expressing the T-cell activation/memory phenotype $CD62L^{lo}CD44^{hi}$. Flow cytometric analysis of cell phenotype and measuring of expression of T-cell activation/memory markers may provide important information for differentiating allergen- and irritant-induced proliferative responses in the draining lymph nodes (DLNs) of chemically treated mice[14,15]. Coquette et al. published that divergent *in vitro* interleukine (IL) IL-1α and IL-8 release profiles characterize the reconstructed human epidermis response to irritants and sensitizers. For the first time, it was demonstrated that the combination of dose-dependent cell viability measurements, with IL-1α and IL-8 quantification, could provide enough information to allow, in a single assay, in vitro detection and discrimination of irritant and sensitizing agents. Whether these initial and early molecular responses of the keratinocytes are sufficient to detect and discriminate all types of compounds, which are sensitising and/or irritating to skin remains to be determined in further studies[16].

Despite these differences, there is evidence that in response to both ACD and ICD Langerhans cells are mobilized and induced to migrate from the skin via afferent lymphatics to DLNs the site of contact[17]. With a growing appreciation of the cellular and molecular events that initiate and regulate immune responses to chemical allergens and the induction of sensitization, there have emerged new opportunities to design *in vitro* models. The induction phase of skin sensitization is dependent on the activity of cutaneous DC and, in particular, of epidermal LC. Topical exposure of mice (or humans) to skin sensitizing chemicals causes the mobilization of LC and their directed movement from the skin. These cells, many of which bear high levels of antigen, move via afferent lymphatics to skin DLNs where they accumulate in the paracortical regions as immunostimulatory DC[18].

DC and LC – markers for sensitization pathways

The induction of tyrosine phosphorylation following stimulation with contact sensitizers has been examined in both human[19] and murine[20] LC. Whereas freshly isolated human LC failed to demonstrate changes in phosphotyrosine (p-tyr) following exposure to the strong hapten 5--chloro-2-methyl-isothiazolinone plus 2-methylisothiazolinone (MCI/MI), 24-h cultured LC demonstrated a significant increase in p-tyr by flow cytometric quantitation[19]. Neisius et al. reported similar increase in p-tyr in murine LC following *in vitro* stimulation with the strong contact sensitizers trinitrochlorobenzene (TNCB) and MCI/MI but not with the irritants SLS or benzoic acid. Although poorly defined, the mechanisms of signalling pathways in LC during hapten-mediated activation may serve as a basis for the development of an *in vitro* test system[20].

The potential for changes in expression by DC of messenger RNA (mRNA) for other cytokines or chemokines to serve as useful markers for sensitization testing has recently been explored by Verheyen et al.[21]. Following exposure of $CD34^+$-progenitor derived DC to allergens or irritants, mRNA expression for IL-1β, IL-6 and IL-8, and the chemokines (CCL) CCL2, CCL3, CCL3L1, and CCL4 was examined by real-time polymerase chain reaction (RT-PCR.) Significant interindividual variations in mRNA expression in response to chemical treatment were observed. Based on these results, it was concluded, as did Pichowski et al.[22], that allergen-induced IL-1β mRNA expression in DC was not an appropriate indicator of sensitizing potential. Neither IL-6 nor IL-8 was able to discriminate clearly allergens from irritants. However, at the 24-h time point, mRNA levels for CCL2, CCL3, and CCL4 displayed a two-fold or greater increase relative to control for the allergens, but not for the irritants. The authors suggest that further investigation of these chemokine genes is warranted. Using LC-like DC generated from $CD34^+$ cord blood cells, Rougier et al.[23] consistently observed increased expression of cell markers HLA-DR, CD83, and CD86, and decreased levels of E-cadherin (cell adhesion protein) following treatment with a strong allergen but not with an irritant. Tuschl and Kovac[24] examined CD86, CD54, and HLA-DR cell surface expression in parallel with the induction of intracellular expression of IL-1β in peripheral blood mononuclear cells (PBMC)-derived DC. An up regulation of these surface markers was observed in the majority of donors following culture with allergen but not with irritant. However, no clear results were obtained for the induction of intracellular IL-1β.

Searching for novel markers by holistic expression profiling

Microarrays are the most frequently used technology for transcript profiling allowing detection of thousands of mRNAs in cells or tissue. They have been used to study various aspects of DC biology and function such as differentiation, maturation, and migration[25], as well as to elucidate DC intracellular signalling pathways for application to potential immunomodulation strategies[26]. While expression profiles induced by various stimuli such as viral and bacterial pathogens[27], lipopolysaccharide[28,] and cytokines[29] have been monitored; there are limited reports of genome-wide analysis of the changes induced in human DC upon contact with skin sensitizers.

In addition the genes not previously associated with skin sensitization or DC biology, such as AKR1C2, DUSP6, and QPCT, were identified and theirs role in those mechanisms was established, therefore widening the pool from which potential markers can be selected. It is anticipated that similar genomics studies using LC surrogates with different allergens and irritants may provide additional gene targets not previously discovered. However, genes that are selected as markers for skin sensitization must fit the criterion for dynamic range, robustness, sensi-

tivity, and selectivity for a predictive model and cover a range of chemical classes[30].

PBMCs-DCs were treated for 24h with various concentrations of chemicals and in each instance the expression of up to 60 genes was examined by RT-PCR analysis. Consistent allergen-induced changes in the expression of many genes were observed and further prioritization of the targets was conducted by analysis of the same genes in DCs treated with non-sensitizing chemicals to determine their specificity for skin sensitization. RT-PCR analyses of multiple chemical allergens, irritants, and non-sensitizers have identified 10 genes that demonstrate reproducibly high levels of selectivity, specificity, and dynamic range consistent with providing the basis for robust and sensitive alternative approaches for the identification of skin sensitizing chemicals[31].

Conclusion

There is currently some enthusiasm for the application of microarray technology in identifying new gene candidates which expression could serve as potential markers of sensitization effect of chemicals. The ideal candidate in this context would be a gene that displays dramatic increase in expression following encounter of DC with a chemical allergen, fails to show similar changes in expression in response to other stimuli (irritants), and codes for a protein that correlates mechanistically and quantitatively with the acquisition of skin sensitization.

It remains to be seen whether the novel genes will be identified that provide the transcriptional dynamic range and selectivity required for use in *in vitro* prediction models.

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L06

HEALTH RISK EVALUATION OF EXPOSURE TO MIXTURES OF CHEMICALS IN OCCUPATIONAL AIR

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Key words: occupational environment, chemical mixtures, biological effects, interactions, risk evaluation

Introduction

Workers are regularly exposed to multiple chemical agents[1]. The health criteria relating to these exposures in workers generally do not take into account the possibility of interactions between these contaminants, which may result in a change in toxicity. However, priority should be given to this factor[2]. Regarding general environment, both the U.S. Environmental Protection Agency[3], and the Agency for Toxic Substances and Disease Registry[4] published guidance documents on this topic in recent years.

The central question involves the nature of the possible interactions. Regulations and common industrial practices address the question of interactions by hypothesizing, that toxic effects are additive. In some cases, this hypothesis may lead to an underestimation or an overestimation of the actual risk[5]. As in the ACGIH approach[6] (American Conference of Governmental Industrial Hygienists), the Czech regulation prescribes that when two or more hazardous substances are present in workplaces and have similar effects on the same organ of the human body, their effects should be considered additive, unless established otherwise. The mechanism of toxic action is not taken in account.

The power of additive effect is estimated according following formulae:

$$Rm = K_1 / PEL_1 + K_2 / PEL_2 + \ldots + K_n / PEL_n$$

where *Rm* is the mixture exposure index (the sum of the *K / PEL* ratios from the individual components of the mixture), *K* the measured concentration of a substance at a workplace, *PEL* the permissible time-weighted average exposure value in accordance with Czech regulation, *1*,*2*,...*n* the indices designating the various individual substances in the mixture. If *Rm* is greater than one, the time-weighted average exposure value of the mixture of the substances is exceeded.

Presented work is a part of wider research project. The project is oriented on completion and validation of user-friendly Czech toxicological database (CTD) for identification the possible additive effects in mixtures presented in Czech occupational environments and listed in Czech occupational regulation.

Methodology

The Quebec toxicological database (QTD) was used as a starting point for formation of CTD[7,8]. Totally of 350 important substances were selected from QTD. For those selected compounds which were listed in QTD but not in Czech regulation, the ACGIH limits were accepted. Both databases include information on toxicokinetics and target organs. This information was taken mainly from secondary sources: ACGIH[6], OSHA[9], "Proctor and Hughes' chemical hazards of the workplace"[10], "Toxicologie industrielle et intoxications professionnelles"[11], NIOSH[2], and WHO/ IPCS/ILO[12]. To evaluate carcinogenic properties, the data from IARC[13] and from the DFG[14] were taken into account. The "CSST's Service du répertoire toxicologique", a toxicological on-line database developed and maintained by the Quebec Occupational Health and Safety Commission (CSST)[15] was consulted as well.

In relation with QTD, the types of effects on the organs and the mechanisms of toxicity were also determined[7]. The target organs in QTD were identified in relation to the exposure concentration of the substances in the air, when this information was available. In humans, the target organs and effects were determined only for realistic exposure concentrations corresponding, as a maximum, to the STEL (where the "Short-Term Exposure Limit" is the maximum concentration to which workers can be exposed for a period of 15 minutes), to the ceiling value (CV), or to 5 times the TWA (time weighted average, which represents the average concentration of a given chemical to which workers can be exposed for normal 8-hour workdays, 5 days a week). Animal data were used when no human data were available. In this case, the target organs and the effects were determined only for exposure concentrations corresponding to a maximum 100 times the TWA or the CV (factor of 10 for extrapolation of the LOAEL [lowest observed adverse effect level] towards the NOAEL [no observed adverse effect level] and 10 for the differences between the species)[7]. For CTD, all data mentioned above were modified according guidelines for Czech regulation, e.g. for PEL (TWA) and NPK (CV).

To facilitate the assessment of the possibility of toxicokinetic interactions, the data on the main metabolites

(with their CAS number when available), on the absorption and elimination rates, on the distribution, and on the accumulation of the substances in the organs were included. According to its toxicological characteristics, each substance was assigned to one or more of the 32 classes of similar biological effects[7]. This concept of QTD was necessary to accept because it constitutes the basis for the aditivity rule in calculating the Rm.

Computer program was developed for creating work sheets as well as data sheets for the user. The software used for CTD was Microsoft® Access 2003 and Microsoft® Internet Explorer 6. The database disposes of several tables: names of substance, CAS numbers and toxicokinetic characteristics, effects of substances on the body and target organs or systems, as well as intermediate tables that link the main tables. All information in CTD is in both, Czech and English language. By choosing the database, as well as its structure as a tool, easy export is possible in an Internet-adaptable format. Eventually, it will be possible to consult the database via the Internet.

Results and discussion

Since relevant human toxicological data are rare, it is not surprising that the procedures adopted by different countries to define the admissible exposure values (AEVs) differ. This is why the Canadian authors and consequently the Czech authors took the toxicological data from several secondary references, despite the fact that the majority of Czech AEVs are based on the ACGIH's TLVs (ref.[6]).

The mechanism of non-carcinogenic effects for many substances is rarely described in the secondary references. In addition, this description is quite often rather general. Since one of specific objectives of databases was to determine the classes of similar effects from the list of selected effects, more general effects (for example, damage to the lower respiratory tract) had to be combined with more specific effects (for example, pulmonary edema) since the authors could not disregard any possibility of interaction between two substances for the simple reason that they do not currently know a given substance's specific effect on an organ. For evaluation of carcinogenic effects primarily the information from ACGIH and IARC were used.

From the list of selected effects (overall 83 effects) the list of 32 classes of similar biological effects was constructed. The effects retained do not all have the same degree of specificity, and the list contains rather specific effects, or rather general ones. Furthermore, the general effects often encompass effects that are more specific. As a precaution, the definition of the similarity of effects in the organs must therefore be broad enough to cover, for example, a possible interaction between a substance for which detailed human toxicological data are available, and another substance for which even animal toxicological data is scarce and for which the TLV was estimated by physico-chemical analogy with another substance. This is why CTD have defined all effects on an organ or a system as being similar, irrespective of the degree of specificity of this effect. According to its toxicological characteristics, each substance is assigned to one or more of the 32 classes of similar biological effects.

Specific attention has been given to Class no. 32, which contains all the carcinogenic and mutagenic effects. This is because for the majority of carcinogens, information is lacking on the human organs affected, since data on their carcinogenicity come from animal studies. It is difficult to predict which organs may be affected in humans based on results of animal studies. In the majority of cases, tumors are observed in multiple sites in animals. When multiple sites exist, we simply identified the generic effects as *CANCER*. The additional problem that is raised involves aditivity between the carcinogenic and non-carcinogenic effects on a given organ or system. The calculation of an Rm in this case could suggest that the concentrations of any of the substances could be reduced in the workplace in order to reach an Rm value of less than 1 and thus comply with the Czech regulation. A specific efforts must, however, always be made to reduce exposure to carcinogens. Consequently, QTD/CTD did not consider it appropriate to allow the software to calculate an Rm for a mixture of two or more carcinogens or for a mixture of carcinogens and non-carcinogens affecting the same anatomic site. When the user queries the computer system about such mixtures, a warning is displayed indicating that one or more of the substances in the mixture are carcinogenic. At the same time, the tool is deactivated for calculating the Rm.

The information entered in the different tables in the database was organized to create a separate data sheet for each of selected substance. The application essentially consists of a series of HTML pages. The main page is the search form. There are twelve drop-down zones on this page, and from each, a substance can be selected. Once a selection has been made, the application shows the PEL (TWA) level or the NPK (CV, if applicable), as well as the list of classes for each of the substances. Finally, the exposure value measured in the workplace is entered. In this way, the application not only determines whether the aditivity rule should apply or not, but also calculates all the possible combinations of Rm in relation to the chosen substances. Hyperlinks also provide very easy access to the list of classes as well as to the individual toxicity data sheets for the substances.

Conclusion

The database is intended for practitioners working in branches of industrial hygiene, industrial toxicology and occupational medicine. The database provides basic toxicological characteristics, predicts the potential aditivity among components of particular mixture and enables identification of substances for which ***Rm*** formula (mixture exposure index) should be applied. If applicable, the database calculates correspondent Rm. Prediction of possible additive effects can markedly improve assessment of occupational health risks.

To the future we assume to extent the number of substances (approximately 200 other compounds) and specify the type of interaction for mixtures most likely to be found in the occupational environment, for which primary literature data are available. In addition the usefulness of the tool will be tested in selected industrial settings.

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L07

CHROMOSOMAL ABERRATIONS AND CHROMIUM BLOOD LEVELS IN RELATION TO POLYMORPHISMS OF *GSTM1*, *GSTT1* AND *GSTP1* GENES IN WELDERS

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Key words: chromium exposure, chromosomal aberrations, and polymorphisms in *GSTM1*, *GSTT1* and *GSTP1* genes

Introduction

Welders are exposed to chromium (III) and (VI) and to a lesser extent to polycyclic aromatic hydrocarbons (PAHs). Hexavalent form is used in many industrial processes, including corrosion inhibition, chrome plating, stainless steel production and metal welding[1,2]. Experience with excessive exposure at the work place has shown that Cr can act as an acute irritant, as an allergen and as a carcinogen[2,3]. In our previous study[4] we focused on lung cancer frequency in relation to occupational and environmental chromium exposure and we found out that chromium increases the lung cancer occurrence especially in chromate workers. Cr(VI) produces DNA strand breaks, DNA-DNA and DNA-protein cross-links and modifies nucleotides, such as 8-hydroxyguanine. The latter mode of action indicates oxygen radical formation[5,6]. Highly reactive intermediates such as Cr (V) and Cr (IV) formed due to cellular Cr (VI) reduction are primarily responsible for the observed genotoxicity[7]. Cellular reducing agents that may be important for Cr (VI) reduction include ascorbate and sulfhydryl compounds such as cysteine and glutathione[8-12]. Glutathione transferases catalyze the reaction of glutathione with a wide variety of compounds and this reaction is a first step in a detoxification process. Some studies report relationship between level of chromosomal aberrations and polymorphisms of glutathione S-transferase (*GST*) genes. *GSTT1-null* genotype was associated with significantly higher aberration frequencies[13].

Genetic changes are considered as one of the most important steps in carcinogenesis[14]. Cytogenetic analysis of peripheral lymphocytes is traditionally used to evaluate an exposure to clastogens[15] and increased levels of cytogenetic parameters such as chromosomal aberrations (CA) may reflect an increased risk of cancer[16]. The purpose of this study was to conduct a biomonitoring study in welders by employing CAs in peripheral blood lymphocytes as a marker of genotoxic effect and the blood levels of chromium as a marker of internal exposure. Association with genetic polymorphisms in genes encoding metabolising enzymes GSTs as biomarkers of individual susceptibility to procarcinogens was assessed as well.

Material and methods

Subjects and sampling

The study was performed on a population of 31 welders and 31 control individuals that were not exposed to any known carcinogens or mutagens. The detailed characteristics of members of observed groups are shown in Table I. Each person included into the study signed informed consent. Ethical approval based on the Declaration of Helsinki was provided by the Institute of Medical Biology, Jessenius Faculty of Medicine in Martin, Slovak Republic.

Chromosomal aberrations assay

CAs were analyzed in peripheral blood lymphocytes in two separate tubes using previously described method[17]. Structural CAs include chromosomal breaks and exchanges visible in arrested metaphase-stage cells and they were divided into chromosome-type aberrations (CSAs) and chromatide-type aberrations (CTAs).

Determination of chromium in the blood

Chromium analysis in the blood was performed using the atomic absorption spectrophotometer AAS Varian Spectr. AA 30 P. All samples were assessed in duplicate or in triplicate, if significant differences occurred.

Assessment of genetic polymorphisms in genes encoding GSTs

The *GSTM1* (deletion), *GSTP1* (alleles ile/val in codon 105 of exon 5) and *GSTT1* (deletion) polymorphisms were analysed by the multiplex PCR method[18].

Table I
Characteristics of observed groups

	Exposed group (N=31)	Control group (N=31)
Age±S.D. (years)	39.83±2.07	37.72±2.71
Employment±S.D. (years)	9.33±1.66	–
Gender M	31	31
Smoking S/NS	19/12	19/12

M – males, S – smokers, NS – non-smokers, S.D. – standard deviation

Statistical analysis

Statistical analysis was carried out with the use of Statgraphics program, version 7 (LEAD Technologies, Manugistics, Cambridge, MA). For data without normal distribution Mann-Whitney U-test were used to test the differences between the groups. Spearman correlation analysis was employed to calculate the correlation between individual parameters. Kruskal-Wallis test was performed to evaluate associations between biomarkers and specific genotypes. Heterozygous (ile/val) and variant (val/val) genotypes were considered as one group because of low number of variant (val/val) individuals.

Results

There was no significant difference (P>0.05) in the number of aberrant cell between the exposed 1.93±0.17 % (±SE) and control 1.54±0.12 % groups. Chromatide type - aberrations (CTA) were higher in the control group 1.03±0.10 % in comparison with exposed one 0.70±0.13 %. The difference was not significant (P>0.05). Biologically more serious chromosome type - aberrations (CHSA) were significantly higher (P<0.005) in the exposd group 1.22±0.20 % versus control group 0.54±0.12 %. Frequencies of CAs, CTAs and CAs total observed in exposed and control groups are presented in the Table II.

Frequencies of CSA in individuals with wild-type ile/ile GSTP1 genotype were significantly lower (p<0.05) than in those with variant val/val and heterozygous ile/val genotypes (1.4%±0.13 versus 1.88±0.20) (Table III).

Polymorphisms in *GSTM1* and *GSTT1* did not modulate the frequencies of CAs, CTA and CSA. We did not find any correlation between chromium level in the blood and chromosomal aberrations.

Discussion

Our previous studies[4,12] as well as many other epidemiological studies[19,20] showed that workers in ferrochromium industry exhibit an excessive risk for chromosomal injury and lung cancer. Other studies relate this ability to evidenced genotoxicity of chromium and one mode of genotoxic action may proceed via reactive oxygen species that are formed during chromium activation[21]. Glutathione may act as an antioxidant, participating in reduction of chromium species and in diminishing formation of oxygen radicals. The aim of this study was to conduct a biomonitoring study in welders by employing CAs in peripheral blood lymphocytes as a marker of genotoxic effect in relation to genetic polymorphisms of genes encoding principal metabolizing enzymes GSTM1, GSTT1 and GSTP1 as biomarkers of individual susceptibility to procarcinogens.

Our results showed no significant difference in frequency of CAs, CTA and CSA among different genotypes of *GSTM1*and *GSTT1*. These findings are in agreement with previous conclusions of Lee et al.[22]. They studied the relationship between differences in plasma antioxidant capacity, and genetic polymorphisms in detoxification (*GSTM1*, *GSTT1*, and NQO1) and DNA repair (*OGG1*, *XRCC1*) genes and levels of sodium dichromate-induced DNA damage. In their hands *GSTM1* and *GSTT1* genotypes had no significant effect on levels of either background or dichromate-induced DNA damage suggesting that neither GSTM1nor GSTT1 play a rate–limiting role in metabolic detoxification or activation of sodium dichromate. Alternatively compensatory mechanisms may exist to overcome GST deficiency, and their study cannot com-

Table II
Types of structural chromosomal aberrations and chromium level in the blood in studied populations

	CAs±SEM [%]	CTA±SEM [%]	CSA±SEM [%]	Chromium±S.D. [μmol l^{-1}]
Exposed group (N=31)	1.93±0.17	0.71±0.13	1.22±0.2**	0.05±0.04
Control group (N=31)	1.54±0.12	1.33±0.11	0.54±0.12	0.03±0.02

CAs – chromosomal aberrations (total), CTA – chromatide-type aberrations, CSA – chromosome-type aberrations, ** (P<0.005), SEM – standard error of mean, S.D. – standard deviation

Table III
Total chromosomal aberrations, chromatide-type and chromosome-type aberrations stratified for polymorphism of *GSTM1*, *GSTT1* and *GSTP1* genes

		Exposed group		
		CAs±SEM [%]	CTA±SEM [%]	CSA±SEM [%]
GSTM1	*plus*	2.01±0.27	0.81±0.21	1.19±0.28
	null	1.93±0.26	0.61±0.16	1.46±0.31
GSTT1	*plus*	2.03±0.19	0.78±0.15	1.22±0.23
	null	1.50±0.29	0.25±0.25	1.25 ±0.25
GSTP1	ile/ile	1.87±0.27	0.47±0.31	1.40±0.13*
	ile/val, val/val	1.99±0.24	0.86±0.23	1.88±0.20

SEM – standard error of mean, * (P<0.05)

pletely exclude a role for GSTM1 and GSTT1 in the metabolic activation of Cr(VI).

On the other hand we found that frequencies of CSA in individuals with wild-type ile/ile GSTP1 genotype were significantly lower than in those with variant val/val and heterozygous ile/val genotypes. It could indicate the possible role of GSTP1 in detoxification of oxidative stress products. Correlation between genetic polymorphism of GSTP1 and oxidative stress markers was published by Vibhuti[23] and complies with our findings.

The goal of most studies dealing with polymorphism of detoxification and DNA repair genes is to look for the tools for monitoring of individual susceptibility to mutagens and carcinogens in order to protect susceptible individuals. However, functional consequences of the studied polymorphisms have not yet been explored. Our study represents a piece in the puzzle of knowledge needed for developing of optimal and effective monitoring of environmental and occupational exposure in respect to individual characteristics.

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L08
RISK - PROTECTION OF VITAMINS

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Key words: vitamin, micronutrient, recommendation, daily intake, deficiency

Introduction

Vitamins are organic substances, which the organism needs for its function. They are needful for optimal enzymatic activity. Many of them are indispensable activators of enzymatic systems; they are part of enzymes or directly react in metabolic pathways. Vitamins have essential importance for appropriate development and function of organism. Another use of vitamins is in protection before harmful effect of free radicals, in enhancement organism resistance towards stress and also towards infections. Intake of vitamins could influence health condition of population. Incidence of "civilization disease" has close relationship to nutrition, its quantitative and qualitative aspect[1].

Variety of names for recommendation intake are used such as Recommended Daily Amounts, Recommended Daily Allwances (RDA), Recommended Daily Intakes, Recommended Dietary Intakes and Recommended Nutrient Intakes (RNI). The RNI is the intake level sufficient to meet the daily nutrient requirements of almost all (97.5 %) apparently healthy individuals in a specific life-stage and gender group. RNI does not included recommendation for condition with increasing demand such as illness physical and psychical stress, but it is recommendation for homogenous group of population whereas really demand of individual could vary from average. It is based on an estimated average nutrient requirement (EAR) plus two standard deviations above the mean: $RNI = EAR + 2SD_{EAR}$[2]

Water-Soluble Vitamins[3–7]

To group of water-soluble vitamins belong B-complex vitamins and vitamin C. By the toxicity water-soluble vitamins are safe.

B-complex vitamins

There are Vitamin B_1 – *Thiamin,* Vitamin B_2 – *Riboflavin*, Vitamin B_3 – *Niacin,* Vitamin B_5 – *Pantothenate,* Vitamin B_6 – *Pyridoxine,* Vitamin B_{11} – *Folate*, Vitamin B_{12} – *Cyanocobalamin* and Vitamín H – *Biotin* in this group of B-vitamins. All of these vitamins have important function in metabolic pathways.

All of vitamins B-complex has important function in metabolic pathways. Classical deficient syndromes, for examples Beri-Beri (thiamin deficiency) or Pellagra (niacin deficiency) is relatively rare in developed countries. But there is evidence of widespread sub-clinical deficiency of these vitamins including these symptoms: fatigue, apathy, nausea, diarrhea, cheilosis, angular stomatitis, glossitis, seborrhea dermatitis, muscular pain, anemia, peripheral neuritis, depression, and convulsion. High doses of folate may cause moderate gastrointestinal disorders. Chronic high doses of niacin (therapeutically useful in lowering serum cholesterol) can lead to hepatotoxicity as well as dermatologic manifestations. So it was determine upper limit of 35 mg day^{-1}. Niacin could be synthesized from dietary tryptophan. About 60 mg dietary tryptophan is equivalent to 1 mg preformed niacin.

Vitamin C – Ascorbic Acid

Vitamin C involved in oxidation and reduction reactions in metabolic pathways of carbohydrates, amino acids, fatty acids, prostaglandins, steroid hormones and metals. Classical deficient syndromes, scurvy is relatively rare in developed countries. A common feature of vitamin C deficiency is anaemia, due impaired iron and perhaps folate metabolism. A lesser degree of hypovitaminosis C can manifest in symptoms such as weakness, lassitude, fatigue, increased susceptibility to infections. Intoxication is extremely rare. High doses (10–20 g day^{-1}) may cause at some individuals disquiet, sleep disturbances and oxalate kidney stones[3].

Fat-Soluble Vitamins[3–7]

Absorption of these vitamins is depending on absorption of lipids. Occurrence of fat-soluble vitamins deficiency is rather as a consequence of malabsorption (for example: gliadin intolerance, gastrointestinal I^1 inflammatory, obstructive icterus) than their insufficiency in food.

Vitamin A – Retinol

Vitamin A is an essential nutrient needed in small amounts by humans for the normal functioning of the visual system, growth and development, maintenance of epithelial cellular integrity, immune function and reproduction. These dietary needs for vitamin A are normally provided for as preformed retinol and provitamin and carotenoids. Efficiency is expressed as Retinol equivalent (RE): 1 μg RE = 1 μg retinol = 0.6 μg β-carotenoids = 3.3 IU.

Table I
Recommended Nutrient Intakes for water-soluble vitamins part 1 *

Recommendation of FAO/WHO		Thiamin [mg]	Riboflavin [mg]	Niacin [mg] (a)	Pyridoxine [mg]	Pantothenate [mg]
Infants	0–6 months	0.2	0.3	2 (b)	0.1	1.7
	7–11 months	0.3	0.4	4	0.3	1.8
Children	1–3 years	0.5	0.5	6	0.5	2
	4–6 years	0.6	0.6	8	0.6	3
	7–9 years	0.9	0.9	12	1.0	4
Adolescents	10–18 y. Males	1.2	1.3	16	1.3	5
	10–18 y. Females	1.1	1.0	16	1.2	5
Adults	19–65 y. Males	1.2	1.3	16	1.3/1.7(b)	5
	19–50 y. Females	1.1	1.1	14	1.3	5
	51–65 y. Females	1.1	1.1	14	1.5	5
Older adults	65+ Males	1.2	1.3	16	1.7	5
	65+ Females	1.1	1.1	14	1.5	5
	Pregnancy	1.4	1.4	18	1.9	6
	Lactation	1.5	1.6	17	2.0	7

(a) niacin equivalent NE : 1 mg NE = 60 mg dietary tryptophan = 1 mg preformed niacin, (b) 1.3 mg for 19–50 years and 1.7 mg for 50+ years

Table II
Recommended Nutrient Intakes for water-soluble vitamins part 2 *

Recommendation of FAO/WHO		Biotin [μg]	Folate [μg]	Vit. B_{12} [μg]	Vit. C [mg]
Infants	0–6 months	5	80	0.4	25
	7–11 months	6	80	0.5	30
Children	1–3 years	8	160	0.9	30
	4–6 years	12	200	1.2	30
	7–9 years	20	300	1.8	35
Adolescents	10–18 y. Males	25	400	2.4	40
	10–18 y. Females	25	400	2.4	40
Adults	19–65 y. Males	30	400	2.4	45
	19–50 y. Females	30	400	2.4	45
	51–65 y. Females	30	400	2.4	45
Older adults	65+ Males	–	400	2.4	45
	65+ Females	–	400	2.4	45
	Pregnancy	30	600	2.6	55
	Lactation	35	500	2.8	70

* For the purpose of these composite tables of RNI values, the body weights used was derived from the 50th percentile of NCHS data until adult weights of 55 kg for females and 65kg for males were reached. The weights used are the following: 0–6mo = 6 kg, 7–12mo = 8.9 kg, 1–3ys = 12.1 kg, 4–6ys = 18.2 kg, 7–9ys = 25.2 kg, 10–11ys M = 33.4 kg, F = 34.8 kg, 12–18ys M = 55.1 kg, F = 50.6 kg, 10–18ys M = 55.1 kg, F = 50.6 kg, 19–65ys M = 65 kg, F = 55 kg

Hypovitaminosis A is often expressed as only nausea. Other typical symptoms like night blindness, xerophalmia, hyperkeratosis, hepatosplenomegaly, metaplasia of mucous membrane and anaemia are rare in developed countries. Vitamin A as well as vitamin D is the only vitamin which may cause a hypervitaminosis. Hight doses of vitamin A are toxic and cause visual disorders; they are teratogenic (dosage of 7500 μg day^{-1}). Upper limit is 2800–

Table III
Recommended Nutrient Intakes for fat-soluble vitamins *

Recommendation of FAO/WHO		Vit. A(a) [μg] RE	Vit. D [μg]	Vit. E [mg] α-TE	Vit. K(d) [μg]
Infants	0–6 months	375	5	2.7 (c)	5
	7–11 months	400	5	2.7 (c)	10
Children	1–3 years	400	5	5	15
	4–6 years	450	5	5	20
	7–9 years	500	5	7	25
Adolescents	10–18 y. Males	600	5	10	35–65
	10–18 y. Females	60	5	7.5	35–65
Adults	19–65 y. Males	60	5/10 (b)	10	65
	19–50 y. Females	500	5	7.5	55
	51–65 y. Females	500	10	7.5	55
Older adults	65+ Males	600	15	10	65
	65+ Females	600	15	7.5	55
	Pregnancy	800	5	(c)	55
	Lactation	850	5	(c)	55

(a) Vitamin A values are "recommended safe intakes" instead of RNIs. Recommended safe intake as μg RE/day; 1 μg retinol = 1 μg RE = 0.6 mg β-carotenoids = 3.3 IU, (b) 5 μg for 19–50 years and 10 μg for 50+ years, (c) For pregnancy and lactation there is no evidence of requirements for vitamin, (d) The RNI for each group is based on a daily intake of 1 μg kg^{-1} of phylloquinone

Table IV
Referential range for vitamins C, A, E, D - upper tolerable intake level

	Vitamin C – UL [g day^{-1}]	Vitamin D – UL [μg day^{-1}]	Vitamin E – UL [μg day^{-1}]	Vitamin A – UL [μg day^{-1}]
Children to 3 years	400	50	200	600
Children to 8 years	650	50	300	900
Children to 13 years	1200	50	600	1700
Adolescents	1800	50	800	2800
Pregnancy	1800	50	800	2800-3000
Adults	2000	50	1000	3000

3000 μg day^{-1}.

Vitamin D – Cholecarciferol

Vitamin D is required to maintain normal blood levels of calcium and phosphate that are in turn needed for the normal mineralization of bone, muscle contraction, nerve conduction and general cellular function in all cells of the body.

The early signs of vitamin D deficiency are decreased serum concentration of calcium resulting from depressed absorption of calcium from the intestine leading to depletion of calcium from bones. Later signs are inadequate skeletal mineralization (rickets or osteomalacia), bone pain, severe bone deformities and alterations in muscle metabolism and respiratory function. Symptoms of acute and chronic vitamin D intoxication include nausea, diarrhea, polyuria, weight loss, hypercalcemia, hypercalciuria and eventually nephrocalcinosis, decreased renal function or calcification of soft tissues. Upper limit for vitamin d intakes is 50 μg day^{-1}.

Vitamin E – Tocopherol

The biological action of vitamin E results principally from its antioxidant properties, it prevents propagation of the oxidation of unsaturated fatty acids by trapping free radicals. Vitamin E also has anti-inflammatory effect and modulates immunity respond.

Vitamin E deficiency causes oxidation of PUFA, which leads to structural and functional damage to cellular membranes. Vitamin E has very low toxicity.

Vitamin K – Fylochinon

Vitamin K is essential cofactor for synthesis blood

clotting factors. The most common type of deficiency is from malabsorption or using antibiotics or anti-vitamin K drugs (warfarin). Deficiency results in a bleeding syndrome. Clinical features may range from mild bruising to severe life-threatening haemorrhage. No adverse signs have ever been reported from large oral doses of vitamin K.

Conclusion

Vitamins are essential nutritional elements which affect different biochemical and metabolic processes in the human organism. Adequate concentrations of individual vitamins in biological tissues are a necessary pre-requisite for metabolism of basic nutrients – glucose, lipids and proteins and for energetic metabolism. In addition they influence many physiological functions and intervened pathobiochemical processes. Besides positive projective vitamin effects on health, possible health risks of overhelming consumption of some vitamins must be emphasized.

A recommended vitamin intake in the Czech Republic, EU as well as the US is provided in recommended dietary allowances (RDA).A definition of upper tolerable intake level (LA), NOAEL and LOAEL allowances are included into the recommended vitamins intake (Tables I–IV).

Supported by VZ MOO FVZ 0000502.

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L09

USE OF PHOTOAFFINITY LABELING FOR STUDY OF CYTOCHROMES P450, TOXICOLOGICALLY IMPORTANT ENZYMES

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Key words: Cytochrome P450, photoaffinity probe, metabolism, access channel, three dimensional structure

Introduction

Cytochromes P450 (CYPs) comprise a superfamily of b-type hemeproteins, characterized by their cysteinyl-heme ligation. CYPs belong to the most extensively studied enzymes involved in the metabolism of foreign compounds (xenobiotics) e.g. drugs, pollutants, dyes, carcinogens, as well as endogenous compounds (steroid hormones, fatty acids, prostaglandins)[1]. Because of the clinically important role of CYPs in the human body, understanding of the principle of CYP substrate binding is crucial. Moreover, as the carcinogen activation is mediated by CYPs, the research of CYP structure-function relationships is important to reveal the first stage of the carcinogenesis process at the molecular level. Recent knowledge of this process is limited to three-dimensional (3D) structures for mammalian CYPs derived from X-ray crystallography of truncated chimeric CYP protein constructs. Therefore, the possibility exists that in the CYP crystal the CYP native structure is perturbed by the protein changes prior the microsomal CYP crystallization (e.g. elimination of N-terminal transmembrane domain, addition of C-terminal 4xHis tag) and/or caused by the process of crystallization itself (e.g. CYP dimerization). Hence, it is necessary to utilize other approaches to validate structure-function data of mammalian enzymes.

Photoaffinity labeling, a chemical modification technique, was proved to be useful for structure study of membrane-bound proteins, difficult to examine by other techniques. It makes use of the highly reactive intermediates generated by photolysis from photolabile substrate analogues. Azides, diazirines, and benzophenones are most frequently introduced into the substrate molecule, providing the photoaffinity probe. Several photolabile substrates have been used in the past as effective photoaffinity ligands of mammalian and bacterial CYPs[2]. Recently, CYP2B4 putative access channel was identified by a heterobifunctional photoaffintity probe, *N*-(*p*-azidobenzyl)-*N*--methyl-*p*-aminophenethylamine (***I***) (for structure see fig. 1) and the 3D model of this CYP verified[3]. In the present study, we examined ***I*** as photolabile probe for labeling of the access channel region of CYP3A6, the CYP that serves as a model of highly homologous human microsomal cytochrome CYP3A4. In the human body, this CYP is involved in metabolism of a majority of drugs and a large number of carcinogens. Although crystals of N-terminal modified CYP3A4 chimeras were prepared, and 3D structure models developed[4], the problem, how the substrate reaches the active center deeply buried in the CYP macromolecule remains to be solved.

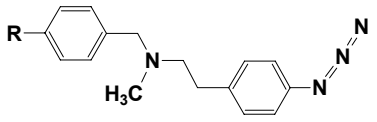

Fig. 1. **Structure of desmethylbenzphetamine bifunctional photolabile probe**. Probe ***I*** - R = -NH_2; probe ***II*** - R = -N_3

Materials and methods

Cytochrome P4503A6 preparations

Liver microsomal (Ms) samples were prepared from rabbits treated with rifampicin (RIF). Cytochrome CYP3A6 was purified from the microsomal fraction basically as described by Haugen and Coon[5]. The final electrophoretically homogeneous preparation had a specific content of 7.6 nmol of CYP/mg protein as determined on the basis of the absorbance of reduced CYP complex with CO at 450 nm (ref.[6]).

Cytochrome P450 difference spectra

CYP difference spectra with photolabile probes[7], ***I*** and ***II*** (see fig. 1), were recorded on a Specord M-40 spectrophotometer (Carl Zeiss, Jena, Germany). CYP3A6 samples were diluted with the phosphate buffer (0.1 M K/PO_4, pH 7.4, with 20% glycerol) to 2 μM. The contents of the sample cuvette was treated with a gradually increasing amount of the probe tested (final concentration 2–700 μ M), while the same volume of solvent (water) was added to the reference cuvette. The difference spectra were recorded from 350 to 500 nm.

Inhibition studies

The inhibitory effect of the probe ***I*** (10–400 μM) on CYP3A6 specific metabolic activity: *O*-dealkylation of dibenzylfluorescein (DBF)[8], *O*-dealkylation of 7-benzyloxy-4-trifluoromethylcoumarin (BFC)[8] and 6β-hydroxylation of testosterone[9] was determined with Ms-RIF and purified CYP3A6 in reconstituted system. Samples were diluted with the phosphate buffer (0.1 M K/PO_4, pH 7.2 with 20% glycerol) to the final CYP concentration of 10–200 nM. The reaction mixtures containing up to 600 nM DBF, 93 μM BFC or 50 μM testosterone (final concentrations) were incubated at 37 °C for 30, 10 or 15 minutes, respectively. Reaction products of BFC and DBF assays were analyzed using a Perkin–Elmer LS-5B spectrofluorimeter. The amount of 6β-hydroxytestosterone produced from testosterone was determined on a C18-HPLC column (Nucleosil 100-5, Macherey-Nagel) using 70% (v/v) methanol as a mobile phase.

Homology modeling and docking

The 3D structure of rabbit CYP3A6 was built based on the crystal structures of CYP3A4 (PDB code – 1TQN) using Modeller 6.2 and Clustal X software[3]. Probe ***I*** was docked into the CYP3A6 model as described previously[3]. Briefly, for the docking Autodock 3.05 software has been employed, using the genetic algorithm method, with 27 000 000 generations and 200 populations, with 20 runs for the heme anchored probe ***I***. The conformation having the lowest energy has been chosen as the result.

Results and discussion

Our previous study proved that the heterobifunctional desmethylbenzphetamine probe ***I***, containing azido- and amino-groups on the opposite end of the molecule, is useful for mapping the substrate access channel region of CYP2B4 (ref.[3]). The probe ***I*** is designed to be anchored via its amino-group to the heme while its azido-group is able (after photoactivation) to modify amino acids in a defined distance from the heme. To use this probe ***I*** for CYP3A6 structural studies, the active center binding, as well as the heme ligation of the probe, should be examined.

In order to estimate the probe ability to enter the active site of the CYP3A6, its inhibitory potential towards three marker catalytic activities of the CYP3A subfamily was examined. The assays were performed with the microsomal fraction as well as with a reconstituted system consisting of purified CYP3A6 and NADPH:CYP reductase. All assays were conducted with the probe ***I*** and ketoconazole, a CYP3A selective inhibitor. The inhibitory potential of tested compounds was expressed as IC_{50} values (see Table I). All assayed systems with exception of DBF dealkylation in microsomes had low IC_{50} values (in nM range) for ketoconazole. Thus, they are specific for CYP3A6 activity. The probe ***I*** showed a concentration dependent inhibition of all CYP3A6 specific activities. As judged form its IC_{50} values, the probe ***I*** is a moderate CYP3A6 inhibitor. The detected inhibitory ability of the probe ***I*** suggests its binding in the active center of CYP3A6. Moreover, these experiments supported ketoconazole to be a proper competing compound to expel the probe from the binding site of CYP3A6 for competition studies.

To further characterize the interaction of the probes ***I*** with CYP3A6, experiments using the difference spectroscopy were carried out. The binding of probes ***I*** and ***II*** (aminoazide and diazide) in RIF-induced liver microsomes was examined. From the difference spectra (not shown) it is clear that both probes interact with CYP present in microsomal sample. While probe ***II***, used for comparison, elicited formation of a typical Type I substrate spectrum

Table I
Data of inhibition experiments

CYP assay	CYP sample	Tested compound	CYP [nM]	Substrate [μM]	IC_{50} [μM]
DBF dealkylation	Ms	ketoconazole	10	0.20	10.80
DBF dealkylation	Ms	probe ***I***	10	0.20	30.40
DBF dealkylation	RS	ketoconazole	40	0.60	0.04
DBF dealkylation	RS	probe ***I***	40	0.60	31.10
BFC dealkylation	Ms	ketoconazole	50	27.00	0.03
BFC dealkylation	Ms	probe ***I***	50	27.00	105.00
BFC dealkylation	RS	ketoconazole	120	93.00	0.90
BFC dealkylation	RS	probe ***I***	120	93.00	n.d.
Testosterone hydroxylation	Ms	ketoconazole	200	50.00	0.09
Testosterone hydroxylation	Ms	probe ***I***	200	50.00	110.30

BFC – 7-benzyloxy-4-trifluoromethylcoumarin; CYP – cytochrome P450; DBF – dibenzylfluorescein; IC_{50} – inhibitor concentration eliciting 50 % inhibition; Ms – microsomal fraction; RS – reconstituted system; n.d. – not determined

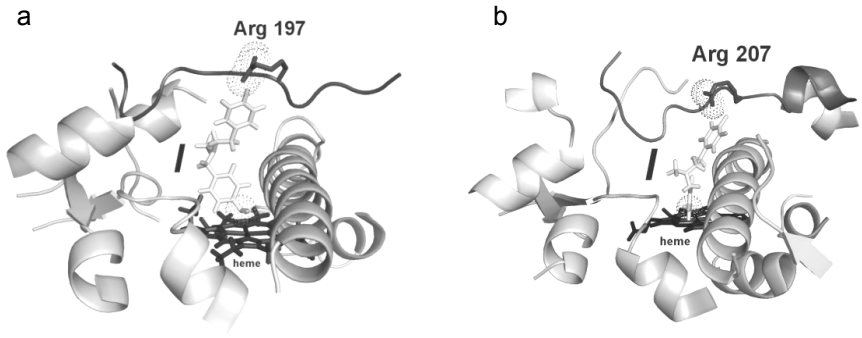

Fig. 2. **Docking of probe *I* to model structures of CYP2B4 (A) and CYP3A6 (B)**. In both structures the probe ***I*** is anchored via its amino-group to the heme iron. Arg residues of CYP2B4 (already detected as modified) and of CYP3A6 (predicted in a close vicinity to reactive probe nitrene) are highlighted

(absorbance maximum at 387 nm and minimum at 422 nm), probe ***I*** showed the ligand Type II spectrum with an absorbance maximum at 438 nm and minimum at 418 nm. Thus, the probe ***I*** proved its amino-group to provide the heme iron ligation. In other words, the probe ***I***, in contrast to probe ***II*****,** is oriented in the CYP active center in a desired orientation, necessary for photoaffinity experiments.

As the probe nitrene was previously shown to specifically modify Lys and Arg residues[10], the presence of these residues within the probe ***I*** reaction radius in the CYP3A6 structure should be examined. The experimentally defined binding and orientation of the probe ***I*** in the CYP3A6 active center was compared with results of docking computational experiments. At first, the 3D molecular model of rabbit CYP3A6 was constructed by homology modeling based on the known crystal structure of highly related CYP3A4. The CYP2B4 structure with the probe ***I*** in an Arg 197 adduct productive orientation as well as the probe ***I*** docked to the putative access channel of the CYP3A6 model is presented in fig. 2. Of the amino acid residues close to the probe ***I*** nitrene, Arg in the sequence position 207 is likely to be modified with the photoactivated probe.

Conclusion

The *N*-(*p*-azidobenzyl)-*N*-methyl-*p*-aminophenethylamine (***I***) heterobifunctional compound was examined as a potential photolabile probe for mapping the access region of CYP3A6. Metabolic inhibitory studies with various CYP3A6 specific substrates and difference spectroscopy measurements revealed the probe ***I*** to be bound in the CYP active center in the orientation allowing the heme iron ligation. In addition, docking experiments with CYP3A6 structure predict Arg in the sequence position 207 to be a potential target of the probe ***I*** modification.

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Abbreviations

BFC	7-benzyloxy-4-trifluoromethylcoumarin
CYP	cytochrome P450
DBF	dibenzylfluorescein
IC_{50}	inhibitor concetration eliciting 50% inhibition
Ms	microsomal fraction
RIF	rifampicin
3D	three-dimensional

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L10
EFFECTS OF BORNEOL, A BICYCLIC TERPENE, ON HUMAN CELLS OF DIFFERENT ORIGIN

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Key words: borneol; comet assay; hydrogen peroxide; human cells HepG2, VH10, Caco-2

Introduction

Various industries are now looking for alternative, more natural and environmentally friendly antimicrobials, antibiotics, antioxidant and crop protection agents as well as an alternative route for the substitution of synthetic chemicals, side effects of which are always in question. The possibility of utilizing volatile oils is now being investigated as, although their biological activity has been known for centuries, their mode of action was not fully understood. For this, the essential oils and the extracts of many plants have been prepared and screened for their activities. Borneol belongs to monoterpenoid alcohols that represent a class of natural compounds that are widely present in the environment as component of plants and are often occuring in the production of oils, perfumes, and foods. In addition, borneol has been reported to have an antimalarial[1], antimicrobial and antifungal activities[1–3]. Interestingly, borneol has been used as a medicine by many Asian cultures. Some investigators suggested that borneol had the potential to be used as an ophthalmic[4] and nasal[5] penetration enhancer. Borneol is also used in folk remedies for various purposes, such as the treatment of abdominal pain, particularly stomachache[6]. Our study compares cytotoxic, DNA-damaging and possible DNA-protective effects of borneol on human cells of different origin.

Material and methods

Cell lines: HepG2 and Caco-2 cells were obtained from Prof. A.R. Collins (University of Oslo, Norway) and VH10 cells from Dr. A. Kolman (Laboratory of Radiobiology, University of Stockholm, Sweeden). The cells were cultured at 37 °C in a humidified atmosphere of 5 % CO_2 in William's medium (HepG2) or Dulbecco's modified minimum essential medium (VH10, Caco-2) supplemented with 10 % fetal calf serum and antibiotics (penicillin 200 U ml^{-1}, streptomycin and kanamycin 100 μg ml^{-1}).

Borneol (Sigma-Aldrich Co., Steinheim, Germany) were kept at room temperature, dissolved in 96% ethanol (1 mol l^{-1}) and diluted in complete culture medium to the concentrations 0.1–6 mmol l^{-1} immediately before use.

Chemicals: Hydrogen peroxide – H_2O_2 (Sigma-Aldrich Co., Steinheim, Germany) stock solution (10 mol l^{-1}) was kept at 4 °C and diluted immediately before use in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) at 4 °C. Control cells or cells pre-treated with borneol were embedded in 0.75 % LMP agarose and treated by 250 μmol l^{-1} H_2O_2 for 5 min on ice in the dark.

Cytotoxicity testing

Cytotoxic effects of borneol were in all human cells evaluated by the trypan blue exclusion technique. Cells were exposed to different concentrations of borneol for 24 h. Control cells were kept in a fresh complete culture medium containing 0.1% ethanol for 24 h. The assayed cells were washed with PBS (Ca^{2+}- and Mg^{2+}- free), trypsinized, stained by 0.4% trypan blue and the number of viable and dead cells were scored.

Single cell gel electrophoresis (cometassay)

The procedure of Singh et al.[7] was followed with modifications of Slameňová et al.[8] and Gábelová et al.[9]. Briefly: The assayed cells embedded in 0.75 % LMP agarose were immersed in ice-cold lysis mixture (2.5 mol l^{-1} NaCl, 0.1 mol l^{-1} Na_2EDTA, 0.01 mol l^{-1} Tris-HCl, pH 10, 1 % Triton X-100) for 1 h. The slides were then transferred to an electrophoresis solution (0.3 mol l^{-1} NaOH, 1 mmol l^{-1} Na_2EDTA, pH>13) for 40 min at 4 °C. A current of 25 V (0.3 A) was then applied for 30 min. The slides were neutralized with 0.4 mol^{-1} Tris-HCl (pH 7.5) and stained with ethidium bromide (5 μg ml^{-1}). For each sample, 100 "comets" were evaluated and scored with an Olympus fluorescence microscope and computerized image analysis (Komet 5.5, Kinetic Imaging, Liverpool, UK) for determination of % DNA in tail.

Statistics

The results represent a mean of two or three independent experiments ± standard deviation (SD). The significance of differences between samples was assessed by Student's *t*-test.

Results and discussion

Cytotoxicity and genotoxicity testing on human cells

The trypan blue exclusion assay showed that human fibroblastoid VH10 cells were the most sensitive to the toxic effects of a 24 h treatment with borneol (0.1–3 mmol l^{-1}) among all human cell lines studied. HepG2 and Caco-2 cells reacted on a 24 h treatment with borneol (0.15–6 mmol l^{-1}) similarly (figs. 1A,B,C-insets). Measuring of DNA strand breaks induction in human cells of all three types treated for 24 h with different concentrations of borneol (figs. 1A,B,C-black columns) by the comet assay showed that at concentrations $<IC_{50}$ borneol induced no or only slight increase in DNA damage. Our results on assessing genotoxicity of borneol correlate with the findings of Azizan et al. who observed no mutagenic activity of short-term (20 min) or 120 min borneol-treatment in Ames Salmonella/S9 microsomal assay[10].

DNA−protective effects of borneol

The comet assay was used also for evaluation of possible protective effects of borneol against DNA-damaging effects of H_2O_2 (fig. 1 – stripped bars). As borneol reduced significantly the level of H_2O_2-induced DNA strand breaks in human hepatoma HepG2 cells and slightly but significantly in human fibroblastoid VH10 cells, it is evident that borneol protects some types of cells against the DNA-damaging effect of H_2O_2. On the other side borneol had no DNA-protective effects against DNA damage induced by H_2O_2 in Caco-2 cells (fig. 1C – stripped bars). These distinctions among cytotoxic, genotoxic and mainly DNA-protective effects of borneol in different human cells are at present unclear, but we cannot exclude that they could be connected with different antagonistic action of borneol on signal transduction systems of cells of different origin[11,12].

Conclusion

The cytotoxic, genotoxic and DNA-protective effects of borneol were studied in human cells of different origin (human hepatoma HepG2, human fibroblastoid VH10 and human colon carcinoma Caco-2 cells). Cytotoxicity testing was secured by the trypan blue exclusion technique. The levels of DNA damage were determined using alkaline single cell gel electrophoresis (comet assay). The trypan blue exclusion technique showed that borneol was cytotoxic in increasing concentrations on all cells tested, VH10 cells being the most sensitive. Borneol itself was slightly genotoxic at concentrations $<IC_{50}$ only on HepG2 and Caco-2 cells. Borneol protected human hepatoma HepG2 cells and partially human fibroblasts VH10 against H_2O_2-induced DNA damage at concentrations $<IC_{50}$, but it manifested no effect on human colon carcinoma Caco-2 cells. These differences in effectiveness of borneol found in cytotoxicity, genotoxicity and DNA-protectivity testing among human cells of different origin are not explained at present.

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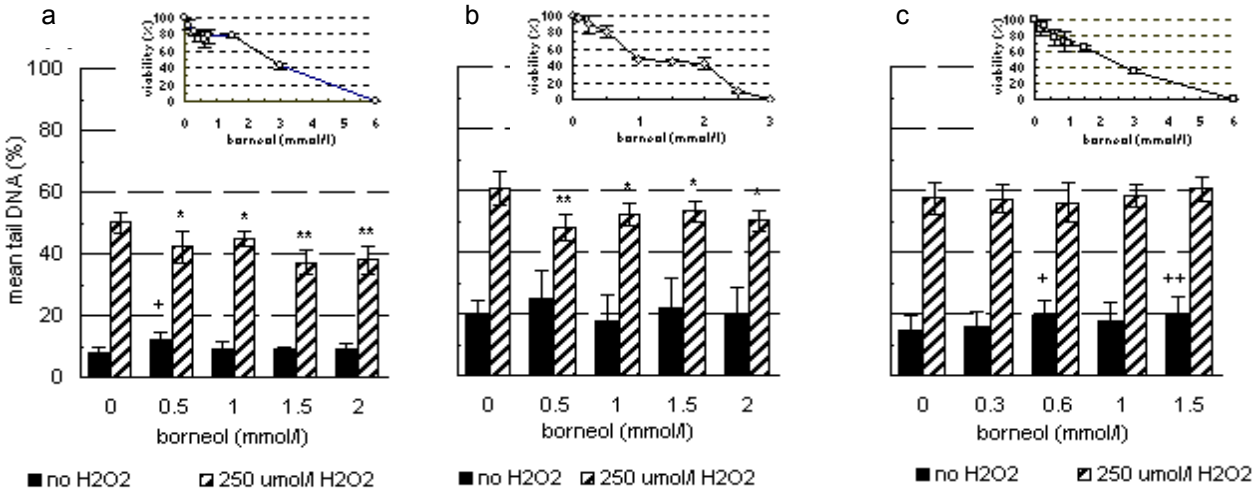

Fig. 1. **Levels of DNA damage in HepG2 (A), VH10 (B) or Caco-2 (C) cells treated with borneol** for 24 h, with 250 μM H_2O_2 for 5 min or pre-treated with borneol and then treated with H_2O_2. Data represents three independent experiments with three replicate samples ± SD. Statistically different from control treated with 0.1% ethanol or from value for hydrogen peroxide $^{+;}$ * $P<0.05$; $^{++;}$ ** $P<0.01$; $^{+++;}$ *** $P<0.001$. Insets: Viability of HepG2 (A-open circles), VH10 (B-open diamonds) and Caco-2 (C-open squares) treated for 24 h with different concentrations of borneol. Data represent means of two determinations (with two parallels each) ± SD

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L11
POTENTIAL ANTIMUTAGENIC EFFECT OF POLYSACCHARIDE *N*-(2- CARBOXYETHYL) CHITOSAN

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Key words: DNA-topology, plasmid DNA, *Salmonella typhimurium*, DPPH radicals, OH radicals, carboxyethyl chitosan

Introduction

Every year some new natural substances have been isolated. These compounds can positively influence human health, and due to their potential antigenotoxic and antioxidant effects they could be used in the cancer prevention[1,2].

Chitosan is a cationic polysaccharide made from alkaline *N*-deacetylation of chitin. Chitin is contained in the cell wall of fungi, exoskeletons of insects and crustaceans. Over the past decades, chitin and chitosan have found many applications in agriculture, textile and paper industry, cosmetics, waste treatment, and food processing. Due to their biodegradability, non-toxicity, and antioxidant properties the chitin derivatives have been attracted attention for their possible application in human medicine and pharmacy as a biological material, with unique biological activities, which include antitumor, immuno-enhancing effect, and antibacterial activities[3–6]. The applications of chitosan are limited because of the insolubility in water at neutral or high pH region. One of the most popular way to provide a hydrophilic character to chitosan is carboxyethylation[7].

In this work antimutagenic/bio-protective and antioxidant effects of CECh were evaluated with a special emphasis on the study of its potential mechanism of action. The possible antimutagenic activity of carboxyethyl chitosan was assessed in the Ames assay on histidine-deficient bacterial *Salmonella typhimurium* strain TA100. In the DNA-topology assay bio-protective activity of CECh on pBR322 plasmid DNA was evaluated. The potential antioxidant activity of CECh was evaluated in the DPPH assay and the hydroxyl radicals scavenging assay.

Material and methods

N-(2-carboxyethyl) chitosan (CECh) was obtained by reaction of a low molecular weight chitosan with a low degree of acetylation and 3-halopropionic acids under mild alkaline media (pH 8–9, $NaHCO_3$) at 60 °C. It is the derivative of chitin, synthesized by Skorik et al.[7] from coarse ground crab. CECh with molecular weight about 70 kDa, and 1.5 degree of substitution was used[7].

Salmonella typhimurium mutagenicity/antimutagenicity (Ames) assay

For mutagenicity/antimutagenicity of *N*-(2-carboxyethyl) chitosan (CECh) the Ames pre-incubation assay with 1 hour pre-incubation was performed. The *Salmonella typhimurium* tester strain TA100 was obtained from Czech Collection of Microorganisms (Brno, Czech Republic). As a positive control direct mutagen sodium azide (NaN_3) was used.

The assay was conducted in test tubes containing 0.1 ml of the overnight bacterial culture (approximately 10^8 cells ml^{-1}, cultivated in 50 ml of LB medium), sterile water and the tested CECh (750, 500, 250 μl of 1 mg ml^{-1} per plate). Test tubes containing CECh were incubated for 1 h with bacteria. After 1-hour-incubation direct-acting mutagen and the top agar were supplemented. The content was mixed, and plated on minimal bottom agar plates. *His*$^+$ revertants were counted after 72 h of incubation at 37 °C in Biotran III Colony Counter (New Brunswick Scientific Co.). The data were analysed using the Student´s *t*-test.

DPPH radical scavenging assay

The potential antioxidant activity of CECH was measured in terms of 1,1-diphenyl-2-picrylhydrazyl free radical scavenging ability, with slight modifications[8]. Ascorbic acid was used as the reference compound. The highest concentration of ascorbic acid was considered as 100 % of scavenging activity. A methanolic solution of CECh (50 μl) at different concentrations was placed in a cuvette and 1 ml of 23.7 μg ml^{-1} methanolic solution of DPPH radical was added followed by 30 min incubation. The decrease in absorbance at 517 nm was determined with the Spekol 221 spectrophotometer. All determinations were performed in three replicates.

Hydroxyl radical scavenging assay

The hydroxyl radicals (•OH) were generated in an L-

ascorbic acid/$CuSO_4$ system by reduction and were assayed by the oxidation of cytochrome c[9]. In this experiment, the •OH were generated in 1 ml of 7.5 mM sodium phosphate buffer (pH 7.4) containing 5 mM L-ascorbic acid, 5 mM $CuSO_4$, 0.6 mM cytochrome c, and the samples of CECh to be tested at different concentrations (0.01 mg ml^{-1}, 0.025 mg ml^{-1}, 0.05 mg ml^{-1}, 0.1 mg ml^{-1}). The mixture was incubated at 25 °C for 15 min. Change in transmittance caused by a color change of cytochrome c was measured at 550 nm using Spekol 221.

D N A - t o p o l o g y a s s a y

The plasmid pBR322 was purchased from Advanced Biotechnologies Ltd. (Units B1-B2 Longmead Business Centre, Blenheim Road, Epsom, Surrey KT 199QQ, U.K.) The method of electrophoretically monitored DNA damage and DNA protectivity was described in detail by Rauko et al.[10]. Briefly, the reaction mixture (final volume 20 ml) contained 200 ng plasmid DNA in buffer. The plasmid DNA was exposed either to Fe^{2+} (10 μM), or H_2O_2 (1 mM) alone or in combination with CECh (1 mg ml^{-1}, 0.75 mg ml^{-1} a 0.5 mg ml^{-1}).

DNA single-strand breaks were assayed by measuring the conversion of supercoiled DNA (form I) to relaxed circular DNA (form II). Topological changes of DNA molecules correspond with the electrophoretical mobility of DNA topoisomers. Analysis of DNA modifications was made by agarose gel electrophoresis (1.5 % agarose, 45 min/60 V). The DNA was made visible by staining with ethidium bromide (1 mg ml^{-1}, Sigma, USA) and UV illumination (Ultra-Lum Electronic UV Transilluminator, USA). Percentages of supercoiled, relaxed and linear DNA forms were calculated by a computer program (Uthcsa, Image Tool for Windows, Version 1.27).

Results and discussion

In this paper investigation of the potential antigenotoxic and antioxidant properties of newly synthesized carboxyethyl chitosan were presented.

In the Ames assay no mutagenic effect was found, and also antimutagenic effect of CECh against mutagenicity induced by NaN_3 on the *S. typhimurium* strain TA100 was not statistically significant (fig. 1).

The results of the DNA-topology assay suggest that polysaccharide CECh did not induce single- and double-strand DNA breaks in the plasmid DNA. In the presence of Fe^{2+} CECh reduced the plasmid DNA relaxing in the concentration-dependent manner. In the presence of H_2O_2, CECh stimulated the plasmid DNA relaxation in the concentration-dependent manner. In the simultaneous incubation CECh with oxidative agent Fe^{2+}, CECh exerts DNA protective activities, while in combination with H_2O_2, polysaccharide CECh increased damage to the plasmid DNA (fig. 2 and 3).

The antioxidant activity of the CECh was assessed using two different methods. The first method used was the DPPH assay. On the basis of the results obtained we can conclude that due to the high molecular weight of CECh used (70 kDa) it did not show any antioxidant effect (fig. 4). Also the results of other researchers documented that the scavenging activity of CECh is depending on the molecular weight[11]. While, the 30 kDa chitosan exhibited a strong scavenging activity, antioxidant activity of 120 kDa chitosan was considerably low.

The second method for antioxidant detection used was the hydroxyl radical scavenging assay. The scavenging rate of CECh increased in the concentration range from 0.01 mg ml^{-1} to 0.1 mg ml^{-1} (fig. 5). The scavenging activity may by attributed to its metal-bonding capacity also Xue et al.[12], reported that water-soluble chitosan may che-

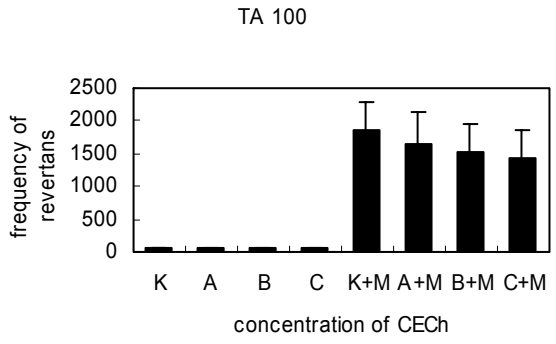

Fig. 1. **Effect of CECh on NaN_3-induced mutagenicity in *S. typhimurium* strain TA100 using the Ames 1 h pre-incubation assay;** C = control, A = 0.75 mg ml^{-1}, B = 0.5 mg ml^{-1}, C = 0.25 mg ml^{-1}, M = mutagen (NaN_3)

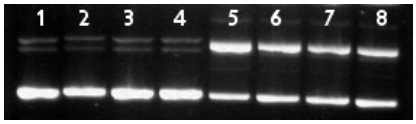

Fig. 2. **Electrophoretic monitoring of the DNA-topology of the plasmid DNA treated with CECh line 2–4, and CECh with Fe^{2+} line 6–8**

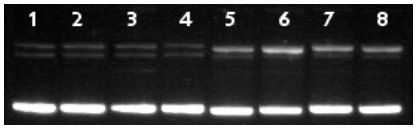

Fig. 3. **Electrophoretic monitoring of the DNA-topology of the plasmid DNA treated with CECh line 2–4, and CECh with H_2O_2 line 6–8**

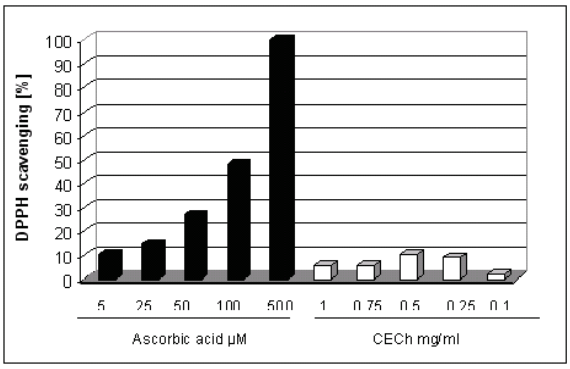

Fig. 4. **Antioxidant activity of CECh determinated by the DPPH radical scavenging assay;** The values represent the data from three independent experiments (S.D.< 5 %). Ascorbic acid was used as a reference compound

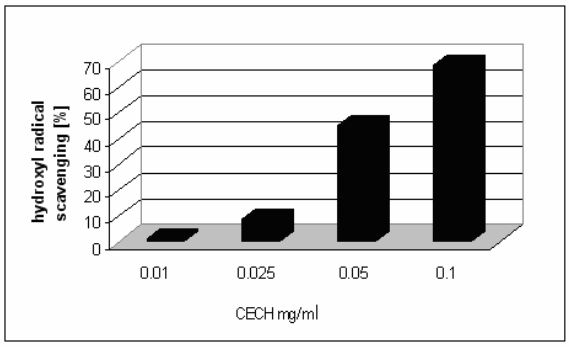

Fig. 5. **Antioxidant activity of CECh determinated by the OH radical scavenging assay;** 25 µg ml^{-1} of thiourea = 100 % of scavenging activity

late metals.

In conclusion, the results suggest that antimutagenic and antioxidant effects of this polysaccharide are dependent on concentration, way of application and method used. Comparing two procedures used for the CECh antioxidant activity assessment we can conclude that owing to molecular weight of CECh used in our experiments, the hydroxyl radical scavenging assay was more convenient and sensitive than DPPH scavenging assay.

Conclusion

Our research was aimed at the CECh mutagenicity exclusion, and potential antimutagenic effect assessment, using the Salmonella/microsome assay. We also aimed at the evaluation of CECh DNA protective activity, using the DNA-topology assay, and at the antioxidant properties of CECh, using the DPPH assay and the hydroxyl radical scavenging assay. On the basis of results obtained we can conclude that CECh may be applied in the biomedicine and due to its antioxidant properties it may be useful in cancer prevention.

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L12
DOSE-VARIABLE EFFECT OF NITRENDIPINE ON THE DISTRIBUTION OF GALANTHAMINE INTO THE BRAIN OF THE LABORATORY RAT

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Key words: galanthamine, nitrendipine, pharmacokinetic interactions

Aim

Effect on the concentration of the cholinomimetic agent (galanthamine) in the target sites of the central nervous system by means of pharmacokinetic interaction with nitrendipine.

Introduction

In the present therapeutic strategy of cognitive deficient conditions (e.g., senile dementia of Alzheimer type – SDAT), pharmacological stimulation of the central cholinergic transmission /e.g.,[1]/ outlasts. Galanthamine (GAL), a parasympathomimetic agent with a dual mechanism of effect, ranks among the second generation of SDAT drugs. In previous experiments[2], the present authors succeeded to partially strengthen the cerebral anticholinesterase activity of GAL by means of L-carnitine (an agent influencing transport mechanisms). Hypothetic strengthening of GAL-efficiency by means of nitrendipine (NITR) is based on other types of pharmacological interactions: the inhibitory action of NITR on calcium channels (and a subsequent cascade of reactions resulting in an influence on cytosol release of calcium) and the intervention of NITR into the activity of P-glycoprotein transporters (which influence the kinetics of a number of xenobiotics by the efflux mechanism).

In order to determine suitable doses and suitable time intervals, in the introductory experiment the animals were administered a single dose of GAL (based on previous studies[2]) and subsequently the kinetics of plasma concentrations of GAL and its three metabolites, *O*-desmethylgalanthamine (ODMGAL), *N*-desmethylgalanthamine (NDMGAL) and chirally converted epigalanthamine (EPIGAL) was determined.

The proper study consisted in repeated medication with two different doses of NITR, subsequent single-dose administration of GAL, and determination of GAL concentrations (in its steady state of the pharmacokinetic phase) in purposefully selected biological samples: in the brain tissue (= the pharmacologically targeted tissue), in the hypophysis (= lying outside the hematoencephalic barrier), in the liver tissue (= representing the biotransformational and peripheral pharmacokinetic compartments), in blood plasma (= the central distributional compartment). For the supplementing verification of the relationship of the nitrendipine dose and the degree of influence on galanthamine biodistribution, NITR levels were determined in identical biological samples.

Methods

Experimental animals: rats – Wistar males, weight range 210–255 g, six individuals in each experimental group.

Drug administration: galanthamine hydrobromide (Janssen Pharmaceutica, Belgium): 10 mg kg^{-1} i. m.; nitrendipine (European Pharmacopoeia Catalogue): 5 mg kg^{-1} or 20 mg kg^{-1} p. o. (vehicle: polyethylene glycol : water = 1 : 1).

Bioanalytical techniques: determination of galanthamine and its metabolites: HPLC + detection: fluorescence, mass-spectrometric, and on the photodiode field[3]; nitrendipine determination: GC + ECD detection[4].

Design of the experiment for the determination of the time course of plasma levels of galanthamine and its metabolites: single-dose i. m. administration of 10 mg kg^{-1} GAL, withdrawals of samples of blood plasma from the 5th to the 120th minute from the incannulated *v. jugularis.*

Design of the biodistribution study of GAL under the influence of nitrendipine: repeated administration of NITR (5 mg kg^{-1} or 20 mg kg^{-1}) for two days; on the third day, administration of another dose of NITR (5 mg kg^{-1} or 20 mg kg^{-1}), after 30 minutes i. m. administration of galanthamine (10 mg kg^{-1}), and after another 30 minutes withdrawals of biological samples (plasma, brain, hypophysis, liver) from animals under general anesthesia; in a portion of samples determination of GAL concentration, in another portion NITR concentration.

Results

1) Demonstration of T_{max} plasma levels of GAL between the 10th and 30th minute (fig. 1) made it possible to determine the steady state phase and the withdrawal biodistribution interval for further experiments at 30 minutes after GAL administration. As the levels of GAL metabolites were low they were not examined in further experiments.
2) A comparison of inter-organ distribution ratios of GAL (fig. 2) clearly shows higher levels in the tissues against plasma levels (in the hypophysis ca. 6.5 times,

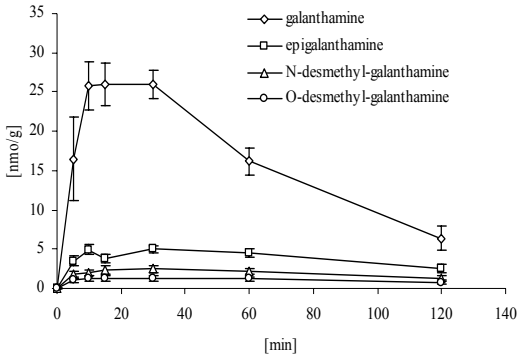

Fig. 1. **Rat: plasma levels of GAL + its metabolites** (after single – dose 10 mg kg^{-1} i.m. administration of GAL)

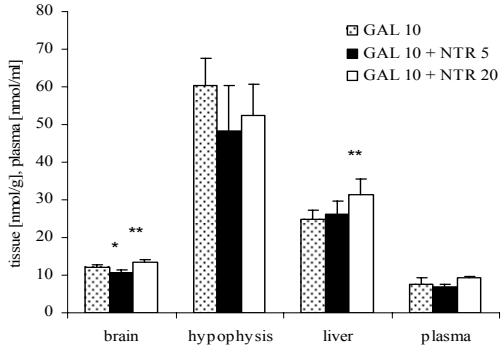

Fig. 2. **Rat: effect of repeated p.o. doses of NITR** (5 mg kg^{-1} or 20 mg kg^{-1}) on the biodistribution of GAL (after 10 mg kg^{-1} i.m. administration)

Table
Rat: effect of NITR-dose (5 mg kg^{-1} or 20 mg kg^{-1}) on its organ distribution

	Nitrendipine conc. [ng ml^{-1}]		
NITR-dose [mg kg^{-1}]	plasma	brain	liver
5	88.75±57.51	105.65±57.62	225.95±67.02
20	203.72±85.71	175.07±69.45	1208.05±103.70

in the liver 3.2 times, in the brain 1.4 times).

3) Effect of nitrendipine on the biodistribution of GAL is dose-dependent (fig. 2):
   - in the brain tissue after doses of 5 mg kg^{-1} of NITR there occurs a statistically significant decrease in GAL levels, after doses of 20 mg kg^{-1} of NITR there is a statistically significant increase in GAL levels,
   - in the hypophysis, GAL levels insignificant decrease by the action of NITR,
   - in the liver tissue, after doses of 5 mg kg^{-1} NITR, GAL levels are unchanged, after doses of 20 mg kg^{-1} NITR, GAL levels are increased in a statistically significant manner,
   - in blood plasma there is a statistically insignificant similar trend in the shifts of GAL levels as in the brain.
4) Nitrendipine levels in dependence on its dose change in a nonlinear manner (Table).

Discussion

The finding of a relatively intensive extravasal "outflow" of GAL and its different distribution ratios between the brain tissue and hypophysis (and also the liver) confirms the importance of hematoencephalic barrier for this distribution.

Dose-dependent interactions of NITR and GAL can be interpreted as follows:

- in the case of lower doses of NITR by the predominance of its vasodilatory action and a subsequent probable increase in the supply of GAL towards bioelimination compartments,
- in the case of higher doses of NITR by the predominance of its effect on the efflux carrier mechanism in the distribution of GAL into the brain (or the liver).

This interpretation is also based on a probable direct biodistribution interaction between NITR and GAL, i.e., from the variable tissue distribution of NITR in dependence on its dose. Whereas the ratio of the NITR-doses is 1 : 4, the levels in the brain are higher only ca. 1 : 1. 4, in plasma only 1 : 2.3, but in the liver 1 : 5.4.

Conclusions

- In the steady state of the pharmacokinetic phase, galanthamine levels are higher in the tissues than in blood plasma, and mutual tissue biodistribution ratios suggest an effect of the hematoencephalic barrier on the penetration of GAL into the brain.
- Nitrendipine in a repeated dose of 5 mg kg^{-1} decreases the distribution of GAL into the brain (influence of nitrendipine vasodilatory effect?), in a repeated dose of 20 mg kg^{-1} it increases the distri-

bution of GAL into the brain and the liver (nitrendipine effect on P-glycoprotein efflux?).

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L13

THE USE OF TERRESTRIAL BACTERIA WITH NATURAL BIOLUMINESCENCE IN ENVIRONMENTAL TOXICOLOGY

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Key words: terrestrial bacteria, bioluminescence, environmental toxicology

Introduction

The toxicity of a substance is the measure of its incompatibility with normal biological function. Toxicity measurements are usually performed by quantifying the alteration of given biological characteristics from the genetic to the molecular, cellular, tissue, and organism levels (e.g. gene expression, enzyme functionality, fertilization efficiency, growth, behavior) upon exposure to known added concentrations of a specific substance. Biomonitoring programs use a battery of bioassays to screen such a variety of routes of contamination, involving a diversity of organisms (bacteria, crustaceans, bivalves, fishes) possibly at different stages of development (embryo, larvae, adult). Because of the complexity and cost of such an endeavor, there is a strong interest in simplifying toxicity assessment. A common alternative is to use microorganisms with natural bioluminescence (BL). Actually, bioluminescent microorganisms have become increasingly used in environmental toxicity testing due to their ease of use and the potential to rapidly report on toxic events. The assumption is that decreases in bioluminescence, i.e. the biological production of visible light with an ecological function, reflect the toxicity of a given condition, because light emission is a measure of the health and metabolism of the luminescent organism[1–4].

From the organisms involved in the toxicity measurements the most commonly used are bacteria with the natural BL. The light of several hundred cells may be determined with a luminometer. Bacterial bioluminescence is actually chemiluminescence that requires an enzyme luciferase. Although amino acid sequences of bacterial luciferases differ, they all emit light with maximum at 490 nm and the emission requires both reduced flavin mononucleotide and a long-chain aliphatic aldehyde[5]. Chemophysical and biological toxicants that affect cell respiration, the rate of protein or lipid synthesis, cell's integrity and especially membrane function, have a strong effect on *in vivo* luminescence. Hence, toxicants of different characteristics such as pesticides, herbicides, chlorinated hydrocarbons, heavy metals etc., exert a dramatic and measurable effect on the bacterial luminescence system.

Presently, at least fourteen species of BL bacteria in four genera (*Vibrio, Photobacterium, Shewanella and Photorhabdus*) are known to produce visible luminescence. They are found predominantly in the marine environment and according to our knowledge only marine luminescent bacteria are used for the assessment of water pollution in a number of commercial assays (Microtox, ToxScreen, Biotox). It is well known that the use of marine BL bacteria also brings some imperfections. One of them is the need for addition of salt to all freshwater samples being assayed which may inadvertently alter the toxicant properties of the sample, and hinders the development of potentially more advanced *in situ* toxicity monitoring systems. Moreover, the temperature optimum for the cultivation of marine bacteria is relatively low (15 °C for *Vibrio fischeri*) and bioluminescence of marine bacteria is relatively sensitive to the changes in temperature which is another imperfection.

On the other hand, terrestrial BL bacteria of the genus *Photorhabdus* are not used for the toxicity measurement. The genus *Photorhabdus* (previously designated as *Xenorhabdus*) was proposed by Boemare *et al.*[6] and includes terrestrial gram-negative bacteria of the family *Enterobacteriaceae*, which are mainly found in association with entomopathogenic nematodes *Heterorhabditis sp*. Besides species found in nematodes, *Photorhabdus asymbiotica* was obtained from human clinical specimens only. *Photorhabdus* species are the only known terrestrial bacteria with natural BL, all other bioluminescent bacterial species were isolated from marine sources[7].

The aim of the study was to analyse the BL emitted by *Photorhabdus* species and subspecies and to verify whether bioluminescence of *Photorhabdus* sp. can be very sensitive indicator of the presence of toxicants in water or water solutions.

Material and methods

Type strains: Eight presently known bioluminescent *Photorhabdus* species and subspecies were kindly provided by Czech Collection of Microorganisms (CCM, Brno, Czech Republic): *P. asymbiotica* CCM 7074^T, *P. asymbiotica* subsp. *australis* CCM 7293^T, *P. luminescens* subsp. *akhurstii* CCM 7075^T, *P. luminescens* subsp. *kayaii* CCM 7294^T, *P. luminescens* subsp. *laumondii* CCM 7076^T, *P. luminescens* subsp. *luminescens* CCM 7077^T, *P. luminescens* subsp. *thracensis* CCM 7295^T and *P. temperata* CCM 7078^T. *Photobacterium leiognathi* (TANI1) was purchased as a part of the commercial kit CheckLight ToxScreen On-Site Test (CheckLight, Haifa, Israel).

The effects of temperature, salinity and pH on the bacterial BL were tested in bacteria cultivated on medium

Table I
Bioluminescence [RLU] of *Photorhabdus temperata* and *P. luminescens* subsp. *thracensis* after 6, 24 and 48 h incubation at different temperatures

Incubation [hours]	*P. temperata*			*P. luminescens* subsp. *thracensis*		
	20 °C	25 °C	30 °C	20 °C	25 °C	30 °C
6	771	834	2240	597	708	495
24	5851	6696	4277	1893	3061	10880
48	16440	14300	13250	2561	3732	21300

Table II
The effect of salinity on bioluminescence [RLU] of bacteria after 30 min cultivation of bright cells

	Salinity [%]			
	0.5	1.0	1.5	2.0
Photorhabdus temperata	4325	4827	4918	4465
Photorhabdus luminescens subsp. *thracensis*	1064	1230	1049	1017

DSMZ 423. Salinity and pH were adjusted using NaCl, HCL and NaOH. For testing the effects of metal ions, bacteria were cultivated at 25 °C in Hank's Balanced Salt Solution (5.40 mmol l^{-1} KCl; 0.44 mmol l^{-1} KH_2PO_4; 0.81 mmol l^{-1} $MgSO_4 . 7 H_2O$; 0.14 M NaCl; 4.17 mmol l^{-1} $NaHCO_3$; 0.36 mmol l^{-1} $Na_2HPO_4 . 12 H_2O$; 5.05 mmol l^{-1} glucose; 1.26 mmol l^{-1} $CaCl_2$; pH 7.4). The concentration of bacteria was adjusted to optical density of 1.0 at 400 nm (corresponding to $2.4 \cdot 10^8$ cells ml^{-1}).

Luminometrical analyses: BL of bacterial suspensions was measured using Luminometer Orion II (BERTHOLD Detection Systems, Germany). The kinetics of changes in BL activity were analysed within 30 minutes in 1 min intervals.

Results and Discussion

In our first experiments, *Photorhabdus* species and subspecies (43 h old cultures with bright cells, initial bacteria concentration = $2.4 \cdot 10^8$ cells ml^{-1}) were cultivated on medium DSMZ 423 at the temperature range of 20–30 °C and the BL activity of bacterial suspension was measured after 6, 24 and 48 hours of cultivation. All strains emitted reliably measurable BL, however among *Photorhabdus* species and subspecies tested, BL of *P. temperata* and *P. luminescens* subsp. *thracensis* reached the brightest BL intensity (Table I). BL of *P. temperata* was independent on the cultivation temperature, BL of *P. luminescens* subsp. *thracensis* reached maximum at 30 °C. The highest BL was emitted by both bacteria after 48 h cultivation. It is in agreement with literature data. Cultures of *Photorhabdus* sp. are capable of logarithmic growth in complex media, with doubling time of approximately 1.5 h at 30 °C. During the early stage of growth, there is a very low BL because of limitation for aldehyde and low levels of luciferase. In the late logarithmic growth, rapid rise in BL coincides with a burst of luciferase and aldehyde synthesis, a pattern that is consistent with autoinduction. Bacteria are emitting the highest BL intensity during the stationary phase, which begins from 30 to 40 hours of cultivation[8,9].

Cultures of *P. temperata* and *P. luminescens* subsp. *thracensis* were selected for following experiments where the effect of salinity and pH was tested. Exact concentrations of bright bacterial cells collected from the cultures of selected two strains in stationary phase ($2.4 \cdot 10^8$ cells) were prepared. The salinity was adjusted using NaCl to 0.5, 1.0, 1.5 or 2.0 % and bacterial BL was measured continuously for the time period of 30 minutes. Longer time intervals were not tested because the decrease of BL after 15 or 30 minute co-cultivation of bacteria with metal ions is usually used for the calculation of EC_{50}[4]. As shown in Table II, BL of both *Photorhabdus temperata* and *P. luminescens* subsp. *thracensis* was quite stable and independent on the salinity. While no effect of salinity was observed, BL signal of bacteria cultivated in pH 6.2 was about 30 % lower in comparison with bacteria cultivated in pH 8.2. It was the reason why we decided to use HBSS in further experiments. HBSS is the buffer withour aldehyde (sometimes

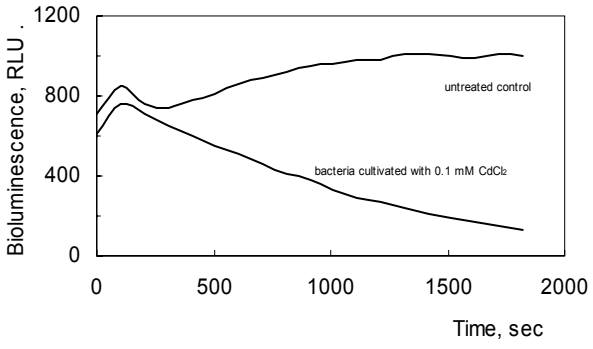

Fig. 1. **The bioluminescence of *Photorhabdus temperata* cultivated in the presence of 0.1 M $CdCl_2$ in comparison with untreated control**

Table III
Bioluminescence [RLU] of bacterial strains after 5–30 minutes incubation at 25 °C with 0.1 mM $CdCl_2$

Time of incubation [min]	*Photobacterium leiognathi*		*Photorhabdus temperata*		*Photorhabdus luminescens* subsp. *thracensis*	
	Control	$CdCl_2$	Control	$CdCl_2$	Control	$CdCl_2$
5	5017	3170	760	649	954	751
15	6261	1462	910	377	1208	465
30	6850	561	1028	134	1564	241

used as an external enhancer of BL) which does not interfere with BL emitted and ions and glucose present in the buffer ensure the viability of bacteria and their proper metabolism[7].

In the final set of experiments, effect of $CdCl_2$, $CuSO_4$, $NiSO_4$, $HgCl_2$, and $K_2Cr_2O_7$ was tested. Bacteria *P. temperata* and *P. luminescens* subsp. *thracensis* were sensitive to metal ions and BL of these strains was decreased in the presence of metal ions similarly in both strains. The effect of 0.1 mM $CdCl_2$ on the BL of *P. temperata* is shown in fig. 1. It is quite obvious that the proper time for the calculation of EC_{50} value is 30 minutes after the beginning of cultivation. Ulitzur and co-authors[4] used Check-Light ToxScreen On-Site Test containing bacteria *Photobacterium leiognathi* and reached EC_{50} for cadmium ions about 100 times lower in comparison with our results. Such high sensitivity is caused by the presence of the special buffer in the kit which favours the detection of heavy metals and amplifies their bioeffects. In the last set of experiments we used the pure culture of *Photobacterium leiognathi* (from the kit) without the buffer. The effect of $CdCl_2$ on BL of *Photobacterium leiognathi* was quite comparable with effects reached using *P. temperata* and *P. luminescens* subsp. *thracensis* (see Table III). It proves that BL of terrestrial bacteria can be affected by metal ions comparably with marine bacteria.

It can be concluded that *P. temperata* and *P. luminescens* subsp. *thracensis* are sensitive indicator of the presence of inorganic toxicants in water or water solutions. These terrestrial bacteria could be potentially used for routine testing of water toxicity.

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L14
MYCOTOXINS AND BIOTERRORISM

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Key words: mycotoxins, toxicity, bioterrorism

Introduction

Mycotoxins are toxic metabolites produced by a diverse groups of fungi, namely by those of ubiquitous Aspergillus, Penicillium, Fusarium, Alternaria, Claviceps genera[1]. Some mycotoxins could be used as bioterrorist weapons. The risk of such an attack is now considered to be real because the exposition to mycotoxins can have a wide range of adverse biological effects including hemorrhagic, hepatotoxic, nephrotoxic, neurotoxic, estrogenic, teratogenic, mutagenic and carcinogenic ones. Mycotoxins would be suitable as chemical warfare agents for the actions of sabotage, because their use is not associated with any primary alarming signs such as blast, fire or suspicious odors and because there are no real-time detection and alarm systems for these toxins. Therefore, there would be no reason to escape and then it would be too late to do so after the signs of intoxication become evident[2]. The abuse of mycotoxins for purposes of the biological warfare does not seem to be a purely theoric threat. There is some evidence that Iraqi scientists were developping aflatoxins as part of Iraqi biowarfare programme during the 1980s[3]. The potent acute toxicity and chemical stability of mycotoxins Aflatoxin B_1 (AFB_1), fumonisin B_1 (FB_1), ochratoxin A (OTA), T-2 toxin and deoxynivalenol (DON) make them apt to be weaponized for bioterrorism[2,3]. The prospect of their abuse is all the more higher that publicly available literature includes information on the production of these mycotoxins by fermentation and on their extraction and purification[2].

Therefore, the surveillance of mycotoxins seems justified. In the Czech Republic, this surveillance is being assured by the National Authority for Nuclear Safety under the Act No. 281/2002 of the Czech Rep. Law Collection. The prevention of bioterrorist acts implies the introduction of various measures ranging from keeping the evidence on plants and toxins via guaranteeing their traceability to controls of handling with mycotoxins and inspections to laboratories. The human exposure to mycotoxins can be generally proved by determining the intake of mycotoxins and by analyzing the biomarkers of mycotoxins in human blood plasma, serum or urine. In this regard, the fact that OTA in serum and AFM_1 (metabolite of AFB_1) in the urine of blood donors are monitored in the common Czech population provides an undoubtful advantage.

Aflatoxins (AF_s)

AF_s are nontoxic but they are converted into highly toxic forms through oxidative metabolism by cytochrome P 450[2]. Aflatoxin B_1 is the most potent hepatocarcinogen[3,4] although its effects vary with species, age, sex, and general nutrition[1]. The liver is the primary organ affected for the acute toxicity of AF[3,4]. Acute aflatoxicosis results in death; chronic aflatoxicosis results in cancer, immune supression, and other pathological conditions[1]. The main cause of death from acute intoxication is liver hemorrhage, fatty infiltration. In 1974, the acute lethal dose for adults was determined to be approximately 10 to 20 mg of aflatoxins[3,4]. The hepatocarcinogenicity of AFB_1 is associated with its biotransformation to highly reactive electrophilic epoxide that forms covalent adducts with DNA, RNA and proteins. Damage to DNA is thought to consist in the initial biochemical lesion resulting in the expression of the pathologic tumor growth[2,4]. After oral uptake AFB_1 is efficiently absorbed and metabolized prior to excretion by fecal and urinary route[3,4] , e.g. aflatoxin M_1 (ref.[4]). Oltipraz may prevent carcinogenesis and acute intoxication[2,5].

Increased incidence of death from lung cancer among workers who were exposed to grain dust particles of peanut contaminated with AF_s indicate that inhalation could also become an important route of exposure during attacks with AF-containing weapons[2,4]. The inhalation is the most effective and hazardous route of exposure[2,4–6]. The serious effects of exposure to AF via the respiratory tract was demonstrated by the rapid appereance (30 min post-exposure) of AF-DNA adducts in rat liver DNA[2].

Ochratoxin A (OTA)

Acute (or chronic) OTA toxicity is derived from its ability to inhibit protein synthesis[5] . OTA inhibits the reactions in which phenylalanine is implicated 5. These interactions seem to explain the effective protection from OTA intoxication by the artificial sweetener phenylalanine or aspartame[7]. OTA is suspected to have nephrotoxic, immunosuppressive, genotoxic, carcinogenic and teratogenic

effects on humans[5]. The IARC/WHO classifies OTA as a possible human carcinogen, included in category 2B[5]. Most of the nephrotoxic effects[5–7] and the genotoxicity (mutagenicity) induced by OTA were prevented by aspartame which is effective as a prophylactic and for flushing out the toxin even 2 weeks after intoxication[7]. Toxicokinetic studies on animals have demonstrated that at least of 90 % OTA administered orally or intraperitonneally are bound to plasmatic proteins, that´ s why OTA has very long half–live of excretion (35 days)[5]. Free OTA is readily excreted through urine[5]. Findings of high OTA levels in serum of the patients suffering from Balkan endemic nephropathy (BEN) led to hypotheses about the association between the nephrotoxicity of OTA and BEN and also the incidence of renal system tumours in the population of the affected Balkan regions[5,8].

Nevertheless, there a case of inhalation exposure to OTA has also been reported and inhalation of OTA was considered to be the reason of the acute renal failure within 24 h (ref.[2]).

Fumonisin B_1 (FB_1)

The structural similarity between sphinganine and FB_1 led to hypothesise that the adverse effects of this mycotoxin in human liver, kidney, lung, and nervous system are due to a disruption of sphingolipid metabolism or of the function of sphingolipids[2,5].

In pigs, acute forms of toxicity include cardiotoxicity and pulmonary edema[2]. FB_1 induces an increase in the concentration of sphingosine and sphinganine in the left ventricle and the aorta, affecting cardiac output and decreasing arterial oxygen without changes in pulmonary cappilaries[2–5]. The current theory is that the increased concentrations of sphingosine and sphinganine inhibit L-type calcium channels[2].

Trichothecenes

Trichothecenes constitute another important group of mycotoxins highly relevant to human health. T-2 toxin is an example of trichothecenes, type A, and DON is an example of trichothecenes, type B[5,9]. DON is metabolized *in vivo* by de-epoxidation to DOM-1[5,9] and to DON-glucuronide[10]. After an oral administration of deoxynivalenol some 7 % of unmetabolized compound was recovered in urine of sheep[5]. Acute toxicity of DON is characterized by intestinal disorders and emesis. Dermatological lesions, hematological changes (hemorrhage), immunosuppressive, teratogenic as well as nephrotoxic effects have also been reported[5,6,9]. Humans seem to be quite sensitive to DON[2]. At low doses, DON causes anorexia and at higher doses it induces vomiting by unknown mechanisms. Anorexia and emesis could result from its neurotoxicity[2].

In humans, T-2 toxin causes alimentary toxic aleukia[1] and induces apoptosis in progenitors of megakaryocytes[2]. Clinical symptoms of intoxications are e.g. inflammation and hemorrhaging of digestive tract, edema, leucopenia, degeneration of the bon marrow and even death. In an outbreak of T-2 toxicosis in China, victims suffered from nausea, diziness, vomiting, chills, abdominal distension, thoracic stuffiness and diarrhea, with a period of latency from 10 to 30 min (ref.[2]). T-2 toxin is 10- to 50-fold more toxic when inhaled than when taken orally[2].

In mice, inhalation of T-2 toxin for 10 min was sufficient to induce death in less than 5 hours (ref.[2]). T-2 toxin is also readily absorbed through intact skin[2].

Results and discussion

Monitoring the levels of mycotoxins in the Czech Republic

In the Czech Republic, the data on this normal exposure of the population are available thanks to programme of monitoring the mycotoxins in biological materials. OTA and AFM_1 have been monitored in serum and urine of blood donors since the 1990s. The validated and accredited method of reversed phase HPLC with fluorescence detection has been employed for purposes of the OTA detection and quantification. As for OTA, of 2, 408 samples (investigated in 1994–2005) 2, 246 (93.3 %) samples of blood serum turned out to be positive (with levels ≥ 0.1 μg l^{-1} /LoQ/), the mean was 0.28 μg l^{-1},the median was 0.2 μg l^{-1}, and the 90 % percentile was 0.5 μg l^{-1}. The ochratoxin A levels ranged from 0.1 to 13.7 μg l^{-1} of sera[11]. As for AFM_1, the cleaning of AFM_1 in urine has been carried out using immunoaffinity columns. The validated and accredited immunochemical method ELISA has been used for quantification of AFM_1 in human urine. From 205 samples (investigated in 1997–1998) 118 samples (58 %) were positive (with levels ≥ 125 pg l^{-1} of urine /LoQ/). When calculated to a level of creatinine in urine, the average was 391 pg g^{-1}, the median was 127 pg g^{-1}, and the 90 %percentile was 585 pg g^{-1}. The aflatoxin M_1 levels ranged from 19 to 19,219 pg g^{-1} of creatinine[11].

Moreover, in order to face the bioterrorist challenges, two analytical methods have been developped for needs of the National Authority for the Nuclear Safety of the Czech Republic: one for the determination of AFM_1 in human urine by HPLC with fluorescence detection and another for the determination of DON in human urine by HPLC with UV detection after cleaning and separation of these analyts on immunoaffinity columns.

Mycotoxins are impractical as tactical weapons. Table I shows that very large amounts of pure mycotoxin required to produce even 10 % of an effective tactical weapon are dwarf when compared with even larger amount of fungal culture required for the production of mycotoxins, reaching absurdity in the case of DON[2,12]. Furthermore, method for chemical decontamination and effective destruction of mycotoxins (e.g. aflatoxins, OTA) can be simply based on the use of household bleach (5.5 % sodium hypochlorite in water)[2]. On the other hand, the production of fungal weapons does not require elaborate

Table I
Mycotoxins as tactical weapons[2]

Toxin	Species	LD50 [mg kg^{-1}]	10 % of tactical amount [a]	Medium	Yield	Amount of culture needed
AFB_1	human	0.15–0.3	48–96 [t [b]]	liquid medium	300 [mg l^{-1}]	$1.4 \cdot 10^7$ [l]
OTA	mouse	50	16,000 [t]	wheat solid culture	4 [g kg^{-1}]	$4 \cdot 10^6$ [t]
FB_1	monkey	0.11 [c]	35 [t]	corn solid culture	32 [g kg^{-1}] mycelium	1, 100 [t]
DON	mouse	78	25,000 [t]	corn solid culture	175 [mg kg^{-1}]	$1.4 \cdot 10^8$ [t]
T-2 toxin	mouse	0.25	80 [t]	rice solid culture	357 [mg kg^{-1}]	$2.2 \cdot 10^6$ [t]
Ricin	mouse	0.0025	800 [kg [e]]			
Botulinum toxin	mouse	2.5 [ng kg^{-1}]	0.80 [kg [e]]			

[a] The amount needed to cause 50 % lethality over 10 km^2, assuming that the acute toxicity for humans is similar to that for mice. The calculation is based on the linear relationship between the LD_{50} in mice and that of Franz and Zajtchuk[2], [b] Calculation according to LD_{50} in humans, [c] No observable effect level in monkeys, [d] 7.5 % of the world annual grain consumption ($1.904 \cdot 10^9$ tons), [e] Values[2] were adapted to 10 km^2 from information of Franz and Zajtchuk[12], where calculations were done for 100 km^2

facilities for the growth of fungi, sophisticated equipment for the purification of the toxins or highly trained personal. Toxigenic fungi almost always produce several toxins at the same time. The toxicology of mixtures of toxins is more difficult to evaluate which may complicate the design of countermeasures[2]. Poisoning of water or food might be used by bioterrorists but poisoning of crops in the field or of grains in granaries and poisoning of central water reservoirs would probably be ineffective because large amounts of mycotoxins would be required. In general, inhalation of mycotoxins is more dangerous than their ingestion. Inhalation of spores of mycotoxigenic moulds containing mycotoxins has adverse effects on humans and animals[1,2]. Therefore, dispersion of mycotoxins through the ventilation systems of public buldings or subway stations might be a method of choice for bioterrorists´ attacks because it could result in poisoning of many people within a short time. With regard to the existence of these threats, precise knowledge of the normal exposure of the population is one of prerequisites necessary for detection of potential attacks. At present, the health risk resulting from the exposure to mycotoxins from foodstuffs is estimated to be relatively low in the Czech Republic[11].

Conclusion

Mycotoxins could be theorically used by small poor terrorist organizations to poison food and water sources. Crude concentrated or dried extracts of readily grown fungal cultures could be used and preferred as weapons because toxigenic fungi almost always produce several toxins at the same times. Their action is often synergical and they mutually potentiate their effects. However, it is the dispersal of mycotoxins in indoor air which appears to be the most effective method for a bioterrorist attack. That's why precise knowledge of the symptoms of intoxication with mycotoxins, biochemical mechanisms of their action, new methods for rapid determination of mycotoxins´ biomarkers (e.g. aflatoxin M_1 and DON or its metabolites in human urine by HPLC) is necessary.

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L15
THE RISKS OF AUTOIMMUNE DISEASES THERAPY IN PREGNANCY

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Key words: sclerosis multiplex, inflammatory bowel disease, embryotoxicity

Introduction

Chronic autoimmune diseases represent risk of negative pregnancy outcome, which is caused either by used drugs or chronic conditions. Management of treatment has changed from recommendation of pregnancy termination to tolerance of pregnancy. However physicians are still afraid from the drug usage during pregnancy for their presumed teratogenic potential, and they risk relaps of disease during *non-treated* pregnancy or in early postnatal period. In our study we present pregnancy outcomes of women suffered from idiopatic bowel diseases (Crohn´s disease and ulcerative colitis) and treated and non-treated cases of multiple sclerosis. We collected the cases of patiens, that contact CZTIS (Czech Teratology Information Service). The diseases were not complicated by hypertension or endocrine dysbalance that perform an important risk themselves. Immunosuppressants in combination with corticosteroids are the most frequent therapy in inflammatory bowel disease reported by clinicians. Drugs combined were antibiotics, aminosalicylates, nutritive intervention or immunomodulatory cytokines. According to data, both active form of disease and all drugs used in inflammatory bowel disease treatment are associated with increased risk of premature births and low birth weight following intrauterine growth retardation (IUG)[1]. However, our CZTIS small sample of pregnancy outcomes is not consistent with reported embryotoxicity risk nor with reported type of therapy. Monotherapy of mesalazine followed by combination therapy (prednison + azathioprine + mesalazine) were the most frequent queries on Crohn´s disease treatment. The lowest birth weight of healthy newborns was 2480 g in the 40th week of pregnancy. Treatment of ulcerative colitis in followed pregnacies was mesalazine monotherapy. Pregnancy outcomes of women with this type of bowel disease were even 3300–4050 g all in term deliveries. In small number of multiple sclerosis treatment during pregnancy, we did not record significant increase of embryotoxicity. Although our sample of pregnancy outcomes is limited, it could contribute to the findings, that when the disease is maintained in remission, the treatment itself does not increase the embryotoxicity risk.

Idiopatic bowel disease (IBD) and multuple sclerosis (MS) – characteristics of diseases

The first peak of onset is in young age, around the age of 20, the second peak is recorded after the age of 50. years. Typical is North – West geographic predominance, with prevalence 20 (IBD) – 100 (MS) 100 000^{-1}. Etiology is multifactorial, genetic backround in combination with environmental factors.

Multiple sclerosis

Multiple sclerosis (MS) is autoimmune demyelinating disease of central nervous system. Myelin antigens are supposed to be target structure, resulting in myelin sheath damage followed by diffuse axonal damage. Pregnancy is a factor significantly decreasing number of relapses (PRIMS, prospective study). Prevalence in the Czech republic is 70 100 000^{-1}. Pregnancy should be planned to clinically stabilized period, 6-12 months after last attack. It is recommended to stop any treatment 3 to 6 months before pregnancy. If the attack occurs they use low-dose of corticosteroids or intravenous immunoglobulins. Treatment should be started immediately after delivery otherwise risk of attack increase two times[2].

Idiopatic bowel diseases(IBD)

Crohn´s disease is chronic non-specific inflammatory disease of small and large intestine. It manifests with diarrhoea, pain in abdomen, absorption impairment and malnutrition. Chronic bleeding may cause anemia. Stenosis of intestine often requires surgical treatment. Crohn´s disease in pregnancy results in increase risk of low birth-weight, spontaneous abortion, prematurity and perinatal complications. However, when it is in remission, the risk of negative pregnancy outcome is not increased.

Ulcerative colitis is chronic non-specific inflammatory disease of rectum and/or sigmoid part of large intestine. It manifests predominantly with diarrhoea, rectal bleeding, obstipation, fever, and weight loss. It resembles intestinal infection. It is connected often with extraintestinal symptoms (uveitis, erythema nodosum, arthritis, pyodermia). The risk factors perform dietary practice. Pregnancy with ulcerative colitis is often without any complications. When it is inactive during conception, it remains inactive during whole pregnancy. In active form of disease is increased risk of spontaneous abortions and preterm deliveries.

Treatment of IBD perform immunosupressants, corti-

costeroids, aminosalicylates, antibiotics, alternatively new drug, anti- TNF, Infliximab[3].

Characteristics of drugs used

Risks for drugs used by our patients that are announced for treatment during pregnancy[4-7].

Mesalazine (acetyl-5-aminosalicylic acid) was not genotoxic in vitro. It has no influence on ductus arteriosus as other NSAIDs. Prospective study following 165 pregnancies exposed to mesalazine, 146 of them were exposed during first trimester, did not demonstrate any embryotoxicity. There was slightly increased probability of preterm delivery. Mesalazine in recommended doses does not increase embryotoxicity risk in comparison with non-exposed population. Mesalazine in pregnancy is considered as drug of choice in therapy of inflammatory bowel diseases (in doses up 3 g day^{-1}).

Prednisone and Methylprednisone are the most frequent used glucocorticoids. Both as other corticoids induce palate cleft in mice. However the results of epidemiological studies are not consistent. It is not possible to exclude any risk of cleft palate, as well as IUGR and preterm rupture of fetal membranes. However absolute risk of cleft is not higher as 1 %. Common dosage is 10–40 mg, in serious conditions till 100 mg per day. Higher risk results from very high dosage by the lupus erythematodes treatment.

Azathioprine is anti-metabolite used in combination with other drugs for the immunosuppressive treatment. It is metabolized into 6-merkaptopurine. Both, original substance as well as metabolite is cytotoxic purine analogs with strong embryotoxic effect. Control epidemiological study was not done because result of azathioprine is combined with other drugs and basic disorder. However results from smaller studies prove that treatment by azathioprine in common dosage (1-2 mg per day) does not represent a risk of malformations but it increases significantly risk of prematurity and IUGR (till 40 %). Chromosomal aberrations in fetus are not supposed.

Infliximab is monoclonal antibody anti- TNF alfa used for treatment of Crohn´s disease unresponsible to the corticoids and immunosupressants. In animal studies was without negative effect on fertility, however this antibody does not react with TNF of rodents therefore this experiment is not valid for the assessment of human toxicity. Reported outcomes of pregnancies exposed during first trimester were in 3 cases spontaneous abortion and 6 healthy children. The most recent study including exposed 97 pregnancies does not demonstrate higher risk. It seems that risk is not high even the information is not sufficient.

Amantadine is an antiviral drug that is also used in Parkinsonian-like disorders. Amantadine was found to increase the incidence of congenital malformations in rats in dose 10 times higher than therapeutical. No malformations occurred in rabbits and rats at lower maternal doses. There were described individual cases of the exposure to amantadine during pregnancy and one small study involving 64 pregnancies with first trimester prescription. In this population, five births with defects were reported, which was higher than expected (risk 7.8 %) One defect involved the heart, therefore Amantadine is not recommended during pregnancy.

Glatiramer is a mixture of synthetic polypeptides composed of four naturally occurring amino acids, L-alanine, L-glutamic acid, L-lysine, and L-tyrosine. Its molecular weight, 5000 to 9000 Daltons approximately, probably may prevent transport cross placental barier. It is indicated in patients with multiple sclerosis. It is believed to induce the formation of antigen-suppressor cells specific for myelin-based protein and thereby inhibit some of the immunologically mediated damage. The manufacturer reports that reproduction studies using doses up to 18 and 36 times higher than human dose in rats and rabbits, respectively, did not uncover signs of adverse effects on fetal development. There were published only few cases and a series of more than 400 pregnancies in women who were treated with glatiramer acetate. Six (3.1 %) of 193 liveborn infants are said to have had congenital anomalies, which were different in each case. Its potential to decrease attack frequency prevails possible risk for fetus.

Endobulin (IVIG) is a mixture of normal polyvalent immunoglobulins obtained from healthy donors. It is used for treatment of immunodeficiency and for immuno-modulation and treatment of inflammation. Immunoglobulins cross pla-

Table I
Crohn´s disease was treated by the mesalazine monoterapy (4×) or by azathioprine (1×), in other cases by combination above mentioned drugs with prednisone or infliximab. Except for one abortion and one case with hypotrophy, 8 cases resulted in birth of healthy newborn in term with normal birth weight

Crohn´s disease				
Treatment	**Pregnancy outcomes**			
	Vol.abort	Abortion	Prematurity/hypotrophy	Normal/ healthy
Mesalazine	1	0	0	3x in term > 2500g
Azathioprine	0	0	0	1x in term > 2500g
Azathioprine, prednisone	0	0	0	1x in term > 2800g
Azathioprine, prednisone, mesalazine	0	0	1x in term; 2480/45	2x in term > 2500g
Azathioprine, mesalazine, anti-TNF	0	1	0	0
Mesalazine, anti-TNF	0	0	0	1x in term > 2500g

Table II
Ulcerative colitis was treated by mesalazine alone or in combination with prednisone or completed moreover by azathioprine. Infliximab was not used. Pregnancy resulted in 5 cases in birth of healty newborn with normal birth weight. Treatment by combination three drugs resulted in spontaneous abortion

Ulcerative colitis				
Treatment	**Pregnancy outcome**			
	Vol.abort	Abortion	Prematurity/hypotrophy	Normal/healthy
Mesalazine	0	0	0	4x in term > 2500g
Mesalazine, prednisone	0	0	0	1x in term; 3750/54
Mesalazine, prednisone, azathioprine	1	1	0	0

Table III
Pregnancy women suffered sclerosis multiplex resulted in all cases except for one abortion in birth of healty newborn with normal birth weight. That was slighly higher in patients without any treatment. Abortion may be considered as a result of intensive treatment or it may be a natural prenatal lost during pregnancy. It is not possible to postulate valid conclusion on the base of single case. There were observed no malformation or intrauterine growth retardation

Sclerosis multiplex – prospective study				
Treatment	**Pregnancy outcomes**			
	Vol.abort	Abortion	Prematurity/hypotrophy	Normal/healthy
Without treatment	0	0	0	0
Prednisone (attack, dose 2g)	0	0	0	3x in term > 2500g
Prednisone, azathioprine	0	0	0	2x in term > 2500g
Prednisone , azathioprine, amantadine	0	0	0	1x in term > 2500g
Prednisone, amantadine, interferon	0	1	0	0
Prednisone, glatiramer	0	0	0	1x in term > 2500g
Sclerosis multiplex – retrospective study				
Treatment	**Pregnancy outcomes**			
	Vol.abort	Abortion	Prematurity/hypotrophy	Normal/healthy
Without treatment	0	0	0	6x in term; mean 3300g
Prednisone (attack)	0	0	0	2x in term > 2500g

centar barrier however no negative impact of pregnancy was observed. Alergic reaction may be risky, only.

Results

Mesalazine followed by azathioprine was the most frequent treatment of idiopatic bowel diseases. Another recorded therapy was combination of azathioprine and corticoids or azathioprine, prednisone, and mesalazine. Pregnacy outcomes were deliveries of healthy newborns with normal birth weight (more than 2500 g). We recorded only two pregnancies terminated by spontaneous abortion in pregnancies exposed to Infliximab in combination with mesalazine or azathioprine (Tables I, II).

Comparing our group of MS patients (prospective study following treated pregnancies) with the group of patients, that delivered babies at the Clinical Department of Gynaecology and Obstetrics, 3rd Faculty of Medicine (retrospective study comprising non-treated pregnancies), we revealed no significant difference between both groups. Pregnancy outcomes in both groups were deliveries of healthy newborns with normal (more than 2500 g) birth weight. Birth weight of newborns of non treated mothers was even 3400 g on the average (Table III).

Conclusions

Almost all outcomes of our followed pregnancies were deliveries of healthy newborns, in term, with birth weight more than 2500 g. These findings are not consistent with published data, presenting risk of preterm labour, prematurity and IUGR. We did not record any inborn developmental defect. Both, cases of spontaneous abortion and low birth weight could be assigned to more severe course of chronic disease, requiring more intensive treatment with Infliximab (anti-TNF). More cases of low birth weight in newborns of women affected by Crohn´s disease are probably caused by absorption impairment resulting in malnutrition.

In group of mothers suffering from MS, we did not

record any significant difference between treated and non treated pregnancies. Our study does not support recommendations of the Centre of demyelinisating diseases, to terminate all the therapy 3 months before conception. We agree only with increase of embryotoxic risk in case treated by cytotoxic drugs (cyclophosphamide, methotrexate, amantadine) during the first trimester. However, in the case that pregnancy does not occur within 3 months, all period of non treated disease can continue more than a year, that considerably increase the risk of attack requiring more intensive treatment. Mesalazine as drug of choice in the treatment of IBD is followed by corticoids and azathioprine. Glatiramer, if necessary, can be used in attack control in the 2nd and the 3rd trimester. We recommend an extended prenatal diagnosis with a detailed fetal ultrasound. For possible temporal hypofunction of suprarenal gland of newborn after exposition of high doses of corticoids at perinatal period, detailed examination of newborn should be required. Nevertheless, the risk for mother and baby remains the disease itself. Treatment interruption during pregnancy increases risk of relapses during pregnancy and after delivery. However, our results, in general consistent with other larger studies, are still insufficient for statistical embryotoxicity risk assessment for small number of cases.

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L16

COMPARISON OF SOLID PHASE EXTRACTION AND LIQUID-LIQUID EXTRACTION IN AMPHETAMINE- LIKE DRUGS ANALYSIS OF HUMAN URINE USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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Key words: amphetamines, designer drugs, solid phase extraction, liquid-liquid extraction, GC-MS analysis

Introduction

Amphetamines are powerful central nervous system stimulants. Chronic abuse of amphetamines causes hallucinations and psychosis, in addiction to dysphoria and depression upon withdrawal[1]. Designer drugs of methylenedioxyphenylalkyl amine type are abused as recreational drugs at all-night techno dance parties. Users of these drugs describe feelings of understanding, euphoria, communicativeness, and emphaty, almost without showing hallucinogenic effects[2–5]. These drugs have been mentioned as psychoactive chemicals by Shulgin[4] and on www.sites (e.g. http://www.erowid.org., http:// www.lycaeum.org). Abuse of amphetamines and amphetamine derived designer drugs remains a serious social problem worldwide. These drugs have not only led to intoxications, but they have also caused driving impairments[6–8]. Gas chromatography (GC) or liquid chromatography (LC) methods have been used for the determination of amphetamines and amphetamine derived designer drugs in biological matrices[9–15], SPE extraction (solid phase extraction) and L-L extraction (liquid-liquid extraction) have been applied as a pre- analytical steps [16–19] of GC-MS or LC-MS assays (MS mass spectrometry).

Material and Methods

MAMP-d_5 deuterated methamphetamine internal standard (IS) was obtained from Lipomed AG (Arlesheim, Switzerland). Sulphate of AMP amphetamine and hydrochlorides of MAMP methamphetamine, EPH ephedrine, NOREPH norephedrine, MDA 3,4-methylenedioxyamphetamine, MDMA 3,4-methylenedioxymethamphetamine (ecstasy), MDEA 3,4-methylenedioxy-*N*-ethylamphetamine and MBDB *N*-methyl-benzodioxolylbutanamine were purchased from UNDCP United Nations International Drug Control Programme (Vienna, Austria). HFBA heptafluorobutyric anhydride was obtained from Fluka (Stenheim, Germany). Solvents were obtained from Penta (Chrudim, Czech Republic), and chemicals from Lachema (Brno, Czech Republic) and were of analytical grade. Isolute Confirm HCX cartridges (130 mg, 10 ml) were obtained from International Sorbent Technology IST (Mid Glamorgan, UK).

Blank human urine samples were obtained from volunteers. Authentic human urine samples were submitted to laboratory for toxicological analysis.

SPE extraction: 2 ml of urine samples were processed in accordance with IST SPE Application Note[20] after addition of 5 μl (20 ng μl^{-1}) IS MAMP-d_5. The analytes were eluted with 2 ml of methanol-aqueous ammonia (98:2 v/v) into glass vial. Before elution, tartaric acid solution 0.1 ml (0.1 mg ml^{-1}) was added. The eluates were evaporated to dryness under a stream of air at 40 °C. L-L extraction: 2 ml of urine samples were after addition of 5 μl (20 ng μl^{-1}) IS MAMP-d_5 and 0.5 ml M-NaOH extracted with 3 ml of benzene. After 5 min of extraction and centrifugation 2 ml of organic layer were separated into glass vial. The eluates were evaporated as described above. Derivatization was carried out after addition of 50 μL of HFBA at 70 °C 30 min. After cooling, the derivatization extracts were mixed with 0.1 ml of cyclohexane, the mixtures were centrifuged, and 0.2 ml of an aqueous 0.5 M-Na_3PO_4 solution was added. The organic layers were transferred to new vials and 1 μL was injected to GC-MS system[11].

GC-MS analysis

HP-5 MS capillary column (30 m × 0,25 mm i.d. × 0,25 μm film thickness) and Finnigan MAT Magnum ion trap GC-MS system (A200S GC autosampler, 3400 gas chromatograph (Varian Instrument Group)) were used. GC conditions were as follows: SPI injection mode, carrier gas helium, flow rate 1 ml min^{-1}, column temperature initially 65 °C for 2 min and increased to 250 °C at 20 °C min^{-1} and was held at this temperature for 10 min. MS conditions were as follows: transfer line heater 270 °C, electron ionization (EI) mode, full scan, ionization energy 70 eV, mass range *m*/*z* 35 to 500, seconds/scan 0.500.

The presence of analytes was screened for by extraction of characteristic fragment ions from the total ion chromatograms (*m*/*z*): 240 for AMP, NOREPH; 254 for MAMP, EPH, MDMA; 135 for MDA; 268 for MDEA, MBDB; 258 for MAMP-d_5 IS.

Quantification was carried out by comparison of peak area ratios (analyte versus IS) with calibration curves in which peak area ratios of spiked calibrators were plotted against their concentrations.

Assay evaluation for urine analysis

Aliquots of drugs free urine were spiked with 5 μl (20 ng μl^{-1}) IS MAMP-d_5 and the corresponding analytical

standard solutions to obtain calibration samples at concentrations of 0, 5, 10, 20, 50, 100, 500, 1000 and 2000 ng ml^{-1} of each analyte. Samples at each concentration were analyzed in doublets by SPE and L-L extractions. The regression line was calculated using a weighted [1/(concentration)^2] least-squares regression model. The means and standard deviations (SD) of slopes and intercepts as well as the coefficients of determination (R^2) were calculated.

Samples for repeatability study (n=6) at concentrations 50 ng ml^{-1} and 500 ng ml^{-1} were prepared by spiking 2 ml drugs free urine with the corresponding analytical standard solutions and extracted by SPE and L-L methods. After SPE and L-L extraction 5 µl (20 ng μl^{-1}) IS MAMP-d_5 was added to each eluate, extracts were evaporated, the residues were derivatized and analyzed as described above. Repeatability was calculated (as RSDs).

Samples for extraction study (n=6) at concentrations 50 ng ml^{-1} and 500 ng ml^{-1} were prepared by the same manner as the samples for repeatability measurment. The control samples (n=6) were prepared at concentrations 50 ng ml^{-1} and 500 ng ml^{-1} by adding the analytical standard solutions with 5 µl (20 ng μl^{-1}) IS MAMP-d_5 into vials and evaporated. After evaporation, the residues of extraction and control samples were derivatized and analyzed as described above. Extraction efficiencies (means and SDs) were estimated by comparison of the peak area ratios (analyte vs IS) from extraction samples and control samples for each analyte at each concentration.

Limit of detection (LOD) and limit of quantification (LOQ) were based on a signal-to-noise (S/N) ratios. Drugs free urine samples spiked by mixtures of standards used for determination of calibration parameters were assayed to determine LOD (S/N greater than 3:1) and LOQ (S/N greater than 10:1).

Results and Discussion

The analytes were isolated from 2 ml urine by a mixed-mode SPE extraction and L-L extraction. The evaporation carried out at 40 °C and the most volatile analyt AMP proved loss at a low concentration levels. The use of HFBA for derivatization can cause high background levels and degradation of the stationary phase of the GC column. Therefore, the heptafluoro (HFB) derivatives were extracted into cyclohexane and any excess reagent was removed from the organic phase by washing with sodium phosphate solution[11].

GC-MS analysis

The characteristic ions for HFB derivatives were chosen from the corresponding full-scan mass spectra. The presence of analytes were screened for by characteristic fragment ions. Peaks, that apeared in the extracted fragmentograms of the screening ions were checked. Quantification was carried out by comparison of the peak area ratios (analyte vs IS) with calibration curves obtained with spiked calibrators.

Assay evaluation for urine analysis

Doublets of matrix calibrators at eight different concentrations from 5 to 2000 ng ml^{-1} were analyzed. A weighted linear regression model was used to account for unequal variances across the calibration range. In Table I (SPE extraction) and Table II (L-L extraction), slopes, intercepts (means ± SDs) and coefficients of determination of calibration curves are shown. The SDs of the slopes corresponded to RSDs values for all analytes, showing, that callibration curves were reproducible. The measurement of calibration dependence allowed to determine extent of linearity in the concentration range from 10 to 2000 ng ml^{-1} for all analytes for SPE extraction and extent of linearity from 5 to 2000 ng ml^{-1} for L-L extraction, except AMP. Correlation coefficients of amphetamines and derived drugs exceeded 0.98 for both extraction methods.

Repeatability and extraction efficiency for all analytes in urine samples was tested on concentration levels 50 and

Table I
Slopes, y-intercepts and coefficients of determination of calibration curves in doublet of GC-MS assay for amphetamines and amphetamine derived drugs (SPE extraction)[19]

	Linearity of calibration curves		
	slope	y-intercept	R^2
AMP	0.0003±0.0001	0.0018±0.0003	0.9725
MAMP	0.0024±0.0002	0.0107±0.0218	0.9998
EPH	0.0111±0.0012	0.4575±0.0425	0.9819
NOREPH	0.0046±0.0003	0.6769±0.0917	0.9957
MDA	0.0052±0.0002	0.0852±0.0515	0.9999
MDMA	0.0009±0.0001	0.0225±0.0112	0.9960
MDEA	0.0012±0.0001	–0.0015±0.0181	0.9969
MBDB	0.0003±0.0001	–0.0025±0.0138	0.9987

Table II
Slopes, y-intercepts and coefficients of determination of calibration curves in doublet of GC-MS assay for amphetamines and amphetamine derived drugs (L-L extraction)

	Linearity of calibration curves		
	slope	y-intercept	R^2
AMP	0.0005±0.0001	–0.0011±0.0003	0.9949
MAMP	0.0005±0.0001	0.0067±0.0023	0.9992
EPH	0.0072±0.0001	–0.2119±0.0036	0,9975
NOREPH	0.0018±0.0008	0.1478±0.0907	0.9819
MDA	0.0047±0.0002	–0.0346±0.0289	0.9992
MDMA	0.0010±0.0001	0.0077±0.0005	0.9990
MDEA	0.0012±0.0001	0.0013±0.0023	0.9984
MBDB	0.0012±0.0001	0.0018±0.0078	0.9951

Table III
Extraction efficiencies and repeatability of GC-MS assay for amphetamines and amphetamine derived drugs at concentration levels 50 and 500 ng ml^{-1} (SPE extraction)[19]

	Extraction efficiency (mean±SD) [%]		Repeatability RSD [%]	
	50 ng ml^{-1} (n=6)	500 ng ml^{-1} (n=6)	50 ng ml^{-1} (n=6)	500 ng ml^{-1} (n=6)
AMP	60.3±11.8	89.2±7.8	9.7	7.7
MAMP	88.4±8.6	96.2±4.8	8.8	4.9
EPH	94.3±7.6	98.2±4.6	6.9	3.6
NOREPH	92.2±7.4	94.5±6.2	5.4	2.8
MDA	96.4±4.2	98.3±3.2	3.8	2.3
MDMA	96.2±4.3	99.3±3.6	3.2	1.2
MDEA	92.4±6.8	91.4±7.6	4.7	2.2
MBDB	95.5±6.4	96.4±3.8	3.9	1.7

Table IV
Extraction efficiencies and repeatability of GC-MS assay for amphetamines and amphetamine derived drugs at concentration levels 50 and 500 ng ml^{-1} (L-L extraction)

	Extraction efficiency (mean±SD) [%]		Repeatability RSD [%]	
	50 ng ml^{-1} (n=6)	500 ng ml^{-1} (n=6)	50 ng ml^{-1} (n=6)	500 ng ml^{-1} (n=6)
AMP	58.3±12.6	85.5±6.8	10.4	8.5
MAMP	83.2±9.1	94.8±5.2	8.1	5.2
EPH	92.3±8.2	96.7±4.8	7.2	4.4
NOREPH	91.3±7.9	94.0±6.2	5.9	3.8
MDA	95.8±5.2	97.2±3.9	4.4	2.9
MDMA	95.4±4.8	98.6±4.2	3.9	2.2
MDEA	93.5±7.4	92.0±7,3	5.3	2.8
MBDB	96.7±8.3	96.9±4.5	4.8	2.5

500 ng ml^{-1} (n=6). The criteria for repeatability were filled for all analytes (<15 % RSD). The results are shown in Table III (SPE extraction) and Table IV (L-L extraction). The contents of target drugs in extracted samples were compared with of contents of target drugs in control samples. Extraction recoveries for all analytes in urine samples were tested at concentration levels 50 and 500 ng ml^{-1} and established in range 60–99 % (SPE extraction) and 58–99 % (L-L extraction). Limits of detection for all analytes except AMP were established at 5 ng ml^{-1} and limits of quantification for MAMP, EPH, NOREPH, MDA, MDMA, MDEA, MBDB at 10 ng ml^{-1} (SPE extraction). Limits of detection for all analytes were established below 5 ng ml^{-1} and limits of quantification at 5 ng ml^{-1} except AMP (L-L extraction). LOD for AMP was established at 20 ng ml^{-1} and LOQ at 50 ng ml^{-1} for SPE extraction and L-L extraction.

Conclusion

In this study, GC-MS method is presented for screening as well as identification and quantification of amphetamines and /or designer drugs of ecstasy type in human urine. The mixed-mode solid phase extraction and liquid-liquid extraction methods are both suitable to use as a pre-analytical step of amphetamines and amphetamine derived designer drugs GC-MS assay. The effectivity of both extraction methods is comparable. This procedure should be suitable for the confirmation of immunoassay positive results for amphetamines and amphetamine derived designer drugs of ecstasy type.

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L17
IDENTIFICATION AND DETERMINATION OF THE TOXINS OF *Amanita muscaria* AND *Amanita pantherina*

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Key words: ibotenic acid, muscimol, muskarine, *Amanita muscaria*, *Amanita pantherina*

Introduction

Over the last year the abuse of the fly agarics *Amanita muscaria* and *Amanita pantherina*, attained of novel expansion, mainly among young people. Psychotropic effect of this mushrooms is caused by isoxazole derivatives ibotenic acid and muscimol and by muscarine[1,2]. Amounts and mutual proportions of those toxins show considerable variability and are dependent on the growing season, on the locality of growth and number by other factors. Such complexity significantly increases the risk connected with abuse of this mushrooms.

Toxins of *Amanita pantherina* and *Amanita muscaria*

Amanita pantherina and *Amanita muscaria* are morfologically and chemically very similar[3]. The poisoning syndrome due to this fly agarics has been called „mycoatropinic", as the symtoms are similar to those induced by atropin-containing plants[4–6]. The principal toxins, responsible for the hallucinogenic effect are ibotenic acid[7], muscimol[8] (decarboxylation produkt ibotenic acid) and muscarine. Others are muscazon[9], whose pharmacological activity is minor compared to the previous substances, and stizolobic and stizolobinic acids[10], whose pharmacological activities are still unclear[11,12] (fig. 1).

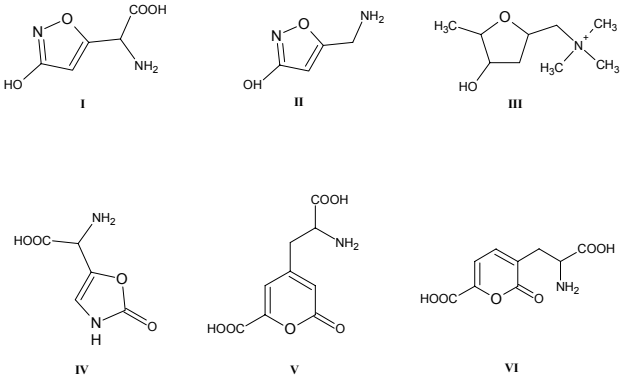

Fig. 1. **Chemical structure of the ibotenic acid (I), muscimol (II), muscarine (III), muscazon (IV), stizolobic acid (V) and stizolobinic acid (VI)**

Ibotenicacid and muscimol

Ibotenic acid and muscimol have the structural similarities with glutamic acid and γ-aminobutyric acid (GABA). Both of them react as false neurotransmitters[12–14]. Latency of the abuse is 30 min to 3 or 4 h after ingestion. The symptoms caused by this toxins are tiredness, convulsions, dizziness, euphoria, hallucinations, mydriasis, depression, muscle twitches, slow transition to coma[4,15–17].

Muscarine

Muscarine, due to its structure bind on the cholinergic receptors, acts on the part of the peripheral parasympathetic nervous system, which uses acetylcholin as a mediator[18]. Symptoms of the abuse start 30 min to 2 h after ingestion. They involve miosis, visual and auditory aesthesia, enhanced perspiration, salivation and lacrimation (so called PSL syndrome) and asthmatic respiration[4,15–17].

Methods of the determination of the toxins of *Amanita pantherina* and *Amanita muscaria*

High-performance liquid chromatography (HPLC)

There are a sensitive HPLC ion-interaction method for the separation and determination of muscimol and ibotenic acid in the extract from *Amanita muscaria*. The toxins of the mushroom were extracted with water and sonication and then injected into the chromatographic system equipped with a C18 column and UV detector[19]. In other works muscimol and ibotenic acid were determined in the methanolic extract from *Amanita muscaria* by the HPLC with a C18 column and UV detector[20] or by HPLC/DAD as derivatives after dansylation[21].

Gas chromatography-masss pectrometry (GC-MS)

Analysis of diastereomers of muscarine in Amanita muscaria was performed by gas chromatography[22]. In the study[23], followed the older work[24], hallucinogenic constituents were determined in *Amanita* mushrooms by gas chromatography-tandem mass spectrometry. After evaporation of the methanolic extracts from the mushroom, the residues were derivatized with a mixture *N*,*O*-bis (trimethylsilyl)trifluoroacetamide (BSTFA), 10 % trimethylchlorosilane (TMCS) and ethyl acetate and then injected into the chromatographic system.

Liquid chromatography-mass spectrometry (LC-MS)

There are several reports on the contents of muscimol, ibotenic acid or muscarine by LC/MS. The methanolic extracts containing the toxins were derivatized with dansyl chloride followed by ethylation and then were determined by LC-ESI/MS[21]. Dansyl chloride reacts with primary and secondary amino group and provides very stable derivates with good retention to reversed-phase columns. The detectable concentration of ibotenic acid and muscimol was in the units of ppm. Also muscarine, muscimol and ibotenic acid were determined by LC-MS/MS from *A. muscaria*[25]. The aqueous extracts of the mushrooms were directly injected into the chromatographic system and separated on C18 column. The detectable concentration of ibotenic acid and muscimol was in the units of ppm too. Hydrophilic interaction liquid chromathography (HILIC) on amide-based stacionary phase in combination with ESI tandem MS/MS was employed to separate polar mushrooms toxins – amanitins and phallotoxins (toxins from *Amanita phalloides*) and muscarine[26].

Conclusions

In the cited works, concentration of the toxins (ibotenic acid, muscimol and muscarine) were higher in the cap of the mushrooms then in the stems.

Although there are the articles on the concentrations of ibotenic acid, muscimol and muscarin from *Amanita muscaria* and *Amanita pantherina*, practically there are not the work describing economically and time-saving analytical method to the identification and determination of this toxins from biological matrix as urine, blood or gastric content. Until nowadays diagnosis of poisoning is maked by a microscopic examination.

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L18
BIO-MODULATORY EFFECT OF MICROBIAL POLYSACCHARIDE CARBOXYMETHYL GLUCAN

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Introduction

Polysaccharide constituents of fungal, plant and certain bacterial cell walls, belong to the naturally occurring agents with a broad spectrum of bio-modulatory activities[1–3]. The β-D-glucans belong to the class of compounds known as biological response modifiers (BRMs), which stimulate the immune system of the host and exert a wide range of immunopharmacological activities that enhance protection against viral, bacterial, fungal and parasitic infections. The β-glucans also exhibit radioprotective, immunoadjuvant, antioxidant, antimutagenic and anticarcinogenic effects[4–8]. Due to the need to identify new bio-protective agents and to determine their mechanisms of action, the aim of the present research was to assess the potential DNA-protective activity of carboxymethyl glucan (CMG) using the DNA-topology assay and compare its bio-modulatory potential with that of other polysaccharides included among BRMs. Moreover, the DNA excision repair deficient strain *uvs12* of the unicellular green alga *Chlamydomonas reinhardtii* was used for bio-protective activity assessment against methylmethane sulphonate induced cytotoxicity and mutagenicity.

The DNA-topology assay responds sensitively to a metal occurrence and free radical generation in a reaction medium. The assay is based on the electrophoretic detection of changes induced in a plasmid DNA topology through a conversion of a supercoiled DNA (form I) to relaxed DNA (form II) or to linear DNA (form III). These changes in the DNA topology are related to the induction of single- or double-strand DNA breaks by genotoxic/oxidative agent. When appropriately applied, this technique can be also used for monitoring of natural compounds with the DNA bio-protective activities[9]. Similarly as in bacteria and yeast, the collection of repair deficient strains of *C. reinhardtii* is available[10], and can be used for genotoxicity/antigenotoxicity assessment.

Materials and methods

Chemicals

All polysaccharides: carboxymethyl glucan (CMG); carboxymethyl chitin glucan (CMChG); sulphoethyl chitin glucan (SEChG); glucomannan (GM); sulphoethyl glucan (SEG); carboxyethyl chitosan (CECh) were isolated at the Institute of Chemistry of Slovak Academy of Sciences in Bratislava. Insoluble derivatives of β-D-glucan were solubilized by carboxymethylation, sulphoethylation which was detailed described by Machová et al.[11]. Methylmethane sulphonate (MMS) (Aldrich) was of the highest purity available. Analytical purity grade iron (II) sulfate heptahydrate ($FeSO_4 . 7 H_2O$) and hydrogen peroxide (30 % H_2O_2) were commercially available.

DNA-topology assay

Two variants of experiments were performed. In both the potential cleavage of a supercoiled plasmid DNA was made in the 20 μl reaction mixture of sodium phosphate buffer (10 mM, pH 7.0) containing the sample of a supercoiled plasmid DNA (200 ng). In the 1st variant of experiments aimed at comparison of 6 polysaccharides to induce DNA breaks, the supercoiled plasmid DNA was exposed for 30 min at 37 °C in the dark to CMG (1 mg ml^{-1}); CMChG (1 mg ml^{-1}); SEChG (1 mg ml^{-1}); SEG (1 mg ml^{-1}); SEChG (1 mg ml^{-1}); GM (1 mg ml^{-1}); CECh (1 mg ml^{-1}). In the 2nd variant of experiment the plasmid DNA was exposed either to Fe^{2+} (10 μM), or H_2O_2 (1 mM) alone or in combination with CMG (0.5 mg ml^{-1}, 1 mg ml^{-1}, 2 mg ml^{-1}). The sample was exposed to Fe^{2+} with CMG, for 15 min at 37 °C in the dark, and to H_2O_2 with CMG for 30 min at 50 °C. The induced single-strand breaks were assayed by measuring the conversion of supercoiled DNA (form I) to relaxed circular DNA (form II), while double-strand breaks were estimated on its conversion to linear DNA (form III). Plasmid DNA isomers migrated as discrete bands in agarose gel during electrophoresis (1.5 % agarose, Sigma, USA; 60 min/60 V) in the minigel apparatus. The DNA was made visible by staining with ethidium bromide (1 μg ml^{-1}, Sigma, USA) and UV illumination (Ultra-Lum Electronic UV Transilluminator, USA). Percentage of supercoiled, relaxed, and linear DNA forms was determined by densitometry using a computer program (UTHSCA, Image Tool for Windows, Version 1.27)[9].

Algal (*Chlamydomonasreinhardtii*) bio-protectivity assay

Algal (excision repair deficient) strain *uvs12* of the unicellular green alga *Chlamydomonas reinhardtii*, iso-

lated at the Department of Genetics, Faculty of Natural Sciences, Comenius University, Bratislava[11] was treated with MMS (0.1–0.5 %) and simultaneously with MMS and CMG (10^{-6} M) for 30 min in the dark, and then plated on agar dishes. Survival was evaluated by microscopic method which enabled to distinguish algal cells died due to cytotoxic and due to mutagenic (lethal mutations) effect of MMS. (Results are means of five experiments.) For statistic analysis Wilcoxon's two sample test was used.

Results and discussion

Similarly as a measurement of DNA strand breaks by the modified alkaline comet assay[2]; the Trolox equivalent antioxidant capacity assay[1]; the electron paramagnetic resonance spectroscopy[12], also the DNA-topology assay has its advantage because it may provide specific information about DNA damage, and possible mechanism of action of the biologically active polysaccharide which can either increase or reduce the level of DNA strand breaks.

Results on electrophoretic monitoring of the DNA topology are shown in fig. 1. The plasmid DNA was exposed to CMG and other polysaccharide preparations (CMG, line 2; SEChG, line 4; SEG, line 5; GM, line 6; CECh, line 7) and with exception of CMChG they did not induce DNA breaks and any changes in the DNA topology. Moreover, a resulting gel lines looked like these of the untreated control plasmid DNAs (fig. 1, lines 1 and 8), and percentage of their DNA forms (Table I) were also similar to these in the controls.

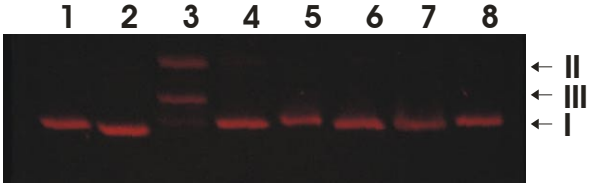

Fig. 1. **Electrophoretic monitoring of the DNA topology of the plasmid DNA** treated with CMG line 2; CMChG line 3; SEChG line 4; SEG line 5; GM line 6; CECh line 7; control – lines 1 and 8

In addition, the effects of CMG on suppression of DNA relaxation (during co-incubation with Fe^{2+}) due to iron chelatation, and on stimulation of DNA relaxation (during co-incubation with H_2O_2) were verified in the dose-dependent experiments (fig. 2). These results correspond with results obtained in experiments with another biologically active fungal polysaccharides: SEChG[13] and GM[3], both potent iron chelators.

Results obtained after simultaneous treatment of algal excision repair deficient strain *uvs12* with MMS and CMG documented that it exerted bioprotective effect (Table II), because cytotoxicity and frequency of lethal mutations induced by MMS were reduced in the presence of CMG. Antigenotoxic effect of CMG may be explained either by MMS inactivation (acting as desmutagen) or by stimulation of non-damaged repair mechanism(s), probably recombination repair (acting as bioantimutagen).

The established antimutagenic activity of CMG demonstrated by a suppression of the mutagenic and toxic effects of cyclophosphamide in mice[5] and documented on bacterial, yeast and plant genetic models in our previous experiments[14] as well as in experiments using repair deficient alga *C. reinhardtii* in this work, spoke in favour of the possibility to use it as a chemopreventive agent. However, biological and consequential medicinal importance of CMG is based on the nature and mode of action of the counteracting genotoxic compound as well as the experimental model system used.

Conclusion

Two experimental techniques, the DNA-topology assay and the algal bio-protectivity assay were used to evaluate the bio-modulatory effects of CMG - biologically active carboxymethylated derivative of β-D-glucan, isolated from the cell wall of yeast *Saccharomyces cerevisiae*. In the DNA-topology assay CMG like other biologically active polysaccharides (SEChG, SEG, GM, CECh) did not induce any damages to DNA. Only CMChG exerted DNA breakage and subsequent increase of the DNA form II content. In the DNA-topology assay, CMG protected plasmid DNA from breaks induced by Fe^{2+} due to its chelating activity and slightly enhanced damage induced by H_2O_2. Antigenotoxic effect of CMG revealed in the algal bioprotective assay may be explained by its desmutagenic

Table I
Levels of the DNA topological forms after treatment with biologically active polysaccharides

	Sample of polysaccharide						
Line	1	2	3	4	5	6	7
Molecular weight	Control	CMG [250 kDa]	CMChG [59 kDa]	SEChG [40 kDa]	SEG [240 kDa]	GM [30 kDa]	Control
Form I, %	94.59	95.94	17.56	89.18	97.30	100.00	95.07
Form II, %	5.41	4.06	39.21	10.82	2.7	0.00	4.93
Form III, %	0.00	0.00	43.23	0.00	0.00	0.00	0.00

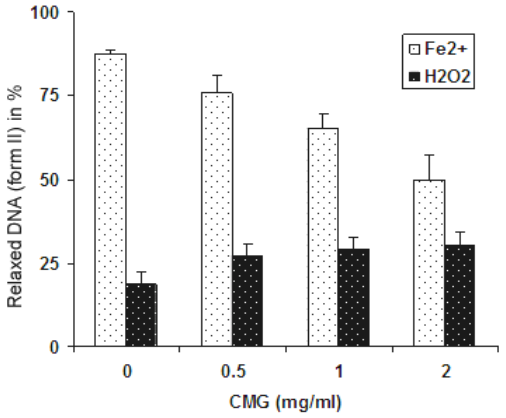

Fig. 2. **Concentration-dependent activity of CMG** (0.5–2.0 mg ml^{-1}) on suppression of DNA relaxation during co-incubation with Fe^{2+} and on stimulation of DNA relaxation during co-incubation with H_2O_2

Table II
Bio-protective effect of CMG against MMS-induced cytotoxicity and mutagenicity in excision repair deficient strain *uvs 12* of *Chlamydomonas reinhardtii*

Algal strain *uvs12*	Survival [%]	
Concentration of MMS [%]	–CMG	+CMG
Control	100.00	100.00
0.1	82.27	83.52
0.2	23.73	21.77
0.3	7.68	11.95
0.4	2.88	6.89
0.5	1.22	3.96

and/or bioantimutagenic activity. The results imply that CMG is a polysaccharide with marked biological activities and suggest its potential application as a promising natural chemopreventive agent.

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L19

CHROMOSOMAL ABERRATIONS AND URINARY LEVEL OF THIOETHERS IN RELATION TO POLYMORPHISMS IN *GSTM1*, *GSTT1* AND *GSTP1* GENES IN TIRE PLANT WORKERS

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Introduction

Workers in tire plants are exposed especially to 1,3--butadiene (BD) and to a lesser extent to styrene. Black sooths parts, contain polycyclic aromatic hydrocarbons (PAHs). According to IARC, BD is classified as probable human carcinogen (group 2A). Genotoxic effects of individual above chemicals were described in several studies[1–6]. BD induces chromosomal changes *in vivo* in blood cells of mice[7–8], as well as increases micronuclei frequency in bone marrow of mice[9]. Both the 1,2-epoxy-3-butene (EB) and 1,2,3,4-diepoxybutane (DEB) metabolites induce chromosomal aberrations (CAs) in rats[10]. In summary, the BD metabolites are mutagenic and clastogenic in all systems following the order of DEB > EB > EB-diol[11–13]. The BD-exposed workers who were carriers of glutathione S-transferase (GST) *GSTT1-null* genotype had significantly higher aberration frequencies[14], suggesting importance of inter-individual differences in metabolic and DNA-repair activity in humans. Such differences may be caused by polymorphisms in genes encoding biotransformation and DNA-repair enzymes[15]. GSTs catalyze the reaction of glutathione with a wide variety of organic compounds to form thioethers. A higher frequency of chromosome-type breaks was observed in *GSTT1-null* smokers. The effect of *GSTM1-null* and polymorphism in *XRCC1* codon 399 on the frequency of CAs was modified by smoking[16]. Mercapturic acids have proved to be very suitable biomarkers for human exposure to a variety of industrial chemicals. When mercapturic acid metabolites were first proposed as biomarkers for occupational exposure to BD[17], assays were developed to measure 1-hydroxy-2-(*N*-acetylcysteinyl)-3--butene and 2-hydroxy-1-(*N*-acetylcysteinyl)-3-butene (i.e. -isomeric mixture of both forms M2) and 1,2-dihydroxy-4--(*N*-acetylcysteinyl)-butane (M1). End-of-shift excretion of global thioethers after styrene exposure was found to significantly correlate with styrene exposure as well as with inhaled cigarette smoke[18].

The purpose of this study was to conduct a biomonitoring study in tire plant workers by employing CAs in peripheral blood lymphocytes and the urinary levels of thioethers. Association with genetic polymorphisms in genes encoding principal metabolizing enzymes GSTM1, GSTT1 and GSTP1 as biomarkers of individual susceptibility to procarcinogens was assessed as well.

Materials and methods

Characteristic of workers

We analyzed relationship between total chromosomal aberration frequencies (CAs), chromatide-type (CTA) and chromosome-type of aberrations (CSA) in peripheral blood lymphocytes, the urinary levels of thioethers and genetic polymorphisms in glutathione S-transferase genes *GSTM1*, *GSTT1* and *GSTP1* in 65 tire plant workers. They were classified according to exposure to important xenobiotics into high exposure Group I (N=42), and low exposure Group II (N=23). Group I consisted of weighers and operators of mixing machines. Group II was recruited from workers in the inner tube press operation as confectioners of inner tubes in tires. The detailed characteristics of individual groups are shown in Table I.

Chromosomal aberrations assay

CAs were analyzed in peripheral blood lymphocytes in two separate tubes using previously described method[19]. Structural CAs include chromosomal breaks and ex-

Table I
Characteristics of study population stratified for occupational exposure

	Group I	Group II
Age±S.D.	45.4±8.2	43.8±7.6
Employment±S.D.	10.8±6.5	9.2±7.2
Gender M/F	42/0	17/6
Smoking S/NS	22/20	8/26

M – males, F – females, S.D. – standard deviation,
S – smokers, NS – non-smokers

changes visible in arrested metaphase-stage cells and they were divided CTAs and CSAs.

Determination of urinary thioethers

Thioethers (mercapturates) were identified in the urine samples by photometric method. Concentration of thioethers in μmol was corrected by concentrations of the concomitantly excreted creatinine in mmol for finally concentration in μmol mmol^{-1} creatinine[20,21].

Assessment of genetic polymorphisms in genes encodIng GSTs

The *GSTM1* (deletion), *GSTP1* (alleles Ile/Val in codon 105 of exon 5) and *GSTT1* (deletion) polymorphisms were analyzed by the multiplex PCR method[22].

Statistical analysis

The analysis was performed using Statgraphics program, version 7 (LEAD Technologies, Manugistics, Cambridge, MA) with employment of non-parametric tests (Mann-Whitney, Kruskal-Wallis and Spearman correlation).

Results

The highest frequency of CAs was found among the mixing operation staff, i.e. Group I., where it reached 2.21±0.16 % (SEM) and was significantly higher than that identified among the inner tube press operation staff, i.e. Group II 0.78±0.20 % (P<0.001). In the Group I were detected significantly higher 1.33±0.18 % CTA types of CAs (P<0.001), Table II. The levels of thioethers were higher in group II in comparison with group I. The frequencies of CAs in individuals with *GSTM1-plus* genotype were significantly higher (P<0.01) than in those with *GSTM1-null* genotype (2.73±0.31 % vs.1.38±0.28 %). The same significantly higher frequencies (P<0.05) we detected in CTA and CSA types of CAs. The frequencies of CSA types of CAs for individuals with *GSTT1-null* genotype were statistically higher than in individuals with *GSTT1-plus* genotype (1.56±0.36 % vs. 0.70±0.17 %; P<0.05). Individuals with wild-type Ile/Ile *GSTP1* and as well with variant Val/Val genotype had the higher frequency of CAs as compared to those with heterozygous Ile/Val genotype (Table III.). The exposure to high concentrations of xenobiotics in the tire plant results in significantly higher CAs frequency, whereas the *GSTM1-plus* polymorphism seems to be associated with more effective clearance of intermediates arising from the chemicals. We did not observe relationships between CAs frequency and the urinary levels of thioethers in Group I and II, and so in polymorphisms examined genes.

Discussion

Epidemiological studies show the close connections of the occupational BD exposure with the increased risk of leukaemia[23]. BD induces the DNA aducts[24,25] and haemoglobin adducts[26]. The detoxification process of reactive BD metabolites is of particular importance. Especially the conjugation pathway with glutathione catalyzed by GSTs attracts attention, since about 60 % of reactive BD metabolites are processed via this route[27]. On the contrary to BD, only 1% of styrene oxide, a reactive metabolite of styrene is metabolized via conjugation with glutathione[28]. Results on the possible association of *GSTT1* and *GSTM1* polymorphisms with cytogenetic biomarkers in BD-exposed workers have also provided some support for the *in vitro* findings. An elevated frequency of CAs was observed in *GSTT1-null* workers[14]. Our data support these observations. The CAs were significantly higher in the Group I, with high exposure (P<0.001). We evaluated levels of urinary thioethers as terminal products of metabolism after conjugation of the electrophilic substances with glutathione. Levels of thioethers in the urine were higher in the Group II, with low exposure, and associated with the *GSTM1* polymorphism. The urinary thioethers levels were higher in *GSTM1-plus* carriers (P=0.026) in comparison with *GSTM1-null* ones. Regression analysis (R=0.316; P=0.006) and analysis of variance (F=0.16; P=0.006) confirmed the relationship between *GSTM1* polymorphism and levels of urinary thioethers. We may assume that faster elimination of the toxic substance results in its lesser harm for the organism. Haufroid[29] pointed out the inter-individual differences between final products of the sty-

Table II
Types of structural chromosomal aberrations [%]±SEM of aberrant cells and urinary thioethers [μmol mmol^{-1} creatinine] ±SEM in study population stratified for occupational exposure

	CAs	CTA	CSA	Thioethers
Group I (42)	2.21±0.16***	1.33±0.18***	0.88±0.18	3.47±0.34
Group II (23)	0.78±0.20	0.39±0.19	0.39±0.26	4.29±0.41

CAs – chromosomal aberrations (total), CTA – chromatide-type aberrations, CSA – chromosome-type aberrations, *** (P<0.001), SEM – standard error of mean

Table III
Total chromosomal aberrations, chromatide-type and chromosome-type aberrations [%]±SEM stratiefied for polymorphisms in *GSTM1, GSTT1* and *GSTP1*

Group1	GroupII	CAs	CTA	CSA	CAs	CTA	CSA	GSTM1 plus
GSTM1	*plus* (26)	2.73±0.31**	1.62±0.32*	1.12±0.23*	*plus* (10)	0.60±0.20	0.40±0.20	0.20±0.12
	null (16)	1.38±0.28	0.88±0.15	0.50±0.18	*null* (13)	0.92±0.21	0.38±0.11	0.54±0.21
GSTT1	*plus* (33)	2.00±0.26	1.30±0.21	0.70±0.17	*plus* (20)	0.75±0.17	0.35±0.11	0.40±0.16
	null (9)	3.00±0.50	1.44±0.61	1.56 ±0.36*	*null* (3)	1.00±0.00*	0.67±0.24	0.33±0.24
GSTP1	*Ile/Ile* (20)	2.45±0.38	1.45±0.37	1.00±0.23	*Ile/Ile* (9)	0.44±0.18	0.22±0.11	0.22±0.11
	Ile/Val (18)	1.89±0.30	1.28±0.26	0.61±0.20	*Ile/Val* (14)	1.00±0.21	0.50±0.16	0.50±0.23
	Val/Val (4)	2.50±0.75	1.00±0.36	1.50±0.83	*Val/Val* (0)	0	0	0

** (P<0.01), * P<0.05)

rene metabolism in the urine (mandelic acid, phenylgyoxalic acid) and specific mercapturic acids. *GSTM1* polymorphism provided the best explanation for the different PHEMA excretion as it was 6-times lower in individuals with the *GSTM1-null* genotype (P<0.0001). Various genetic polymorphisms may have an impact on the incidence of different diseases, partially tumors, and on the genotoxic effects induced by the occupational exposure to genotoxic substances[30]. It would be ideal to develop tools for monitoring of individual susceptibility and set the "positive" and "negative" genotypes in order to minimize the risks associated with exposure in susceptible individuals.

In conclusion, our study showed that individual susceptibility estimated by analysis of GST polymorphisms greatly influences biomarkers of exposure (elimination of thioethers) and biomarkers od biological effect (CAs). Further clarification of puzzled posture brought by this study should help to establish principles of effective monitoring occupational and environmental exposures and minimization of their hazards.

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L20
POTENTIAL FALLOUTS OF ACCIDENTAL INGESTION OF LIMESCALE REMOVERS

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Key words: Limescale remover, titratable acid reserve (TAR), water acidity, Czech Toxicological Information Centre (TIC)

Introduction

Limescale layers on the walls and particularly on the heating coils of a tea-kettle are formed by sedimentation of calcium and magnesium salts, which are present in dissolved form in various concentrations in each drinking or supply water. Their content is expressed by s.c. general water hardness, which is composed of two parts: "carbonate" and "non-carbonate" ("permanent") hardness. The first part corresponds to the amount of hydrogen carbonates which can be removed by boiling. As permanent water hardness, a source of limescale, the content of hydrogen carbonates is denoted, which are chemically transformed into insoluble calcium, magnesium respectively carbonates. The formed limescale layers decrease the efficiency of the heating, increase the energy consumption; they increase the risk of bacterial colonies to grow on very rugged relief. The most frequently used process to remove the limescale are the chemical ways – although speediest and the most effective, from a medical point of view, are potentially corrosive for the wall of the oesophagus and stomach. Various commercial products as a possible source of ingestion are available to every household. Over 35 000 exposures to caustic agents, including 24 known fatalities reported in 1987 in the USA[1]. The majority of cases has been accidental ingestion[1,2]. Injury can result in some cases in death or prolonged hospitalization and lifelong disability due to necrosis of the eosophagus or stomach[2]. Although it has been suggested that pH, viscosity, concentration of consequential ingredients, amount ingested, contact time, and premorbid state of the esophagus all may contribute to the outcome of injury, no marker exists to accurately predict a product's potential for injury[2–4]. pH is often fails to predict the extent of injury after exposure. pH-value 3.0 of a drink is medically important as corrosive damage can be expected below this value. The other important limits represent pH-values 4.5, 6.5, and the neutral value 7.0 (original pH of used drinking water)[5]. pH-value of drinking water must be kept within 6.5–9.5 in the Czech Republic (CR); pH of packed drinking water and water in containers[5] must be higher then 4.5. For comparison, pH values of different beers amount to 2.5–5.0, colas are in range of 2.0–3.0. Thus reliance on pH alone might result in clinical errors in patient management[2]. Therefore parameter denoted as titratable acid reserves (TAR) (or water acidity) was suggested as more appropriate estimate of the corrosive potential[2,4,6]. TAR is defined as the ability of sample to neutralize alkaline reacting compounds, e.g., NaOH. It corresponds to the sum of CO_2 and humin acids in case natural waters. "Total TAR" corresponds to the sum of carbon dioxide and all present acids (humin acids, limescale remover etc.)[7]. The aim of this investigation was to study the factors affecting severity of limescale remover ingestion and to compare some commonly used limescale removers. The research was divided into: *1*) Recherché of remover ingestions in years 2000–06 according to the phone calls to the Czech Toxicological Information Centre (TIC), *2*) Experiments realized to get reliable comparable parameters of the common commercial and chemical agents used for limescale removing in the CR.

Methods

Data on limescale remover ingestion obtained in 2000−2006 was collected from the Czech TIC-database of answered phone calls and medical reports. The severity in terms of reaction of five of the most common (in CR) commercial limescale removers (denoted A to E) and 2 other similar compounds were characterized by comparison of time and temperature changes of pH and their TAR. Their compositions are as follows: Remover A contained: amidosulphonic acid (ASA) 100 % (m V^{-1}); remover B: ASA 5−15 % , ortophosphoric acid (OPA) 5 %, tensides 2 % (0.2 L diluted by 0.8 L of water); remover C: citric acid (CA) 100 % (25 g solved in 1.0 L of water); remover D: ASA acid 5−15 % and remover (0.1 L diluted by 0.3 L of water); E: ASA 5−15 % (0.15 L diluted by 0.5 L of water). pH were determined with a digital pH-meter "WTW inoLab-Level 2" (inoLab, CR) applying a combined glass/calomel reference electrode (Schott − BlueLine). All measurements were performed at room temperature (the samples after heating up to the boiling point were cooled using the water bath).

Results and discussion

Situation in Czech Republic 2000– 2006

Between 2000–2006 the TIC answered 361 calls, i.e., about 50 enquiries p.a. following limescale remover inges-

tion (271 adults and 90 children). Number of both sexes was approximately equal. 91 % of questions concerned diluted solutions, only 9 % dealt with intake of concentrated remover. In all cases unintentional ingestion was noticed. Most enquiries were up to 1 hour after ingestion. At the time of the call (within 1−2 hours after ingestion) 68 % of patients did not exhibit any symptoms. The remaining 32 % had mild symptoms only (burning in oral cavity or retrosternally but rarely causing digestive difficulties). In 32 % a remover containing ASA was ingested, in 21.2 % with CA, in 19.3 % combination of CA + ASA or OPA, in 15 % with ASA and OPA, in 5 % with OPA, and in 10 % the composition of removers was unknown. Information obtained during calls from years 2005 and 2006 generated detailed data from medical reports. In 2005, 48 calls were answered (30 adults and 18 children), 68.4 % patients experienced no symptoms, 36.6 % mild symptoms (burning in oral cavity (58.3 %) or retrosternally (16.7 %), and causing digestive difficulties (25 %). Subsequent oesophagoscopy was performed in 11 % of subjects and no pathological findings were revealed. In 2006, 45 enquires were obtained (30 adults and 15 children). Only 3 patients ingested concentrated remover and subsequent oesophagoscopy was performed: 2 revealed no pathological findings and 1 patient presented with hyperaemia only. 19 subjects were examined otorhinolaryngologically, no pathology was found. 79 % experienced no symptoms, 21 % only mild symptoms (burning in oral cavity (84 %) or retrosternally (15 %), but rarely causing digestive difficulties (1 %). In 73 % the remover solution was consumed with tea, in 11 % with coffee or milk, 10 % as a plain remover solution and 6 % concentrated remover.

Chemical analysis of the most common removers

Water used to prepare tested remover solutions, amounted to pH 7.1 before boiling, and 7.2 after boiling. Coffee prepared from this water exhibited pH 6.6 and tea pH 6.3. If coffee was prepared from remover C (diluted CA) pH 3.1 was determined, if remover D was used (diluted ASA) pH 1.7 was found. Tea prepared from remover C exhibited pH 3.0, when remover D was used, pH 1.7 was recorded. The tea-kettles, their heating elements were slightly covered by limescale at the start of our experiments. Therefore we can suppose that relatively small parts of agents were neutralized by limescale compound. Limescale remover solutions (prepared as recommended by the manufacturer) were placed into the kettle and its pH value was recorded at the temperature 20 °C (fig. 1). pH values were in range of 1.3 to 2.0. The solutions were cooled down up to 20 °C after boiling. The pH-values stayed unchanged or increased slightly (from ΔpH 0 to ΔpH 0.85). In the following 15 hrs pH did not change substantially. In case of remover A (ASA 100 %), pH increased in subsequent two hrs by 0.2 (up to 1.6) and in remover C (5−15 % of ASA, 5 % of OA, and 2 % of tensides), pH increased in following 3 hrs by 0.8 (up to 3.2). It is possible to ascribe this increase to the dissolution of the limescale sediment from the walls and from the heating coil. In the following hours pH of all removers was practically constant or decreasing and reached its original pH-value that was recorded straight after boiling. TAR of remover solutions was examined. The amount of NaOH necessary for achievement of chosen pH in 100 mL of the limescale remover was determined (fig. 2). Titration curves of diluted HCl 1:10 and 1:100 were recorded, too. This acid is commonly used for effective limescale removing. The TAR reaction (pH 6.00) of 3 of the most common limescale removers and of CA with a time interval of up to 24 hrs (ref.[8,9]) was measured. The time changes of the titration curves were recorded. For comparison, TAR of 1 % acetic acid (AA) was measured (Table I). TAR of remover solutions is relatively low; it is comparable with vinegar (8 % AA) in 3 fold dilution with water.

Conclusion

It is clear that remover B has the highest TAR to achieve pH 7, i.e., it may be the most effective as limescale remover. However, at the same time, it might have the highest corrosive potential for the gastrointestinal tract. Remover E exhibited the lowest TAR of all men-

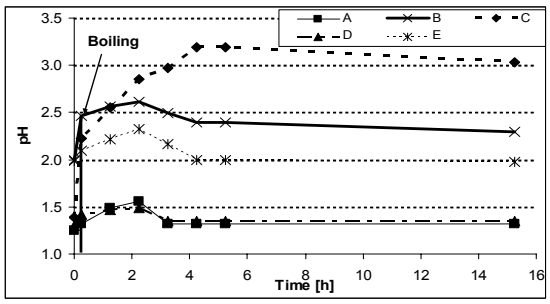

Fig. 1. **Time changes of pH of 5 tested limescale removers** (A-E, composition in "*Reagents and Materials"*)

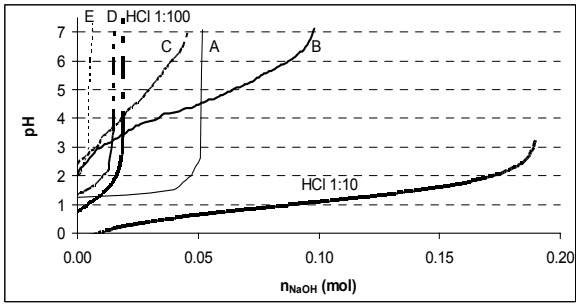

Fig. 2. **Titration curves (realized by NaOH)** of 100 mL of 5 tested limescale removers (A-E, composition "*Reagents Materials"*), and HCl diluted 1:10 and 1:100

Table I
Measurements of TARs up to pH 6 at time intervals of 24 hrs

Remover \ Time	Initial pH	TAR [mmol NaOH 100 mL^{-1} of tested solution]				Percentage change of TAR [%]			
		0	2	4	24	0	2	4	24
Remover A	1.3	33.1	30.2	29.5	29.1	100.0	91.2	89.0	87.9
Remover B	2.3	12.0	11.0	10.9	10.7	100.0	91.7	91.0	89.0
Citric acid (100 %)	2.8	36.0	34.5	33.5	32.5	100.0	95.9	93.1	90.4
Remover D	2.3	88.0	92.1	83.6	46.0	100.0	104.0	95.0	52.3
Acetic acid (1 %)	2.8	11.1	11.3	11.2	11.0	100.0	102.0	101.0	99.0

tioned pH values. Remover A has the second highest TAR. Measurements of pH and TAR of diluted limescale removers A, B, C and D in 4 time intervals up to 24 hrs has shown relatively low values that are either lower or comparable with about 8 % acetic acid, i.e. commercially availably vinegar. It is possible to conclude that the TAR curve course, slope did not change in time substantially. It slightly decreases in time, as part of the remover is consumed by the reaction with limescale. pH values and neutralization capacities could be affected by presence of active compounds, e.g., surfactants. Partially the remover is buffered by the consumables buffering capacity (coffee, tea, soup etc.). In agreement with our previous findings[2,8–11], the pH did not correspond with objective findings (expected corrosive damage typical for such a low pH values). This could explain why the mucous membranes were not seriously injured post-contact with removers. One reasons of the relatively low TAR might be the dissolution of the limescale sediment from the walls and from the heating coil. This finding corresponds with the clinical status of persons who ingested these removers either as pure solution or in a beverage. The number of enquiries following limescale remover ingestion in the TIC database in years 2000–2006 has been relatively constant since 2000 (resulting in approx 50 enquiries p.a.) and no serious course with concomitant complications has been recorded.

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L21
BENZODIAZEPINES IN CLINICAL AND FORENSIC ANALYSIS

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Key words: benzodiazepines, identification, determination, FPIA, GC-ECD, GC-MS

Introduction

Recently, benzodiazepines have become one of the most important groups of so-called toxicologically significant substances. Not only because of their frequent occurrence as positive findings in forensic as well as in clinical toxicogical analyses but also due to the high incidence of abuse. More than 4670 samples were analysed for benzodiazepines over a period of the six years (2001–2006) in our department. Around one third were positive. Thus, benzodiazepines are currently the most frequently found substances in our toxicological routine.

Although identification and determination of benzodiazepines in biological material are analytically well managed[1–32], certain problems sometimes do arise, especially in the interpretation of the results. Such problems in some laboratories can lead to incorrect conclusions.

Experimental

Screening analysis of benzodiazepines (BZD)

Immunoassays have become the most commonly used routine preliminary tests for benzodiazepines in urine in most of toxicological laboratories in the Czech Republic. Their major advantage is that they do not require any sample preparation. In our laboratory we use Fluorescence Polarisation Immunoassay (FPIA) on the AxSYM system (Abbott Laboratories) with a cut-off value set to 200 ng ml^{-1}.

Thinlayer chromatography (TLC)

TLC is suitable for the detection of parent forms of BZD, especially in gastric contents, as well as for screening of some BZD metabolites, particularly in urine. Use of HPTLC plates and the Bratton-Marshall reagent enables sensitive detection of the metabolites of flunitrazepam, clonazepam and nitrazepam as well as some other metabolites (in the form of benzophenones) after acid hydrolysis.

Gas chromatography – mass spectrometry (GC-MS)

GC-MS is suitable for the selective detection of BZD metabolites in urine after enzymatic hydrolysis using beta-glucuronidase, solid phase extraction (SPE) on Bond Elut Certify columns (Varian) and derivatization by silylation.

Instrumentation: Gas chromatograph Trace GC, Mass spectrometer Polaris Q (Thermo Finnigan), capillary column Rtx -5MS (15 m × 0.25 mm × 0.25 μm, Restek), temperature program: 210 °C (1 min), 20 °C min^{-1} to 290 °C (10 min), full scan mode (40–450 AMU).

Gas chromatography with electron capture detector (GC-ECD)

GC-ECD is used for simple, rapid, sufficiently sensitive and cost effective determination of BZD in blood (serum).

Sample preparation ("freeze-out method"): 0.5 ml serum + internal standard (lorazepam) + 0.05 ml 2 M TRIS buffer pH 9 + 0,15 ml butyl acetate mixed, centrifuged and frozen-out (1 hour by –20 °C).

Table I
Summary of analyses of benzodizepines in 2001–2006

	Total number of analyses focused on BZD			Positive results			Quantitative analyses			False negative results		
Year	Dead	Live	Together	Dead	Live	Together	Dead	Live	Together	Dead	Live	Together
2001	160	461	**621**	18	171	**189**	10	53	**63**	1	15	**16**
2002	187	547	**734**	26	197	**223**	20	84	**104**	4	15	**19**
2003	186	609	**795**	27	233	**260**	20	61	**81**	4	11	**15**
2004	233	573	**806**	41	182	**223**	35	64	**99**	8	13	**21**
2005	257	590	**847**	36	194	**230**	29	74	**103**	9	18	**27**
2006	256	617	**836**	46	219	**265**	36	73	**109**	12	56	**68**
Together	1279	3397	**4676**	194	1196	**1390**	150	409	**559**	38	128	**166**

Instrumentation: Gas chromatograph AutoSystem XL (Perkin Elmer) with ECD, capillary column DB 5 (15 m × 0,25 mm × 0,25 μm, J & W), temperature programme: 180 °C (1 min), 20 °C min^{-1} to 250 °C, then 10 °C min^{-1} to 280 °C (10 min).

Results and discussion

Table I shows the total number of analyses on BZD carried out in our department over the six-year period, including the positive findings. The number of false negative results of immunochemical screening with subsequent positive findings is another monitored parameter together with the number of quantitative analyses.

Despite the undeniable advantages of immunoassays for BZD screening of urine there are also some drawbacks. These can be of major importance especially when interpreting the results. The main problems are both false positive and false negative results.

False positivity was sporadically observed in cases when a high concentration of indomethacin, cocaine, methadone, sertraline or trazodone was determined in biological material. For this reason positive results obtained from immunochemical methods should always be confirmed by another independent method. There are, however, some laboratories in our country (mainly biochemical) which do not respect this general rule.

False negativity mainly occurred when the urine contained metabolites of bromazepam, flunitrazepam, nitrazepam and clonazepam for which the assay is less sensitive, but rarely also diazepam and alprazolam gave the false-negative results. This is a significant problem because the false negativity occurs even in cases of intoxication through some of the above mentioned BZD.

Table II shows some interesting examples of false negativity (FPIA results were lower than the cut-off value).

The first part (A) summarizes cases in which both FPIA analysis of urine and subsequent TLC analysis of urine (and – if available – of gastric contents) proved negative. In reported cases therapeutic concentrations of BZD in blood/serum were found and positivity of the findings was not recognized until examination of a blood/serum samples using the GC-ECD method.

The part (B) displays examples where concentrations of BZD were found even above the therapeutic levels in spite of a negative preliminary FPIA results and, in some cases, also after negative TLC analysis of urine and gastric contents.

Conclusion

The results presented here show that despite the well-known advantages of immunoassays in screening analysis of urine for drugs of abuse (including BZD), it is necessary to confirm practically all benzodiazepine results obtained

Table II
Examples of false-negative results in BZD analysis by means of immunochemical and chromatographic methods

Group	AxSYM (U) [ng ml^{-1}]	TLC (U) or GC-MS (U)	TLC (GaC)	GC-ECD (B)	Concentration [mg l^{-1}]
A	24	negative	negative	diazepam	0,02 - therap.conc.
	152	negative	negative	flunitrazepam	0,01 - therap.conc.
	152	negative	negative	bromazepam	0,13 - therap.conc
	43	negative	negative	diazepam	0,12 - therap.conc.
	43	negative	negative	nordiazepam	0,17 - therap.conc.
	76	negative	negative	bromazepam	0,10 - therap.conc.
B	17	7-aminoflunitrazepam	negative	flunitrazepam	0,13 - toxic conc.
	86	negative	negative	clonazepam	0,42 - toxic.conc
	33	negative	midazolam	midazolam	0,60 - therap.-toxic.
	33	negative	midazolam	clonazepam	0,11- toxic.conc.
	134	ACB, MACB	negative	diazepam nordiazepam flunitrazepam	0.06 - therap.conc. 0.07 - therap.conc. 0,06 - toxic.conc
	188	7-aminoclonazepam	negative	clonazepam	0,14 - toxic.conc.
	188	α-hydroxyalprazolam	negative	alprazolam	0,05 - terap.conc.

U – Urine, GaC U – Urine, GaC – Gastric contens, B – Blood, ACB – 2-amino-5-chlorbenzofenone, MACB – 2-methylamino-5-chlorbenzofenone

in this way. Immunochemical screening of urine should be always followed by identification of BZD and their metabolites in urine or better in both urine and gastric contents using e.g. TLC, GC-MS and subsequent BZD confirmation/quantitation in blood, for example using GC-ECD.

Only complex toxicological analysis of biological material (urine, gastric contents and blood) using immunochemical as well as chromatographical methods permits toxicological laboratories to give valid interpretation of results, thus contributing to objective evaluation of the level of intoxication in forensic as well as clinical practice.

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L22

ARACHIDONIC ACID DERIVATIVES IN THE EXHALED BREATH CONDENSATE IN PNEUMOCONIOSES AND THEIR CORRELATION WITH INDIVIDUAL FACTORS

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Key words: leukotrienes, asbestos, silica, breath condensate

Introduction

Asbestosis is an incurable interstitial lung disease caused by the inhalation of asbestos fibers. It is characterized by inflammation and pulmonary fibrosis. It has unpredictable, but mostly progressive development. Additionally, asbestos exposure leads to an increased risk of mesothelioma and lung cancer. There are no markers available to measure the activity and prognosis, and there is no effective treatment for asbestosis[1]. Similarly, silica after chronic inhalation leads to another interstitial lung fibrosis, silicosis. Accordingly, no markers for monitoring underlying events are available and no causal treatment is known. The mechanisms of fibrogenic dusts-induced disease development and progression are not well understood**.** High cytotoxicity and long pulmonary retention of these fibrogenic dusts are major factors responsible for their long-term effects. Defining the mechanisms triggered by exposure to asbestos and silica could facilitate early diagnosis by identifying markers of early diseases and could elucidate novel therapeutic strategies.

Surface radicals on fibrogenic particles and iron-derived reactive oxygen species are considered to be among the key events in the development of fibrosis and lung cancer. After reaching the alveoli, fibrogenic dust particles are phagocytosed by pulmonary macrophages, which become activated and release reactive oxygen intermediates, chemokines, cytokines and arachidonic acid metabolites, such as leukotrienes (LTs) and isoprostanes[1]. We recently described increased 8-izoprostane in exhaled breath condensate, both in patients with asbestos[2] and silica exposure[3].

LTs are pro-inflammatory and pro-fibrogenic mediators derived from the 5-lipoxygenase pathway of arachidonic acid metabolism. LT B_4 is recognized as a potent leukocyte chemoattractant and activator; cysteinyl LTs (C_4, D_4, and E_4), in contrast, are best known for their role in the pathogenesis of asthma.

It has been reported, however, that patients with idiopathic pulmonary fibrosis exhibit overproduction of both LT B_4 and cysteinyl LTs in the lungs[4]. Cysteinyl LTs play an important role in the inflammation and remodeling of the airways. It was recently found that human bronchial fibroblasts may also generate both cysteinyl LTs and LT B_4 (ref.[5]). LTs participate in the pathogenesis of lung fibrosis, and they may do so by direct effects on migration, proliferation and matrix protein synthesis by fibroblasts, as well as by indirect effects, such as suppression of lung mononuclear production of antifibrogenic cytokines[6]. LT B_4 is known to rise during chronic obstructive pulmonary disease exacerbations[7]. Additionally, the influence of recent smoking, another source of reactive oxygen species, on the concentrations of LT B_4 in the exhaled breath condensate has been described[8].

Exhaled breath condensate examination appears to be a simple and non-invasive method for studying lower respiratory tract events *in vivo*[9]. It contains several biomarkers of inflammation and oxidative stress, and collection can be repeated several times without any adverse effects, even in severely ill patients.

Methods

LTs B_4, C_4, D_4, and E_4 were measured in exhaled breath condensate in subjects who came to the Department of Occupational Medicine for regular follow-up due to their past exposure to asbestos or silica.

Ninety-two subjects with previous exposure to asbestos for 24±2 years (mean age 69±2 years) in three asbestos manufacturing plants, mostly in the production of asbestos insulation and textile materials, asbestos-cement roofing, and pipe, were examined. Control group consisted from 46 subjects (mean age 65±3 years). Classification of the occupational cumulative asbestos fiber dust dose in three fiber-year classes yielded the following distribution: 58 (nearly 63 %) subjects were classified in fiber-year class III (100 and more fiber-years), 18 (20 %) in fiber class II (25–99 fiber-years) and 16 (17 %) in fiber class I (< 25 fiber-years). A fiber-year is defined as the cumulative asbestos dust dose in the workplace of $1 \cdot 10^6$ [(fiber m^{-3})× years]. Exposure estimation was based on measurements available since 1949, according to Hagemeyer et al[10].

Sixty subjects with silicosis and mean exposure (24±3 years) to silica were examined (mean age 67±2 years). The control group had 25 subjects, with mean age 65±5

years. There were 37 patients with simple silicosis and 23 patients with complicated silicosis according to ILO classification of radiographs[11]. Exposure was evaluated as medium or high based on the evaluation of the industrial hygienist (considering SiO_2 concentration, duration, and technical equipment).

Subjects of both control groups were employed as hospital technical workers without occupational exposure to fibrogenic dusts. They did not differ from the subjects of the exposed groups in age, sex, level of education and factors potentially influencing oxidative stress, such as smoking, cigarette pack years and alcohol consumption.

Exhaled breath condensate samples were collected using the EcoScreen (Jaeger, Germany); contamination of saliva in the exhaled breath condensate was excluded by colorimetric detection of α-amylase (α-Amylase-Liquid BIO-LA-TEST kit, Pliva-Lachema, Czech Republic). LTs were analyzed by method combining pretreatment method immunoextraction mediated by resin with anchored antibody against LTs and highly sensitive and selective detection method LC-ESI-MS in MRM mode. Quantification was performed using a stable-isotope-dilution assay. LOD, LOQ and recovery for a particular analyte were as follows: LT B4 – LOD = 1 pg ml^{-1}; LOQ = 4 pg ml^{-1}; recovery = 46 %; LT C4 – LOD = 2 pg ml^{-1}; LOQ = 16 pg ml^{-1}; recovery = 55 %; LT D4 – LOD = 1 pg ml^{-1}; LOQ = 6 pg ml^{-1}; recovery = 61 %; LT E4 – LOD = 1 pg ml^{-1}; LOQ = 5 pg ml^{-1}; recovery = 81 %. Cotinine in the urine was determined by gas chromatography/mass spectrometry (SIM mode) after extraction with ethyl acetate at pH 10 (LOQ 50 ng ml^{-1}, linearity fulfilled in the range 50–1000 ng ml^{-1} with the correlation coefficient 0.9994). LTs were also compared with individual factors, such as age, gender, smoking and alcohol consumption, respiratory and systemic diseases.

Student's t-test (for equal variances and for equal means), F-test, ANOVA, Chi^2 and linear regression (correlation coefficient) methods were used for the statistical analysis.

Results

Values of LTs are shown in fig. 1 and 2. As can be seen, LT D_4 was significantly higher in subjects exposed to asbestos compared to the control group (P=0.044). LTs D_4 and E_4 were associated with pleural plaques (P=0.0031 a P=0.0000), and LT E_4 borderline correlated with fiber-years (P=0.0503). An association of the fiber-years category (high, middle, low) was seen for crepitation (P=0.000039), chronic cough (P=0.00066) and dyspnoea (P=0.0001). LT D_4 and E_4 were positively associated with the presence of pleural plaques (P=0.0031, P=0.0000, respectively), LT E_4 also with fiber-years (P=0.0503) and latency since first exposure (P=0.0018).

LT D_4 was also significantly increased in the silicotics vs. the controls (P=0.001). The LT B_4 level in the silicotics correlated negatively with α1-antitrypsine concentration (P=0.024). LT E_4 was associated with the category: simple, complicated silicosis or controls (P=0.0022), level of silica exposure (P=0.0080) and cough (P=0.0015). LT C_4 was not significantly different in silicotics and controls; and it was positively associated with chronic obstructive pulmonary disease (P=0.0405) and correlated positively with urine cotinine concentration (P=0.046).

Only very low probabilities of correlations were recorded between LTs and smoking, daily alcohol consumption, daily vitamins intake, body mass index, lung functions, kidney functions, history of allergic diseases, pleuritis, pneumonia, tuberculosis, kidney, liver, gall bladder disease, hypertension, diabetes, and cerebral stroke or cerebral injury.

Discussion

Among LTs, only LT D4 in exhaled breath condensate was increased in the subjects of both exposed groups, and LT E4 has shown the association with cumulative exposure to both fibrogenic dusts. These finding are in agreement with experimental studies and are biologically plausible given that fibrogenic particles persist in the lungs for decades. In rats, silicotic nodules continued to grow even after inhalational exposure to silica was discontinued[12]. Progressive fibrosis is also true in patients with

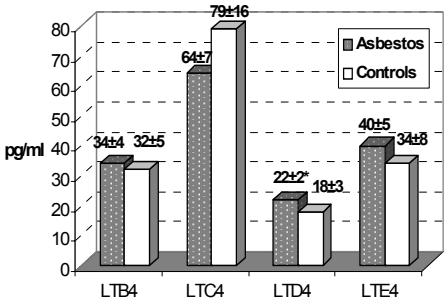

Fig. 1. **Leukotrienes in asbestos-exposed and controls;** *P<0.05

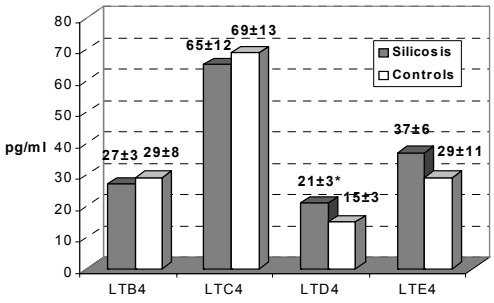

Fig. 2. **Leukotrienes in silicotics and controls;** *P<0.05

chronic silicosis and asbestosis. It thus appears plausible that LTs are increased and could point to the continuing process of fibrosis. Smoking and alcohol consumption have been described to increase markers of inflammation and oxidative stress in blood [13,14]. However, in this study, using exhaled breath condensate, this effect was not seen.

These results support the hypothesis that asbestos and silica are the main cause of increased LT D4 in exhaled breath condensate in our patients. However, additional factors influencing the balance in antioxidant/oxidant status probably play contributing roles. Even if the clinical significance of increased LT D4 in the exhaled breath condensate is not yet sufficiently clear, fibrogenic dusts appear to be the main factors, in addition LT E4 positively correlated with fibrogenic dusts exposure level.

Conclusion

This study suggests that exhaled breath condensate analysis of LTs is useful in fibrogenic dust exposed subjects, especially LTD_4 and LTE_4. LT D_4 was higher both in the subjects with asbestos exposure and in the silicotics and might point to the activity of the fibrogenic process; on the other hand LT E_4 correlated with the level of exposure – asbestos fiber-years and category of silica exposure. On the other hand, LT C_4 correlated with chronic obstructive pulmonary disease. No correlation with daily alcohol consumption, cigarettes/day or systemic diseases was found for LTD_4 and LTE_4, which supports the theory that analysis of exhaled breath condensate reflects solely processes ongoing in the lungs.

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L23

EPIDEMIOLOGY OF ADDICTIVE SUBSTANCES: COMPARISON OF CZECH AND ITALIAN UNIVERSITY STUDENTS´ EXPERIENCIES

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Key words: addictive substances usage, university students, Italy, Czech Republic

Introduction and aim of the survey

This work is a component part of a long-lasting monitoring of students drug usage realized at our faculty. Legal and illegal substances usage can considerably affect young people life. Recent researches point up to stagnation of problematic drug users in contrast to a group of occasional experimenters whose numbers have been increasing[1,2].

Our survey gathers data about addictive drugs attitudes and experiences of students of the University Hradec Kralove (UHK) and Tomas Bata University in Zlin (UTB) in the Czech Republic and of the University of Camerino (UC) in Italy. Aim of this work is to maintain the continuity of data collection and their update regarding the problems of university students' addictive drugs usage as well as their extension to international level.

Material and methods

The monitored population comprises 805 participants: 305 students of UC (45.6 % males, 54.4 % females); 270 respondents from the UHK (28.5 % males, 71.5 % females) and 231 students of UTB (43.7 % males, 56.3 % females) in the Czech Republic.

The average age of Hradec Kralove´s students was 21.2±1.9 years, of Zlin´s students 20.7±1.9 years and of the Italians 23.2±3.1 years.

Data collection was carried out via questionnaires completed by students themselves, it was effectuated during the academic year 2005/2006 and it was absolutely anonymous and voluntary. The questionnaire was comprised of a socio-demographical part, of a part regarding students´ attitudes and experiences with legal and further with illegal substances and finally of a part mapping prevalence of infection diseases allied to addictive substance usage (viral hepatitis and HIV infection). Mainly close questions were attached by several half-open and open ones.

Data evaluation was realized via Microsoft Excel and statistical analysis was carried out via chi-square test.

Results

Socio-demographical part

Students from Hradec Kralove and Zlin were mainly of the Czech nationality (95.0 %), students from Camerino mainly of the Italian nationality (83.9 %). There were 50.9 % Czech and 48.5 % Italian subjects coming from a town with more than 10 000 inhabitants.

Czech students live rather together with parents whereas Italian live alone (P<0.05). This matter can be influenced both by the higher average age of Italian students and by the diverse temper of either nation. Accordingly one tenth of Czech (10.2 %) as well as Italian (10.5 %) students live together with a person using illegal addictive substances.

Parents of Czech participants (60.9 % of their fathers and 61.1 % of their mothers) achieved mostly secondary education, either apprenticeship or finished with leaving examination; analogical Italian education was achieved by 39.6 % of fathers and 41.6 % of mothers.

Legal substances experience

The participants smoked their first cigarette in average age of 13.8±2.9 years (the Czechs), respectively in 15.5±1.8 years in average (the Italians). Smoking attitudes are recorded in Table I.

It was evidenced via chi-square test that there were significantly more non-smokers among Czech students and significantly more regular smokers among the Italians (P<0.05).

The average age of first experience with alcohol was stated by the Czechs in average age of 12.4±2.8 years, by

Table I
Smoking

Smoking	The Italians [%]	The Czechs [%]
Never	46.7	57.7
Ex-smoker	5.9	7.0
Rarely	9.5	12.0
Sometimes	8.9	11.8
Regularly	28.2	11.4

Table II
Alcohol drinking

	The Italians [%]	The Czechs [%]
Abstainer	15.1	2.2
Only rarely	34.1	28.5
Sever times per month	24.3	47.7
Sever times per week	20.0	19.6
Every day	3.6	1.4

the Italians in 15.4±2.0 years in average. Frequency of alcohol drinking is figured in Table II.

Over again the significant differences between these two groups have been found. There were more abstainers and rare drinkers among Italian students in contrast to Czech ones who rather consume alcohol sever times per month (P<0.05).

Further we have dealt with opinions concerning harmfulness of various substances. Students assessed coffee drinking harmless/little harmful (CZE 66.9 %, ITA 79.7 %), alcohol harmful when it is used for a long time (CZE 75.0 %, ITA 62.3 %) and tobacco smoking either harmful when it is used for a long time (CZE 54.7 %, ITA 41.3 %) or very dangerous (CZE 41.1 %, ITA 55.4 %). After the statistic evaluation we have found out that Italian students considered coffee less harmful than Czech (P<0.05) in contrary to tobacco and alcohol which were judged very dangerous by more Italian (27.5 % vs. 14.6 %) (P<0.05).

Illegal substances experience

Accordingly the respondents had to judge harmfulness of various illegal addictive drugs. Particularly we were interested in their appreciation of „drugs which are smoked“ in comparison with their appreciation of tobacco harmfulness. Let's point out that roughly the same percentage of Czech students consider both tobacco and drug smoking harmful when they are used for a long time (54.7 % vs. 54.3 %) but less of them consider drug smoking very dangerous (in comparison with tobacco: 41.1 % vs. 35.9 %). Unlike Italian reckon drug smoking for harmful when they are used for a long time less times than tobacco smoking (30.8 % vs. 41.3 %) and likewise more of them reckon drug smoking for very dangerous (in comparison with tobacco, 59.0 % vs. 55.4 %). This different attitude to substances harmfulness between nations was verified as significant (P<0.05). This is a warning finding which indicates cannabis underrating by Czech young. It is a question if it was promoted by previous repressive political approaches and deterrent theories of some politicians and drug „experts“ in media which could act oppositely: maybe the young „experimenters“ have realized that THC usage do not take the disastrous consequences so that they can take the cannabis usage warnings easy.

The participants usually consider themselves well (CZE 41.1 %, ITA 31.1 %) or partially well informed (CZE 47.7 %, ITA 57.7 %) about drugs related issues. But while they had to state at least one institution or organization helping people with problems related to addictive drugs, only 27.8 % of Italian in comparison with 63.1 % of Czech students were able to enunciate it (P<0.05). This fact can figure possible drug prevention goals.

As for the attitudes to legalization of so called "soft" drugs 4.6 % of the Czechs and 7.8 % of the Italians preferred their absolute legalization, 43.9 % Czech and 35.4 % Italian (P<0.05) students gave preference to free usage but controlled distribution, 26.5 % of the Czechs and 9.2 % of the Italians (P<0.05) prioritised free usage and punishable distribution and finally 23.0 % of the Czechs and 42.3 % of the Italians (P<0.05) were in favour of punishable both usage and distribution. Again the higher Czech benevolence to cannabis substances (e.g. "soft" drugs, "drugs which are smoked") became evident.

Drug classification to „soft“ and „hard“ was used to risk perception assessment. Marihuana and hashish, which risk level is relatively low, are considered to be "soft" drugs[3]. The differences between both nations were confirmed by the chi square test: Italian students are not acquainted with methamphetamine because its "role" as a stimulant is in Italy substituted by cocaine[1] (P<0.05) which was in contrary incorrectly classified by more Czechs (P<0.05); the Italians are not very well informed of solvents abuse risk (P<0.05) but on the other hand they have classified cannabis, LSD, amphetamines, ecstasy, psychedelic mushrooms and methadone among "hard drugs" often than the Czechs (P<0.05). These findings can be interpreted as a result of possible international dissimilarities in drug preventive programmes, history, religion attitudes, political approaches etc.

Concerning personal experiences with addictive drugs, an experience with some substance was found out by 61.7 % Czech and 51.1 % Italian participants (P<0.05). Cannabis drugs clearly predominate among experienced substances, followed by psychedelic mushrooms in the Czech Republic and cocaine in Italy. Experiences with illegal substances are summarized in Table III.

The average age of an illegal substance first experiment was nearly the same between both nations: CZE 16.5±1.5 years and ITA 17.0±1.7 years. The substance mostly used for the first time was marijuana again (CZE: 72.5 % of drug-experienced subjects, ITA: 58.7 % of drug-experienced subjects). The most stated circumstances of first substance use were "with friends" (CZE 27.5 %, ITA 16.7 %) or a party, a celebration (CZE 8.4 %, ITA 14.7 %). Last month prevalence was found out at 10.8 % of Italian and 14.2 % of Czech students and the most used substance was cannabis again. Although some authors claim[4,5], that number of regular cannabis Czech users is smaller than in some other European countries, in our survey the number of students with a 20-fold cannabis experience were similar (CZE 19.2 %, ITA 22.3 %).

Drug procuring is considered to very difficult by only

Table III
Experience with illegal substances

Substance	The Italians [%]	The Czechs [%]
Totally	51.1	61.7
Marijuana+hashish	46.9	59.5
Cocaine	13.1	3.6
Psychedelic mushrooms	7.9	17.6
Ecstasy	6.2	9.8
LSD	3.9	8.0
Methamphetamine	1.3	5.2
Speed, amphetamine	5.2	3.6
Solvents	0.7	4.6
Heroine	1.0	0.8
Methadone	0.7	0.0

0.6 % Czech and 2.6 % Italian students. Negative influence of drug usage was observed only by 3.6 % of Czech and 9.0 % of Italian university students; these data reflect either just usual recreational drug usage among students either fact that many students did not response these questions. Accordingly only few participants had to affront problems with police in relevance with drug usage (4.2 % CZE, 6.4 % ITA).

Discussion

Our results confirm that cannabis is the illegal substance most commonly used in all countries of the European Union (EU), with many countries reporting lifetime experience of the drug by more than 20 % of the general population. The use of cannabis is increasing, mainly among young adults[1,6,7]. The usage extent among males is higher than among females[1], what is confirmed also by our survey: 56.5 % Italian and 69.7 % Czech males have used cannabis whereas "only" 39.2 % Italian and 55.1 % Czech females have tried it (P<0.05). The cannabis usage is to a great extent occasional, or it is quit beyond a certain period[1]. Our study was carried out among young adults and that is one of the reasons, why we have found out such huge lifetime prevalence. As well we can expect that the students of medical sciences can show a higher tendency to drug experiments because of supposed "medicine-wise" and "hardihood".

The population investigations show that amphetamines are the second abused substances next to the cannabis in the EU[1]. The life-time prevalence (LTP) of amphetamines moves between 2 % and 11 % among young adults (15-34 years old). In Italy, the use of amphetamines and ecstasy among young adults is under 1 %, but the cocaine use nears to 2 %. The cocaine usage is abandoned in the age of young adulthood or it is occasional. Its use was confirmed by 5.4 % subjects (15-44 years) in Italy in 2003 (LTP) and 3.4 % of subjects of general population interviewed in 2003 admitted to having used amphetamine derivates at least once in their life[8]. We have evidenced rather higher numbers in Italy: the amphetamines´ LTP of 8.5 % and the cocaine's LTP of 13.4 %. The situation in the Czech Republic differs because of the methamphetamine ("pervitin") spread which substitutes cocaine[1]. Some authors claim that its prevalence among university students rises up to 4.1 % (ref.[2]), in our study there were 5.2 % of students experienced with "pervitin". The experience with cocaine among university students in the Czech Republic is 2.8 % (ref.[2]), in our survey there were 3.6 % of cocaine-experienced participants. As for the other substance quite often used by Czech subjects, psychedelic mushrooms, its prevalence varies around 9 % among Czech students in general[2], what is significantly lower number (P<0.05) than in our study (17.6 %).

The general population studies indicate that the perception of risk relating to the influence of illegal substances use can affect their consumption. Obviously for that specific population segment that considers drugs harmless, the risk of being exposed is much greater[8]. We have confirmed distorted perception of cannabis by the students: this drug is subconsciously classified as a legal one; we presume it so because of 19.1 % Czech and 38.5 % Italian (P<0.05) experienced students who have skipped over the questions determined for subjects with drug experience. The spread of cannabis is associated with a generalized underestimation of actual health risks and psycho-behaviour consequences. Subjects not perceiving cannabis use as dangerous are four times more likely to use it over those who perceive the risk[8]. This hypothesis was supported by our findings which have disclosed experienced students´ opinion tending to the cannabis legalization rather than among inexperienced subjects (P<0.05).

Conclusions

Our results confirm the accretive experimental usage of cannabis drugs among European young people and as well they reflect specifics of drug scenes of both countries.

The young people in Europe have similar experiences with legal and illegal substances; the founded differences can be caused by social, historical and political factors etc.

This survey has contributed to data mapping in the field of drug related problems and it has brought a new comparison of students' attitudes and experiences in the European Union.

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L24
SELENIUM – PROTECTION AND RISK

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Key words: selenium, DDD, NOAEL, antioxidant, toxicity, deficiency

Introduction

Selenium is an essential trace element. It is an integral part of enzymes, which are critical for control of the numerous chemical reactions involved in brain and body functions. Selenium has a variety of functions. The main one is its role as an antioxidant in the enzyme selenium-glutathione-peroxidase. This enzyme neutralizes hydrogen peroxide, which is produced by some cell processes and would otherwise damage cell membranes. Selenium also seems to stimulate antibody formation in response to vaccines. It also may provide protection from the toxic effects of heavy metals and other substances. Selenium may assist in the synthesis of protein, in growth and development, and in fertility, especially in men. It has been shown to improve the production of sperm and sperm motility[1,2].

Selenium forms in foods

Foods contain a number of different selenium forms. In animal foods, there are specific selenium proteins where selenium is incorporated via selenide as selenocysteine, while selenomethionine, and possibly also selenocysteine to some extent, are non-specifically incorporated as analogues to methionine and cysteine in foods both of animal and plant origin[1,3]. Selenomethionine, as well as the inorganic forms selenite and selenate, are the most common forms in food supplements and fodder additives. In addition to these forms a number of uncharacterized forms exit, e.g. in fish (Åkesson and Srikumar), but their contribution to total dietary selenium is unknown (SCF). Fish, shellfish, red meat, grains, eggs, chicken, liver, and garlic are all good sources of selenium. The amount of selenium in vegetables is dependent on the selenium content of the soil. Brewer's yeast and wheat germ, both considered "health foods," are also good sources of selenium[2,3].

Bioavailability of different forms of selenium

Most forms of selenium salts organic bound selenium, i.e. selenomethionine and selenocysteine, are easily absorbed from the gastrointestinal tract. Only a few studies on the bioavailability of selenium have been performed in humans. Selenium in blood or serum is most effectively raised by selenium-rich wheat or yeast selenium (the latter may vary in quality), probably because of non-specific incorporation of selenomethionine into proteins. Inorganic selenium as selenate and selenite can be incorporated specifically into selenium proteins via selenide as selenocysteine and increase seleno-enzyme activity until saturation.

Selenium consumed from fish had no apparent effect on the amount of selenium incorporated into functional selenoproteins and a low effect on general level of selenium in plasma. Given different bioavailabilities and differences in non-specific incorporation of selenium compounds from different sources such as cereals, meat, fish and organic and inorganic supplements, the selenium concentration in whole blood will relate differently to the total intake of selenium[4].

Deficiency

Selenium deficiency is relatively rare in healthy well-nourished individuals. It can occur in patients with severely compromised intestinal function, or those undergoing total parenteral nutrition. Alternatively, people dependent on food grown from selenium-deficient soil are also at risk.

Selenium deficiency can lead to Keshan disease, which is potentially fatal. Selenium deficiency also contributes (along with iodine deficiency) to Kashin-Beck disease. The primary symptom of Keshan disease is myocardial necrosis, leading to weakening of the heart. Kashin-Beck disease results in atrophy, degeneration and necrosis of cartilage tissue. Keshan disease also makes the body more susceptible to illness caused by other nutritional, biochemical, or infectious diseases. These diseases are most common in certain parts of China where the soil is extremely deficient in selenium[5].

Selenium is also necessary for the conversion of the thyroid hormone thyroxine (T4) into its more active counterpart, triiodothyronine, and as such a deficiency can cause symptoms of hypothyroidism, including extreme fatigue, mental slowing, cretinism and recurrent miscarriage[3].

The amount of selenium available in the soil for plant growth and corresponding variations in the intake of selenium by humans varies considerably among regions and countries. In most European countries the mean intake levels are much lower, in the lower range of 30–90 μg Se day^{-1}, except for Finland, that has a somewhat higher mean intake (60 μg Se day^{-1}) due to import of wheat rich in selenium. The margin between the present mean intake, excluding supplements, in the European adult population

and an UL (adult) of 300 μg Se day^{-1} would be between 2, 7 to 10. The mean intakes of non-vegetarian adults in different studies are Belgium 28–61 μg day^{-1}, Denmark 41–57 μg day^{-1}, Finland 100–110 μg day^{-1}, France 29–43 μg day^{-1}, United Kingdom 63 μg day^{-1}, Norway 28–89 μg day^{-1}, Spain 79 μg day^{-1}, Sweden 24–35 μg day^{-1}, and Czech 37 μg day^{-1} (ref.[1,6–9]).

States of mild to moderate Se deficiency have been proposed as contributing to a large number of conditions, such as cardiovascular disease, infertility, ageing, eye disease, diabetic retinopathy and cancer. In none of these has firm link been established, although the potential involvement of a mild impairment in antioxidant activity with reduced glutathione peroxidase levels cannot be discounted. Thus, low Se states have been associated with platelet aggregability and prospective studies have also suggested an increased risk of stroke and coronary heart disease in subject with low serum Se (ref.[10]).

Toxicity

Although selenium is an essential trace element it is toxic if taken in excess. Exceeding the Tolerable Upper Intake Level of 400 micrograms per day can lead to selenosis. Symptoms of selenosis include a garlic odour on the breath, gastrointestinal disorders, and hair loss, sloughing of nails, fatigue, irritability and neurological damage. Extreme cases of selenosis can result in cirrhosis of the liver, pulmonary edema and death.

Elemental selenium and most metallic selenides have relatively low toxicities because of their low bioavailability. By contrast, selenate and selenite are very toxic, and have modes of action similar to that of arsenic. Hydrogen selenide is an extremely toxic, corrosive gas. Selenium also occurs in organic compounds such as dimethyl selenide, selenomethionine and selenocysteine, all of which have high bioavailability and are toxic in large doses.

Selenium poisoning of water systems may result whenever new agricultural runoff courses through normally-dry undeveloped lands. This process leaches natural soluble selenium compounds (such as selenates) into the water, which may then be concentrated in new "wetlands" as it evaporates. High selenium levels produced in this fashion have been found to have caused certain congenital disorders in wetland birds[11,12].

Daily requirements

The amount of dietary selenium (as DL-selenomethionine) required to saturate the selenium need of extracellular GSHPx was used as one of the approaches to define a Dietary Reference Intake for Selenium in the USA in 2000 (55 μg day^{-1} for adult men and women) (NAS). A so-called Population Reference Intake of 55 mg selenium per day for adults, but also other levels of intakes based on other criteria, was established by the Scientific Committee for Food of the European Commission.

A joint FAO/IAEA/WHO Expert Consultation (WHO) gave several modes for the calculation of requirements of the individual and populations. For a 65 kg reference man the average normative requirement of individuals for selenium was estimated to be 26 μg day^{-1}, and lower limit of the need was estimated to be 40 μg day^{-1}. For a 55 kg reference woman were 21 and 30 mg selenium day^{-1}, respectively. The latter value was estimated to increase to 39 μg day^{-1} throughout pregnancy and to attain the values of 42, 46 and 52 μg selenium day^{-1} at 0–3 and 6–12 months of lactation, respectively[1,2,4,12]. In Czech Republic the daily recommendation intake are for organic form of selenium compounds 40–70 μg for male and 45–55 μg for female, 75 μg for lactation and 30 μg, 20 μg, 10–20 μg for 7–10, 4–6 and 1–3 years old children. For inorganic form of selenium compounds RDI are 1.5–2.5 mg for adolescents and adults and 0.4–1mg and 1–1.5 mg for children in age 1–3 and 4–10 years[13].

Our study

In the project there were involved 933 healthy volunteers of the Czech Republic Rescue Fire brigades from selected areas of the Czech Republic. The level of selenium in serum was determined by atomic – absorption spectrophotometer method (AAS Unicam, GB). Serum was mineralized in microwave system (milestone, Italy) provided selenium analysis. In the monitored group, mean serum concentration of selenium was 0.67–0.93 μmol l^{-1} (52.86–73.36 μg l^{-1}) – Praha (0.87 μmol l^{-1}), Beroun (0.67 μmol l^{-1}), Kroměříž (0.93 μmol l^{-1}), Klatovy (0.73 μmol l^{-1}), Ústí nad Labem (0.78 μmol l^{-1}) and Nový Jičín (0.89 μmol l^{-1}), which are incommensurate reference range 1.0–1.9 μmol l^{-1} (fig. 1). The results of the study contribute to information about normal values of serum concentration in the Czech population. The level of selenium was in area of deficiency (Table I). In monitored group, the average serum concentration of selenium shoved a tendency towards lover values of so-called

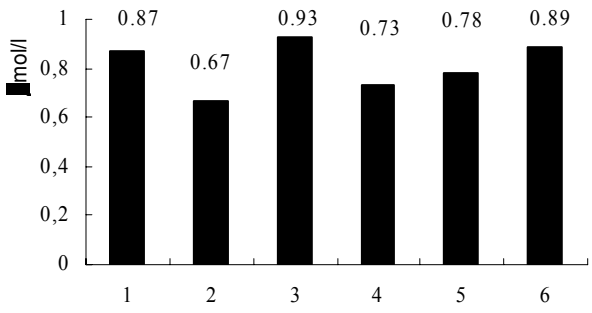

Fig. 1. **The level of selenium in serum in monitored groups;** (1 μmo l^{-1} ~ 78.89 mg l^{-1}), 1 – Praha, 2 – Beroun, 3 – Kroměříž, 4 – Klatovy, 5 – Ústí nad Labem, 6 – Nový Jičín

Table I
Evaluation of serum selenium concentrations in inhabitants of CZ

Evaluation	Se in serum [mg l^{-1}]	Frequency [%]
Pharmacol. level	>140	0.1
Optimal level	100–140	0.7
Marginal deficiency	70–100	8.6
Mild deficiency	55–70	25.6
Deficiency	45–55	28
Severe deficiency	<45	37

physiological range, however, it corresponded with other findings whiting the Czech population[7,8]. No statistically significant relations among the age of examined subject and their serum selenium concentrations were proved. There was found only the tendency of selenium in serum to increase to higher weight, but no statistically significant of these changes was proved.

Conclusion

Selenium is a trace mineral that is essential to good health but required only in small amounts. Selenium is incorporated into proteins to make selenoproteins, which are important antioxidant enzymes. The antioxidant properties of selenoproteins help prevent cellular damage from free radicals. The content of selenium in food depends on the selenium content of the soil where plants are grown or animals are raised. There is evidence that selenium deficiency may contribute to development of a form of heart disease, hypothyroidism, and a weakened immune system. On the other hand Observational studies indicate that death from cancer, including lung, colorectal, and prostate cancers, is lower among people with higher blood levels or intake of selenium. Selenium occurs in staple foods such as corn, wheat, and soybean as selenomethionine, the organic selenium analogue of the amino acid methionine. Selenomethionine can be incorporated into body proteins in place of methionine, and serves as a vehicle for selenium storage in organs and tissues. Selenium supplements may also contain sodium selenite and sodium selenate, two inorganic forms of selenium. Selenomethionine is generally considered to be the best absorbed and utilized form of selenium.

The need of individual nutrients for human organism was and is verified in still new connections in many metabolic studies in people, in experimental work on animals and also in clinical and epidemiological monitoring. This need is quantified in the final phase in the form of reference intake values of individual nutrients that are the basis for quality assessment of our food and our nutrition.

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L25
CZECH UNIVERSITY STUDENTS AND LEGAL ADDICTIVE SUBSTANCES

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Introduction

The use of illegal drugs is a great social, health, economic, legal and politic problem both worldwide and in the Czech Republic. Alcohol together with tobacco belongs to the group of legal addictive substances. However, negative impacts of their consumption on human health have been known for a long time. Alcohol is causally related to more than 60 different medical conditions and 4 % of the global burden of disease is attributable to alcohol[1]. Smoking of tobacco is the main cause of lung cancer[2], leads to the development of 20 different diseases and to the early deaths[3].

The attitudes of adult Czech population toward the use of legal addictive substances (drugs) may be characterised as tolerant or benevolent and their consumption is relatively high in the Czech community. In 2005, average statistical consumption of 100% spirit was 10.2 litters/year per person (including infants) in the Czech Republic[4], which is one of the highest consumption in Europe[5]. 2,275 cigarettes were consumed in the Czech Republic in 2005 (ref.[4]). 26.3 % and 4.1 % of Czech population (age 15–64) were regular and occasional tobacco smokers, respectively[6].

Such atmosphere leads to relatively high and hazardous consumption of legal drugs in the sample of adolescents. According to data from The European School Survey Project on Alcohol and Other Drugs 2003 (ESPAD), the proportion of students who had been drunk during the last 12 months was higher (68 %) than the European average (53 %). 18 % and 13 % of students (age 16–17) had used excessive amount of alcohol and had been drunk at least three times during the last month, respectively. 43 % of students (age 16–17) had smoked in the last month, European average is 35 %. In comparison with year 1995, increased of daily smokers among students (aged 16–17) was observed (from 23 % to 27 % in 2003)[7].

Most surveys on drug use have been restricted to elementary and secondary school students (e.g. ESPAD). However, research has revealed that some university students drink excessive amounts of alcohol and use other addictive substances. Alcohol abuse and dependence was identified in 14 % of Belgium university students[8]. 15 % of drinking university students in the UK (ref.[9]) reported to drink alcohol at the hazardous level. According to Kypri et al., the majority of drinking tertiary students in New Zealand (62 % of men and 48 % of women) reported hazardous or harmful alcohol use[10]. Heavy alcohol use was also found in US and Canadian students with prevalence rates 33 % and 30 %, respectively[11]. In 1995–1999, 11 % of university students in the Czech Republic were abstainers[12]. The survey conducted in the population of Prague (capital city of the Czech Republic) university students revealed that 14 % of students consumed alcohol in a hazardous way in 2003 (ref.[13]).

Smoking of tobacco is not so extended as alcohol consumption in the population of university students. 26 % of male and 25 % of female students in the UK reported current regular smoking of tobacco[9]. 29 % of Croatian medical students[14], 24 % of university students in Brasil[15] and 18 % of university students in Papua New Guinea[16] are regular tobacco smokers. In 1995–1999, 5 % and 17 % of Czech university students were regular and occasional tobacco smokers, respectively[12]. In 2003, percentage of regular smokers among Prague university students was higher – 22 % (ref.[13]).

Our survey was concerned on legal drug use among university students in the Czech Republic in years 2002–2006. Its general aim was to estimate the prevalence rates and examine changing patterns of legal drug use among these students, and to compare results with previous local, multi-regional and international studies among university students.

Methods

The questionnaire-based survey was performed in academic years 2002/3–2005/6 at thirteen faculties of eight universities in seven regions of the Czech Republic. Anonymous standardized questionnaires were personally administered to students during their registration at the beginning of the academic year or during seminars with obligatory participation of students. The questionnaire included questions on the use of alcohol, tobacco and illegal drugs of abuse, general socio-demographic data (e.g. age, sex, residence, financial means), knowledge and attitudes of students towards drugs of abuse.

For the assessment of alcohol intake, students were asked about frequencies of beer, wine and spirits use, the age of the first contact with alcoholic beverages, the age of the first drunkenness and the frequency of drunkenness in the last month. The consumption of any sort of alcoholic beverage at least once a week was stated as the indicator of regular alcohol consumption. Repeated drunkenness (i.e. at least two or three episodes of drunkenness per month) was used as the indicator of hazardous alcohol drinking. For

the assessment of tobacco smoking, students were asked about their current smoking status, the number of cigarettes smoked per week and the age of the first contact with tobacco smoking.

The results are presented as descriptive statistics. Associations between variables were analysed by chi-square test.

Results

Characteristics of the group

A total number of 3,484 university students completed and returned the distributed questionnaires. Average age of students was 20.8 years (range: 18–48). The number of females was higher (73 %) than that of males. For details see Table I.

Alcohol use

Alcohol was the most frequently used addictive substance among university students. 44.1 % of students reported regular consumption of any sort of alcoholic beverage (from 35.1 % of students at the Faculty of Medicine and Dentistry, Palacky University in Olomouc to 65.4 % of students at the Faculty of Architecture, Technical University of Liberec). On average, 30.6 %, 20.8 % and 6.9 % of students reported regular consumption of beer, wine and spirits, respectively. Hazardous alcohol drinking was reported by 14.1 % of university students (from 31.6 % of students at the Skoda Auto University to 65.4 % of students at the Faculty of Architecture, Technical University of Liberec). For details see Table II.

Average age of the first contact with alcoholic beverages and average age of the first drunkenness were 13.1 years (range: 1–24) and 16.4 years (range: 2–25), respectively. Almost 60 % of students reported being drunk for the first time before the age of 18 (legal age limit of alcohol consumption in the Czech Republic).

Tobacco smoking

8.6 % of students were regular tobacco smokers (from 3.8 % of students at the Faculty of Architecture, Technical University of Liberec to 16.9 % of students at the Mathematical Institute, Silesian University in Opava). More than 140 pieces of cigarettes per week was smoked by 2.0 % of regular smokers, most of them (59.7 %) smoked 11–70 pieces of cigarettes per week. 20.2 % of students were occasional tobacco smokers (from 11.9 % of students at the Mathematical Institute, Silesian University in Opava to 26.9 % of students at the Faculty of Architecture, Technical University of Liberec). Occasional smokers smoked usually 1–10 pieces of cigarettes per week. For details see Table III.

Table I
Characteristics of university students

Faculty	University	Abbreviation	Males [%]	Females [%]	Number of students	Average age
Faculty of Pharmacy	Charles University in Prague	FaFUK	16.6	83.3	1338	20.3
3rd Faculty of Medicine	Charles University in Prague	MFUK	42.9	56.5	147	22.5
Faculty of Pharmacy	University of Veterinary and Pharmaceutical Sciences Brno	FaFVFU	12.9	87.1	379	20.2
Faculty of Architecture	Technical University of Liberec	FATUL	46.2	53.8	26	20.0
Faculty of Economics	Technical University of Liberec	FHTUL	35.0	65.0	140	19.7
Faculty of Mechatronics	Technical University of Liberec	FMTUL	90.0	10.0	100	19.8
Faculty of Education	Technical University of Liberec	FPTUL	33.7	66.3	486	21.8
Faculty of Textile Engineering	Technical University of Liberec	FTTUL	19.9	80.1	267	20.6
Faculty of Medicine and Dentistry	Palacky University in Olomouc	MFUP	27.9	72.1	111	21.9
Faculty of Economics and Administration	University of Pardubice	FESUP	36.1	63.9	108	21.2
Faculty of Business Management and Administration	Skoda Auto University	SkodaAuto	64.4	35.6	118	20.6
Mathematical Institute	Silesian University in Opava	MISU	45.8	54.2	59	21.5
Faculty of Education	University of Hradec Kralove	PdFUHK	28.8	71.2	205	21.4
Total			26.8	73.1	3484	20.8

Table II
Prevalence of non-drinking, regular and hazardous drinking of alcohol in university students

Faculty	Regular drinking***	Hazardous drinking***	Non-drinking***
FATUL	65.4	3.8	0.0
FMTUL	61.0	18.0	5.0
SkodaAuto	56.8	31.6	1.7
MFUK	54.4	29.3	6.8
FHTUL	47.9	18.6	6.4
MISU	47.5	25.4	3.4
FESUP	46.3	17.6	5.6
FaFVFU	45.0	12.5	6.4
FPTUL	44.7	12.3	5.3
FaFUK	41.3	11.4	4.2
FTTUL	40.1	11.2	3.0
PdFUHK	40.0	9.3	1.0
MFUP	35.1	21.6	10.8
Total	44.1	14.1	4.7

*** $P<0.001$, chi-square test. For abbreviations see Table I

Table III
Prevalence of non-smoking, occasional and regular tobacco smoking in university students

Faculty	Non-smoking [%] ***	Occasional smoking [%] ***	Regular smoking [%] ***
MISU	71.2	11.9	16.9
MFUK	63.9	21.1	15.0
SkodaAuto	68.6	17.8	13.6
FMTUL	65.0	22.0	13.0
MFUP	67.6	19.8	12.6
FPTUL	67.5	20.4	12.1
FESUP	76.9	13.0	10.2
FTTUL	65.2	24.7	10.1
FHTUL	69.3	22.1	8.6
FaFVFU	71.5	21.1	7.4
FaFUK	75.2	19.2	5.6
PdFUHK	71.7	23.4	4.9
FATUL	69.2	26.9	3.8
Total	71.2	20.2	8.6

*** $P<0.001$, chi-square test. For abbreviations see Table I

Average age of the first contact with tobacco smoking was 14.2 years (range: 2–26).

Associations

Sex differences in use of legal drugs were found, both regular and hazardous drinking patterns as well as tobacco smoking were reported by higher proportion of males than females ($P<0.001$, chi-square test).

There was a statistically significant association between the financial situation of students (i.e. financial means per week) and use of legal drugs. Increasing amount of financial means per week was related to the increasing proportion of regular and hazardous drinking and regular tobacco smoking ($P<0.001$, chi-square test).

Discussion

44.1 % and 14.1 % of university students reported regular and hazardous alcohol drinking, respectively. Such situation is compatible with the state of alcohol use in the Czech Republic. Consumption of alcohol in the Czech Republic belongs to the highest both worldwide and in Europe[5]; more over 33 % men and 14 % women in the Czech Republic (age 18–64) consumed alcohol in a way hazardous for health[17]. High and hazardous consumption of alcohol was also found in the population of university students both in the Czech Republic[12,13] and worldwide[8-12]. However, adequate comparison of our findings with those from other studies is problematic mainly because of different measures of alcohol intake, different definitions of hazardous alcohol drinking and differences in the age distribution across samples.

8.6 % and 20.2 % of university students were regular and occasional tobacco smokers, respectively. In comparison with the surveys in the population of Czech high school students (27 % of regular smokers in 2003, ref.[7]) and in the general Czech population between the ages 15 and 24 years (29.9 % of regular smokers in 2005, ref.[6]), the percentage of regular smokers among university students was lower. This situation can be explained by the long-term observed trend in the Czech Republic, that the highest percentage of regular smokers is in the group of persons with primary or incomplete secondary education[6]. However, the part of the current occasional tobacco smokers among university students meets the criteria of regular daily smoking; they smoke more than 10 pieces of cigarettes per week and can become regular smokers in the future.

Findings of both, significant sex differences and associations between financial situation of students and their use of legal addictive substances are consistent with earlier reports on alcohol and drug use in university students[9,12,13].

The reliability and accuracy of the data can be subjected to discussion. We suppose that voluntary, anonymous and confidential principle of our survey improved reliability of students' responses. Furthermore, question-

naires were personally administered to the students during situation with their obligatory participation and completed by almost all students.

Conclusion

Hazardous alcohol drinking and tobacco smoking is strongly associated with a wide range of negative health and psychological consequences[1-3]. Our study among university students in the Czech Republic provides worrying data mainly about their alcohol consumption and supports the need of continuous education also in university students to advise them on the risks of drug and alcohol misuse.

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P01

EFFECT OF BENZO[*A*]PYRENE TREATMENT ON HEPATIC CYTOCHROME P450 AND NADPH:CYTOCHROME P450 REDUCTASE (POR) LEVELS IN MICE WITH LIVER-SPECIFIC DELETION OF THE *POR* GENE

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) including benzo[*a*]pyrene (BaP) are produced by incomplete combustion or pyrolysis of organic matter[1,2]. A number of them, such as BaP, are mutagenic and carcinogenic[1,3–5]. PAHs are ubiquitous in the environment, leading to measurable background levels of exposure in the general population[1]. Beside the inhalation of polluted air, the main routes of exposure are through tobacco smoke, diet[1,3,4] and occupational exposition throughout e.g. coal, coke or coal-tar processing and use of coal-tar products[1].

Before the reaction with DNA, BaP requires metabolic activation, which is an essential step in the mechanism by which BaP exerts its genotoxic effects. The typical 3-step activation process with contribution of CYP1A1 or CYP1B1 and epoxide hydrolase leads to the formation of the ultimately reactive species, benzo[*a*]pyrene-7,8--dihydrodiol-9,10-epoxide (BPDE) that can react with DNA, forming adducts preferentially at guanine residues[2]. CYP1A1 and CYP1B1 are widely accepted as the most important enzymes in the metabolic activation of BaP[2]; however current studies show that BaP-induced genotoxicity (DNA adduct formation) was increased in mice lacking CYP1A1, indicating that *in vivo* the role of CYP1A1 is in detoxification and protection against BaP toxicity[6,7].

PAHs affect the expression of numerous enzymes involved in metabolism of xenobiotics (including CYP1A1) mainly *via* the aromatic hydrocarbon receptor (AhR). AhR-dependent inducibility was correlated to the predisposion to some types of cancer[8,9].The detailed knowledge in enzymes participating on PAHs activation and detoxication as well as in PAHs-mediated enzyme induction could make possible to modify the susceptibility of humans to the carcinogenic action of PAHs found in the environment.

Materials and methods

HRN™ ($Por^{lox/lox}$ + Cre^{ALB}) mice on a C57BL/6 background (CXR Bioscience Ltd., Dundee, UK) used in this study were derived as described[10]. Mice homozygous for loxP sites at the *Por* locus ($Por^{lox/lox}$) were used as wild-type (WT). Groups (n = 3) of female HRN™ and WT mice (3 months old, 25–30 g) were treated with 125 mg kg^{-1} body weight of BaP once daily up to five days by i.p. injection. Animals were sacrificed 24 hours after last injection. Pooled hepatic microsomes from HRN™ and WT mice untreated or treated with BaP were isolated as described[11]. The activities of hepatic microsomal CYP1A1/2 (7-ethoxyresorufin *O*-deethylation, EROD) and POR (using cytochrome *c* as a substrate) as well as the protein levels of these enzymes (Western Blot) were determined using ordinary methods[12].

Results and discussion

Constitutive CYP1A and POR levels in livers of HRN™ mice

The HRN™ (Hepatic Cytochrome P450 Reductase Null) mouse line with conditionally deleted cytochrome P450 oxidoreductase (POR) specifically in the liver[10] is in present a remarkable *in vivo* model to evaluate the role of hepatic *versus* extra-hepatic drug metabolism and its pharmacokinetics. The deletion of POR as the unique electron donor to CYP enzymes results in the loss of essentially all hepatic CYP function[10]. As shown by immunoanalyses,

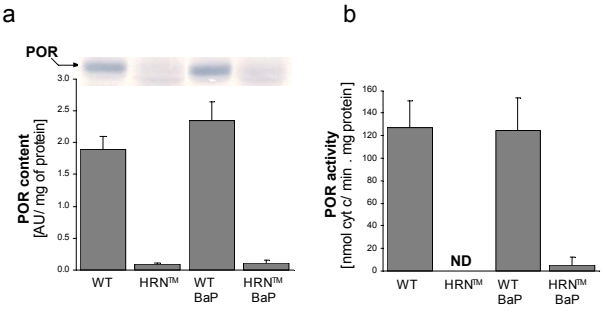

Fig. 1. **POR protein levels (a) and activity (b) in hepatic microsomes of HRN™ and WT mice, untreated or treated with BaP.** Inset in a): immunoblots of microsomal POR from all mice groups, stained with chicken polyclonal antibody against rabbit POR. Hepatic microsomal samples were pooled from 3 animals, values are given as means ± SD (n = 3)

the POR protein expression in HRN™ mouse liver is negligible as expected, but still detectable under the conditions used in the experiments (fig. 1a). CYP1A1 and CYP1A2 enzymes are constitutively expressed in livers of HRN™ and WT mice, having 1.23-fold higher protein levels found in HRN™ mice (fig. 2a). The POR-dependent reduction of cytochrome *c* (fig. 1b) as well as the CYP1A- and POR-dependent EROD activity (fig. 2b) in liver microsomes of HRN™ mice was under the detection limit of the used method. All results mentioned above are in agreement with previously published studies[10].

Effects of BaP treatment on CYP1A and POR levels

As expected, treatment by BaP caused pronounced induction of CYP1A protein expression, more remarkable in the case of HRN™ mice: a 9- and 17-fold increase in CYP1A protein levels was found in consequence of BaP-treatment in microsomal fractions of WT and HRN™ mice, respectively (fig. 2a).

Surprisingly, in addition to CYP1A enzymes, expression of hepatic POR was also increased in both mice after BaP treatment (fig. 1a); we found a 1.2- and 1.4-fold increase in hepatic POR expression in WT and HRN™ mice treated with BaP, respectively. In the case of BaP-treated HRN™ mice, the increase of POR protein levels is accompanied by an increase in POR specific activity towards cytochrome *c*.

In spite of POR deficiency, we detected relatively high EROD activity in liver microsomes from HRN™ mice treated with BaP, representing 73-fold increase towards activity in uninduced WT mice. As a result, this CYP1A- and also POR-dependent activity in HRN™ mice treated with BaP approach (representing more than 30 %) to the activity in BaP-induced WT mice (fig. 2b).

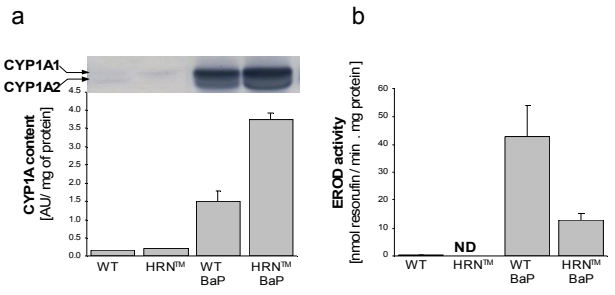

Fig. 2. **CYP1A protein levels (a) and EROD activity (b) in hepatic microsomes of HRN™ and WT mice, untreated or treated with BaP.** Inset in a): immunoblot of microsomal CYP1A from all mice groups, stained with chicken polyclonal antibody against rat recombinant CYP1A1. Hepatic microsomal samples were pooled from 3 animals, values are given as means ± SD (n = 3)

Conclusion

The findings presented in this study suggest that in animals with conditionally deleted hepatic POR, the repeated treatment with BaP and maybe some other CYP- and POR-inducers can result in almost normal CYP-dependent activity.

Abbreviations

HRN™	Hepatic Cytochrome P450 Reductase Null
CYP	cytochrome P450
POR	NADPH:cytochrome P450 reductase
BaP	benzo[*a*]pyrene
PAHs	polycyclic aromatic hydrocarbons
EROD	7-ethoxyresorufin *O*-deethylation
WT	wild-type

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P02

FREQUENCY OF MICRONUCLEI IN ALVEOLAR MACROPHAGES OF RATS AFTER EXPOSURE TO INDUSTRIAL MINERAL FIBRES DUSTS AND IN COMBINATION WITH CIGARETTE SMOKE

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Key words: alveolar macrophages, micronuclei, fibres dusts, cigarette smoke

Introduction

The influence of industry fibrous dust on respiratory system is a permanently topical problem. Asbestos is a naturally occurring fibrous silicate mineral that has been mined for its useful properties such as thermal insulation, chemical and thermal stability, and high tensile strength. Amosite asbestos is classified as carcinogenic to humans and animals[1,2] and it is suggested to be genotoxic, causing DNA damage and chromosomal aberrations in *in vitro* systems[3]. Previous studies with living cells have shown that asbestos causes specific mitotic disturbances and, as a result of such effects, micronucleus formation[4,5]. Man-made mineral fibres (MMMFs) are widely used at different worksites. MMMF products can release airborne respirable fibres during their production, use and removal. MMMFs have some physical similarities to asbestos; in particular, their fibrous character gives them similar aerodynamic properties and leads to their deposition throughout the respiratory tract. The potency of the induced adverse effects depends on biopersistence of the fibres.

The micronucleus (MN) assay is a widely used short-term screening test for determining the induction of spindle or chromosome damage by estimating the frequency of MN. MN are formed spontaneously or in response to the genotoxic exposure from acentric chromosomal fragments or from whole chromosomes that are excluded from the daughter nuclei following cell division. The MN test has been extensively used in rodents for detecting potentially carcinogenic agents.

Methods

We studied the DNA damage in alveolar macrophages of Albino Wistar rats by micronucleus test after exposure to selected types of mineral fibres – amosite (asbestos, A), glass (GF) and refractory ceramic fibres (RCF).

In the time dependence experiment we studied changes in MN frequency in the dependence on the length of exposure. Groups of 3 male rats were intratracheally instilled with fibres (2 × 2 mg fibres animal^{-1}); control groups (C) were treated in the same way using the saline solution (0.2 ml animal^{-1}). The animals were sacrificed 48 hours, 1 month and 3 months after the last instillation. The lung was removed, the macrophages were isolated by bronchoalveolar lavage (BAL)[6] and they were diluted to the density of 10^5 cells ml^{-1} PBS. A volume of 250 μl of the cell suspension was dropped onto microscopic slides using a cytocentrifuge (6 min/1400 rpm) and air dried. After 24 h the slides were fixed with methanol and stained with 5 % Giemsa-Romanowski for 10 min. In every animal group 18,000 cells were evaluated for MN frequency[7].

In the combined exposure experiment we studied the effects of the intratracheal exposure to fibres (2 × 2 mg fibres animal^{-1}) combined with repeated inhalation exposure to cigarette smoke (85 mg of total particulate matter/m^3 air for two hours daily) on changes in MN frequency. Six groups of 3 male rats were treated as follows: *1*) intratracheally instilled by saline solution (2 × 0.2 ml animal^{-1}); *2*) intratracheally instilled by A; *3*) intratracheally instilled by RCF; *4*) exposed only to cigarette smoke (S); *5*) exposed to A+S; *6*) exposed to RCF+S. After 6 months the animals were sacrificed and the BAL was performed. Microscopic slides we prepared and MN frequency was evaluated by the same way as in the first experiment.

Statistics: The Kruskal-Wallis test for comparison of two independent samples and the Friedman test for comparison of paired samples at the significance level α=0.05 was used for statistical analysis.

Results and discussion

The time dependence experiment: Compared to control animals we observed statistically significant (P=0.05) differences in the frequency of MN only in animals sacrificed 48 hours after the last instillation of asbestos or glass fibres but not at later intervals, nor in animals instilled by refractory ceramic fibres. The results of the influence of length of exposure on the frequency of MN are summarized in Table I.

The combined exposure experiment: Compared to the control animals we observed a statistically significant (P=0.05) difference in the frequency of MN in macrophages of animals exposed to asbestos, to cigarette smoke alone, as well as in those after combined exposure to mineral fibres and cigarette smoke. We have not found a difference in the frequency of MN in animals exposed to refractory ceramic fibres alone, as compared to the control.

Table I
The influence of length of exposure on MN frequency

Length of exposure	Exposure	Number of animals	Number of analysed cells	Number of MN	MN/1000 cells
48 hours	Control	3	18000	6	0,33
48 hours	Amosite	3	18000	18 [a]	1,00 [a]
48 hours	Glass fibres	3	18000	16 [a]	0,89 [a]
48 hours	RCF	3	18000	12	0,67
1 month	Control	3	18000	11	0,61
1 month	Amosite	3	18000	17	0,94
1 month	Glass fibres	3	18000	10	0,56
1 month	RCF	3	18000	3	0,17
3 months	Control	3	18000	8	0,44
3 months	Amosite	3	18000	21	1,17
3 months	Glass fibres	3	18000	12	0,67
3 months	RCF	3	18000	24	1,33

[a] P=0.05 compared to the control

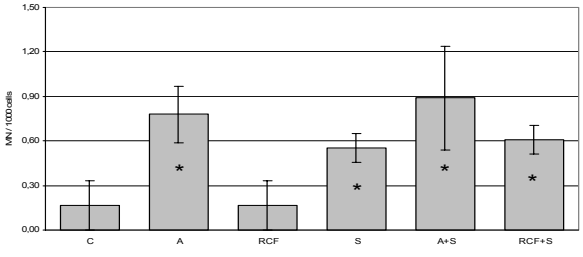

Fig. 1. **The influence of combined exposure on MN frequency;** **P*=0.05 compared to the control. The error bars represent S.D.

There were statistically significant differences in the frequency of MN between animals exposed to amosite and refractory ceramic fibres, as well as between those exposed to cigarette smoke alone and RCF alone. The results of the combined exposure experiment are illustrated in fig. 1.

Conclusion

Two experiments evaluating the genotoxic damage of fibrous dusts were made in rats. The time dependence experiment showed at 48 hours a statistically significant chromosomal i.e. genotoxic damage after i.t. instillation of 2 × 2 mg amosite or glass fibres. The combined exposure experiment showed that a repeated cigarette smoke exposure for 6 months can cause statistically significant additive chromosomal damage to that caused by amosite and it can turn the non-significant response to refractory ceramic fibres (RCF) to a significant damage in rats receiving these fibres intratracheally in a similar dose 2 × 2 mg per animal. The exposure to amosite or RCF can add genotoxic damage to animals exposed only to cigarette smoke. Generally, the RCF tended to show less genotoxicity than amosite asbestos.

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P03
INCIDENCE OF FATAL BENZODIAZEPINE INTOXICATIONS IN BRNO REGION

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Introduction

Benzodiazepines have been one of the largest, most important and most frequently used groups of substances in therapy for several decades. Their number and a variety of effects make them currently an indispensable group of drugs. They appear daily in medical prescriptions, which also implies the negative effect of rather frequent incorrect prescription, or not quite precise observance of maximum doses, routes of administration, or respect for undesirable side-effects, in particular contraindications (especially with regard to the age of patients).

Benzodiazepines are also one of the most commonly used and misused psychopharmaceuticals. They are abused mostly in conjunction with other substances (e.g. with alcohol, etc.) as a supplementary drug. Such use is rather dangerous since benzodiazepines, if used with another inhibitory substance, may cause death. After their long-term use, the withdrawal condition in overcoming dependence may also be fatal[1].

In the literature on forensic toxicology, where substances are assessed in terms of their potential to cause intoxication or death, benzodiazepines are placed in group No. 34, subgroup 3412-nonbarbiturate hypnotics within the framework of classification of poisons (according to Gonzales)[2].

The objective of this study was to make an attempt at a statistical view on the incidence of benzodiazepines as the cause of fatal intoxications, namely during the °10-year period in the region of Brno.

Methods

In order to process results for a period of 10 years it was necessary to go through more than 13 000 records of toxicological examinations and select from them only those in which the lethal level of the relevant substance resulted in death, i.e. cases in which the death was due to the intoxication by the relevant substance or a combination of substances.

Results

In 1994–2003, there were 613 cases, 194 females and 419 males, meeting the criterion of fatal poisoning in the catchment area of the Institute of Forensic Medicine in Brno. Benzodiazepines were involved in 131 cases. In individual years the contribution of benzodiazepines to the total number of fatal intoxications was uniform, higher number in 2002 corresponds to the higher overall number of intoxications in the given year (see fig. 1). In the total number of fatal intoxications caused by benzodiazepines, the proportion of both sexes is highly balanced – 65 cases in females and 66 in males (see fig. 2). The tendency to combined intoxications was relatively small (13 cases out of 131 – i.e. slightly less than 10 %) – most frequently in conjunction with alcohol or antidepressants (see fig. 3). The average age of the dead was 45.2 years.

In the field of prevention of the abuse of benzodiazepines in general, some measures have already been taken by competent authorities. For example, the legislative measure adopted a few years ago allows to dispense flunitrazepam (Rohypnol) and buprenorphine (Subutex) only on the so-called "opioid prescription", i.e. medical prescription with a blue stripe.

In complications occurring during the substitute treatment of heroin abuse, the deaths caused by combining buprenorphine (Subutex) with benzodiazepines (e.g. toxicological examination of urine for the detection of benzodiazepines is suitable) were descried[3].

The above mentioned legislative measure will not only make it more difficult for the unauthorized persons to obtain a prescription or to fake it, but it also shortens its validity from 7 to 5 days and requires the above mentioned

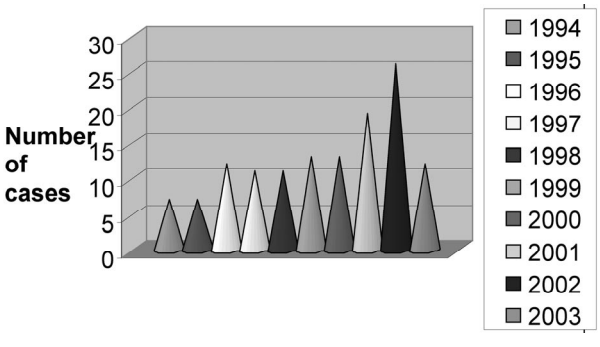

Fig. 1. **Number of fatal benzodiazepine intoxications in 1994–2003;** in 1994 – 7 cases; in 1995 – 7 cases, in 1996 – 12 cases, in 1997 – 11 cases, in 1998 – 11 cases, in 1999 – 13 cases, in 2000 – 13 cases, in 2001 – 19 cases, in 2002 – 26 cases, and in 2003 – 12 cases

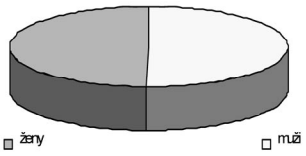

Fig. 2. **The proportion of men and women in the incidence of fatal benzodiazepine intoxications in 1994–2003;** 1 – men: 66 cases, 2 – women: 65 cases

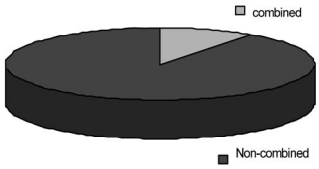

Fig. 3. **Proportion of combined and non-combined benzodiazepine intoxications in 1994–2003;** 1– non-combined intoxications – 13 cases, 2 – combined intoxications – 118 cases

drugs to be kept in a safe, imposing on pharmacies an obligation to record and keep prescriptions with a blue stripe for 5 years. In addition, the stock receipt in pharmacies is subject to stricter rules and records. The measure in fact does not decrease the drugs availability to the patients to which they are prescribed but it will reduce the possibility of obtaining them illegally, lowering the possibility of abusing them.

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P04

EFFECT OF THE NEWLY SYNTHESIZED COMPOUND 44BU ON ACONITINE INDUCED CARDIOTOXICITY – COMPARED WITH LIDOCAINE AND PROPAPHENONE

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Key words: experimental toxicology, aconitine mortality, lidocaine, propaphenone, ventricular fibrillation

Introduction

The newly developed compound hydrochloride of 2-hydroxy-3-(butylaminopropyl-4-{(butoxycarbonyl) amino} benzoic acid (draft name 44Bu) is an original compound that was synthesized by the staff at our Faculty of Pharmacy[1]. No pharmaceutical company has taken part in its development. Aconitine is a neurotoxin that causes several changes in biophysical properties of voltage-gated sodium channels in excitabile tissues. The inhibiton of inactivation of sodium channels result in their persistent opening and thus in permanent depolarization of membranes[2]. Thus, it is not surprising that life-threatening cardiac arrhythmias are reported under the effect of aconitine[3]. This type of arrhythmia is considered to be quite resistant to antiarrhythmic drugs[4]. No specific antidote has been found yet and treatment of the aconitine intoxication is just supportive. The aim of this work was to compare the antiarrhythmic effect of 44Bu compound with two antiarrhythmics with rapid initiation of action – lidocaine (class 1b antiarrhythmic drug) and propaphenone (class 1c) in experiment *in vivo* on male Wistar laboratory rats.

Materials and methods

Induction of total anaesthesia and aconitine intoxication

The animals were anaesthetised by i.m. administration of 1% ketamine (Narkamonâ inj.) and 2% xylazine (Rometarâ inj.) in a dose 5 ml kg^{-1}. Intoxication was induced by aconitine administering into the uncovered *vena jugularis* at a dose of 30 μg kg^{-1}. Tested compounds (44Bu or lidocaine or propaphenone were administered at the same way in a dose 1.5 mg kg^{-1} 40 sec after aconitine. Control animals obtained only aconitine.

Tested groups and numbers of animal: Control group (n=16); 44Bu compound (n=12); lidocaine (n=8); propaphenone (n=7).

Monitored parameters

Animals were monitored continuously on a Seiva Praktik ECG machine (SEIVA, CR) for 15 min. Each experimental group evaluated on:

1. the percentage of occurrence of the given types of arrhythmia
2. the overall mortality of the animals
3. the change in the width of the QRS complex in the course of the intoxication

Statistical analysis

Changes in the width of the QRS complex and the changes were analysed using the statistical package Unistat 5.1. Data with homogeneous variances were subjected to a one-way ANOVA and subsequently to the Tukey-HSD test. Data with heterogeneous variances were subjected to the Kruskal-Wallis ANOVA. The statistical significance of the frequency of occurrence of the individual types of arrhythmias in the groups was assessed using the χ^2 (chi square) test of two variables.

Results

Percentage of occurrence of the given types of arrhythmias

In the control group the signs of increased atrial and ventricular excitability arose after the administration of aconitine. Excitability disorders were accompanied by a blockade of sinoatrial and atrioventricular (AV) conduction of the 1st degree and later the 2nd degree AV block. These disorders appeared in very rapid sequence.

After administration of tested compounds the occurrence of ventricular fibrillation significantly decreased. After 44Bu administration from 94 % to 8 %, after lidocaine administration from 94 % to 50 % and after propaphenone administration from 94 % to 29 %. Statistical differences of the percentage of the occurrence of the certain types of arrhythmia against control group are given in Table I. 44Bu was significantly more efficient in suppressing ventricular fibrillation (P<0.05) than lidocaine and propaphenone. Effect of the lidocaine and propaphenone is the same.

Table I
Percentage of occurrence of the given types of arrhythmia

Type of arrhythmia		Control [%] *n*=16	44Bu [%] *n*=12	Lidocaine [%] *n*=8	Propaphenone [%] *n*=7
SVPB	(supraventricular premature beats)	69	67	38	43
AF	(atrial fibrillation)	50	75	50	43
VPB-DS	(ventricular premature beats – discrete or in salvos)	94	75	100	57
Bi-Tri	(bigeminies, trigeminies)	63	67	88	71
VT	(ventricular tachycardia)	100	83	63 **	57 **
VF	(ventricular fibrillation)	94	8 **□ ◆	50 *	29 *
BL	(AV-blockade of conduction)	75	67	88	0
Mortality		100	25 **	63 **	29 **

100 % = total number of experimental animals in the given group (*n*), * = significancy of all groups vs. control group; □ = significancy of 44Bu group vs. Lidocaine group, ◆ = significancy of 44Bu group vs. Propaphenone group; 2× sign. = $P<0.01$; 1× sign. = $P<0.05$

The overall mortality of the animals

The most frequently animals died of ventricular fibrillation. Another reason of death was the total blockade of impulse conduction. In the control group all animals died. After administration of 44Bu compound the overall mortality of the animals dropped from 100 % to 25 %, after administration of lidocaine from 100 % to 63 % and after propaphenone administration from 100 % to 29 %. All these decreases were analysed using the χ^2 (chi square) test as highly statistical significant against control group (see Table I). The average survival time in the control group K was 5.1 min. The average survival time of the dead animals was 3 min longer after 44Bu administration. After lidocaine administration this survival period increased to 14 min and after propaphenone administration to 7.45 min.

Change in the width of the QRS complex in the course of the intoxication

QRS complex was significantly widened due to aconitine administration and reached up to 230 % of its initial value. After the 44Bu administration of aconitine marked widening of the QRS complex occurred also (maximum 218 % of the initial value). Nevertheless, there was a gradual retreat to the initial value on the contrary to the control group. Regarding the QRS width changes, lidocaine and propaphenone seemed to be more effective directly after the administration while the effect of 44Bu appeared later. At the end of experiment, the values of the QRS complex width converged in both tested groups. After lidocaine administration the QRS complex width ranged from 100 % to 146 % of the initial value, after propaphenone administration from 98 to 112 % of the initial value.

Discussion and conclusion

Treatment of the aconitine-induced ventricular arrhythmias is just supportive in clinical practice because no specific antidote has been found yet. Propaphenone or lidocaine (previously) are frequently chosen for treatment. As discussed above 44Bu seemed to be more efficient in suppressing the aconitine-induced arrhythmias in comparison with both standard antiarrhythmic drugs. All tested compounds statistically highly significant decreased the overall mortality of animals intoxicated by aconitine.

Suggested experimental results confirmed that 44Bu compound is more efficient in suppressing aconitine-induced ventricular arrhythmias than lidocaine and propaphenone.

The underlying cause of this antiarrhythmic effect is the ability of 44Bu to completely block the depolarization fast sodium current I_{Na} (100% blockade). This compound also has pronounced blocking effect on the repolarization outward potassium current Ito (50% blockade) *in vitro*[5].

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P05

MONITORING OF PRO-INFLAMMATORY CYTOKINES IN THE DEVELOPMENT OF ADJUVANT ARTHRITIS

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Key words: adjuvant arthritis, IL-1α, IL-4, TNF-α, MCP-1, IL-6, pro-inflammatory cytokines, flowcytometry

Introduction

Clinically and histologically, adjuvant-induced arthritis (AIA) is comparable to human rheumatoid arthritis (RA), which is assumed to be autoimmune in nature and initiated by autoimmune CD4+T cells. Relevant data indicate that many immune-mediated diseases (especially rheumatic diseases) display abnormal regulation of several cytokines. These pathological events are generally manifested either by defective production of immunosuppressive factors or by overproduction of pro-inflammatory cytokines. Cytokines are involved as main mediators of inflammation in rheumatoid arthritis (RA). T_H2 cytokines (e.g. IL-4 and IL-10) possess protective properties, while T_H1 cytokines (such as IL-2 and IFN-γ) support the pro-inflammatory local microenvironment in joints from patients with RA. RA patients showed a more general increase in pathognomic cytokines, with much higher levels of IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-13, IFN-γ, G-CSF, GM-CSF, MCP-1, and MIP-1beta compared with controls[1]. Experimental *in vitro* and *in vivo* investigations in a mouse model have reported that TNF-α, IL-12, IL-15 and IL-18 participate in the pathogenesis of erosive inflammatory arthritis[2]. In the present study, plasmatic levels of relevant cytokines were determined in a rat model of RA.

Material and methods

Adjuvant arthritis was induced by an intradermal injection of *Mycobacterium butyricum (MB)* in incomplete Freund´s adjuvant to Lewis rats. The blood for cytokine analysis was withdrawn from the retroorbital plexus before MB injection (controls) and on monitored experimental days 14, 21, and 28. On these days also the main disease parameters − change of body mass (CBM) and hind paw volume (HPV) were measured. CBM was calculated as the difference of body weight measured on the given days and the body weight measured at the beginning of the experiment. The HPV increase was calculated as the percentage increase of HPV on the given days in comparison to the beginning of the experiment. A multiplex fluorescent bead immunoassay for quantitative detection of rat IL-1α, IL-4, TNF-α and MCP-1 was done by immunoflowcytometry (rat Cytokine Flowcytomix, Bender MedSystems). For IL-6 evaluation ELISA determination was used (rat IL-6 Quantikine®, R&D Systems). The data for all parameters were expressed as arithmetic mean and SD and/or SEM. For significance calculations ANOVA test was used with $*P<0.05$ (significant);$**P<0.01$ (very significant); $***P<0.001$ (extremely significant). The data from different days were compared with those from the beginning of the experiment.

Results and discussion

Several experimentally induced rat and mouse models of autoimmune erosive arthritis are currently widely used to provide insight into etiopathogenetic mechanisms of RA. An intradermal injection, at the base of the tail, with

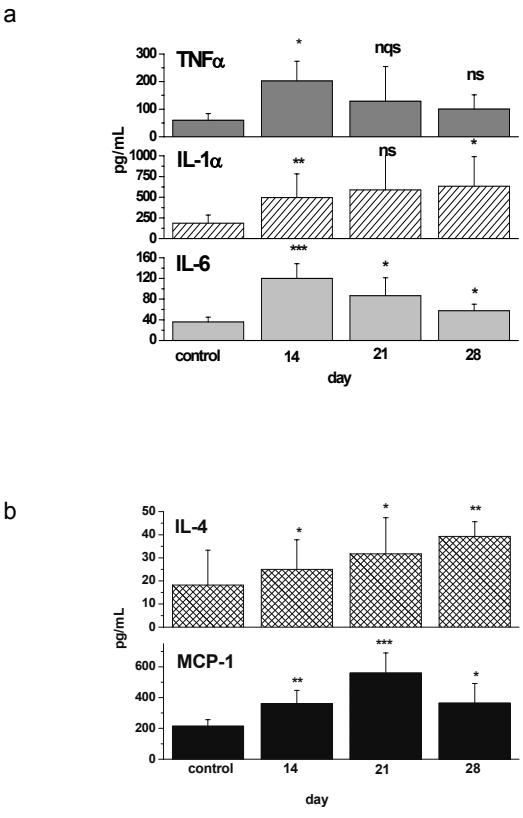
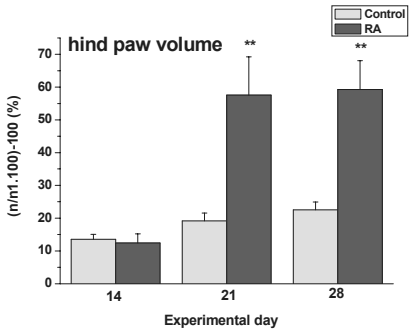


Fig. 1. **Time profiles of measured cytokines;** (mean ± standard deviation)

a



b

Fig. 2. **Time profiles of main disease parameters,** (mean ± standard error)

heat-killed MB in incomplete Freund´s adjuvant results in destructive arthritis within 14 days in susceptible Lewis inbred rat strains. Increased synthesis of tumor necrosis factor α (TNF-α), interleukin 1 (IL-1α) and interleukin 6 (IL-6) was detected as early as day four after adjuvant injection[3]. In the present experiment the most significant reactive increase of pro-inflammatory cytokines TNF-α (P<0.05), IL-1α (P<0.01) and IL-6 (P<0.001) was observed on the 14th experimental day, followed by statistically significant increase of monocyte chemoattractant protein 1 (MCP-1) on the 21st experimental day (P<0.001), expressed in a response to previously induced pro-inflammatory cytokines TNF-α, IL-1α and IL-6. On the 28th day the most significant increase of pro-T_H2 interleukin IL-4 was observed (P<0.01). The maximal plasmatic peak of IL-4 was accompanied by selective decrease of pro-inflammatory cytokines TNF-α, IL-1α, IL-6, as a result of inhibitory action of IL-4. All observed significant changes in cytokines levels (excluding IL-4 and MCP-1) occurred prior to the significant changes of disease parameters, i.e. CBM and HPV (fig. 1, 2).For these parameters the differences were most pronounced on days 21 and 28. This observation was made both in this experiment and in our previous experiments with AA (ref.[4,5]). We conclude that monitoring of clinical parameters and plasmatic levels of TNF-α, IL-1 α, IL-4, IL-6 and MCP-1 may be a very useful tool for exact study of new anti-rheumatics, including anti-cytokines. Understanding of cytokines regulation in RA could ultimately lead to novel and specific treatments. Moreover, cytokines concentrations are good indicators of the development of RA and may contribute to the understanding of RA pathogenesis.

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P06

THE EFFECT OF DELTAMETHRIN ON ACTIVITY OF GLUTATHIONE *S*-TRANSFERASE OF COMMON CARP (*Cyprinus carpio)*

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Key words: fish, pyrethroides, Decis, glutathione *S*-transferase

Introduction

Pollution of aquatic environments by chemicals is widerspread, possibly affecting the biological integrity of ecosystems as well as the physiological functions of individual organisms. Exposure of aquatic organisms to pollutants has been typically determined by measuring external level of contaminants in the surrounding water or sediments, but this determination is considered insufficient in order to establish the quality of these ecosystems, and it is often unable to predict toxicological consequences. To clearly assess the quality of the aquatic environment, the impact of contaminants in terms of biochemical response reflecting their potential for impairment of physiological processes in different organisms has been examined. Research on effective biomarkers is a very relevant point of interest[1]. They are many different parameters that can be used as markers of environmental contamination, the presence of an inductory or inhibitory process of phase I (monooxygenases) and II (conjugation) biotransformation enzymes could be an effective and sensitive biomarker of aquatic pollution[2,3].

The glutathione *S*-transferases (GST), the major phase II xenobiotic-metabolizing enzymes, constitute a multifunctional family of dimeric biontransformation enzymes that are involved in the detoxification and excretion of physiological and xenobiotic substance. The enzymes function by catalyzing the nucleophilic addition of the triol of reduced glutathione (GSH) to electrophilic center in organic compounds. The final GSH-conjugates have increased hydrophilicity, which facilitates their further metabolism and elimination[4]. The GST enzymes, mainly located in the cytosolic fraction of the liver, may have a great importance in order to determine their potential use as biomarkers in different protocols of ecotoxicology to assess chemical pollution of the environment[5]. These enzymes can help to indicate the exposure of aquatic organisms in the wild to xenobiotic compounds and to evaluate the degree and risk of environmental contamination[1]. The GST enzymes are very often used as a indicator of exposure to pesticides and metalloids[6].

The synthetic analogs of the pyrethrins, extract from the ornamental *Chrysanthemum cineraiaefolium*, have been developed to circumvent the rapid photodegradation problem encountered with the insecticidal natural pyrethrins. The pyrethroides are widely used in field pest control and household use and as veterinary and human pediculicides and are among the most potent insecticides known[7,8]. Pyrethroides are very toxic to insects, amphibians and fish and are of a very low order of toxicity to birds and mammals[9]. Based on the symptoms produced in animals receiving acute toxic doses, the pyrethroids fall into two distinct classes of chemicals. These classes are known as type I („*T syndrome"*) and type II („*CS syndrome"*)[2]. While type I pyrethroids affect sodium channels in nerve membranes, producing repetitive neuronal discharge and prolonged negative after-potential, type II pyrethroids produce even longer delay in sodium channels inactivation leasing to a persistent depolarization of the nerve membrane without repetitive dicharge. They are more hydrophobic in nature[10] and their target site is biological membrane. In addition, type II syndrome implicates the central nervous system, while type I involve the peripheral nerves[11].

One of the pyrethroids that has found wide acceptability is deltamethrin[12]. Deltamethrin, synthetic type II pyretroid insecticide and acaricide, belong to the most effective pyrethroid preparations. It is the active substance of an insecticide formulation Decis flow 2.5.

The goal was to assess the effect of Decis flow 2.5 (active substance 25 g l^{-1} of deltamethrin) on activity of hepatic glutathione *S*-transferase of common carp.

Materials and methods

For the test, carp of 566±53 g (mean ± SD) body weight and 329±38 mm body length were used. Examination was performed on 14 control and 15 experimental specimens of two-year-old common carp after 96 h of exposure to Decis flow 2.5 (Bayer Crop Science GmbH, Germany) in a concentration of 0.13 mg l^{-1} (3.25 μg l^{-1} of deltamethrin).

At the end of the assay, all fish were killed by bisecting the brain and abdomino-thoracic ganglion and hepatopancreas (liver) excised for subsequent measurement of GST activity. The samples were kept frozen at –80 °C in Eppendorf test-tubes until analysis.

The liver samples were extracted with phospate buffer (pH 7.2). The homogenate of liver was centrifuged (9000 rpm for 10 min, 4 °C) and supernatant was used for

determination of GST (ref.[13]) and protein concentration. The enzymatic activity of GST was measured spectrophotometrically according to modified method[12] using biochemical analyzer Cobas Emira (at 340 nm). Supernatant with other reagent – phospate buffer; 0.02 M 1-Cl-2,4-dinitrobenzene and 0.1 M GSH was pipetted into the cuvette of analyzer. The protein concentrations were determined using Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich) using bovine serum albumin.

The statistical differences between control and experimental group were analyzed by one-way analysis of variance ANOVA. The software used for mathematical operations was STATISTICA 7.0. The level of significance was calculated at the $P<0.01$ and $P<0.05$ levels.

Results

During the experiment of deltamethrin poisoning in experimental common carp the following clinical symptoms of choreoathetosis were observed: accelerated respiration, loss of movement and coordination, for example fish laying down at the bottom of the tank and moving on one spot was a common observation. All two-year-old common carp survived in control and experimental group. In common carp, 96h exposure to 0.13 mg l^{-1} of Decis flow 2.5) caused a significant ($P<0.01$) increase in activity of GST. The GST activity (fig. 1) in control and experimental group was 31.07±11.90 and 44.29±12.48 nmol GSH min^{-1} mg $protein^{-1}$, respectively.

Discussion

It is well documented that the GST expression levels in many species can be significantly increased by exposure to foreign compounds, suggesting that they could form part of an adaptative response to chemical stress, which could determine their availability to be used as effective biomarkers of aquatic contamination[5]. In *Channa punctatus*, 96h exposure to 0.75 μg l^{-1} of deltamethrin caused a significant increase ($P<0.01$–0.001) in activity of GST in liver and kidney, while there was a significant decrease ($P<0.001$) in activity of GST in gills[12].

In our study we detected significantly higher level of activity glutathione *S*-transferase enzyme after acute exposure to deltamethrin. We confirmed that deltamethrin-based preparations are very toxic for aquatic organisms and may be referred to possible damage of liver. As is common with many pyrethroids, deltamethrin has a high toxicity to fish under laboratory conditions. However, in the field, under normal conditions of use, deltamethrin does not exhibit the same level of toxicity in fish. This may be due partly to rapid adsorption of deltamethrin in sediment, uptake by plants and evaporation in the air[14].

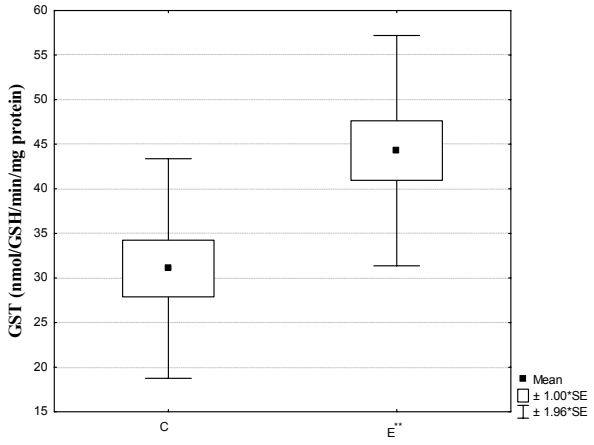

Fig. 1. **Effect of acute exposure to Decis flow (0.13 mg l^{-1}) on activity of GST in common carp;** C – control group, E – experimental group; $^{**}P< 0.01$

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P07
EFFECT OF URIC ACID ON OXIDATIVE DAMAGE

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Key words: uric acid, lipid peroxidation, Fenton reaction

Introduction

Uric acid (UA) has been proposed to be the dominant low-molecular weight plasma antioxidant because of its relatively high concentration (150–500 μM). Uric acid has peroxynitrite ($ONOO^-$), aqueous peroxyl radicals ($ROO^•$), and hydroxyl radical ($^·OH$) scavenging activity. On the other hand, uric acid may behave as prooxidant during LDL oxidation promoted by copper or hydrophilic ROO (ref.[1,2]).

The aim of this study was to determinate the antioxidant capacity of uric acid with use of Fenton reaction and to evaluate the effect of uric acid on lipid peroxidation induced by Fenton reaction in bovine aortic endothelial cells (BAECs). The degree of lipid peroxidation was expressed as concentration of malondialdehyde and 4-hydroxy-2-nonenal per μg protein.

Materials and methods

Scavenging of hydroxyl radical

Scavenging of hydroxyl radical was measured luminometrically. 10 μl of uric acid (final concentration 50–600 μM) were added to the mixture of cell culture medium M199, luminol (1 mM), and $FeSO_4$ (100 μM). The reaction was started by adding of H_2O_2 (1 mM). The measurement lasted for 20 minutes. The integrals of the resultant kinetic curves were used to evaluate the scavenging activity and expressed as relative light units (RLU). The reaction mixture with distilled water instead of the sample was used as a control.

Effect of uric acid on lipid peroxidation

Bovine aortic endothelial cells (BAEC) were cultured in M199 (Sigma) containing MEM vitamins solution (Sigma), MEM non – essential amino acid solution (Sigma), 5 % of fetal bovine serum and 5 % of newborn calf serum. 24 hours after plating the culture medium was replaced with serum free medium. The cells were treated with uric acid (50, 100, 200, 300, 600 μM) for 1 hour. Than $FeSO_4$ (100 μM) and H_2O_2 (1 mM) were added. Control incubations were performed without uric acid (control 1, control 2) and without Fe^{2+}/H_2O_2 (control 1). After 2 hours the medium was removed, the cells were once washed with PBS and scraped to cold distilled water containing EDTA (10 mM) and desferroxamine mesylate (600 μM). The cells were sonicated and centrifuged (at 12 000 rpm for 15 min, 4 °C). The supernatants were used for HPLC analysis. The pellet was resuspended in SDS lysing buffer and used for protein analysis. Protein content in the pellet was measured with BCA protein assay kit (Pierce).

Cell lysate samples were sonicated and then hydrolysis of protein bound MDA and HNE was achieved by incubating samples with 500 μl 0,25N hydrochloric acid in 60 °C water bath for 30 min (in the presence of 10 mM butylhydroxytoluene). Then, protein was precipitated with 500 μl of 35 % trichloracetic acid, and mixture was centrifuged (20 000 g, 15 min, 4 °C). For derivatization, 400 μl of DNPH reagent (5 mM solution in 2 M-HCl) was added to 2 ml of supernatant. This reaction mixture was incubated for 40 min at room temperature protected from light. Samples were extracted with three 2 ml aliquots of hexane. Extracts were evaporated to dryness under nitrogen and reconstituted in 300 μl of acetonitrile:water (1:1) mixture[3]. 100 μl volume of acetonitrile/water extract was directly injected onto Agilent Eclipse XDB-C18 column (5 μm, 4.6×150 mm I.D.). Chromatography was performed using Agilent 1100 series and DNPH derivates of aldehydes were detected with Agilent 1100 photo-diode detector at 310 nm (MDA) or 350 nm (HNE) at flow-rate 1ml min^{-1} with an isocratic elution acetonitrile-water-acetic acid (40:60:0.1, v/v/v/) (for MDA-DNPH determination) and with linear gradient of acetonitrile-water-acetic acid (50:50:0.1) to acetonitrile-water-acetic acid (80:20:0.1) in 20 min (for HNE-DNPH determination). The amounts of MDA and HNE were quantified by performing peak area analysis using external calibration curve. The concentra-

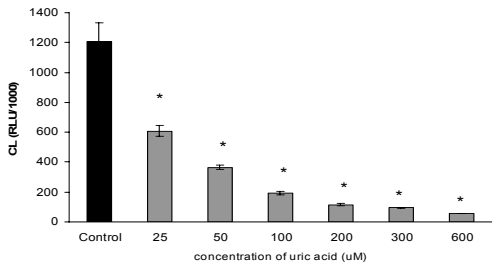

Fig. 1. **Scavenging of radicals formed in Fenton reaction by uric acid measured luminometrically;** the values represent integrals of resultant kinetic curves expressed as relative lights units/1000

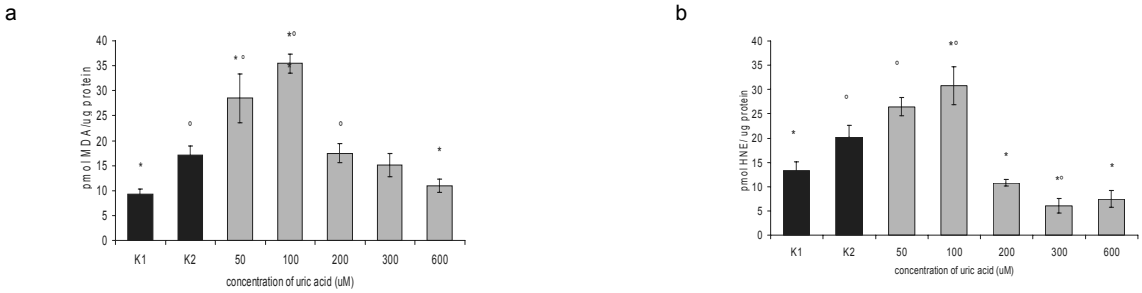

Fig. 2. **Effect of uric acid on lipid peroxidation expressed as MDA concentration (a) and HNE concentration (b);** values are the mean±S.E.M. of four different experiments and are expressed as pmol mg^{-1} protein. Data were analyzed with Student t-test, **P*<0.05 vs. control 2(K2); °*P*<0.05 vs. control 1 (K1)

tions of MDA and HNE in cell samples were expressed as pmol μg^{-1} protein.

Statistics

Data are reported as mean ± S.E.M. from four independent experiments. For comparison of control and uric acid treated cells a Student t-test was employed and statistical significance was assumed when $P<0.05$.

Results

Scavenging of hydroxyl radical

As shown in fig. 1, the concentration dependent antioxidant effect of uric acid against radicals formed in Fenton reaction was found using chemiluminiscence method.

Lipid peroxidation

Lipid peroxidation was enhanced by low uric acid level (50–100 μM), as indicated by the increased MDA (fig. 2a) and HNE (fig. 2b) levels. In contrast, an antioxidant effect was observed at higher uric acid concentration (300–600 μM).

Discussion

It has been previously shown that urate is an efficient antioxidant in plasma[4,5]. In the present study, we demonstrated that uric acid has concentration dependent antioxidant effect against radicals formed in Fenton reaction in chemical system using chemiluminescence measurementod. In contrast we found out prooxidant effect of uric acid on lipid peroxidation induced by Fenton reaction in bovine aortic endothelial cells. Concentrations of uric acid between 50 and 100 μM exerted a prooxidant behavior whereas 600 μM uric acid acted as antioxidant. These results are consistent with previous report in which a shift from pro to antioxidant activity was observed at 200–400 μM uric acid concentration[6]. Previous study demonstrated generation of Cu^+ by uric acid in reaction analogous to Fenton´s. This Cu^+ may greatly facilitate the decomposition of preformed LOOH in LDL, giving rise to lipid alkoxyl radicals ($LO^•$) capable of initiating oxidative processes in LDL (ref.[1]).

In addition the aminocarbonyl radical, a urate-derived free radical, has been demonstrated as the species responsible for the effect of urate in amplifying $ONOO^-$ – mediated LDL oxidation[7]. Thus further rapid reaction of the urate radical with some suitable co-antioxidant (e.g. ascorbic acid) will enhance the ability of urate to act in a beneficial manner and regenerate urate[4]. It was found out that ascorbic acid, which is present in human plasma at much lower concentrations than urate, prevents prooxidant effects of urate, when added to oxidizing low density lipoprotein simultaneously with urate, even at 60-fold molar excess of urate over ascorbate[8].

Our data indicate that the effect of uric acid on lipid peroxidation may be determined by a balance between its pro- and antioxidant activities.

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P08

CHANGES IN THE CYTOTOXIC PARAMETERS OF BRONCHOALVEOLAR LAVAGE OF RATS AFTER 6 MONTHS EXPOSURE TO REFRACTORY CERAMIC FIBRES, AMOSITE ASBESTOS AND CIGARETTE SMOKE

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Key words: amosite, refractory ceramic fibres, smoking, cytotoxicity, lung

Introduction

Refractory ceramic fibres (RCF) have excellent insulating properties (low heat storage, low thermal conductivity, resistance to thermal shock, chemical resistance) supporting their use as asbestos substitute[1]. Currently produced RCF contain fibres with the diameter distribution within the respirable range[2]. RCF belong to the fibres with biodurability higher than some other man made vitreous fibres[3] but lower than amosite (A)[4] . They are considered to be rodent carcinogens[5] and possibly carcinogenic to humans[6].

Despite the well known impact of cigarette smoke (S) on the human health, about thousand million of people are still active smokers[7]. That is the reason, why the possibility of the effect of combined exposure to cigarette smoke and another respirable substance has to be taken into account. Cowie et. al.[8] found in smokers inverse relation between pulmonary function and exposure to RCF. Maxim at al.[1] mentioned possible negative synergism between RCF exposure and smoking.

The aim of our work was to study the effects of exposure to RCF and RCF + S and to A and A + S on some chosen cytotoxic parameters in rat bronchoalveolar lavage (BAL).

Material and methods

Male Albino Wistar rats (VELAZ, Prague) weighing 210.9 ± 21.94 g at the beginnig of the exposure were used in this experiment. Animals were fed by standard laboratory chow (ST1 TOP DOVO Horné Dubové).

The animals were randomly divided into 6 groups: control group (n=12), group exposed to A (n=6), group exposed to RCF (n=6), group exposed to S (n=12), group exposed to A + S (n=6) and group exposed to RCF + S (n=6). The fibrous dusts (4mg/animal) and the saline solution (control group) were applicated intratracheally in two doses[9]. The origin, size, distribution and composition of the used RCF and A fibres were described in our previous studies[9–11]. The inhalation exposure to cigarette smoke started the next day after the last instillation. The inhalation procedure and equipment was the same as in the previous study[11].

Six months after starting the inhalation exposure the animals were sacrificed[12]. The BAL procedure and the separation of cell-free BAL fluid (cfBAL) and BAL cell sediment from the BAL fluid were described in previous works[9,12]. The aliquots of cfBAL and cell sediment were

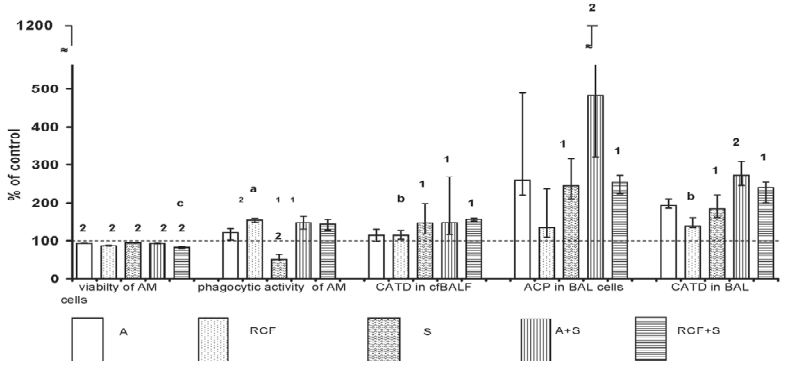

Fig. 1. **Viability of alveolar macrophages, phagocytic activity of alveolar macrophages, activity of cathepsin D** in cell-free bronchoalveolar lavage fluid, activity of acid phosphatase and cathepsin D in bronchoalveolar lavage cells after 6 month exposure to amosite, refractory ceramic fibres, cigarette smoke, amosite and cigarette smoke and refractory ceramic fibres and cigarette smoke. Comparison with the control group: [1]P <0.01; [2]P<0.001; comparison between the RCF and A groups: [a]P<0.05; [b]P<0.01; comparison between the RCF + S and A + S groups : [c]P < 0.01. A – amosite; RCF – refractory ceramic fibres, S – cigarette smoke, AM – alveolar macrophages, CATD – cathepsin D, cfBALF – cell-free bronchoalveolar lavage fluid, ACP – acid phosphatase, BAL – bronchoalveolar lavage. Values represent medians and 25th and 75th percentiles

stored at −75 °C till analysis. Following BAL parameters were estimated: viability and phagocytic activity of alveolar macrophages (AM) (ref.[9]), activity of lactate dehydrogenase (LDH) in cf BALF and activity of acid phosphatase (ACP) in cfBALF (ref.[12]), activity of cathepsin D in cfBALF (ref.[18,19]), activity of ACP and cathepsin D in BAL cells[12–15]. Mann-Whittney´s test was used for the comparison of chosen groups.

Results and discussion

The most important results of this experiment are in fig. 1.

Each exposure significantly decreased the viability of AM. This confirmed the duration of cytotoxic effect of A as well as that of RCF. The differences between results after exposure to A and after exposure to RCF were not significant. Higher decrease of viability after combined exposure RCF + S (in comparison with S or RCF) indicates the possibility of amplification of RCF effect by cigarette smoke. The viability after eposure to RCF + S was significantly lower than after exposure to A + S. The phagocytic activity of AM was significantly suppressed by smoking. It is in accordance with our previous results[18]. Exposure to A and RCF stimulated the phagocytic activity. Stimulation after exposure to RCF was significantly higher than after exposure to A. After combined exposures the suppressive effect of S was eliminated and the phagocytic activity of exposed group was significantly higher than that of control group. No differences were found between the results of exposure to A + S and exposure to RCF + S.

Increase of the LDH and ACP activity in cfBALF in this experiment were found only after exposure to smoking. Activity of cathepsin D in cfBALF increased after exposure to S and after combined exposures to A + S and to RCF + S .The results indicate that the increase was caused rather by exposure to cigarette smoke than by exposure to fibrous dust. There were no significant differences between the results after exposure to A + S and the results after exposure to RCF + S.

Activities of ACP and CATD in BAL cells significantly increased after exposure to S and after combined exposures. Differences between results after exposure to S and after combined exposures indicate amplification of the effect of fibrous dusts by the cigarette smoke. Activity of CATD after exposure to RCF was significantly lower than after exposure to A. The most expressive changes were found in ACP and cathepsin D activity in BAL cells. This experiment confirmed our previous results describing the cathepsin D activity as a very sensitive indicator of the changes in lung tissue after exposure to fibrous dusts and especially to smoking[12].

Conclusions

- the expected cytotoxicity of RCF was confirmed (decrease of viability, increase of activity of lysosomal enzymes),
- smoking influenced all of the examined parameters,
- smoking amplified the effects of exposure to examined fibrous dusts, but the additivity of effects was not confirmed,
- cathepsin D was the most sensitive indicator of the effects of exposure to smoking and of the effects of combined exposures,
- the effect of RCF in this study was comparable with the effect of amosite.

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P09
INTERACTION OF NATURAL ANTIOXIDANTS WITH INDIUM *IN VIVO*

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Key words: curcumin, alpha-lipoic acid, indium, oxidative stress

Introduction

Indium (In) is a metal of the IIIA group of the periodic table. Indium is used in radiodiagnostic and also widespread in semiconductor manufactory bringing a potential risk for human exposure. Intoxication with this metal has been associated with affection of liver, kidneys, reproductive organs and lungs. The toxicity of indium comprises oxidative tissue damage[1]. Although the increased use of this metal, there is a little information regarding the affection of indium-induced oxidative damage. Natural antioxidants e.g. curcumin or alpha-lipoic acid (LA) are able to counteract the effect of metal ions, which participate in oxidative stress generation. Curcumin (CUR), diferuloylmethan, is a natural phenolic compound and the major component of *Curcuma longa* L. extract. It is known for its antioxidant, antitumoral and anti-inflammatory activity and is considered to be a potent chemopreventive agent[2]. Alpha-lipoic acid (LA), 6.8-dithiooctanoic acid, is a naturally occurring dithiolic compound and an essential cofactor of mitochondrial bioenergetic enzymes. Its antioxidant properties consist e.g. in free radical quenching, metal chelating or antioxidant recycling[3]. In our laboratory, the antioxidant effect of curcumin[4] and antioxidant effect of alpha lipoic acid[5] on cadmium-induced oxidative liver damage in mice was demonstrated. In this comparative study, the effect of curcumin and α-lipoic acid on indium-induced oxidative damage of liver was monitored in the experiment in mice.

Experiment

Animals and Treatment

Male CD mice (SPF, Velaz Prague, CZ) weighing 20–24 g b.w. were used in this experiment. They were housed in the temperature and humidity controlled room with 12 h-light/dark cycle and free access to diet and drinking water. For the experiment, they were randomly divided into 6 groups of eight to nine animals per group. The following groups were used: I. Control, II. $InCl_3$, III. $InCl_3$ + curcumin, IV. $InCl_3$ + α-lipoic acid, V. curcumin, VI. α-lipoic acid. The antioxidants were administered by gastric gavage (0.1 ml/10 g body weight), dispersed in 0.3% methylcellulose, for 3 days at following doses: curcumin (Sigma, USA) 50 mg kg^{-1} b.w., alpha lipoic acid (Sigma, USA) 100 mg kg^{-1} b.w. The control group received an equivalent volume of methylcellulose. One hour after the last dose of antioxidant, indium chloride was administered ip at a single dose of 7.5 mg kg^{-1} b.w. to animals in group II-IV. Twenty-four hours after indium injection animals were sacrificed by decapitation. Liver were collected, rinsed in ice-cold saline and used immediately or stored frozen at −70 °C until analysis. The experimental protocol was approved by the local Animal Care and Use Committee.

Methods

In the liver homogenates, the lipid peroxidation (LP) expressed as malondialdehyde production formed in thiobarbituric acid reaction[6], the level of reduced glutathione (GSH) estimated by Ellman´s reagent[7], the activities of catalase[8] (CAT) and that of glutathione peroxidase[9] (GPx) were measured. Indium and trace elements concentrations were determined in the liver tissue by atomic absorption spectrometry (SpectrAA 220 FS, Varian Australia Ltd.). The data are presented as mean ± SD and were statistically evaluated using non-paired Student's *t*-test.

Results and discussion

Indium intoxication caused the increase in lipid peroxidation to 128 %,(P<0.01) compared to control group, the depletion of glutathione level to 55 % of the level in control animals (P<0.001), the decrease of catalase activity to 70% of controls (P<0.001) and the decrease of glutathione peroxidase activity to 72 % of controls (P<0.001) (fig.). In the liver of indium-only treated animals the cumulative action of indium and the elevated levels of zinc and cupper comparing to control group (P<0.001) were founded (Table).

The pretreatment with curcumin completely prevented In-induced increase in LP (P<0.05); however other parameters of antioxidant status remained unaffected. Both antioxidants, curcumin and α-lipoic acid, pretreatment increased the indium concentration in the liver tissue compared to In-only treated group (P<0.05). This fact is probably due to the possible chelation effect of these antioxidants. The elevated Zn and Cu hepatic levels in indium exposed group remained unaltered by curcumin as well as by α-lipoic acid pretreatment.

Antioxidants, curcumin and α-lipoic acid, administered alone showed the hepatic levels of LP, GSH and CAT comparable to control animals. After curcumin and α-lipoic acid traetment, the activity of GPx was inhibited in comparison to control group.

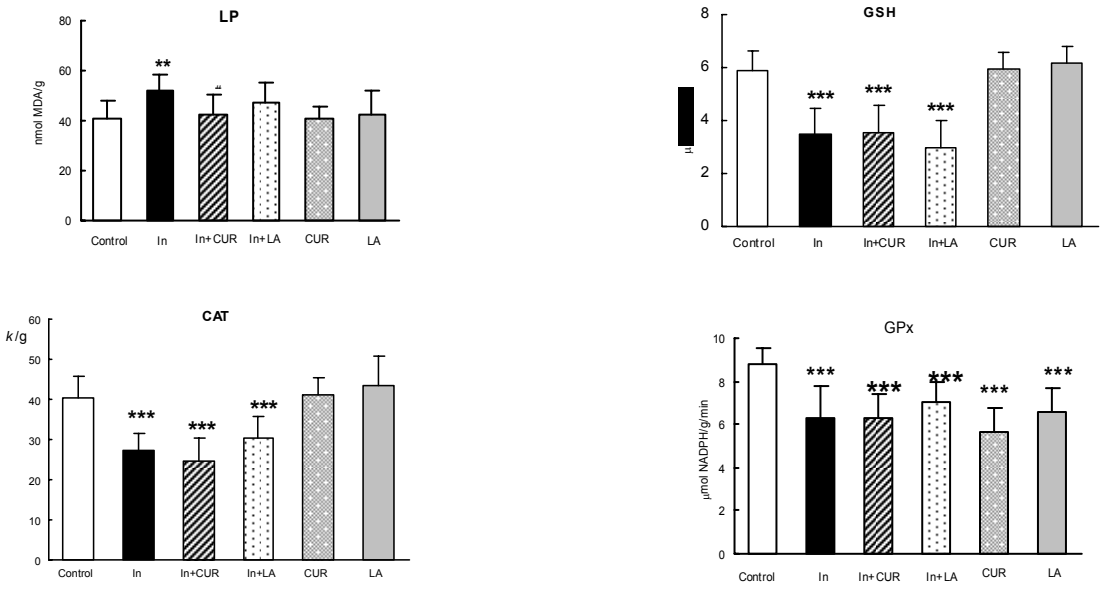

Fig. **The effect of curcumin (CUR) and alpha-lipoic acid pretreatment on hepatic lipid peroxidation (LP) and glutathione (GSH) levels and activities of catalase (CAT) and glutathione peroxidase (GPx) in In-treated mice;** significant difference: *** P<0.001, ** P<0.01 vs. Control group, # P< 0.05 vs. In-only treated group

Table
The effect of antioxidant pretreatment on indium and trace elements concentration in liver tissue of In-treated mice

Group	*N*	In [μg g^{-1}]	Zn [μg g^{-1}]	Cu [μg g^{-1}]
Control	8	<0.2	24.1 ± 3.3	3.64 ± 0.18
In	9	9.6 ± 2.6***	40.4 ± 6.9 ***	5.63 ± 0.77 ***
In+Curcumin	9	12.6 ± 2.0 #	40.8 ± 9.8 ***	5.17 ± 0.83 ***
In+α-lipoic acid	9	12.0 ± 1.4 #	36.8 ± 3.4 ***	5.32 ± 0.49 ***
Curcumin	8	—	22.5 ± 1.2 **	4.09 ± 0.62
α-lipoic acid	8	—	23.7 ± 2.0	3.89 ± 0.22 *

Significant difference: *** P<0.001, ** P<0.01, * P<0.05 vs. Control group, # P<0.05 vs. In-only treated group

Conclusion

The indium-induced oxidative liver damage was approved in experiment in mice. The data show that curcumin would be a better antioxidant than alpha-lipoic acid in indium-induced oxidative damage. Neverthelles, other studies for understanding of antioxidant activity mechanisms of this compound are desired.

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P10
CASPASE-3 AND OTHER MARKERS IN HEPATOCYTE APOPTOTIC AND NECROTIC STUDIES

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Key words: caspase-3, alfa GST, pentoxifylline, silymarin, rat hepatocytes, apoptosis

Introduction

The research in the field of liver cell apoptosis is evolving during the last years. Apoptosis has been reported in several hepatopathological states[1,2]. Key regulatory and metabolic steps have been identified in the apoptotic cascade. In hepatocytes, some of the three important concepts that underlie apoptotic cells are initiation by intracellular events or by extracellular signals often through the activation of death receptors, transmission of signals through the mitochondria or extramitochondrial and the execution of the apoptotic process that depends on specific proteases (caspases). Other important signal, which plays a role in apoptosis, is nitric oxide. Pentoxifylline (PTX) is used in a few non-hepatological indications[3]. It decreases total peripheral resistance of extracellular fluid and its viscosity, consequently protects several organs and tissues and was found to inhibit TNF alpha and other cytokines. Silymarin is considered as a standard for studying hepatoprotective drugs. D-Galactosamine and *tert*-butylhydroperoxide were used as model hepatotoxins. The aims of the present work were to study the in vitro effect of both hepatotoxic drugs namely D-galactosamine (D-GalN) and *tert*-butylhydroperoxide (t-BH) after pretreatment with pentoxifylline (PTX) or silymarin (SM) – as potential protectants – in rat hepatocyte cultures, and to explore the involvement of nitric oxide (NO) in this process.

Materials and methods

Hepatocyte isolation and culture

Hepatocytes were isolated from male Wistar rats (Velaz-Lysolaje, 200–300 g) using the standard two phase collagenase perfusion method. Cells were maintained in complete medium (William´s medium E, Penicillin/ Streptomycin 1%, Glutamine 1%, Insulin 0.06%, FBS – Fetal Bovine Serum 5%) for the first 24 hours and then cultivated in fresh medium with or without the respective drug for the next 24 hours (24–48 h). Hepatocytes were treated with medium alone, t-BH (1 mM), D-GalN (5 mM), with or without PTX (concentrations 1 mM and 5 mM) and SM (0.005 mM) pretreatment.

Determination of caspase-3 like activity

Hepatocytes were harvested from each treatment and washed twice in ice-cold PBS (phosphate buffered saline) by spining down at 500 xg for 5 minutes at 4 °C. Caspase-3 like activity in the cytosol was estimated following the respective manufacturer´s instructions. The total protein contents of all cytoplasmic extracts were determined using the BioRed Protein Assay Kit.

Determination of urea synthesis, ALT (alanine aminotransferase) leakage, alpha GST (glutathione S-transferase) leakage, mitochondrial function (MTT test) and NO production

Urea and ALT concentration in the medium samples were measured using customized diagnostic kits according manufacturer's instruction. Hepatocytes were evaluated for the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-difenyltetrazolium bromide (MTT test). Medium NO_2 – the stable end-product of NO oxidation, was determined spectrophotometrically by using Griess reagent. Alfa GST a highly sensitive biomarker of hepatocyte injury released was also estimated following the respective manufacturer's instructions.

Statistical analysis

Data were expressed as means ± SEM (standard error of mean). Comparisons were analyzed by ordinary one-way ANOVA (analysis of variance) followed by Bonferroni Multiple Comparisons test. *P*-value less than 0.05 was considered to be significant.

Results and discussion

Effects of pentoxifylline on biochemical marker of apoptosis – caspase 3 – like activity

As shown in the fig. 1a, the concentration of PTX 5 mM per se significantly increased casp-3 like activity compared to both control and PTX 1 mM groups. On the other hand, there is a decreasing trend in D-GalN pretreated groups for PTX and SM. In case of t-BH group, there were more necrotic cells at the moment of harvest which contributed to low levels of caspase-3 like activity (fig. 1b).

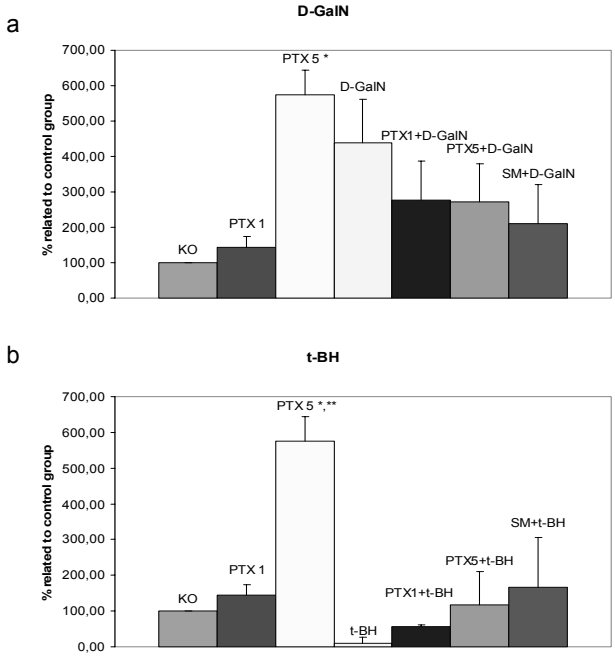

Fig. 1. **Caspase-3 like,** KO – control group – complete medium, D-GalN – D-galactosamine 5 mM in monotherapy, t-BH – *tert*-butylhydroperoxide 1 mM in monotherapy, PTX 1 – pentoxifylline 1 mM in monotherapy, PTX 5 – pentoxifylline 5 mM in monotherapy, PTX 1+ D-GalN – D-GalN 5 mM + 30 minutes pretreatment by pentoxifylline PTX 1 mM, PTX 5+ D-GalN – D-GalN 5 mM + 30 minutes pretreatment by pentoxifylline PTX 5 mM, PTX 1+t-BH – t-BH 1 mM + 30 minutes pretreatment by pentoxifylline PTX 1 mM, PTX 5+t-BH – t-BH 1 mM + 30 minutes pretreatment by pentoxifylline PTX 5 mM, SM+ D-GalN – D-GalN 5 mM + 30 minutes pretreatment by silymarin SM 0.005 mM, SM+t-BH – t-BH 1 mM + 30 minutes pretreatment by silymarin SM 0.005 mM, * significant compared to control group, ** significant compared to group of D-GalN resp. t-BH. All deviations are displayed as standard errors of mean

The metabolic activity and cell viability of cultured hepatocytes pretreated by pentoxifylline

Interestingly PTX 5 mM per se increased ALT leakage significantly compared to control group while significantly reduced D-GalN induced increase in ALT leakage in pretreatment compared to D-GalN (data not shown). TNF alfa inhibition might be concerned with this effect[10]. The effect of PTX 1 mM is comparable to silymarin.

The alfa GST (as a marker of early hepatocyte damage) leakage under D-GalN shows non significant increasing trend under various treatments (data not shown). Increase of alfa GST leakage in t-BH group was significant related to control group while there was a trend of reduction of both alfa GST and ALT leakage under PTX and SM pretreatments.

Urea production demonstrates certain positive influence of pentoxifylline either in non-pretreated or in pretreated groups without statistical significance vs. control group (data not shown). PTX 1 mM and PTX 5 mM significantly increased ureagenesis related to t-BH positive control (data not shown).

MTT tests exhibited variable and non-conclusive results.

Effects of pentoxifylline on spontaneous NO production

Pentoxifylline 5 mM per se increased significantly NO concentration compared to all other groups with no other significant changes in NO levels (data not shown).

Conclusion

1. PTX 1 mM concentration seems to be a reasonable concentration for studying potential hepatocyte protective action.
2. There is a big variation in the study outcome of MTT, urea synthesis, NO production on one hand and caspase-3 like activity, ALT and alfa GST leakage on the other hand at PTX 5 mM.
3. Pentoxifylline 5 mM increased significantly NO level compared to all other samples. With its multifaceted effects, NO might play definite role in modulating several intracellular signals (e.g caspases, $[Ca^{2+}]i$, TNF alfa, free radicals etc.) related to the toxic effects of the high dose of PTX alone or combined with other hepatotoxins.
4. Pentoxifylline and silymarin modulatory effects on D-GalN and t-BH induced hepatotoxicity are comparable and should be thoroughly re-evaluated in various *in vitro* experimental conditions.

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P11
DELAYED TOXIC ENCEPHALOPATHY AFTER TWO MONTH TOLUENE EXPOSURE – CASE REPORT

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Introduction

Organic solvent inhalation is a common form of substance abuse in children and young adults. Toluene is the major component of organic industrial solvents that is thought to cause the neurotoxicity, toxic effects on kidney, liver, and heart seen either in solvent abusers or in the workers occupationally exposed to a mixture of organic solvents.Recently it has become apparent that toluene intoxication is an important cause of encephalopathy in children. Permanent neurological damage may be more common than recognised[1]. We report the case of a young man with progressive encephalopathy, hemiparesis, convulsions, cerebellar ataxia and dementia manifested suddenly four years after a two month period of toluene inhalation exposure.

Case report

A 19-yr-old man was admitted to the department of neurology because of hemiparesis and convulsions. Medical history was notable for 2 month period of toluene sniffing in the age of fifteen. He had no history of neurological or psychological alterations before toluene abuse. The family history was negative for neurological disease. There was no recent contact with infectious disease, exposure to drugs or toxins was denied. One year after sniffing the patient was admitted to psychiatry for suicidium and delinquent behavior. During next three years after toluene exposure neurological abnormalities were not present.

The neurological examination on admission showed cerebellar ataxia, dysarthria, horizontal nystagmus and pyramidal signs. He developed bilateral optic atrophy. Normal results were obtained for the complete blood count, urine analysis, serum electrolytes, liver function test, blood urea nitrogen, serum creatinine and chest radiographs. An electroencephalographic mapping revealed mild cortical dysfunction of the right frontal lobe. Brainstem auditory evoked potential were abnormal. Electromyelogram was normal. Magnetic resonance imaging (MRI) revealed atrophy of the cerebrum with abnormal intensity areas on MRI in temporoparietal area of the cerebral cortex, basal ganglia, internal capsule (especially posterior limb), brain stem and middle cerebellar peduncle. Furthermore, MRI (T2-weighted images) showed reduced signal intensity in bilateral thalamus (fig.), symmetrical hyperintensity lesions in the corticospinal tracts and ventral part of the pons, in deep cerebral white matter including the centrum semiovale, periventricular white matter, posterior limb of the internal capsule, and ventral part of pons, middle cerebellar peduncles, and cerebellar white matter surrounding the dentate nuclei. In cerebrospinal fluid pleocytosis, proteinorachy and high level of lactate was found. Acute disseminated encephalomyelitis and mitochondrial neurogastrointestinal encephalomyopathy were excluded.

Cognitive dysfunction deteriorated progressively and the level of conciousness varied between sopor and coma. The ventilatory support was initiated because of aspiration pneumonia, tracheostomy and percutaneous endoscopic gastrostomy were performed when symptoms of bulbar motor dysfunction appeared. Patient died due to nosocomial pneumonia with septic shock after 10 month of intensive care unit stay.

Discussion

This case reports adverts to possible hazard of short term sniffing of toluene. The mechanism of toluene toxicity is unknown. The main toxic impact of toluene is on the central nervous system, probably explained by high cerebral perfusion and the affinity of toluene for lipid rich tissues, from which it is slowly released[1]. The posibility

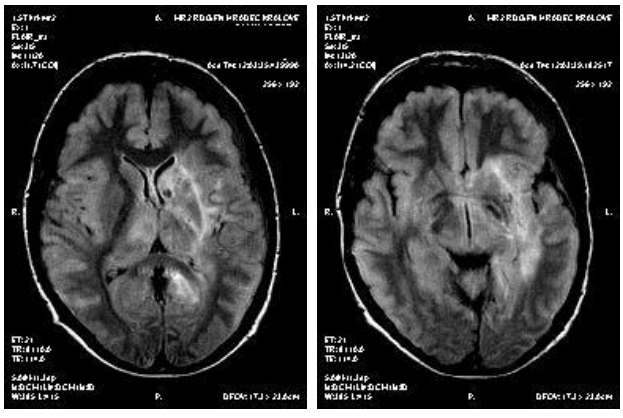

Fig. **MRI (T2-weighted images) showed reduced signal intensity in thalamus**; symmetrical hyperintensity lesions in the corticospinal tracts and ventral part of the pons, in deep cerebral white matter including the centrum semiovale, periventricular white matter, posterior limb of the internal capsule, and ventral part of pons, middle cerebellar peduncles, and cerebellar white matter surrounding the dentate nuclei

that chronic abuse of volatile substances can cause permanent neurological, psychiatric, and intellectual sequelae was critically reviewed elsewhere[2]. The syndrome of depression and loss of concentration occured significantly more frequently among spray painters[3]. Chronic toluene abuse may affect not only the cerebral and cerebelar cortex, brainstem, but also the subcortical cerebral white matter. MRI is considered a sensitive tool for severity and prognosis evaluation of the neurological syndrome resulting from the toluene abuse. A solvent induced toxic encephalopathy is considered a progressive disease comparable with presenile dementia and the effects on the central nervous system probably persist even when exposure has ceased[4].

Conclusion

This report suggests that even short-term abuse of toluene may induce invalidating brain syndromes. The initiation of first neurological signs could occur several years after sniffing. The diagnosis of a solvent induced toxic encephalopathy should be suspected when psychiatric symptoms and pathological psychometric performance is combined with a history of organic solvent exposure. This demands awareness of the possibility of toluene sniffing in any person who presented with coma, convulsions, ataxia, or behaviour disturbance.

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P12

CHEMILUMINESCENCE AS A MEASURE OF OXIDATIVE STRESS IN PAWS AND SPLEEN OF RATS WITH ADJUVANT ARTHRITIS

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Key words: chemiluminescence, adjuvant arthritis, spleen, joint

Introduction

Due to the increasing knowledge on the role of reactive oxygen metabolites (ROM) in the development and progress of rheumatoid arthritis, the approaches aimed at their detection have been extended. The luminol-enhanced chemiluminescence (CL) method is widely used as a sensitive assay for monitoring oxidant production in various systems (cells and cell free systems) and for testing antioxidant activity of drugs[1–3].

The aims of this study were *i*) to verify the luminol-enhanced CL method for measuring oxidant production in tissue samples (paws, spleen) obtained from untreated and methotrexate and *Boswellia serrata* treated rats with adjuvant arthritis (AA), *ii*) to compare the results of (*i*) with CL of whole blood, and disease parameters of AA – hind paw volume and relative spleen mass.

Material and methods

After the approval of the local ethics committee, AA was induced in Lewis rats by a single intradermal injection of *Mycobacterium butyricum*[4]. The experiments included healthy animals as controls, arthritic animals without any drug administration, and arthritic animals with methotrexate administration-twice a week in the oral dose of 0.5 mg kg^{-1} b.w., and administration of *Boswellia serrata* (*Boswellia serrata* extract standardised to boswellic acids – 23 %) twice a week in the oral dose of 50 mg kg^{-1} over a period of 28 days. On day 28, generation of ROM in the spleen and hind paw joint (cartilage and soft tissue without bone) was determined by a modified method of luminol-ehanced CL[2,5]. Briefly, the pieces of approximately 20 mg (spleen) and 450 mg (joint) wet weight were dissected. The samples were placed for 30 min (spleen) or 45 min (joint) into preoxygenated (95 % O_2 and 5 % CO_2) physiological saline solution-PSS (composition in mmol l^{-1}): NaCl (122.0), KCl (5.9), $MgCl_2$ (1.2), $CaCl_2$ (1.25), $NaHCO_3$ (15.0), and glucose (11.0), pH 7.4, and transferred into a cuvette containing 1.0 ml PPS with luminol (final conc. 400 μmol l^{-1}) immediately prior to assessment of ROM generation.

CL responses were measured at 37 °C and recorded continuously for 10 min in alumi-aggregometer model 500 (Chrono-log. Corp., USA) at appropriate sensitivity setting. The wet weight of samples was recorded at the end of experiment. Data were evaluated as the peak of CL curve expressed as mV/100 mg wet weight of joint and mV/1 mg wet weight of spleen. The production of ROM in whole blood stimulated with PMA (0.01 μM) was measured by luminol-ehanced CL in microplate luminometer Immunotech LM-01T (Czech Republic). Data were based on integrated values of CL over 3600 s (RLU x s; RLU-relative light units)[6,7]. Disease parameters were determined on day 28. Hind paw volume increase was calculated as the percentage increase of hind paw volume on day 28 in comparison to that from the beginning of the experiment, and relative spleen mass was calculated as the ratio of spleen mass (g) to body mass (g) x 100[8].

All values are given as means ± SEM. The statistical significance of differences between means was established by Student´s t-test and P values < 0.05 were considered statistically significant. The arthritis group was compared to healthy control animals, the treated arthritis group was compared to the untreated arthritis group.

Results

In rats with AA, increased CL was observed in samples of spleen and joint in comparison with healthy controls (P<0.01, P<0.05) (fig. 1, Table I). The same trend was observed in the CL of whole blood and in the disease parameters-hind paw volume and relative spleen mass. Methotrexate decreased CL of joint and whole blood, as well as hind paw volume and relative spleen mass. On the other hand, CL of the spleen was potentiated by methotrexate. CL of joint, spleen, and whole blood was reduced by *Boswellia serrata*, but the disease parameters were not affected (Table I).

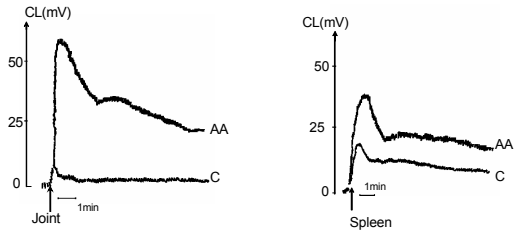

Fig. 1. **Chemiluminescence curves representative of 14 -15 separate samples of tissue**

Table I
Spleen, joint and whole blood CL and disease parameters of healthy, untreated and methotrexate and *Boswellia serrata* treated rats with AA, on day 28

	Joint CL [mV/100 mg]	Spleen CL [mV/1 mg]	Whole blood CL [RLU*s]	Increase of hind paw volume [%]	Relative spleen mass
Control	0.92 ± 0.12 *n*=14	1.49 ± 0.14 *n*=15	60 412 ± 2557 *n*=6	22.58 ± 2.37 *n*=6	1.87 ± 0.05 *n*=6
AA	7.55 ± 1.27** *n*=14	2.94 ± 0.66* *n*=15	543 700 ± 61 389** *n*=6	59.29 ± 8.77** *n*=6	3.02 ± 0.21 *n*=6
Methotrexate	3.38 ± 0.88 $^{+}$ *n*=11	3.85 ± 0.71 *n*=11	128 200 ± 18 435^{++} *n*=5	15.38 ± 2.14^{++} *n*=5	2.18 ± 0.16 *n*=5
Boswelia serrata	6.06 ± 1.49 *n*=6	1.60 ± 0.19 *n*=6	213 633 ± 17 821^{++} *n*=6	61.22 ± 8.06 *n*=6	3.11 ± 0.10 *n*=6

n=number of animals in experimental group, **P<0.01, *P<0.05 vs control rats, ^{+}P<0.05, ^{++}P<0.01 vs arthritic rats

Discussion

In addition to direct damaging effects on tissues, free radicals trigger the accumulation of neutrophils in the tissues involved, and thus aggravate tissue injury indirectly through activated neutrophils[8,9].

Using the luminol-enhanced CL method, we found increased levels of ROM in joint and spleen tissues of rats with AA in comparison with healthy controls. These results were in agreement with the increase of CL of whole blood and disease parameters-hind paw volume and relative spleen mass. Rheumatoid arthritis is characterized by increased numbers of neutrophils in the inflamed synovium, peripheral blood, and spleen[10]. Moreover, neutrophils are assumed to have the highest participation in cartilage degradation and bone destruction[11]. We suppose therefore that the increased CL of the spleen and joint was mainly the result of ROM generation by activated neutrophils, reflecting both local and systemic inflammatory responses of the organism. To verify the luminol-enhanced CL method for testing antioxidant properties of substances, we used methotrexate and the natural product *Boswellia serrata,* known to possess antiarthritic effects[12,13]. In our experimental conditions, methotrexate decreased CL of the joint, whole blood, as well as disease parameters, but increased CL of the spleen. The potentiation of spleen CL by methotrexate in rats with AA might be the result of the immune response of the organism, but on the other hand, this drug induces oxidative injury in many organs[9]. Methotrexate did not affect spleen CL from healthy rats (data not shown), thus oxidant-induced spleen injury appears to be questionable. However, to exclude spleen injury by methotrexate, we will complete our results with histological investigation and determination of myeloperoxidase activity, which reflects recruitment of neutrophils in tissues and provides information on tissue injury. The mechanism of action of the natural product *Boswellia serrata* is due to some boswellic acids and it is related to components of the immune system. The most evident action is the inhibition of 5-lipoxygenase, however, other factors such as leukocyte elastase, cytokines and oxygen radicals might also be targets[13]. In experimental adjuvant arthritis and in pilot clinical studies, *Boswellia serrata* was found to reduce knee swelling. In our experiments, disease parameters were not affected after 28 days of *Boswellia serrata* therapy, yet CL of whole blood, joint and spleen was decreased. It is possible that manifestation of the effect of *Boswellia serrata* on disease parameters would require a longer period than 28 days.

The presented results showed that the luminol-enhanced CL method might be a useful tool for measuring ROM generation and for studying the effect of a given therapy in tissue samples from rats with AA.

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P13
GLUCOMANNAN ISOLATED FROM *Candida utilis* MODULATES THE ACTIVITY OF HUMAN PHAGOCYTES

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Key words: glucomannan, CD11b, phagocytes, biological activity

Introduction

As a part of their cell wall, yeasts and fungi produce glucomannans that belong to the group of substances known as biological responses modifiers with broad range of activity. These polysaccharides consist of glucose and mannose units joined together by glycosidic linkages in different manners and ratios. Purified glucomannans possess wide range of biological activities. Glucommanans have been shown to stimulate various facets of immune responsiveness in humans, including antitumor, anti-infective activities against fungal, bacterial, viral and protozoal infections. These properties are mediated via the activation of leukocytes by stimulating their activity and the production of cytokines and other inflammatory mediators. The first step in the modulation of immune system (by soluble as well as particulate glucomannans) seems to be binding of glucans to specific cell surface receptors on macrophages, monocytes, polymorphonuclear leukocytes, NK cells and microglial cells. However, parameters, such as primary structure, degree of branching, molecular weight, solubility, conformation in solutions and ionic charge were suggested to play a role in biological activity of these molecules[1,2]. Despite high interest in this field for several decades, the relationship between the structure of glucomannans and their biological activity remains still unclear. The structural variability of polysaccharides obtained from various yeasts and fungi and by various biotechnological procedures significantly influences their biological activity.

In our previous study we observed strong immunostimulatory effects of two different polysaccharides schizophyllan and carboxymethylglucan isolated from *Schizophyllum communae* and *Saccharomyces cerevisiae* cell walls, respectively[3]. Herein, a characterization of the biological activity of biotechnologically produced glucomannan (GM) isolated from *Candida utilis* is described. GM isolated from *Candida utilis* consists of the α-(1→6)-D-mannopyranosyl backbone carrying mannooligosaccharidic side chains (1−5 units) containing α-(1→2) linkages, where some of the side chains are terminated with non-reducing D-glucopyranosyl residues[4] (fig. 1). Molar mass of this polysaccharide can vary in the range between 30−70 kDa, the mannose / glucose ratio is 2−3:1 according to our previous measurements. Briefly, GM bound to the cell wall of Candida utilis was isolated by sodium hydroxide hydrolysis. After the hydroxide digestion, suspension was cooled and cell debris was discarded. Following the pH equilibration, GM was precipitated using isopropylalcohol, homogenized and dried. The presence of endotoxins was tested using PyroGene® Recombinant Factor C Endotoxin Detection System (Cambrex, USA) which did not detect any significant amount of endotoxin (less than 0.01 EU ml^{-1}).

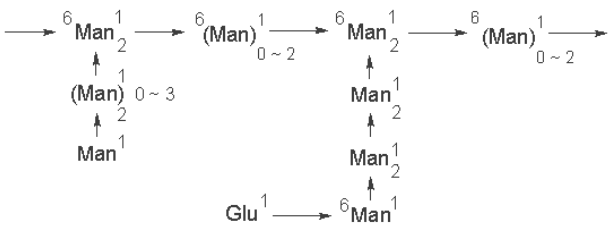

Fig. 1. **Structure of tested GM. α-(1→6)-D-mannopyranosyl backbone carrying mannooligosaccharidic side chains** containing α-(1→2) linkages, some of the side chains are terminated with non-reducing D-glucopyranosyl residues

Materials and methods

Biological activity of GM was evaluated based on the measurement of changes in the expression of polysaccharide surface receptor CD11b (widely accepted marker of phagocyte activation) and the production of selected pro-inflammatory mediators using human blood *in vitro*. Heparinized (50 IU ml^{-1}) blood was obtained from the cubital vein of eight healthy volunteers. The blood samples were incubated with different concentrations of glucomannan (100, 500 and 1000 μg ml^{-1}) at 37 °C. Samples without glucomannan were used as a control sample. The modulation of blood phagocyte activity by expression of CD11b on phagocytes and monocytes was analyzed using combination of anti-CD11b and anti-CD14 monoclonal antibodies (Caltag Laboratories, USA) and flow cytometer FACSCalibur (Becton Dickinson, USA) after 4 hours of incubation as described previously[5,6]. Production of pro-inflammatory cytokines was determined in samples by enzyme-linked immunosorbent assay for human IL-8 and TNF-α (Modul Sets, BenderMedSystems, Austria) after 24 h of incubation[7,8].

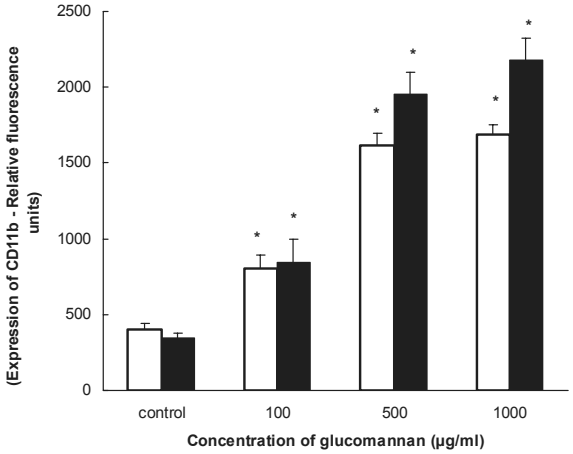

Fig. 2. **Flow cytometric detection of CD11b expression both on monocytes (white bars) and PMNL (black bars)** incubated with GM (100, 500 and 1000 μg ml^{-1}) for 3 hours; data are expressed as relative fluorescence units (mean ±SEM, n=8). Asterisks indicate statistically significant differences (P<0.05) compared with control evaluated by Student t-test

Results and discussion

Compared to untreated blood samples GM of all selected concentrations significantly and dose dependently increased the expression of CD11b molecule both on monocytes and PMNL (fig. 2).

After 24 hours of incubation, glucomannan at the concentration range from 500 to 1000 μg ml^{-1} markedly induced the production of both IL-8 and TNF-α, markers of leukocytes activation, as demonstrated by Elisa method (fig. 3).

Together, these results demonstrate the tested GM could be used as a potent activator of immune system due to the ability to stimulate blood phagocytes. Our previous experiments have also shown a photo protective effect of GM on human keratinocytes (unpublished results). Thus, the combination of the ability to protect human skin against UV irradiation together with the immunostimulatory effect makes GM suitable for pharmaceutical as well as for cosmetic applications.

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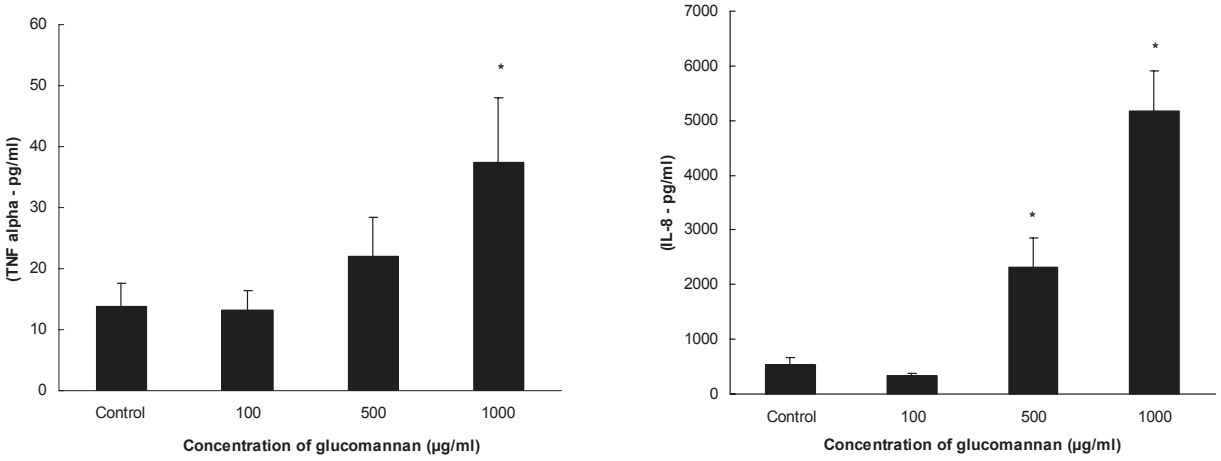

Fig. 3. **Production of TNF-α and IL-8 in the whole blood incubated with GM (100, 500 and 1000 μg ml^{-1}) for 24 hours;** data are expressed as concentration of cytokines (pg ml^{-1}) in plasma (mean ±SEM, n=8). Asterisks indicate statistically significant differences (P<0.05) compared with control evaluated by Student t-test

P14
RISK OF CHEMICAL COMPOUNDS IN INDOOR ENVIRONMENT

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Key words: indoor risk factors, cancer of lung, kidney, oesophagus, pancreas, multinational study

Introduction

A large multinational hospital-based case-control study of lung, kidney and oesophageal cancers coordinated by the International Agency for Research on Cancer (IARC) and Czech study on pancreatic cancer are in progress to identify some of the risk factors that may be involved.

In comparison with the figures pertinent to the Western world the incidence of non-infectious diseases in the countries of Eastern and Central Europe appears to be generally higher[1]. That includes several types of cancers. Even more alarming are the differences in mortality from cancers of different types. Consequently, levels of lifestyle-related risk factors as well as those of occupational and environmental exposure to carcinogens are likely to be elevated in Eastern and Central European countries relative to the West. So far, however, the level and effect of such exposures have not been studied on a large scale[2]. In the present study we considered three different types of cancers as exemples for the overall pattern of different patterns and risks: cancer of the lung, kidney, esophagus and pancreas.

While the incidence of lung cancer in the Czech Republic is rather high though not the highest in Europe, the incidence of kidney cancer appears to be the highest not only in Europe but also worldwide. There is no obvious risk factor for kidney cancer which could help explain such dramatic incidence of this disease in the Czech Republic. Oesophagus cancer is a rare form of cancer, especially when compared to lung cancer but with bad prognosis. Also pancreatic cancer has bad prognosis, probably the worst of above mentioned, and incidence in Czech Republic is on of the highest all over the world, although much lower than this of lung cancer.

To help understand the problem, a large hospital-based case-control multinational study of lung, kidney, pancreatic and esophageal cancers is in progress to identify some of the risk factors that may be involved. It is aiming for a study size of at least 2000 case-control pairs. This paper reports some results from the study on behalf of the Prague center.

Methods

One of the drawbacks of using a longitudinal approach to investigate the cause(s) of the disease with low incidence is that large and lengthy studies may be required to obtain adequate statistical power. For this study we used a case-control design as an alternative which avoids this difficulty[3].

Discovering the risk factors related to the indoor environment was the aim of the first part of the lifestyle questionnaire. Heating and cooking systems were classified into 5 categories: gas, central heating, coal, wood and other (e.g. sawdust, electricity, etc).

At the time of writing all analytical work has not been finished, however some publications have been presented either at international conferences, or published in international journals[4].

Results

A number of risk factors for cancers, such as smoking, are very well known. The aim of our study was, however, to take a look at other, perhaps less obvious factors. In this paper our primary focus is on the risk factors for lung cancer, particularly in relation to indoor environment. Since smoking is a major established risk factor for lung cancer, the results we provide are all stratified according to subject's smoking status. Only in cases where the summary characteristics (e.g. odds ratios) in the two strata appeared homogeneous, the results were combined to produce an overall summary of the association between the risk factor and the outcome (e.g. Mantel-Haenszel common odds ratio estimate).

Although smoking is an undisputed risk factor related to the incidence of several types of cancers, the effect of secondary smoking in the Czech population has not been studied on a large scale so far. Due to small number of smoking partners among the study participants who themselves smoked, statistically significant association between secondary smoking and lung cancer incidence was only observed among non-smokers.

Factors considered as contributing to the overall risk may take up a different meaning when viewed from a different perspective. Living in large cities used to be considered stressful to its inhabitants and stress was assumed to constitute an important risk factor **for a** number of civilization illnesses, including cancers. This assumption was not verified using our data, at least not in terms of these cancer site incidences in the Czech Republic and its relation to the community size where the subject spent his or her early years of life. The incidence of lung, kidney, esophagus & pancreatic cancer decreases steadily with increasing size of the community where the subject lived

during the early stage of his or her life.

Growing up in a larger community, particularly in the big cities, seems to be associated with reduced odds especially of lung cancer incidence. This may be, for instance, due to a better access to health care, increased health awareness, higher average economic status as well as improved nutritional habits and higher level of exercise in families living in larger cities. No adverse effect of higher pollution of the environment in big cities.

Solid fuels have also been considered a risk factor for several types of cancers, lung cancer in particular[5]. The odds of developing the lung cancer were largely increased when coal or wood were used for heating as compared to other type of heating methods. This finding remained consistent across the two strata defined by smoking status of the subject.

The preliminary results of our study have confirmed some well-known facts. There is a positive correlation between smoking and incidence of several types of cancers, lung cancer in particular. In connection with the indoor environment, a positive correlation between the lung cancer incidence and partner smoking was identified in non-smokers. Involuntary smoking is a proven carcinogenetic factor, although not so strong as an active smoking. The incidence of lung cancer was shown to vary according to the heating method used in the home environment. It was found to be significantly elevated when wood or coal was used for heating, irrespective of smoking status of the inhabitant. The heating method reported represents on average the method used during the subject's childhood and adolescence. Growing up in a larger city appeared to be associated with reduced odds of incidence of several cancer sites. The decreasing trend was very much consistent among both smokers and non-smokers in lung cancer. As mentioned before, this finding may be associated with a better access to health care, increasing health awareness among the people living in the larger communities, and last, but not least, a higher flexibility in adopting new lifestyle habits related to nutrition, exercise etc. Finally, the socio–economic status expressed via education level attained also appeared to be very much associated with cancer incidence, especially in lung and esophagus cancer. The trend in association was shown to be significant in smokers, among whom the incidence appeared to drop steadily with increasing level of education attained.

Conclusions

Our initial assumption that people from rural areas embrace healthier lifestyle relative to city population because of less chemicals in the environment indoors & outdoors, and would thus be expected to have fewer health problems and lower cancer incidence in particular, was not confirmed. The association of some well-known risk factors (e.g. smoking) with the incidence of lung cancer was confirmed. We have also identified other potential risk factors for lung cancer, such as secondary smoking and heating method, where using solid fuels (coal or wood) for heating appeared to be associated with increased lung cancer incidence. Social-economic status expressed via education status appeared to be a strong determinant of lung & esophageal cancer incidence in smokers.

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P15

INFLUENCE OF COMBINED EXPOSURE TO CIGARETTE SMOKE AND REFRACTORY CERAMIC FIBRES ON THE SELECTED BRONCHOALVEOLAR LAVAGE PARAMETERS IN EXPERIMENT

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Introduction

Refractory ceramic fibers (RCFs) – amorphous alumino silicates – are used as one kind of asbestos substitutes. RCFs has been classified as Group 2B (ref.[1]). According to IARC (2002): The increased lung diseases including lung cancer risk of asbestos-exposed workers is further increased by tobacco smoking[2]. Some epidemiological studies suggest that the risk from combined exposure is more than additive (in many cases combined exposure induces synergistic effect)[3]. Tobacco smoking and tobacco smoke as well as involuntary smoking (exposure to secondhand or „environmental" tobacco smoke) are carcinogenic to humans (Group 1)[2].

The aim of study was: *1*) to find and compare the effects of subchronic exposure to RCF, cigarette smoke (CS) and combined exposure to RCF+CS by inflammatory and cytotoxic parameters of bronchoalveolar lavage (BAL), *2*) to find out if smoking amplifies the possible adverse effect of RCF as it is known after combined exposure to asbestos + CS.

Material and methods

Four group of Wistar rats (mean weight 192.3 g) were treated: *1*) intratracheally instilled by saline solution (0.4 ml animal^{-1}) – control group; *2*) intratracheally instilled by 4 mg animal^{-1} of RCF; *3*) exposed only to CS (85 mg of total particulate matter m^{-3} air) for two hours daily; *4*) combined exposure to RCF+CS. The animals (6 rats in each group) were fed by standard laboratory pellets and water ad libitum. The period of smoking (8 cigarettes/ animal/day, except Saturdays and Sundays) lasted 6 months. Standard research cigarettes of the 1R1 type were used in experiment. A whole-body actively ventilated exposure chamber was built applying a smoking machine and pumps (THRI) allowing the animals to breathe diluted main-stream tobacco smoke at the target concentration 85 mg of total particulate matter (TPM) m^{-3} air for two hours per daily exposure requiring to burn eight cigarettes. After 6 months the animals were exsanguinated (under i. p. thiopental narcosis) and the bronchoalveolar lavage (BAL) was perfomed. Following BAL parameters were examined: BAL cell and alveolar macrophage (AM) counts; the differential cell count – (% of AM, % of polymorphonuclears – PMN, % of lymphocytes – LY); the proportion of immature AM and the percentage of binucleated cells; the viability and the phagocytic activity of AM. Fiber biopersistence is defined as the retention of fibers in the lung, over time, with regard to their number, dimensions, surface chemistry, chemical composition, surface area, and other physical characteristics[4]. Chemical composition of CRF: SiO_2 45−60 %; Al_2O_3 55−40 %. Detailed description of mentioned methodics is in the paper Hurbankova and Kaiglova[5]. All results were statistically evaluated by Mann – Whittney`s test.

Results

1) BAL cell and AM counts were significantly changed neither in RCF, CS nor combined RCF+CS group.
2) Differential cell count (% of AM, PMN and LY): Exposure to RCF, CF and RCF+CS significantly decreased % of AM (P<0.01); The AM were the most suppressed after combined exposure. In comparison with the control group % of PMNL and LY was statistically significantly increased, the most after combined exposure.
3) Immature form of AM were significantly increased after exposure to RCF, CS and RCF+CS (P<0.01) but mostly after combined exposure.
4) Binucleated cells were significantly increased only after combined exposure RCF+CS (P<0.05).
5) Viability of AM was depressed by every type of exposure but in the group with RCF+CS the changes were the most readible.
6) Phagocytic activity of AM was significantly increased only in the presence of RCF. In the group treated with RCF+CS was this parameter also increased but not significantly. On the contrary, cigarette inhalation significantly decreased phagocytic activity.

Discussion

The inhaled tobacco smoke is in 90 % of incidents in causal relationship with the genesis of lung cancer, and chronical obstructive lung diseases[3]. Combined effect of smoking and asbestos substitute fibres is less known and therefore it is necessary to find new data. In many cases, the cellular constituents obtained in the lavage provide a good indication of lung injury. Differential cell counts and an estimate of total cell count are important when reporting the results of cell analysis based on BAL. Alveolar macrophages play a significant role in the response to fibrous dust or tobacco smoke. In addition to being phagocytes, the AM are also important immuno-regulatory cells involved in the defense mechanisms[6]. AM also play a critical role in cigarette smoke and fibrous dust-related pulmo-

Table
Inflammatory BAL parameters 6 months after last i. t. instillation of RCF (4 mg animal^{-1}), after 6 month smoking, after 6 month combined exposure to RCF + cigarette smoke

BAL PARAMETERS	Control group (0.4 ml saline solution animal^{-1})	Refractory ceramic fibres (RCF) (4 mg RCF in suspension animal^{-1})	Cigarette smoking (8 cigarettes day^{-1})	Refrac. ceramic fibres + cigarette smoking (4 mg RCF + 8 cigarettes day^{-1})
AM, %	98.00 (98.00; 98.00)	↓ ** 75.50 (72.00; 86,50)	↓** 86.50 (85.25; 87,75)	↓** 57.50 (56.00; 64.25)
PMNL, %	1.00 (1.00; 1,00)	↑** 19.00 (9.50; 22.50)	↑** 9.50 (7.50; 10.75)	↑** 34.00 (29.25; 35.00)
LY, %	1.00 (1.00; 1.00)	↑** 4.00 (3.25; 6.25)	↑** 5.50 (4.00; 4.75)	↑** 8.50 (6.50; 9.00)
Immature AM, %	8.50 (7.25; 9.75)	↑** 21.50 (15.25; 27.75)	↑** 22.50 (18.75; 30.00)	↑** 23.50 (19.25; 28.50)
Binucleated cells, %	2.50 (2.00; 3.00)	↑ 4.00 (2.5; 4.75)	↑ 3.00 (3.00; 3.75)	↑* 5.00 (4.00; 8.25)
Viability of AM, %	90.00 (89.25; 90.75)	↓** 84.17 (83.25; 85.5)	↓** 81.50 (78.5; 83.0)	↓** 75.50 (72.75; 77.5)
Phagocytic activity of AM, %	23.50 (20.75; 28.5)	↑** 38.50 (37.25; 39.75)	↓ ** 12.50 (12.0; 13.75)	↑ 36.00 (32.25; 39.75)

Values represent medians and 25th and 75th percentil; * P< 0.05; ** P< 0.01; ↓ decrease or ↑ increase of values compared to the control

nary diseases. They are the predominant cells present in BAL and changes in their number or function are factors in determining lung injury and characterizing the pathogenesis of such a response. Decrease in macrophage count, viability or phagocytic capacity may result in impaired clearence of inhaled materials and thus can lead to an increase of the effective dose of the potentially injurious agent[6,7]. A significant reduction in proportion of AM percentage has been observed also after combined exposure (RCF+CS) in our case. Our results also suggest important finding regarding the phagocytic activity of AM which was significantly increased only in the presence of RCF. In the group treated with RCF+CS was this parameter also increased but not significantly. On the contrary, cigarette inhalation significantly decreased phagocytic activity. In comparison with the control, percentage of viability of AM was significantly decreased, mostly after combined exposure. Peripheral leukocytosis and increases in bronchoalveolar lavage neutrophil and total inflammatory cell counts have also been demonstrated in cigarrete smokers[8]. Higher than control values of the proportions of PMN, of lymphocytes in the BALF indicate the presence of inflammation in the lung at sacrifice. In our study the highest increase of these parameters we found out after combined exposure.

Conclusions

The results of our work suggest:

- the hight biopersistence of the RCF,
- serious inflammatory and cytotoxic changes in lung parameters after subchronic,
- exposure to RCF or cigarette smoke and
- amplification RCF effect by cigarettte smoke,
- great changes in BAL inflammatory and cytotoxic parameters we found out mainly after combined exposure. That means RCF+CS exposure has had similar amplified effect on the selected lung inflammatory parameters as asbestos exposure and smoking.

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P16

INHIBITION OF NEUTROPHIL OXIDATIVE BURST WITH ARBUTIN. EFFECTS *IN VITRO* AND IN ADJUVANT ARTHRITIS

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Key words: neutrophil, arthritis, arbutin, chemiluminescence, reactive oxygen species

Introduction

Reactive oxygen species produced by activated neutrophils participate substantially in joint erosion in arthritis. They can induce cartilage degradation, depolymerise hyaluronan and decrease its lubricative properties, and they can reduce the protective antioxidant and antiproteinase capacity of synovial fluid. Downregulation of neutrophil functions (and particularly of their oxidative burst) might thus increase the effectiveness of standard therapy and result in disease improvement[1–3]. In this study, arbutin was tested as a potential inhibitor of radical formation in neutrophils and its activities were investigated *in vitro* and in experimental arthritis.

Arbutin is a glycosylated benzoquinone, isolated from the leaves of bearberry (*Arctostaphylos uva ursi L.*, *Ericaceae*), wheat, pear skins, majorana, coffee or tea. It was found to possess antiseptic and diuretic properties, the ability to decrease allergic-reaction-induced inflammation and the capacity to potentiate antiinflammatory effects of indomethacine and corticosteroids[4]. Arbutin counteracted oxidative stress of mouse skin during tumour promotion, including leukocyte infiltration[5]. However, its effects on radical formation in neutrophils have not been analysed in detail.

Materials and methods

Arbutin (hydroquinone β-D-glucopyranoside), luminol, isoluminol, PMA (4β-phorbol-12β-myristate-α13-acetate) were from Sigma-Aldrich, Germany.

Fresh blood was obtained at a blood bank from healthy male donors who had not received any medication for at least 7 days. Whole blood chemiluminescence enhanced with luminol and stimulated with PMA (0.05 μmol l^{-1}) was measured in a microtitre plate computer-driven luminometer[6]. Chemiluminescence of isolated neutrophils was recorded separately outside and inside neutrophils, using isoluminol (extracellular) or luminol in the presence of superoxide dismutase and catalase (intracellular) as luminophores[7,8].

Adjuvant arthritis was induced in male Lewis rats by a single intradermal injection of heat-killed *Mycobacterium butyricum*[9]. Arbutin (50 mg kg^{-1}) was administered daily p.o. over a period of 28 days after arthritis induction. Then 10 μl of blood was taken by tail venepuncture and chemiluminescence (spontaneous and stimulated with 0.005, 0.01 or 0.05 μmol l^{-1} PMA) was measured. Formation of chemiluminescence signal was initiated by addition of 50 μl of blood (200× diluted) to the reaction mixture, which consisted of luminol (250 μmol l^{-1}), horseradish peroxidase (8 U ml^{-1}), PMA and phosphate buffer, in 50 μl aliquots. Plasma concentration of interleukin-6 was measured using the ELISA kit on days 0, 14, 21 and 28 after induction of arthritis. Results from three groups of animals were compared – healthy („control"), arthritic without any medication („arthritis") and arthritic animals treated with arbutin („arthritis + arbutin").

Mean integral values of chemiluminescence over 3600 s (whole blood) or 1800 s (isolated neutrophils) are presented. Statistical significance of differences between means was established by Student's t-test and *P* values below 0.05 were considered statistically significant.

Results

Effects of arbutin *in vitro*

In whole human blood, arbutin decreased neutrophil oxidative burst dose-dependently, starting with the concentration of 1 μmol l^{-1} (Table I). A more detailed analysis performed on isolated human neutrophils showed that arbutin potently decreased the external oxidant concentration (significant inhibition started at 0.01 μmol l^{-1}) without reduction of the oxidative burst arising inside neutrophils.

Table I
Effect of arbutin (Arb, [μmol l^{-1}]) on chemiluminescence of human neutrophils. RLU – relative luminescence units. Mean ± SEM, n=6, *P<0.05 (vs 0)

Arb	Chemiluminescence [millions of RLU*s]		
	Whole blood	Extracellular	Intracellular
0	1.53 ± 0.29	17.39 ± 4.61	1.10 ± 0.21
0.0 1	1.50 ± 0.24	13.72 ± 3.39*	1.16 ± 0.22
0.1	1.48 ± 0.23	12.88 ± 3.20*	1.17 ± 0.26
1	1.14 ± 0.19*	11.43 ± 3.07*	1.13 ± 0.22
10	0.35 ± 0.04*	3.21 ± 0.85*	1.17 ± 0.27
100	0.11 ± 0.01*	0.07 ± 0.01*	1.10 ± 0.21

Effects of arbutin in arthritis

Adjuvant arthritis was accompanied by an increase in the number of neutrophils in blood (from 8583±802 to 22 883±1240 μl^{-1}) and by a more pronounced spontaneous as well as PMA stimulated chemiluminescence (fig. 1a). Whereas the arthritis- related alterations in neutrophil count and in spontaneous chemiluminescence were not modified by arbutin, the increased reactivity of neutrophils to PMA was found to be less evident in arbutin-treated animals. This inhibitory effect of arbutin was reversible and declined with increasing concentrations of PMA. Values of chemiluminescence calculated per one neutrophil (Fig. 1b) are confirming the enlarged intensity of oxidative burst in the arthritic group, as well as the potency of arbutin to weaken priming of neutrophils during the inflammatory process.

Besides the inhibition of neutrophil activity, arbutin was found to decrease the concentration of the pro-inflammatory cytokine IL-6. The control plasma level of interleukin-6 was 29.4±2.6 pg ml^{-1}. On day 14 and 21 of arthritis, it increased to 119.9±14.3 and 101.3±11.3 pg ml^{-1}, respectively. In the presence of arbutin, the respective IL-6 concentrations were 72.8±12.0 and 35.5±8.8 pg ml^{-1}.

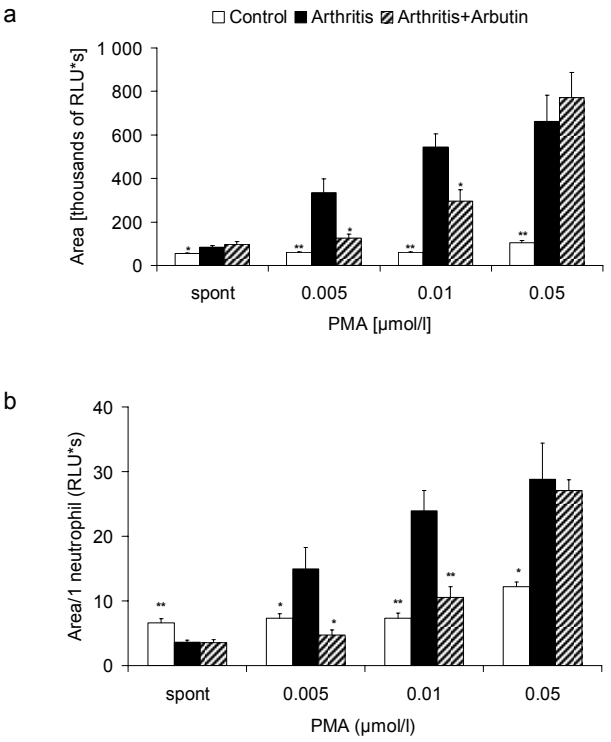

Fig. 1. **Arbutin and arthritis-induced alterations in whole blood chemiluminescence;** a – absolute values, b – chemiluminescence calculated per one neutrophil. Mean ± SEM, *n*=6,

Discussion

Adjuvant arthritis, a rat model resembling rheumatoid arthritis in humans, mimics the latter's immunological and biochemical features. Self antigens are recognised as foreign, the inflamed joint is characterised by proliferation of synovial cells and by infiltration of leukocytes to form pannus, which progressively invades and replaces the cartilage[2]. In our experiments, adjuvant arthritis was accompanied by more than twofold increase in the number of neutrophils and by an evident priming of these cells by the inflammatory process. Arthritic neutrophils excessively responded to PMA and produced six to nine-times more radicals than controls. In the presence of arbutin the hyperreactivity of neutrophils was significantly suppressed.

Since the inhibition of neutrophil reactivity was reversible and declined with increasing concentration of PMA, it does not seem to result from the damage of neutrophils by arbutin. The reduction of radical concentration is likely to be due to the antioxidative and free radical scavenging effects of arbutin[10], which were ascribed to the ability of arbutin to undergo oxido-reduction changes from hydroquinone to quinone and vice versa. The experiments performed *in vitro* showed that arbutin decreased the concentration of extracellular radicals (potentially dangerous for tissues in the neighbourhood of activated phagocytes), without affecting formation of intracellular radicals essential for neutrophil activity. Thus arbutin appears to meet the criteria for an optimal antioxidant, which is expected to minimise tissue damage without reduction of microbial killing.

The removal of existing radicals need not be the sole mechanism involved in arbutin activity. Since arbutin inhibited the PMA stimulated chemiluminescence but did not change the spontaneous one, it seems to interact with some processes involved in activation of neutrophil oxidative burst. The interference with formation of pro-inflammatory cytokines, which function as activators of neutrophils[11], may represent one of the probable mechanisms involved. Decreased plasma levels of interleukin-6 observed in arbutin-treated animals is supporting this assumption.

As neutrophils are considered to be cells with the greatest capacity to inflict damage within diseased joints, they should not be neglected in the search for new arthritis therapies. Arbutin was found to be a potent inhibitor of neutrophil functions both *in vitro* and in experimental arthritis, affecting oxidant production selectively in extracellular space. These effects could explain the previously observed capacity of arbutin to enhance the antiinflammatory activity of indomethacine and corticosteroids[4] and put arbutin to a preferred position among plant medicines which possess the ability to amplify effectiveness of antirheumatic drugs.

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P17

CYTOGENETIC ANALYSIS OF LYMPHOCYTES OF WORKERS OCCUPATIONALLY EXPOSED TO ROCKWOOL AND GLASS FIBRES. MOLECULAR-EPIDEMIOLOGIC STUDY

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Key words: chromosome aberrations, micronuclei, glass fibres, rockwool

Introduction

The production, sale and use of asbestos are no longer permitted in Europe. Some of the properties of asbestos (e.g. as an insulation material) can be substituted by alternative man-made fibres. Rockwool and glass fibres are man-made mineral fibres (MMMF). The last evaluation of the carcinogenic effect of fibres by IARC Monograph Working Groups categorised rockwool to Group 3, i.e. not classifiable as to human carcinogenicity, while glass fibres to Group 2B (possible carcinogenic material)[1,2].

Subject and methods

A biomonitoring study was conducted in two factories producing mineral fibres in Slovakia. We investigated a frequency of chromosome aberrations and micronuclei of workers occupationally exposed to rockwool (R) and glass fibres (GF). Altogether 257 subjects were investigated, 178 exposed (98 R and 80 GF) and 79 controls (43 administrative employees in the same factory for rockwool and 36 for the glass fibres). Workers for at least 5 years occupationally exposed to rockwool and glass fibres were recruited for this study. Participants answered detailed questionnaires and underwent clinical examination. The study was approved by the Ethical Committee of the Slovak Medical University.

Cytogenetic analysis: Conventional short-term lymphocyte cultures were made from whole blood in RPMI medium with L-glutamine (Gibco) supplemented with 20 % foetal calf serum (Gibco) and antibiotics. Lymphocytes were stimulated by phytohaemagglutinin (Murex) and incubated at 37 °C and 5 % CO_2.

Chromosome aberrations: The cells were harvested at 48 h; colchicine (Sigma, 0.75 μg ml^{-1}) was added 2 h before harvest. 100 metaphases per sample were scored for various types of chromosome aberrations[3].

Micronucleus test: Cytochalasin B (Sigma, 6 μg ml^{-1}) was added 44 h after the start of culture and incubation was harvested at 72 h. Micronuclei were identified according to the criteria of Fenech et al[4].

Statistical analysis: SPSS 13.0 software was used for statistical analysis. To test for significant differences between groups, we used the Mann-Whitney U-test, t-test and Bonferroni test. All tests were performed at significance level α=0.05. Pearson (for normally distributed data) or Spearman correlation (for not normally distributed data) were used for analyzing the possible association between studied markers.

Results

We did not find any differences in chromosome aberrations between exposed and control groups in both monitored factories (Table I). Number of aberrant cells in the rockwool exposed subjects correlated positively with age (r=0.2, P=0.05), on the contrary there were negative correlations between number of aberrant cells and age in control subjects (r =−0.34, P=0.03). After dividing exposed and control subjects in both factories on smokers and non-smokers we also did not find any differences in frequencies of chromosome aberrations. Part of this study was already published[5].

There were no differences in frequency of micronuclei between exposed and control groups (Table II). Micronuclei were more frequent in women than in men (P<0.05). Number of micronuclei was influenced by age and sex (P<0.001). Micronuclei are a sensitive marker of aging[6,7,8]. There were no differences in frequencies of micronuclei after dividing exposed and control subjects in both factories on smokers and non-smokers. Analysis of results from the HUMN project[9] confirmed that smokers

Table I
Chromosome aberrations in lymphocytes of exposed to mineral fibres and controls

Group	N	Number of analyzed cells	% of aberrant cells	Break/ cells
Rockwool exposed	97	9700	0.65	0.007
control	43	4300	0.65	0.007
Glass fibres exposed	79	7900	0.59	0.009
control	36	3600	0.53	0.007

Table II
Micronucleus frequency in lymphocytes of exposed to mineral fibres and controls

Group	N	Number of analyzed cells	% of cells with MN	% of MN
Rockwool exposed	98	196 000	0.32	0.34
control	41	82 000	0.45	0.49
Glass exposed	80	160 000	0.21	0.23
control	36	72 000	0.29	0.33

do not experience an overall increase in MN frequency, although when the interaction with occupational exposure was taken into account, heavy smokers were the only group showing a significant increase in genotoxic damage as measured by the micronucleus assay in lymphocytes.

Conclusion

Our study does not show any differences in chromosome aberrations and micronucleus frequencies in lymphocytes of occupationally exposed workers by rockwool or glass fibres compared with control groups.

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P18
BLOOD MORPHINE QUANTITY CONNECTED WITH NATURAL OPIUM AND POPPY HEADS PRODUCTS ABUSE

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Key words: drug addicts, morphine, natural opium, poppy heads

Introduction and aim of the survey

The crucial question for substance dependent people is how to obtain the biggest amount of the drug in the cheapest and the most simply way. One of the possibilities is collection of natural opium and poppy heads, that contain morphine[1,2]. The aim of this study was to figure out the morphine amount in one dose of products that users use to prepare from these sources.

Material and methods

Necessary data and samples were obtained from substance dependent people – clients of Contact Centers (CC) in Olomouc and Prostejov. 32 subjects with a long period of natural opium and poppy heads abusing handed us 6 natural opium samples, 9 samples of dry poppy heads (always at 10 pods) and supplied information via questionnaires.

Results

We specified morphine content in poppy heads in our laboratory (at the Faculty of Pharmacy in Hradec Kralove) via photometry. After analysis of all 9 samples we counted morphine amount in poppy heads in interval 0.20–0.28 %. Natural opium analysis was made via HPLC at the Faculty of Pharmacy in Brno. Morphine content in all 6 samples was determined in interval 24.06–33.9 %. The literature tell about 6–28 % morphine in natural opium and about 0.25–0.6 % morphine in poppy heads grown in our country[1,3].

According to our findings the subjects usually need natural opium from 5 pods for one application by injection usage, from 5–10 pods for smoking and drink – „opium tea“ they made from 15–20 poppy heads pieces. One piece of pod provides average amount about 0.02 g of natural opium, yield after exsiccation of 14.5 % of water[4] is 0.017 g of opium. Bio-availability of morphine after injection application is 100 %, after smoking 70 % and after administration p.o. 30 % (ref.[5]). Base on it we could assess morphine amount in one dose (Table I).

Discussion

Morphine quantity in natural opium given us by drug abusing people exceeds value alleged in the literature search. Nevertheless users claimed they do not to process raw material, they only to prepare it just by injection usage or smoking. On the contrary the morphine quantity in our samples of the poppy heads oscillates near lower bound. It could point up that users boil their drink using fresh poppy heads that contain more morphine than dry poppy heads.

Therapeutic doses for administration p.o. declared in the Czech Codex 2002 advises maximum single dose of powdery opium 0.15 g, maximum daily one then 0.5 g with 10 % morphine amount. It comes to this, that maximum single dose contents 15 mg of morphine and maximum daily one 50 mg of this drug. Maximum single dose in blood circulation (bio-availability 30 %) could be ac-

Table I
Morphine content in blood circulation after one potion application

	Morphine amount in one dose [mg]	
Way of application	Used 6–28 % morphine in natural opium dose and 0.25–0.6 % morphine in poppy heads dose[1,3]	Used 24.06–33.9 % morphine in natural opium dose and 0.20–0.28 % morphine in poppy heads dose (our samples)
Injection	5.1–23.8	20.5–28.3
Smoking	3.6–33.3	14.3–39.6
Drinking	35.3–113.0	28.3–52.8

Table II
Frequency of maximum single dose exceeding and maximum daily dose exceeding in users blood

Way of application	Used 6-28 % morphine in natural opium dose and 0.25-0.6 % morphine in poppy heads dose[1,3]		Used 24.06-33.9 % morphine in natural opium dose and 0.20-0.28 % morphine in poppy heads dose (our samples)	
	Max. Single dose	Max. daily dose	Max. Single dose	Max. daily dose
Injection	5.3×	1.6× or 3.2×	6.3×	1.9× or 3.8×
Smoking	7.4×	2.2× even 6.6×	8.8×	2.6× even 7.8×
Drinking	25.0×	n.a.	11.7×	n.a.

n.a. – not available

cording to the Czech Codex 2002 4.5 mg of morphine, maximum daily one then 15 mg. These ceiling values we can compare with the morphine amount absorbed into users' blood at them declared quantity of the drug. Again we counted with the poppy heads number confided by our subjects and either with morphine level in natural opium and poppy heads resulted from our samples analysis or with morphine value declared in the literature search. Via single injection application the users have probably exceeded maximum single dose of morphine either even in 19.3 mg (calculated with morphine amount declared in the literature search[1,3]) or even in 23.8 mg (calculated with morphine amount in our samples). It is likely, that via smoking our subjects have exceeded it even in 28.8 mg (calculated with morphine amount declared in the literature search[1,3]) or even in 35.1 mg (calculated with morphine amount in our samples). Via „opium tea" drinking users have perhaps exceeded maximum single dose of morphine in their blood either even in 108.5 mg (calculated with morphine amount declared in the literature search[1,3]) or even in 23.8 mg (calculated with morphine amount in our samples). According to the literature a drug dependent person with chronic abusing can endure single dose with even 5 g of morphine without a serious acute intoxication[6]. It is not probable our subjects have achieved this amount.

But also in such way clients of CC have perhaps exceeded maximum single dose of morphine referred-to the Czech Codex 2002. A third of them has admitted injection application 1-time or 2-times a day. Whether we have in mind a day number application and an amount of morphine in natural opium 6–28 % (ref.[1,3]), than we obtained this results: users have perhaps exceeded maximum daily dose of morphine in their blood in 8.8 mg or 32.6 mg depended on usage frequency. Calculating with morphine amount in our samples (24.06–33.9 %) we resulted in 13.3 mg or in 41.6 mg overdosing. A fifth of subjects use to smoke natural opium once to 3-times a day, most often twice a day. Maximum daily dose of morphine has been probably exceeded either in 18.3 mg to even in 85 mg (with morphine amount declared in the literature[1,3]) or in 24.62 mg to even in 103.86 mg (with morphine amount in our samples of poppy heads). Altogether 20 % of them use to cook the „opium tea" , but they did not told how many times they drunk it, it is not possible to find out how many times they could exceed maximum daily dose of morphine. But even one pot of „opium tea" exceed maximum daily dose either in even 98.0 mg (calculated with morphine amount declared in the literature search[1,3]) or in even 37.8 mg (with morphine amount in our samples). You can see it in the Table II.

During one month of the opioid period (June−August) they can apply (according to their frequency of daily usage) 30 even 60 dose by injection/smoking or drink „opium tea" all day. Opioids addictive potential depends on the dosage, frequency of the usage and the users´ sensibility[4,6,7]. Natural opium and poppy heads are usually rated as only season drugs, but in the period of their abusing 40 % of subjects do not use another substances, natural opium and poppy heads become the primary drug than. Nevertheless dependence on opioids is interpreted as the most serious connected with tolerance increasing and the strong syndrome abstinence[8], they are very dangerous also because of overdosing. Opioid tolerance falls during abstinence fast and dose before being off abusing becomes to be mortal if it is used on the beginning of new opium season[9]. This kind of overdosing could be an actual danger menacing our users. Detoxification is very rare; substitution can be appreciated as good effect[5]. None of our subjects has endured reportedly this treatment. Next hazard connected with injection abusing of natural opium is viral hepatitis and HIV infections[10]. Fourth part of clients admit more frequent needle and paraphernalia sharing during opium season than out of it, possibility of so danger diseases transfer increases markedly.

Conclusions

Morphine quantity in users' blood circulation after natural opium and poppy heads products applications seems to be considerable. Gathering from it dependence on opioids and tolerance are developing via existing abusing algorithm. Our subjects consider natural opium and poppy heads only as season drugs, but by contrast of it almost half of them admit it as the primary drug during the opium season. This existing way of abusing could be connected

with so strong dependence that opioids would be the primary drug not only for several months but yearly with all hazards resulted from it.

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P19

PROTECTION EFFECT OF VITAMIN C ON ALCOHOL BINDING TO PHOSPHOLIPID MONOLAYERS

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Key words: monolayer, dipalmitoyl-phosphatidylcholine, Maxwell displacement current technique, ethanol, vitamin C

Introduction

The mechanism of toxic effect of ethanol chronic uptake has been intensively studied[1–3]. The simple model of the biological membrane provides well-controlled lipid monolayers at the air – water interface. The Maxwell displacement current technique (MDC) provides novel approach to conformation study of the membrane models. Ethanol is a clear inducer of oxidative stress. Vitamin C (ascorbic acid) is a coenzyme in many oxidative/reduction reactions, it acts as antioxidant, prevents oxidation of unsaturated fat acids. Protective effects of antioxidants have been confirmed by many studies[4]. Vitamin C is an essential compound for humans; it is required for protein synthesis. Ethanol chronic intake influences many systems in the body. The changes in protein functions, that are the basis of membrane channels, enzymes etc., cause loss of cell energy and decrease its communication ability with the environment. Acetaldehyde and free radicals are created by ethanol metabolism on subcellular level, which evokes oxidative stress.

Simultaneous supplementation of ethanol and vitamin C reduces protein and lipid oxidation in the rat kidney, testes, and lungs[5]. These effects were also observed in human glial cells after simultaneous supplementation of ethanol and vitamin C (ref.[6]). Vitamin C prevents membrane dehydration by ethanol.

The aim of the presented work is the observation of changes in structural and mechanical properties of dipalmitoyl-phosphatidylcholine (DPPC) monolayer situated onto the subphase of methanol-water and ethanol-water mixture after addition of vitamin C. This study reveals another protective effect of vitamin C towards ethanol toxic effect.

Material and methods

The material used in this study as model phospholipid was 1,2-dipalmitoyl-sn-glycero-3-phosphocholine monohydrate (DPPC) purchased from Sigma-Aldrich. Lipid was dissolved in chloroform at the stock concentration 0.5 mg ml^{-1} and spread on the subphase using microsyringer (Hamilton, USA). As subphases were used pure water (bidistilled deionized water, 15 MΩ cm) and solutions of ethanol (spectrophotometric grade purity, Sigma-Aldrich) and vitamin C (Galvex, Banska Bystrica, Slovakia). For alcohol solutions was used concentration of 20 % and 100 mM concentration of vitamin C. All subphases were thermostated to the temperature 17 °C. Monolayers were allowed to equilibrate and solvent to evaporate for 15 minutes. This time was sufficient for chloroform to evaporate and monolayer to stabilize.

Results

Absorption/adsorption of ethanol molecules to phosphocholine (PO_2) group of DPPC molecule occured on DPPC monolayer on the ethanol-water subphase.

After vitamin C supplementation into subphase ethanol-water, it was found that vitamin C molecules influenced interaction between ethanol and DPPC molecules (PO_2 group).

The results of area per molecule measurements revealed that in DPPC monolayer on subphase ethanol-water-vitamin C, the area of DPPC molecule was larger than in DPPC monolayer on subphase ethanol-water.

From the results of elastic modulus measurements of DPPC monolayer on water, subphase ethanol-water and ethanol-water-vitamin C, it was found that elastic modulus of DPPC monolayer on subphase ethanol-water and ethanol-water-vitamin C was increased.

Surface pressure – area isotherms shows similar behaviour of the DPPC monolayer on alcohol-water mixtures independently of the presence of vitamin C. Binding/ adsorption process induces change of electron density distribution across monolayer and thus the molecular dipole moment. We observe small or negligible binding of methanol molecules on oxygen bonds of DPPC.

Discussion

The results of interactions of DPPC monolayer with ethanol and water molecules can change electrical charges on the membrane surface, mechanical properties, permeability, and ion diffusion through the membrane. Patra et al.[7] suggest that ethanol changes the membrane structure. Ethanol causes conformation changes of structures forming membrane proteins. These structures ensure the performance of their physiological function.

Klemm et Williams[8] found that ethanol changes the water molecules arrangement around phosphocholine head (PC). Ethanol molecules bind to PC head, water molecules

move and create accumulation around ethanol alkyl group[9]. Chiou et al.[10] observed via FTIR spectroscopy the phenomenon that after ethanol supplementation to the solution of DPPC reversed micelles, part of water molecules bound to DPPC molecules by hydrogen bonds were replaced by ethanol molecules. The authors stated that interaction between alcohols and PC head of membrane lipid caused weakening of the membrane – water interaction and membrane destabilization. Thus ethanol causes membrane dehydration.

Vitamin C can cause two phenomenon: *a*) vitamin C molecules interact with DPPC molecules or *b*) vitamin C molecules bind preferentially with ethanol molecules and thus prevent binding of ethanol molecules to DPPC molecules. We believe that vitamin C molecules influence DPPC molecules and that vitamin C molecules adsorb to DPPC monolayer.

From the results concerning the area of DPPC molecule we believe that vitamin C molecules act against decreasing of the size of the shell created by water molecules around PC head of DPPC molecules.

Mechanical properties of the cell membrane play a crucial role in many biological processes[11]. Membrane elasticity is criterion of their mechanical stability. Mechanical properties are largely influenced by ethanol molecules, even though they do not absorb to DPPC monolayer. The elastic modulus measurements revealed constant increasing of the membrane stiffness. Vitamin C does not decrease the DPPC monolayer stiffness distinctly after ethanol molecules uptake. The DPPC monolayer on water is more flexible and elastic against mechanical impact than this monolayer on subphase ethanol-water.

The obtained results indicated possible effect of ethanol *in vivo* on membrane function. Here arises the question if ethanol influences the physiological function of membrane *in vivo* with concentration close to concentration in plasma or interstitium and to what extent ethanol can demonstrate its dehydration effect in a living organism. As a model for consideration, the studies dealing with the mechanism of anesthetics effect may be used. The lipid theory assumes changes in functions of membrane under the influence of anesthetics and alcohols[12]. If ethanol has the effect on membrane function and structure in low concentrations, this could be the possible mechanism of ethanol toxicity prior oxidation thereof to acetaldehyde. As described above, the membrane dehydration changes its physical properties (permeability, diffusion) and biological functions derived from changes of physical properties (changes in the protein structure).

Conclusion

The results show that the protective effect of vitamin C may be other than the antioxidant effect. We believe that we may expect similar protection effect *in vivo* and *in vitro*. We used vitamin C in the metabolism-free environment that could have produced free reactive radicals. Thus the antioxidant effect of vitamin C was not demonstrated on relevant level. The question still remains whether ethanol influences the physiological function of a membrane *in vivo* with concentration close to concentration in plasma or interstitium.

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P20

EFFECT OF CURCUMIN AND QUERCETIN ON OXIDATIVE TISSUE DAMAGE INDUCED BY FERRIC NITRILOTRIACETATE (Fe-NTA)

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Key words: curcumin, quercetin, ferric nitrilotriacetate (Fe-NTA), oxidative damage

Introduction

Ferric nitrilotriacete (Fe-NTA), iron(III) chelate of nitrilotriacetic acid, is a known prooxidative compound[1] and a carcinogen, used as a tool in experimental medicine for kidney carcinogenity studies. It is assumed that the mechanism of its nephrotoxicity is a consequence of iron-stimulated production of free radicals which initiate oxidative stress in the tissue[2]. Natural antioxidants, e.g. curcumin (Cur) and quercetin (Que) are able to counteract the effect of metal ions which participate in free radical generations. Curcumin (diferuloyl methane), a phenolic compound and a major component of *Curcuma longa* L., is lately drawing attention for its antioxidant activity and its low toxicity. It is also considered to be a potent cancer chemopreventive agent[3]. Quercetin (3,5,7,3',4'-pentahydroxy flavone) is a naturally occurring flavonoid present in fruits and vegetables, known for its antioxidant and free radical scavenging activities[4].

The aim of this study was to estimate the effect of curcumin and quercetin on lipid peroxidation, iron content and histopathological changes in kidneys of rats subjected to intraperitoneal ferric nitrilotriacetate administration.

Methods

Male Wistar rats (Anlab Prague) weighing 140–150 g were fed standard diet (Altromin C1000) and drinking water ad libitum. After an adaptation period of 6 days, the animals were asigned to four groups of 9 animals as follows: I. Control; II. Fe-NTA; III. Fe-NTA+Cur; IV. Fe-NTA+ Cur, Que. Curcumin was supplemented in the diet (Altromin C1000A, 2500 mg kg^{-1} of diet) for 8 days; quercetin was administered per os (15 mg kg b.w.$^{-1}$, dispersed in 0.5 % methylcellulose) once a day for 8 days. On the 6th day of experiment, the freshly prepared[5] Fe-NTA solution (8 mg Fe kg b.w.$^{-1}$, 1 ml 100 g b.w.$^{-1}$) was injected intraperitoneally to animals in group II-IV. The experiment was finished 24 h after the last dose of antioxidants, ie. 72 h after Fe-NTA injection. Both kidneys were quickly excised, one kidney of each animal was immediately frozen and stored at −70 °C until analyzed. The second kidney was fixed in 4 % formaldehyde, tissue specimens then routinely processed, stained with hematoxylin and eosin and used for histopathological examination.

Lipid peroxidation was measured as malondialdehyde (MDA) production formed in the thiobarbituric acid reaction in kidney homogenates[6]. The level of reduced glutathione (GSH) was estimated in the deproteinized supernatant fraction of kidney homogenate using 5,5'-dithiobis (2-nitrobenzoic acid) and recording absorption at 412 nm (ref.[7]). The iron concentration in the kidney was measured using atomic absorption spectrometry (SpectrAA 220 FS, Varian Australia Ltd.).

The data were expressed as means ± SD. Differences between experimental groups were estimated using unpaired Student's *t*-test. Results were considered statistically significant at $P<0.05$.

Results and discussion

The renal oxidative damage at 72 h after Fe-NTA administration was manifested by a significant increase in lipid peroxidation (fig. 1). Curcumin treatment was found to decrease the level of MDA and quercetin treatment significantly enhanced the protective effect of curcumin ($P<0.01$).

An increase of renal GSH level was found in Fe-NTA treated animals at 72 h following the injection (fig. 2). Curcumin+quercetin treatment increased renal GSH level compared to Fe-NTA only treated group.

As shown in Table I, a significant increase of iron

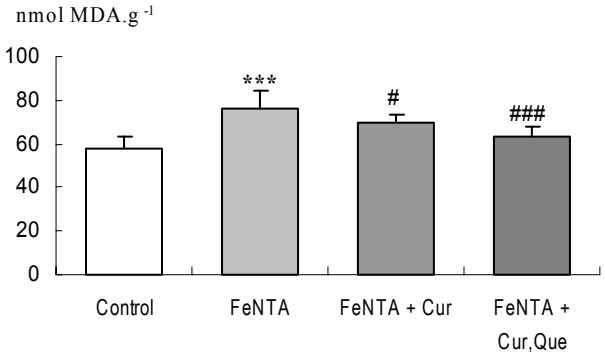

Fig. 1. **The effect of curcumin and quercetin tretment on lipid peroxidation in the kidneys of Fe-NTA exposed rats;** data represent mean ± SD; n=9; *** $P<0.001$ FeNTA group vs. Control group; # $P<0.05$, ### $P<0.001$ FeNTA+antioxidant group vs. FeNTA group

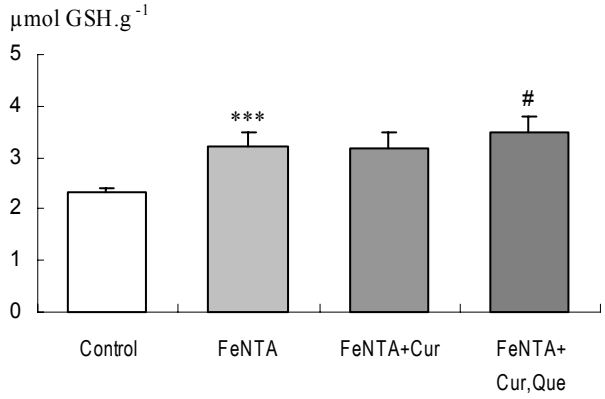

Fig. 2. **The effect of curcumin and quercetin treatment on reduced glutathione (GSH) level in the kidneys of FeNTA-exposed rats;** data represent mean ± SD; *n*=9; *** *P*<0.001 FeNTA vs. Control group; # *P*<0.05 FeNTA+antioxidant group vs. FeNTA group

Table I
Iron concentration in the kidneys of FeNTA and antioxidants treated Wistar rats

Treatment	Fe [µg g^{-1}]
Control	26.7 ± 3.7
FeNTA	39.5 ± 4.9 ***
FeNTA + curcumin	36.4 ± 5.9
FeNTA + curcumin, quercetin	34.7 ± 5.9

Results in µg g^{-1} of wet tissue weight. Mean ± S.D., *n*=9, *** *P*<0.001 vs. Control group

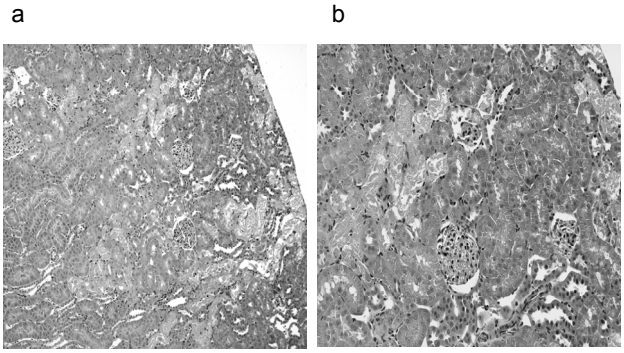

Fig. 3. **Light microscopy of renal tissue from Fe-NTA treated rats;** hematoxylin-eosin staining; (a) intesive zonal necrosis in tubular apparatus, 100×; (b) detail of necrotic changes, 200×

concentration was found at 72 h after a single administration of Fe-NTA. This enhancement was not affected significantly by antioxidants treatment.

Histopathological examination of kidney sections of Fe-NTA treated rats at 72 h after Fe-NTA administration revealed severe tubular epithelial damage with disappearance of distal brush border, without accompanying interstitial changes (fig. 3). The treatment with curcumin alone or in combination with quercetin did not prevent these changes.

Conclusion

The results from this study indicate that the treatment with curcumin and especially the combined treatment with quercetin significantly decreased lipid peroxidation. However, differently from examinations performed by other authors at shorter time period after the Fe-NTA administration (1–12 h)[8,9], no evidence of positive effects of antioxidants on Fe-NTA induced morphogical changes was found in our experiment at 72h after Fe-NTA injection.

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P21
ANTIOXIDANT STATUS IN LUNG OF RATS EXPOSED TO FIBROUS DUST OR CIGARETTE SMOKE

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Key words: lung, antioxidant status, cigarette smoke, fibrous dust

Introduction

The lung is the primary organ at risk of inhaled particles and gases, some of them are contributing factors of various respiratory diseases causation. The evidence of adverse health effects following exposure to asbestos resulted of banning asbetos and replacing it by various types of man-made mineral fibres having suitable technological properties. Cigarette smoke is a complex mixture containing more than 4700 constituents[1] including polycyclic aromatic hydrocarbons, cadmium, arsenic and other oxidants and toxic substances. Exposure to fibres[2] or cigarette smoke has been associated with production of reactive oxygen species generated either from the noxes themselves or by activated inflammatory cells. The lung has various protective mechanisms including an antioxidant system designated to metabolize oxidants. However, antioxidant enzyme systems in the lung may be overwhelmed by increased levels of free radicals[3]. We subjected rats to inhalation of cigarette smoke and to intratracheal instillation of different fibres and evaluated their antioxidant status in lung after finishing the exposures. The effects of different exposures were compared.

Materials and methods

Animals

Male Albino Wistar rats (Velaz, Prague, Czech Republic) were used in all experiments. They were housed under standard laboratory conditions and were given a conventional laboratory diet (TOP-Dovo, Horné Dubové, Slovakia) and tap water *ad libitum*. The study was conducted with the approval of the Animal Committee of the Slovak Medical University and in accordance with the guidelines of the European Convention for the Protection of Vertebrate Animals for Experimnetal Purposes.

Exposure to fibrous dust

Three different fibrous dusts were used in this experiment: amosite (asbestos), glass fibres and refractory ceramic fibres 3 (man-made mineral fibres). Animals were intratracheally instilled 4 mg of fibres resuspended in 0.2 ml saline. The control group was instilled by the same volume of saline. The exposure lasted 48 hours.

Exposure to cigarette smoke. A whole-body actively ventilated exposure chamber with a cigarette smoke generator and pumps (THRI, Lexington, KY, USA) was used. Smoker animals breathed diluted mainstream tobacco smoke from 8 standard research cigarettes of the 1R1 type whose smoke contained defined components. The animals were exposed daily, 5 days a week. The total length of exposure was 6 months.

Preparing of material and biochemical analysis

After finishing the exposures the animals were exsanguinated in anesthesia and bronchoalveolar lavage was performed. The lavage was centrifuged and the cell free fraction was separated and used for analysis. The lung tissue was homogenized in PBS and the 10 % homogenate was centrifuged (30 min, 10 000 rpm). The analyses were done in supernatant. All samples were stored in aliquots at −80 °C till analysis. Superoxide dismutase activity was estimated using Randox kit. Glutathione peroxidase activity was estimated using cumene peroxide as substrate[4], total glutathione was determined using the GSH reductase method[5], ascorbic acid was estimated spectrophotometrically by 2,4-dinitrophenylhydrazine method[6] and protein by Lowry et al[7].

Statistical analysis. The results were evaluated by Wilcoxon test.

Results

The antioxidant status was evaluated by estimation of two antioxidant enzymes (GSH-Px and SOD) and two non-enzymatic antioxidants (GSH and AA) both in homogenate of lung tissue and in cell free fraction of bronchoalveolar lavage. The results of exposure to 3 different fibrous dusts (one asbestos and two man-made fibres) are summarized and compared with those from control animals in Table I. The results showed greater changes in BALF than in lung tissue homogenate, all parameters after exposure to three studied fibrous dusts were lowered, in case of SOD with statistical significance. Fig. 1. demonstrated the changes evoked by inhalation exposure to cigarette smoke. The changes were more pronounced in non-enzymatic antioxidants with statistical significance in the level of AA in lung tissue and GSH in BALF. The effect of exposure was more reflected in BALF than in lung tissue.

Table I
Antioxidant status in lung tissue homogenate and cell free fraction of bronchoalveolar lavage fluid after intratracheal exposure to 4 mg of fibrous dust for 48 hours

Group		GSH-Px [U mg^{-1}prot]	SOD [U mg^{-1} prot]	GSH [nmol mg^{-1} prot]	AA [μmol g^{-1}]
Lung tissue	control	0.128 ± 0.005	8.14 ± 0.73	51.58 ± 6.69	0.87 ± 0.03
	amosite	0,120 ± 0.003	7.24 ± 0.38	66.37 ± 9.59	0.95 ± 0.03
	glass fibres	0.118 ± 0.006	6.84 ± 0.29	64.73 ± 4.40	0.84 ± 0.03
	RCF 3	0.120 ± 0.002	7.41 ± 0.50	48.58 ± 7.57	0.95 ± 0.03
BALF	control	7.27 ± 0.52	0.67 ± 0.03	6.82 ± 0.69	0.085 ± 0.010
	amosite	6.51 ± 1.00	0.48 ± 0.01*	6.63 ± 0.62	0.075 ± 0.005
	glass fibres	6.59 ± 1.24	0.44 ± 0.01**	7.15 ± 0.21	0.066 ± 0.006
	RCF 3	5.77 ± 2.00	0.39 ± 0.06*	7.85 ± 2.64	0.061 ± 0.002

Values are given as means ± SEM (n=6), * P< 0.05, ** P< 0.01

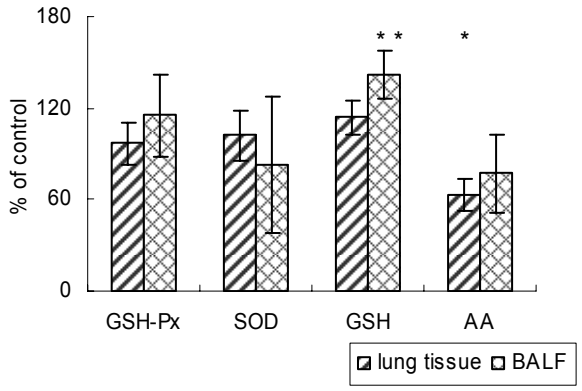

Fig. 1. **The effect of 6 month inhalation exposure of rats** on the antioxidat status in lung tissue homogenate and BAL; * P<0.05, ** P<0.01

Disscusion

Lung is equipped with antioxidant systems enabling to metabolize oxidants. Intratracheal exposure to three fibrous dusts (which differ in their chemical composition, solubility, size distribution and number of particles per dose) showed similar tendency as inhalation exposure to cigarette smoke: the differencies were more pronounced in BALF. BALF contains epithelial lining fluid which is in vital lung on the air-epithelial border[8] and presumed to protect the underlying epithelial cells against oxidative damage[9]. As the response of all tested agents was similar we can speculate that the protective mechanism is universal and focus our next experiments on the role of epithelial type 2 cells which are localized on the epithelial surface and belong from the toxicological point of view to the most important cells.

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Abbreviations

AA	ascorbic acid
BALF	bronchoalveolar lavage fluid
CS	cigarette smoke
GSH	glutathione
GSH-Px	glutathione peroxidase
PBS	phosphate buffered saline
prot	protein
RCF3	refractory ceramic fibres 3
SOD	superoxid dismutase

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P22

INHIBITORS OF NADPH OXIDASE DECREASE ENDOTOXIN MEDIATED INDUCTION OF INDUCIBLE NITRIC OXIDE EXPRESSION IN MOUSE MACROPHAGES

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Key words: lipopolysaccharide, phagocytes, reactive oxygen species, diphenyleneiodonium chloride, apocynin

Introduction

Endotoxin is an important environmental pollutant significantly contributing to wide range of acute and chronic inflammatory diseases including lung inflammatory disorders[1]. Examples of endotoxin are lipopolysaccharide (LPS) or lipo-oligo-saccharide found in the outer membrane of various Gram-negative bacteria. Activation of macrophages by endotoxin leads to production of wide range of inflammatory mediators contributing to pathology of inflammatory process[1]. Among potent inflammatory mediators is nitric oxide (NO) produced by activated macrophages in high quantities due to increased expression of inducible NO synthase (iNOS)[2,3]. Intracellular signaling pathways activated upon the activation of cells by endotoxin engage mediators sensitive to intracellular redox environment[4]. Thus, intracellularly produced reactive oxygen and nitrogen species (RONS) produced by NADPH oxidases and NOS are suggested to be important mediators augmenting the response of cells to endotoxin. Through this mechanisms NO could positively promote NOS expression and further increase of NO production.

The aim of this study was to characterize modulation of endotoxin activated macrophage NO production by the inhibition of RONS producing NADPH oxidases and NOS.

Material and methods

Murine macrophages RAW 264.7 were activated by LPS (100 ng/ml) isolated from *E. colli* (Sigma-Aldrich) for 24 h in the absence and in the presence of NADPH oxidase inhibitor Apocynin and inhibitor of flavoproteins including NADPH oxidases and NOS Diphenyleneiodonium chloride (DPI). Toxicity of these compounds were evaluated based on determination of floating death cells in cell culture media and total amount of protein in cell lysate as a marker of cellular total mass[5]. The production of NO was evaluated based on determination of nitrites (metabolites of NO oxidation) in cell culture media by Griess reagent (Sigma-Aldrich)[2]. Expression of iNOS was determined in cell lysate by Western blot technique using iNOS specific monoclonal antibodies (BD Laboratories)[3]. Values represent mean ± standard error of the mean. The statistic analysis was performed using Mann-Whitney test and differences at $P<0.05$ and $P<0.01$ were regarded as statistically significant.

Results and discussion

Evaluation of toxicity by both protein determination (Table I) and counting of death floating cells (data not shown) showed slight toxic effects of DPI on RAW 264.7 in two highest tested concentrations, however, without statistical significance. Apocynin did not show any toxic effects on RAW 264.7 (Table I) Interestingly, the LPS

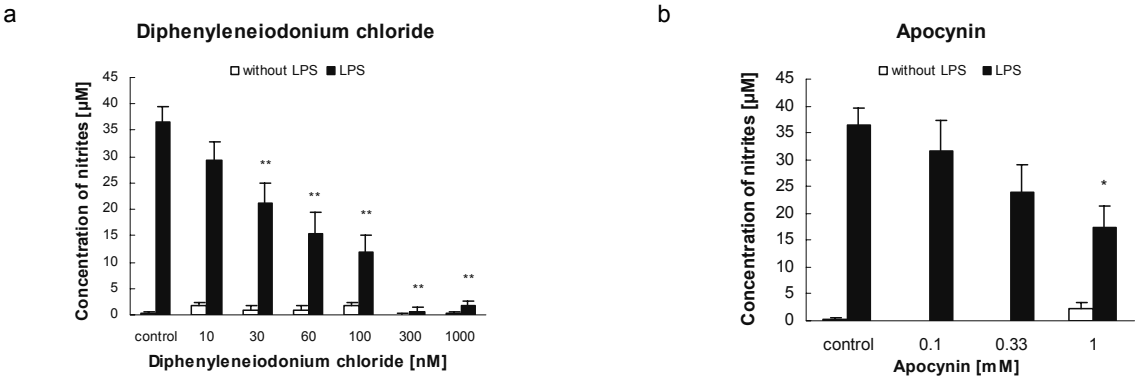

Fig. 1. **Nitrite concentration in culture media** of control (empty bars) and LPS activated (black bars) RAW 264.7 in the absence and in the presence DPI (a) and Apocynin (b). Statistical significance compared to control is marked with asterisk ($P<0.05$) or two asterisks ($P<0.01$)

Table I
Determination of cell viability by evaluation of total amount of protein in cell lysate (n.d. – not determined)

	Control	DPI [nM]						Apocynin [mM]		
		10	30	60	100	300	1000	0.1	0.33	1
Without LPS	1.5 ± 0.1	1.6 ± 0.1	1.4 ± 0.3	1.0 ± 0.5	0.9 ± 0.2	0.7 ± 0.5	0.7 ± 0.5	n.d.	n.d.	1.5 ± 0.2
With LPS	1.1 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	1.1 ± 0.3	1.0 ± 0.2	1.2 ± 0.2	1.1 ± 0.2	1.2 ± 0.3

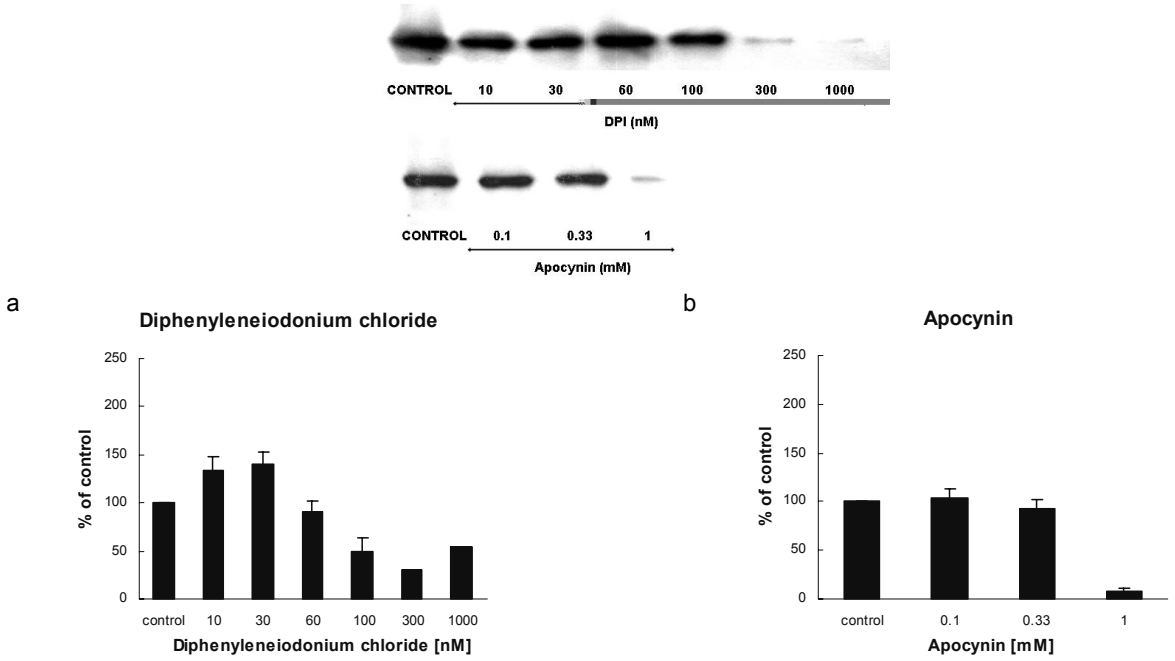

Fig. 2. **Western blot determination of iNOS expression** by LPS activated RAW 264.7 in the absence (control) and in the presence DPI and Apocynin. Picture shows typical example of western blot results and graphs represent densitometric evaluations of 3 repetitions (a and b)

stimulated production of NO by RAW 264.7 was significantly dose dependently down-regulated by both inhibitors (fig. 1a and 1b).

Decreased production of NO by RAW 264.7 could result from different mechanisms. DPI is known as a potent inhibitor of NOS activity, however, together with Apocynin could also modulate expression of iNOS by inhibition of LPS induced signaling pathways. Evaluation of effects of inhibitors on iNOS expression revealed significant inhibitory potential of both DPI and Apocynin on iNOS expression, with higher potential of former (fig. 2a and 2b).

Our data suggest the RONS produced by flavoprotein containing enzymes including NADPH oxidases and NO producing NOS are important for the cell activation by endotoxin. Thus modulation of intracellular RONS formation could be suggested as a potential pharmacological approach for a treatment of the inflammatory diseases connected with the exposure to endotoxin.

The study was supported by a grant No. 524/06/1197 of the Grant Agency of the Czech Republic and research plan AVOZ50040507.

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P23
CONSEQUENCES FOLLOWING INTOXICATION WITH HEPATOTOXIC AND NEPHROTOXIC SUBSTANCES

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Introduction

The aim of our study was to evaluate the severity and consequences of hepatic and kidney damage following intoxication with hepatotoxic and nephrotoxic chemical substances and mushrooms retrospectively in the years between 2000 and 2004 with a focus on their reversibility based on the calls made to the Czech Toxicological Information Centre (TIC).

Methods

A variety of clinical and laboratory parameters were collected. As markers of hepatotoxicity and nephrotoxicity were used: increase of serum alanine aminotranferase level (ALT>0.75 μkat l^{-1}), serum aspartate aminotransferase level (AST>0.75 μkat l^{-1}), serum total bilirubin level (>17.0 μmol l^{-1}), serum creatinine level (>110 μmol l^{-1}) and decrease of prothrombin time (<75 %)[1]. Follow-up evaluation was indicated at the time of discharge if hepatic or renal functions remained abnormal.

Results

From 2000 till 2004 the TIC received in total 43 044 calls. There were 4649 (10.80 %) enquiries with suspicion of poisoning with potentially hepatotoxic or nephrotoxic substances. Intoxications with mixtures or drugs were excluded. Only 269 (0.62 %) calls involved intoxication with chemical substances (ethylene glycol, chlorinated hydrocarbons) or with mushroom (*Amanita phalloides).* Poisoning with *Cortinarius orellanus* was not recorded. The hepatic or renal damage was recorded in 69 (0.16 %) patients. The laboratory markers had twofold to more than hundredfold increase/decrease.

Ingestion of the toxic substances as a route of poisoning occurred in all but two cases. 66years old man inhaled carbon tetrachloride vapours from an old fire extinguisher during his work in the household. He also poured his hands with the toxic liquid and drank one litter of beer. He developed renal failure (serum creatinine 948 μmol l^{-1}) with a necessity of repeated haemodialysis during 14 days. Nevertheless his renal function completely improved till 6 months following intoxication. 29years old woman had been working for 3 months with a mixture of organic solvents containing chloroform and methylene chloride in a small room without ventilation, where she inhaled the vapours of the solvents. She developed jaundice; her AST, ALT and bilirubin levels had tenfold increase above the reference value, but improved until 3 months.

50 adults developed nephrotoxicity following ingestion of ethylene glycol. 74 % of them fully recovered, 20 % did not comply in the follow-up. Hepatic and/or renal damage was recorded in other 17 patients following *Amanita phalloides* ingestion. 5 patients did not comply in the follow-up. In 33 years old woman renal failure persisted with the necessity of haemodialysis continuing even 4 years after intoxication. Other patients fully recovered.

Of 54 adults, who were followed-up, all but 4 recovered completely, mostly (81 %) till 6 months following intoxication.

Discussion

Our follow-up analysis demonstrated that hepatic or renal function impairment was reversible ad integrum in majority of patients. Recovery of their function could be influenced by the dose ingested and time delay between poisoning and admission to the hospital. All 3 subjects that did not recover after ethylene glycol intoxication had a history of disease that could have impaired the kidney function.

All mentioned hepatotoxic substances injure centrilobular cells of the liver acinus. Our results confirmed that this type of liver damage has usually a good prognosis. On the other hand the harmful effect of some other substances (phosphorus, iron) in the periportal area is mostly fatal because the new hepatocytes are created there[2].

Prognosis of poisoning with nephrotoxins, which act in the tubular segment of the nephron (ethylene glycol), is more favourable than a damage of the multipotent progenitor cells from the Bowman´s capsule[3]. This establishment explains the irreversible renal failure with lifelong haemodialysis following ingestion of *Cortinarius orellanus*.

Conclusion

Hepatic or renal function impairment was reversible in almost all patients, usually up to six months following the intoxication. The irreversible damage was observed very rarely, mostly in patients with pre-existing target organ disorders.

This study was supported by Research Project No. MSM 0021620807.

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P24
EFFECTS OF A FLAVONOID STRUCTURE ON CYTOCHROMES P450 INDUCTION

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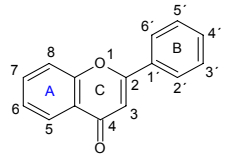
Key words: cytochrome P450, Western blotting, enzyme assay, flavone, flavanone

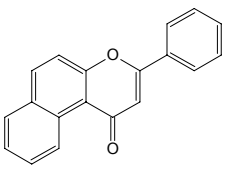
Introduction

Flavonoids are a diverse group of naturally occuring phenolic compounds. They are widely distributed in most plants and are an important component of human diet. It has been reported that flavonoids demonstrate a wide variety of biological activities, such as the enzyme-modifying activity[1], scavenging of reactive intermediates, antioxidant[2], antibacterial, antimutagenic[3] and antiviral properties[4]. On the other hand, it has been suggested that flavonoids may act as mutagens, prooxidants, and enzyme inhibitors and that they exert cytotoxicity at higher concentrations[5].

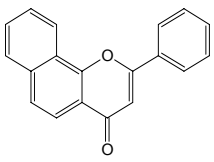
The basic chemical structure consists of two benzene rings (A and C) that are linked by a heterocyclic ring (B) (fig. 1). The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a group differ in the pattern of substitution of the ring A and B. The biochemical and biological properties of flavonoids vary considerably with only minor modifications of the flavonoid structure[6]. The effects of multiple hydroxyl and methoxyl groups substituting the basic flavonoid skeleton have been reported previously[7].



flavone



β-naphthoflavone



α-naphthoflavone

Fig. 1. **Structure of flavones**

One of the mechanism by which these compounds may exert their effects is through the interaction with cytochromes P450, monooxygenases metabolizing xenobiotics (e.g. drugs, carcinogens). In the processes of carcinogenesis, flavonoids might increase CYP-mediated carcinogen activation by inducing CYPs or by stimulating their enzymatic activities[8].

The aim of the present study is to investigate the effects of a series of non-substituted flavonoids (fig. 1) and flavanones (Table I) on the induction and metabolic activities of CYP1A1/2 after *p.o.* administration. For this purpose, their effects were evaluated by (*i*) the activities of ethoxyresorufin-*O*-deethylase EROD (CYP1A1) and methoxyresorufin-*O*-demethylase MROD (CYP1A2); (*ii*) the immunochemical identification of the P450 protein.

Material and methods

Flavonoids (α-naphthoflavone, β-naphthoflavone, flavone, naringenin, naringin, hesperetin, hesperidin, flavanone; Sigma Chemical Co., USA) were administered *p.o.* 60 mg kg^{-1} body weight to male Wistar rats (140–150 g), dissolved in sunflower oil (1 ml), daily for 5 consecutive days. The control group was treated with 1 ml of the sunflower oil. Microsomal fractions were prepared, immediately after sacrificing the rats, by differential centrifugation according to the method of van der Hoeven and Coon[9] from the whole liver. Protein concentration in microsomes was determined by the method of Smith et al.[10]. The CYP1A1/2 protein amounts present in the microsomes were determined by Western blotting on Immobilon-P membrane using specific chicken anti-CYP1A1 antibody. Ethoxyresorufin-*O*--deethylase (EROD) and methoxyresorufin-*O*-demethylase (MROD) activity assays were performed using the method described by Burke and Mayer[11]. Formation of the resorufin product was continuously measured for 10 minutes by monitoring its fluorescence with excitation and emission wavelengths of 530 and 585 nm, respectively. Both these enzyme assays have been performed with PerkinElmer Luminescence Spectrometer LS55.

Table I
Structure of flavanones

Compound	C3’	C4’	5	7	C2-C3
Flavanone	H	H	H	H	single
Hesperetin	OH	OCH_3	OH	OH	single
Hesperidin	OH	OCH_3	OH	rutinose	single
Naringenin	H	OH	OH	OH	single
Naringin	H	OH	OH	rutinose	single

Results and discussion

The aim of this study was to specify the induction effect of natural and synthetic flavonoids on CYP1A subfamily. Our results show that the induction pattern of the flavonoids varies with their structure. Naturally occurring non-substituted flavonoids, flavone and flavanone; two synthetic flavonoids, β-naphthoflavone and α-naphthoflavone; and citrus flavanones, naringenin, naringin, hesperidin, hesperetin, were selected to study the CYP1A1 and CYP1A2 induction. Hesperidin – hesperetin and naringin – naringenin, were selected as representatives of flavanone glycosides and their aglycones. To mimic the human flavonoid intake, the tested compounds were administred *p.o.* to rats and CYP1A1/2 content was determined in isolated liver microsomes.

Flavonoid effects on xenobiotic-metabolizing enzyme activities

We investigated the effects of a number of flavonoids on P450-dependent EROD and MROD activities. All four non-substituted flavonoids increased both monooxygenase activities, EROD and MROD (fig. 2). Synthethic flavonoids, β-naphthoflavone and α-naphthoflavone, enhanced the activity of EROD more than the two other natural flavonoids, flavone and flavanone. Flavone increased both activities more than flavanone. It can be explained by the presence of a C2-C3 single bond (ring C) in flavanone and a double bond in flavones (flavone, BNF, ANF). According to our results, the unsubstituted flavonoids are suggested to be able to function as inducers of several CYP (1A and 2B)[12,13].

The two glycosides, hesperidin and naringin, affected the activity of CYP1A1 in liver microsomes in contrast to their appropriate aglycones. On the other hand, all four flavanones increased the MROD activity. The different affection can be explained by the presence of glycosides in the molecule.

Immunoblotting studies

Immunoblot analyses were carried out to determine CYP1A1/2 protein expression and subsequently to correlate the observed EROD and MROD activities with content of the corresponding isoform. The specific primary chicken antibody against CYP1A1 and the secondary antibody conjugated with alkaline fosfatase were used for detection of CYP1A1 and 1A2. As shown in fig. 3, the CYP1A2 protein is known to be several times more abundant in control rat liver microsomes than the CYP1A1 protein]. From fig. 3, it is clear that beside the model inducer, β-naphthoflavone, the strongest induction effect on CYP1A1 of all natural flavonoids was determined in flavone-treated rats. Whereas, flavanone did not affect CYP1A1, it markedly induced CYP1A2. The induction of CYP1A2 was observed in all four non-substituted flavonoids. The significant induction effect on CYP1A2 was

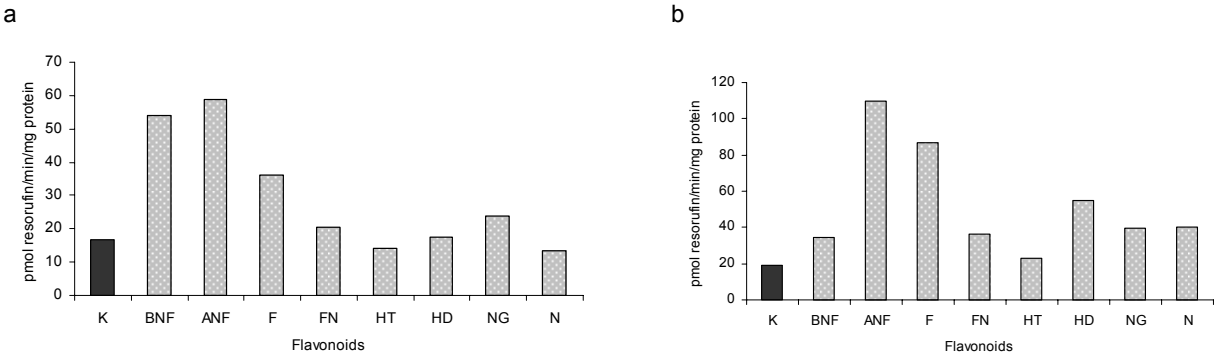


Fig. 2. **Effects of flavonoids on EROD (a) and MROD (b) activities** of CYP1A1/2 in rat liver microsomes after *p.o.* exposure to flavonoids (60 mg kg^{-1} body weight) for 5 days, ±SD≤10 %

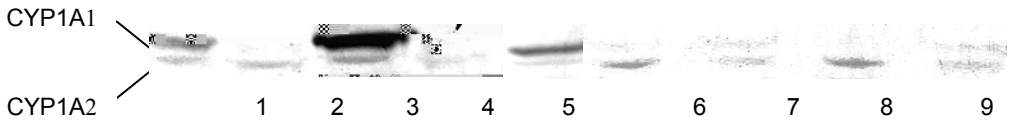


Fig. 3. **Immunodetection of CYP1A1 and CYP1A2 in liver microsomes from flavonoid-treated rats;** electrophoresed microsomal proteins (25 μg) were transferred to Immobilon-P membrane and probed with antibody against CYP1A1 and CYP1A2. Lane 1: flavone; lane 2: flavanone; lane 3: β-naphthoflavone; lane 4: control; lane 5: α-naphthoflavone; lane 6: naringin; lane 7: control; lane 8: hesperidin; lane 9: hesperetin

determined in hesperidin and naringin-treated rats. These results are in accordance with the above mentioned EROD and MROD enzyme assays.

Abbreviations

EROD	ethoxyresorufin-*O*-deethylase
MROD	methoxyresorufin-*O*-demethylase
CYP	cytochromes P450
ANF	α-naphthoflavone
BNF	β-naphthoflavone
N	naringenin
NG	naringin
HD	hesperidin
HT	hesperetin
F	flavone
FN	flavanone

This work was supported by the Czech Science Foundation (Grant No. 303/06/0928 and 303/05/2195) and by the Ministry of Education, Youth and Sports of the Czech Republic (Grant No. MSM 0021620808).

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P25

INFLUENCE OF METHOTREXATE AND L-CARNITINE ON TRANSINTESTINAL TRANSPORT OF MODEL SUBSTANCES (THE RAT SMALL INTESTINE IN SITU PERFUSION)

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Key words: methotrexate, carnitine, transintestinal transport, in situ intestinal perfusion, rat smalt intestine

Introduction

Two substances were selected for the definition of the transport processes and their influence in the small intestine in rat. Methotrexate (MTX) is indicated as cytostatic and imunosupresive drug[1]. Its antiproliferative action temporarily decreases the mucosae cellularity, the villy height and the depth of crypts in small intestine in rat, thus, induces the malabsorption syndrome[2,3]. L-Carnitine (CRT) is naturally occurring endogenous substance which facilitates the transport of the fatty acids through the inner mitochondrial membrane for the purpose of the β-oxidation[4]; thus, we also expect its potential influence on the general transport mechanisms.

Material and methods

Drugs

The representative substances of the transport mechanisms: indapamide hemihydrate (IND) as the representative substance of passive diffusion, galanthamine hydrobromide (GAL) and 7-methoxytacrine lactate (MEOTA) as the representatives of the combination of passive diffusion and carrier mechanism.

MTX and L-carnitine hydrochloride (Sigma AIdrich), IND (PRO.MED.CS Praha a.s.), GAL (Sigma Aldrich), MEOTA was synthesised in the Department of Toxicology, Faculty of Military Health University of Defence in Hradec Králové. [^{3}H]-7-methoxytacrine (specific activity 128.5 GBq mmol^{-1}, radiochemical purity >98 %, tritiated in the 1st Faculty of Medicine, Charles University). The drugs was added into the perfusion medium and was detected in perfusate samples. Detected amount of radioactive labelled MEOTA was consecutively re-counted for the amount of radioactive unlabelled MEOTA.

Animals

Male Wistar Han II rats (six animals in each group) weighing 311±69 g. For 24 hours before the study, the animals were not given solid food. The rats were anaesthesised by urethane (intraperitoneal dose of 1.5 g kg^{-1}) during the whole experiment. The experiment was approved by the Ethica1 committee of the Czech Academy of Sciences.

Application of the intestinal absorption modulating substances

The malabsorption syndrome was induced with intramuscularly application of MTX three days before perfusion in accordance with our previously publicated method[2]. In case of CRT application: the first group of animals was orally administrated with CRT using gastric probe (250 mg kg^{-1}) for three consecutive days. The perfusion with model drug (GAL or MEOTA 36 μg ml^{-1}) was carry out on third day, one hour after the CRT application. In the second group of animals the simultaneous perfusion only was performed with model drug and CRT (18.2 mg ml^{-1} of perfusate). The third group of animals was perfused only with model drug without CRT.

The small intestine *in situ* perfusion

The perfusion apparature, applied perfusates and the surgical procedure were used the same as described previously[2]. For the control of the mucosae physiological state we compared the histology of intestinal barrier among intact animals and animals after the perfusion. After the

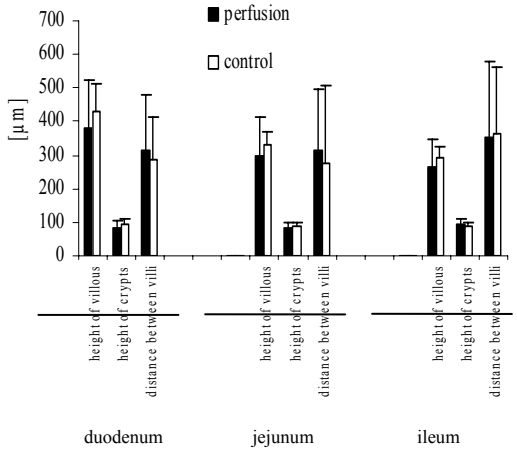

Fig. 1. **Histological examination of the mucosae of small intestine**

overall appreciation of the histological structure of intestine, the heights of villi and crypts and the distance between villi were evaluated using light microscopy. The histological examination of mucosae document that the measured parameters were not significantly altered under the perfusion condition compared to the control animals (fig. 1).

Bioanalytical procedures

The HPLC methods were used for the detection of GAL[5] and IND (UV detection, λ=240 nm, chromatographic column 250×4 mm, PurospherÒ RP-18 endcapped (5 μm), with pre-column 4×4 mm; mobile phase *A:* 0,01 M phosphate buffer (pH 3.4) – acetonitrile (63 : 37, v/v), mobile phase *B* – for the wash of a ballast matters*:* 0,01 M phosphate buffer (pH 3.4) – acetonitrile (20 : 80, v/v); the flow rate of both mobile phases was 1 ml min^{-1}. The radioactive labelled MEOTA was analyzed using scintilation detection. The samples from portal vein were interfused with 1 ml of a scintillation solution. After 24 hours of stabilization, the measuring of an activity was done in the samples with the use of liquid scintillation spectrometry (BECKMAN LS 5000 ID apparatus). Results were expressed as the mean ± standard deviation. F-test and Student' s t-test were used for statistical evaluations.

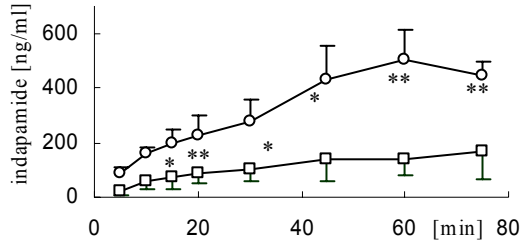

Fig. 2. **Absorption of indapamide (IND) from intestinal lumen.** Control animals (squares), animals with methotrexate induced malabsorption syndrome (circles). The asterisk means the significant statistical difference (* $P< 0.05$, ** $P< 0.01$)

Results

MTX significantly increased the transport of indapamide on the way of passive diffusion (fig. 2), but the transport of MEOTA was not significantly affected by MTX (fig. 3). CRT significantly increased the absorption of MEOTA and GAL after its previous *in vivo* premedication. The lower absorption occurred in the case of simultaneous perfusion of these model drugs with CRT (fig. 3 and 4).

Discussion

In our previous paper we demonstrated that MTX induced the histological changes in mucosae of the small intestine[2]. These findings are in good correlation with results publicated by other authors which demonstrated that MTX induced significant mucosa damage within the duodenum, jejunum and ileum of rats and dose-dependent decrease in villus heights[3]. Small intestine damage is believed to result from hypoproliferation[6] and widespread apoptosis of stem cells in intestinal crypts[7,8]. It is perceptible that MTX induces the malabsorption syndrome, significantly reduces the villus height (thus absorption area) and intestinal wall repair and hences its cellular outfit. It leads to the reduction of the intestinal barrier and thus to the increase the transport of xenobiotics (indapamide) on way of passive diffusion. On the other hand, in our previous experiments[2] we documented that MTX significantly decreases the carrier transport of glucose from lumen of small intestine. The explanation why MTX significantly did not influence the MEOTA and GAL transport may be that both substances are partly transported by passive diffusion and partly by carrier transporting mechanism. The

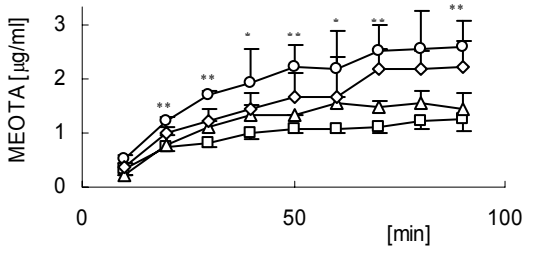

Fig. 3. **Absorption of 7-methoxytacrine (MEOTA) from intestinal lumen**. Control animals – only the MEOTA perfusion (triangles), animals with methotrexate induced malabsorption syndrome (rhombs), simultaneous MEOTA+L-carnitine (CRT) perfusion (squares), CRT premedication (circles). The asterisk means the significant statistical difference control vs. CRT premedication (* $P< 0.05$, ** $P< 0.01$)

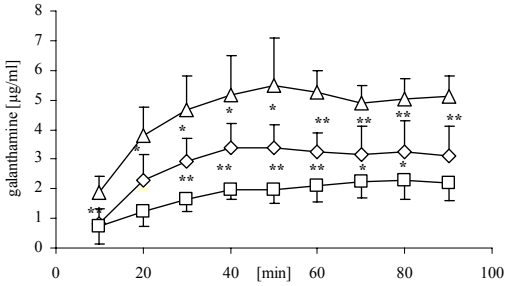

Fig. 4. **Absorption of galanthamine (GAL) from intestinal lumen.** Control animals – only the GAL perfusion (rhombs), simultaneous MEOTA+GAL perfusion (squares), CRT premedication (triangles). The asterisk means the significant statistical difference control vs. CRT premedication (* $P< 0.05$, ** $P< 0.01$)

influence of MTX is finally neutral1 in result. Due to the fact that the CRT absorption occurs as well as MEOTA and GAL part1y via carrier-mediated transport and part1y by passive diffusion[4], it could lead to the over-saturation of carrier system and thereby to the competition upon the absorption of the other compounds. Significantly higher MEOTA and GAL absorption in rats orally premedicated with CRT *in vivo* for a period of three days suggested that CRT have probable some facilitated effect on the transintestinal transport of MEOTA and GAL and maybe some others substances after its incorporating into the cellular metabolism.

Conclusion

The experiments documented the usability of the rat small intestine perfusion method in the study of transintestinal mechanisms in rat. The MTX apllication enable to differentiate the simple diffusion and carrier mechanism and thus the way of transintestinal transport of xenobiotics. MTX and CRT are usable in the study of processes of intestinal absorption modulation.

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P26
BUPRENORPHINE – TRADITIONAL DRUG WITH A NEW POTENTIAL RISK OF ABUSE

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Key words: buprenorphine, substitution therapy, abuse, respiration failure

Introduction

Buprenorfin (BUP) is a conventional analgetic, derived from an alkaloid opiate thebaine. As compared to other opiates, it is considered harmless, since it does not affect largely respiration or mind. Nevertheless, its introduction into therapy of drug addicts brought, together with predicted merits, also new risks. BUP was first semisynthetically prepared in 1968. Traditionally, it has been used as medicament suppressing severe pains and for premedication. At higher doses, it minimizes the need of drug addicts for additional doses of illegal intravenous opiates. Therefore, it was indicated as reportedly harmless peroral substitution treatment of heroin addicts. In Czech Republic, its administration (Subutex) was approved in the 2000.

Pharmacokinetics and pharmacodynamics

Biological accessibility during digestion is relatively low. Therefore, in addition to injections, sublingual tablets are manufactured for oral mucous absorption, which bypasses intestinal degradation and first past effect in liver. BUP is well distributed and as a lipophilic substance easy passes hematoencephalic barrier. A three-compartment model best characterizes its complex kinetics. Its plasma half time ranges between 1–7 hours; the terminal elimination process lasts 20–44 hours. Liver isoenzyme CYP 3A4 catalyzes the BUP conversion to an active metabolite – norbuprenorphine and bought are inactivated by glucuronization. BUP and metabolites are secreted into bile and eliminated by stool (70–80 %), partially by urine (10–30 %).

BUP is an opioid agonist-antagonist. It has a strong affinity to receptors μ and κ (100 times higher than morphine) with a slow reversible dissociation, which causes its long-term effect. It react less with δ receptors and, therefore, exhibits lower euphoric effects, which results in milder risk of addiction and moderate withdrawal symptom. Both experimental and clinical studies confirm the unique pharmacological property of BUP that, in contrast to selective agonists, as morphine and methadone, does not affect significantly the respiratory functions and shows so called "ceiling effect". As a consequence, the severity of over dosage is reduced. Moreover, it acts with κ receptors as a weak antagonist, and in this way can block the effect of other opiates in a degree comparable with antidote naltrexone.

Dosage and indications

BUP is 25 to 50-fold more effective analgesic than morphine and the ordinary doses are therefore low, up to 0.5 mg. The maximal overall dose is not indicated because BUP is considered as a medical preparation with wide-safe therapeutic profile. As analgesic it is registered in Czech Republic as Temgesic in the form of sublingual tablets of 0.2 mg or ampoules of 0.3 mg. For maintenance therapy (substitution) of opiate addicts, BUP is administrated at higher doses in a form of sublingual tablets Subutex of 0.4, 2.0 or 8.0 mg. The medicament has to be administrated only sublingually; once a day for adults and adolescents over 15 years. The dosage is individual in dependence on the addiction. The day dose should not exceed 32 mg.

Substitution therapy

Maintenance substitution is an official method for drug addiction therapy. It represents the first medical help for an addict, which should continue in further programs oriented on abstinence. It consists in administration of a substitute with similar effect as originally used drug but deliberately administrated in a different way than intravenously. It suppresses withdrawal symptoms and leads to a reduction of drug abuse. Consequently it leads to the prospective effect is "harm reduction", i.e., minimizing the health and social impacts of dangerous behavior of addicts as, e.g., communication and social dystrophy and, above all, the criminality. Corporate benefit is the reduction of medical costs spent for the treatment and minimization of incidence of infectious diseases expanded by hazardous injection applications (hepatitis B and C, AIDS).

Side effects and toxicity

Side effects of buprenorphine are negligible and only rarely reported for the high-dosage substitution therapy. They include sedation, dizziness and nausea. As well, poisoning symptoms by BUP are similar, more marked but not serious. Toxic doses are individual and the lethal doses are even not determined. Only a single decease caused by a BUP massive per oral intoxication has been described so far.

Abuse and its risk

Abusers discovered that the sublingual tablets of Subutex could be misused. Crushed tablets applied intravenously elicit an effect comparable to heroine. BUP began to appear at the black market and became more and more popu-

lar drug. In contrast to heroine, it can be obtained legally, it is less expensive, and available in constant and reliable quality. However, in contrast with its reputation of a safe preparation, recently a number of cases were described, in which BUP abuse led to typical symptoms of serious intoxication found with conventional opiates, which leads to patient death caused by respiratory collapse. In Europe, particularly in France and northern states, more than 100 such cases were described till 2006 (ref.[1]). In Singapore counting 4.5 millions inhabitants, 21 such cases was reported during 15 months[2]. In most cases, coincident usage of psychotropic drugs, esp. benzodiazepines (BZD), was documented. It is surprising, that the lethal asphyxia occurred even in the cases with relative small over dosage or even at therapeutic dosages, e.g., combination of buprenorphine and diazepam.

Asphyxic deaths operation

Neither BUP nor BZD alone causes the lethal intoxications. However, it was shown that in an interaction, they show synergic pharmacodynamic effect[3]. BZDs elevate respiratory effort of upper airways and BUP disturbs normal response to this load in attenuation of respiratory centers in brain stem[4]. Apparently, this is the mechanism of alteration protective ceiling affect alteration. The combination of BUP with other sedatives as alcohol and tranqullizers can be similarly dangerous.

Situation in Czech Republic

The report[5] of National Drug Monitoring Center of The Council of the Government for Drug Policy Coordination in 2006 estimates in our countries 32 000 troubled drug users, i.e., persons with long-term, regular or intravenous drug applications. One third of these (11 300) are the persons addicted to opiates. In the last year, sum of 949 were treated by replacement therapy; most of them by methadone and only 375 by buprenorphine. However, as judged from the amounts of distributed Subutex, the total number of BUP users is about 8 times higher (3100) than the number of the officially treated patients. In contrast to methadone, which is prepared from a registered substance and can be obtained only in specialized centers for addiction therapy, Subutex is ordinarily available in pharmacies. Every physician regardless of his professional specialization can prescribe it. Thus, the abusers can visit several physicians, can obtain several packages and sell some of them. Partial restriction was introduced in 2005 since when the blue-strip recipes has to be used for better check-up. Subutex illegal dealing is monitored mainly in Prague, and northern and southern Bohemia.

Deaths connected with opiate abuse

The introduction of the substitution therapy led to a decrease of deaths caused by heroine and other opiates' overdose. In comparison to the years 2001–2005 in our republic, this number decreased more than to a half (from 56 to 24). Among these in 2005, three incidents with methadone were registered while no diagnosis of BUP appeared in official statistics. Nevertheless, in one lethal incident BUP was diagnosed in our laboratory. It is evident that not all the cases can be verified. The analysis is carried out only for the sake of autopsy injunction together with suspicion for Subutex consumption. That is, BUP is not detected in screening test for opiates in urine, nor in ordinary toxicology tests for medicaments. Moreover, in the forms of official Czech statistic, there is no separate column among opiodes, for BUP, as it is the case for methadone.

How to prevent the abuse risk

After introduction otherwise beneficial substitution therapy by BUP, in number of countries new risk arose: BUP abuse as an intravenous drug can have lethal effects. Can we prevent the growth of such incidents?

a) One of the prerequisite is an extension of laboratory diagnostics for Subutex abuse. Urine test for BUP should be included into routine screen testing and its presence should be examined in the lethal incidents caused by unknown substance.
b) Further education among physicians, e.g., seminars organized by The Scientific society JEP for addictive diseases.
c) To restrict illegal dealing with Subutex tablets, State Institute for Drug Control suggests to establish electronic registration of prescriptions for the registered patients. This would prevent multifold prescriptions of the medicament to the same person.
d) To prevent BUP overdose, new preparation Suboxon (combination of BUP and naloxone) is planned for the distribution. The opioide antagonist – naloxone, is present in the tablets in a form, which is not absorbed per os, while applied venously it blocks euphoria and provokes severe withdrawal. This prevents the abuse or overdose. In Czech Republic, it has to be introduced to the market at the end of 2007; in the meantime together with Subutex.

Conclusions

It is necessary to be aware that the abusers' community is inventive. To hamper the negative impact of their activities, all possible measures restricting the abuse of other medical preparations have to be installed, at the best, a step in advance.

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P27
THE EFFECT OF NiO NANOPARTICLES ON IMMUNE SYSTEM OF RATS

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Key words: NiO nanoparticles, Immunotoxicity, Cellular immunity, Sprague-Dawley rats

Introduction

Nickel and its alloys are used in a wide variety of industrial applications. Therefore, there are assumptions of possible contamination and following impact on environment. Data on the influence of these materials on human health, especially on immune system are rare and more studies are needed to examine possible immunomodulatory effect.

Epidemiological studies have demonstrated increased mortality from lung carcinoma and nasal cavities in workers chronically exposed by inhalation to nickel-containing dusts in nickel refinery. The respiratory tract cancers in nickel refinery workers have been associated with inhalation exposures to nickel compounds with low aqueous solubility such as NiO[1]. Our study has monitored the effect of nickel nanoparticles on selected cellular parameters of immune response in Sprague-Dawley rats.

Material and methods

Male Sprague-Dawley rats (ISASZEG, Hungary) with starting weight of 200–220 g were treated by intratracheal instillation with one single dose of 0.5 mg NiO nanoparticles (0.5 mg animal^{-1}). After one and four weeks, the blood samples and spleen were aseptically removed. The following immune assays were performed: (*i*) Proliferative assay – splenocytes and peripheral blood were incubated *in vitro* with mitogens: 5 mg ml^{-1} concanavalin A (ConA), 25 mg ml^{-1} phytohemmaglutinin (PHA), 2.5 mg ml^{-1} pokeweed (PWM) and 100 mg ml^{-1} *Salmonella typhimurium* (STM). The proliferative activity of cells was determined by incorporation of [^{3}H]-thymidine into DNA and measured using beta scintillation counter. (*ii*) Expression of adhesion molecule CD11b on monocytes, lymphocytes and granulocytes was quantified using monoclonal antibodies conjugated to fluorescein isothiocyanate and evaluated by flow cytometry. (*iii*) Differential white blood cells count examined percentage of each type of white blood cells. Analyses were performed on slides where the drop of blood was smeared and stained with contrast dye using microscope. Statistical analysis was done using SPSS. Differences between groups were analyzed using Student T-test or Mann-Whitney test.

Results and discusion

The DNA synthesis in spleen lymphocytes *in vitro* stimulated with PHA, PWM and STM as well as in non-stimulated cells derived from rats exposed to NiO nanoparticles was significantly suppressed in comparison with cultures derived from control rats one week after exposure (Table I). Similarly, proliferative activity of

Table I
Proliferative activity of splenocytes *in vitro* stimulated with mitogens (PHA, PWM, STM) and non-stimulated splenocytes

Parameter		1 week after exposure [dpm min^{-1}]	4 weeks after exposure [dpm min^{-1}]
Proliferative activity of T-lymphocytes *in vitro* stimulated with phytohemaglutinin (PHA)	Control	42760	28829
	Exposed	17356***	25398
Proliferative activity of spleen T-dependent B-lymphocytes *in vitro* stimulated with pokeweed mitogen (PWM)	Control	73610	34199
	Exposed	48152**	39293
Proliferative activity of spleen B-lymphocytes *in vitro* stimulated with mitogen from *Salmonela typhimurium* (STM)	Control	6000	6614
	Exposed	4191**	6140
Basal proliferative response of non-stimulated spleen cells	Control	3805	4428
	Exposed	2432***	4662

Statistical significance ** P<0.01, ***P<0.001

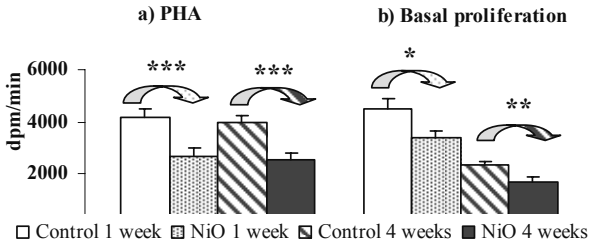

Fig. 1. **Proliferative activity of blood lymphocytes** stimulated with phytohemaglutinin (a) and non-stimulated (b). * P<0.05, ** P<0.01, *** P<0.001

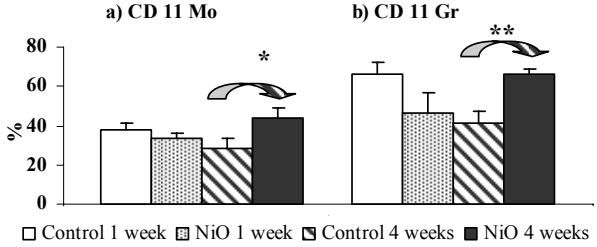

Fig. 3. **The expression of adhesion molecule CD11b on monocytes (a) and granulocytes (b)** in peripheral blood. * P<0.05, ** P<0.01

blood lymphocytes (*in vitro* stimulated with PHA and non-stimulated) taken from NiO exposed rats was significantly decreased in comparison with belonging control rats (fig. 1). Suppression of proliferation was significant in both groups – after shorter and longer exposition time. Our data correspond with study of Haley et al. who found that inhalation exposure of mice to nickel can result in varying effects on the immune system. Particularly, study showed decreased number of spleen antibody forming cells in mice exposed to NiO. These nickel-induced changes may contribute to significant immunodysfunction and effect depends on dose and physicochemical form of the nickel compound[2].

In group of animals with shorter exposure we recorded significantly lower percentage of neutrophils in comparison with control group. The same effect was observed also on percentage of monocytes (fig. 2). Depletion of phagocytic cells might be due to cytotoxic effect of nickel oxide as was described by Takahashi et al.[3].

Four weeks after exposure, the expression of adhesion molecule CD11b on monocytes and granulocytes of NiO exposed rats was significantly higher then in control animals (fig. 3). This enhanced expression of adhesion molecule might be associated with increased cell transfer into region of chronic inflammation. It has been recently shown that the beta(2)-integrin molecule (CD11b) is upregulated on circulating neutrophils in chronic obstructive pulmonary disease (COPD) subjects[4]. Nickel oxide can cause

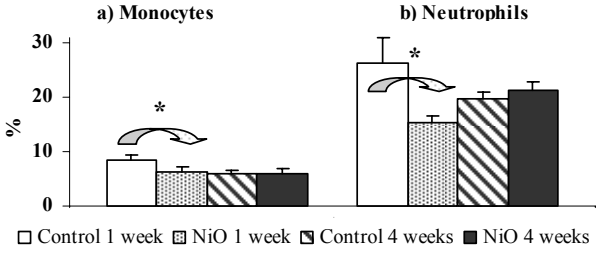

Fig. 2. **Percentage of monocytes and neutrophils** in peripheral blood. * P<0.05

inflammation, hyperplasia, and fibrosis in the lungs of rats, and to a lesser extent in mice, exposed to nickel compounds. Published papers found histopathological changes in the lungs as the most sensitive parameters for nickel toxicity in exposed animals where chronic active inflammation, fibrosis, and alveolar macrophage hyperplasia were associated with nickel exposure[5].

Conclusion

Our results indicate immunomodulatory effect of NiO nanoparticles in exposed rats. Immune changes were observed one week after exposure of animals and most of them persisted also one month after exposure to NiO.

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P28
AMES TEST AS AN ALTERNATIVE METHOD: MUTAGENICITY OF OSTRAVIAN AIR (YEAR 2006)

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Key words: urban air, industrial sites, pollutants measurement, Ames test

Introduction

Genetic toxicology testing is capable of finding substances, or their mixtures, that can damage DNA. These processes could result in the initiation and progression of cancer.

Ostrava region is one of the most polluted regions in the Czech Republic. Air quality is frequently evaluated by comparison of contaminant's concentration with its legislated limit[1]. The real environmental air is a complex mixture of many together acting compounds. The interactions among all of them are complicated not only on the chemical but even more on the biological level[2–4].

The aim of study has been determining the mutagenic potentials of sampled air and levels of selected pollutants (during one year), based on which correlations, if any, between biological (mutagenic) effect of the real air and its components could be evaluated.

Materials and methods

Air sampling has been performed at three industrial sites of the city (coking plant, chemical works, iron and steel industry, vehicle emission) daily (for 24 hours) four times a month during one year.

Chemical analyses of some carcinogenic and mutagenic substances has been carried out: benzene, trichlorethene, styrene, toluene – gas chromatography with FID detector; arsenic, nickel, cadmium, chromium – X-ray spectrometry; eight polycyclic aromatic hydrocarbons (PAHs) including benzo/a/pyrene (B/a/P) – HPLC with fluorescence detection.

Concurrently every month pooled air sample from every site has been tested using the standard plate-incorporation Ames test[5].

Monthly means of individual pollutants (to find correlations with mutagenic potentials) and their annual means (to compare them with residential annual limits) have been calculated.

Statistical evaluation of the mutagenic potentials using Genetox Manager v. 2.1 program and the statistical evaluation of pollutant's concentrations using ANOVA has been carried out. Associations between monitored factors have been analyzed by the correlation and regression analyses.

Results and discussion

Mutagenic activity of air

Mutagenic potentials (per volume of air) of monitored air samples have been found higher during winter period in comparison with summer period at all sites. Levels of indirectly acting substances (for example PAHs) have been observed higher than those of directly acting ones.

Pollutan levels

Annual values of B/a/P concentration exceeds the limit at all monitored sites, annual arsenic value is within the limit at one site while above it at two other sites and the benzene annual concentration exceeds the limit at one site only.

PAHs' and metals' monthly levels display season-related differences during the year in contrast with the monthly levels of volatile compounds.

Correlations between biological effect and presence of pollutants

The closed correlations have been noted at all sites between mutagenic potentials of the air and the presence of B/a/P and all measured PAHs. The weakly one has been found between the levels of air mutagenic activity and concentration of arsenic (only at one site, where the arsenic annual value has been within the limit). Furthermore, at two sites with benzene annual value under the limit, weak correlations between the biological effect and the presence of volatile compounds (trichlorethene, styrene, benzene) have been registered.

Conclusion

Our study confirmed that interactions among components of the complex mixture with a number of potentially genotoxic substances are complicated. Taking into account mutagenic potency of Ostravian air the major contribution seems to occur due to PAHs action.

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P29

COMPARISON OF ACUTE TOXICITY OF POTASSIUM PERMANGANATE TO JUVENILE AND EMBRYONIC STAGES OF *Danio rerio*

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Key words: potassium permanganate, age, LC50, *Danio rerio*, fishes

Introduction

Zebrafish (*Danio rerio*) is one of the model organisms most commonly used in toxicity testing. It is a popular aquarium fish from the family *Cyprinidae* (Cyprinids) which originates from the Ganges river system in the Indian Peninsula[1]. The main advantage of this species is easy availability, low price, and easy rearing. At suitable conditions, one female is able to produce 50–200 eggs per day. Eggs sized 0.8 mm^2 are non-adherent and fully transparent[2].

Potassium permanganate ($KMnO_4$) is a violet, crystalline substance with oxidative effects, well soluble in water. The compound is used as a disinfectant in immersion, short-term or long-term baths[3] to purify water in fishery and as a traditional oxidant. The substance gained its significance and became widely used thanks to its versatile use and easy availability. Therapeutic baths are used worldwide in both fishery and aquarium fish breeding to treat and prevent bacterial skin and gill infections and ectoparasitic and fungal diseases of freshwater fish[4,5]. The compound exerts its powerful oxidative effect on pathogens. It reacts as an oxidative agent when in contact with organic substances including pathogens that cause external diseases of fish[4]. The effective concentration of the active ion (i.e. MnO_4^-) used to control ectoparasitic, bacterial, and fungal diseases is usually 2 mg l^{-1}.

Generally, manganese-containing compounds do not pose any major risk to fish species. The only exception is potassium permanganate whose toxicity to fish is moderate to strong[6]. The toxicity of permanganate was determined in a number of different fish species[7–13] at various factors that affect the toxicity of this chemical in fish. The toxic effect of potassium permanganate is not exactly known. Some authors[14] have confirmed that permanganate or its products such as MnO_2 may cause damage to gill tissue as they precipitate in gills[4] at high pH. Acute toxicity of potassium permanganate was investigated in a number of fish species at different age. Generally, it is assumed that lower developmental stages of fish are more susceptible to toxic effects of chemicals. Some authors report that earlier developmental stages of fish have greater tolerance to toxic effects[15–18].

The main aim of this work is to compare acute toxicity of potassium permanganate in embryonic and juvenile developmental stages of the aquarium fish species *Danio rerio*.

Materials and methods

Acute toxicity tests were performed in the juvenile stages of zebrafish (*Danio rerio*) according to OECD 203 methodology (Fish acute toxicity test). A series of six tests was performed. The fish aged 2–3 months, weighed 0.3±0.1 g and being 30±5 mm long were used in the experiment. The tests were performed using a semistatic method, with solutions being changed after 48 hours. Each vessel contained 10 fish randomly selected from the stock population; 96-hr acute toxicity tests were then performed.

Embryo toxicity tests were performed in zebrafish embryos (*D. rerio*) in compliance with the OECD No. 212 methodology (Embryo toxicity tests). A series of six tests was performed. The tests used a series of six concentrations. 20 fertilized eggs placed in one Petri dish were tested for each concentration and in control. The bath was changed every 48 hrs.

Statistical significance of the difference between LC50 values in juvenile and embryonic stage of *D. rerio* was tested using the Mann-Whitney non-parametric test implemented in the Unistat 5.1 programme.

Results and discussion

Since potassium permanganate is frequently used in fishery, it is necessary to test the toxicity of this substance in order to ensure that suitable therapeutic concentrations

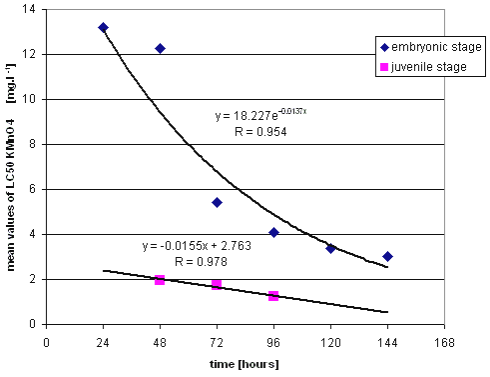

Fig. 1. **Variation in LC50 $KMnO_4$ with the duration of the test** for the embryonic and juvenile stages of *D. rerio*

will be used in the treatment of fish. This topic was addressed by a number of authors whose investigations particularly focussed on freshwater species[4,5].

Acute toxicity of $KMnO_4$ expressed as 96h LC50 varied in a range of 2.09–5.76 mg l^{-1} for the embryonic stage of zebrafish (the mean value of 96h LC50 = 4.075±1.585 mg l^{-1}) and in a range of 1.09–1.53 mg l^{-1} for the juvenile developmental stage of zebrafish (the mean value of 96h LC50 = 1.233±0.174 mg l^{-1}). The value of 144h LC50 $KMnO_4$ for the embryonic stage of zebrafish was found to vary in a range of 2.14–4.07 mg l^{-1} (the mean value of LC50 = 3.021±0.735). The values of 96h LC50 $KMNO_4$ reported for the Cyprinidae family equal to 40 mg l^{-1} in common carp *Cyprinus carpio*[6]. Slightly lower values of 96h LC50 potassium permanganate but still similar to those found in our tests were reported for *Morone saxatilis* (1.58 mg l^{-1})[10], *Pimephales promelas* (2.13 mg l^{-1})[11], *Lepomis macrochirus* (3 mg l^{-1})[7], *Carassius auratus* (3.6 mg l^{-1})[8]. Higher tolerance of toxic effects of $KMnO_4$ were observed in perch (96h LC50=6 mg l^{-1})[6], *Colossoma macropomum* (96h LC50=8.6 mg l^{-1})[13] and the fish species *Angilla rostrata* (96h LC50=21.6 mg l^{-1})[12].

It is generally assumed that the lower developmental stages of fish are more susceptible to the toxic effects of chemicals. A comparison of LC50 mean values for both developmental stages of zebrafish (*D. rerio*) showed that the LC50 in the embryonic stage of zebrafish was significantly higher (P<0.01) than that for the juvenile stage of zebrafish. This result indicates greater tolerance of potassium permanganate in the embryonic stage of zebrafish. It follows from the results of the tests that the resistance of zebrafish embryos (*D. rerio*) to the effects of potassium permanganate decreases with the increasing duration of the test. The exponential relation $y=18.227*e^{0.0137x}$ (r=0.954) between the mean values of LC50 $KMnO_4$ and the duration of the test was found (Graph 1). The highest mortality rate occurred in zebrafish at the juvenile developmental stage within the first 48 hrs of the 96-hr test. Similarly, the tolerance to the tested dose decreased with the increasing duration of the test in embryos, with the linear relation being found between the mean values of LC50 $KMnO_4$ and the duration of the test, i.e. $y = -0.155x+2.763$ (r=0.978).

Greater tolerance in earlier developmental stages of fish was also confirmed by other authors who compared the resistance of embryonic, juvenile, and adult stages of various species of fish[18,15,16] to different pollutants.

Acute toxicity tests in embryos revealed that permanganate at individual tested concentrations affects the hatching time in fish at individual tested concentrations, which is considered to be a side effect of permanganate.

Conclusion

Potassium permanganate has been used worldwide in fishery and aquaculture for treatment and prevention of skin and gill bacterial infectious, fungal infectious and external parasites in freshwater fish. The aim of the study was to compare acute toxicity of potassium permanganate to juvenile and embryonic stages of zebrafish (*Danio rerio*). The semistatic method according to OECD 203 was used in the acute toxicity tests with juvenile fish and OECD 212 methodology was used in the acute toxicity tests with embryonic stages of *D. rerio*. The LC50 $KMnO_4$ values ranged from 1.09 to 1.20 mg l^{-1} in juvenile *D. rerio* fish while LC50 $KMnO_4$ values ranged from 2.14 to 4.07 mg l^{-1} in embryonic stages of *D. rerio*. The study proved statistically significantly higher (P<0.01) sensitivity of juvenile fish to potassium permanganate as compared with embryonic stages.

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P30

SUPEROXIDE GENERATION AND MYELOPEROXIDASE RELEASE IN HUMAN POLYMORPHONUCLEAR LEUKOCYTES TREATED WITH CARVEDILOL

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Key words: carvedilol, superoxide generation, myeloperoxidase, human polymorphonuclear leukocytes

Introduction

Polymorphonuclear leukocytes (PMNL) are one of the main types of inflammatory cells. The production of reactive oxygen metabolites (ROM) including generation of superoxide anions (SO) by activated PMNL is critical for their successful bactericidal action, yet under pathological conditions they may contribute to host tissue injury[1]. Activation of PMNL induces also release of enzymes from PMNL granules which play a crucial role in the destruction and phagocytosis of microorganisms[2], but also in potential tissue damage[3].

Carvedilol (CARV) is a lipophilic vasodilating antihypertensive drug that selectively blocks α_1-receptors and non selectively antagonizes β_1- and β_2-adrenoreceptors. Moreover, CARV was suggested to possess antioxidant properties and have a potential for myocardial and vascular protection[4]. Due to its antioxidant effects, CARV has been suggested to provide greater benefit than β-blockers in the treatment of chronic heart failure[5]. Dandona et al.,[6] described the inhibitory effect of CARV on SO anion release from activated PMNL *ex vivo*. CARV was also found to inhibit luminol-enhanced chemiluminescence of ROM in blood cells *in vitro*[7] and to decrease SO generation and myeloperoxidase (MPO) release from isolated human PMNL activated with FMLP[8].

The aim of the present study was to compare the effect of CARV on SO generation and MPO release by human PMNL stimulated with opsonized zymosan (OZ), a specific receptor activator, and with phorbol-12-myristate-13-acetate (PMA), a receptor bypassing stimulus.

Material and methods

Carvedilol (CARV) − (Slovakofarma, Slovak Republic) was dissolved in tartaric acid (100 μmol l^{-1}) and then diluted in PBS(−). Cytochrome c, Dextran T500 (Pharmacia Fine Chemicals), Lymphoprep (Nycomed Pharma AS), cytochalasin B, PMA (phorbol-12-myristate--13-acetate)and zymosan A (Sigma). All other chemicals used were of analytical grade.

Zymosan A from *Saccharomyces cerevisiae* was opsonized according to Lojek et al.[9].

Polymorphonuclear leukocytes (PMNL) were isolated from blood of healthy volunteers into 3.8 % trisodium citrate. Erythrocytes were removed by dextran sedimentation and centrifugation on lymphoprep by the modified BOYUM´S method[10].

Superoxide dismutase inhibitable reduction of cytochrome c was used to measure superoxide (SO). Suspension of PMNL (10^6 cells/1.5 ml PBS with 1.8 mmol l^{-1} $CaCl_2$ and 0.5 mmol l^{-1} $MgCl_2$) was preincubated for 5 min at 37 °C with CARV (0.1, 1, 10 and 100 μmol l^{-1}) and then stimulated with PMA (1 μmol l^{-1}) for 15 min or with 0.5 mg l^{-1} OZ for 60 min, at 37 °C. Controls were included for the effect of each stimulus and of CARV on cytochrome c reduction. After centrifugation at 4,200 g for 2 min at 4 °C, absorbance was measured at 550 nm using a spectrophotometer Hewlett Packard 8452 A.

For myeloperoxidase (MPO) determination, PMNL were preincubated with cytochalasin B (5 μg ml^{-1}) at room temperature for 5 min. Subsequently the suspension of PMNL (2.10^6 cells/ml PBS) was incubated with CARV (0.1, 1, 10 and 100 μmol l^{-1}) and then stimulated with PMA (1 μmol l^{-1}) for 15 min or with 0.5 mg l^{-1} OZ for 60 min, at 37 °C. Controls for direct interaction of CARV and MPO were included.The activity of MPO was assayed in the supernatant after centrifugation at 983 g for 10 min at 4 °C by determining the oxidation of *o*-dianisidine in the presence of hydrogen peroxide in a Hewlett Packard 8452 A spectrophotometer at 463 nm (ref.[11]).

All data are expressed as the mean ± SEM. The data were analysed by one-way analysis of variance (ANOVA), and *P* values below <0.05 were taken as significant.

Results

Unstimulated cells showed neither SO formation nor enzyme release after preincubation with CARV. Figure 1 shows the effect of CARV (0.1, 1, 10 and 100 μmol l^{-1}) on OZ activated SO generation and MPO release in isolated human PMNL. CARV dose-dependently decreased SO generation and MPO release after OZ stimulation, however a significant decrease was recorded only with CARV concentration of 10 and 100 μmol l^{-1} to 86.70±1.82 % and 90.01±2.27 %, respectively, and MPO release to 62.74±4.40 % and 63.44±3.70 %, respectively.

The effect of CARV on SO generation and MPO release in isolated PMNL stimulated with PMA is given in fig. 2. Its action is also dose-dependent. CARV in the concentration 10 and 100 μmol l^{-1} significantly decreased SO generation to 70.21±4.45 % and 87.53±8.02 %, respectively, and MPO release to 62.04±4.84 % and 91.27±5.56 %, respectively.

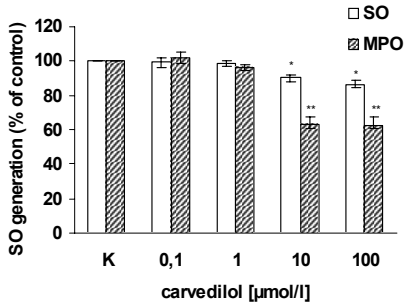

Fig. 1. **Effect of carvedilol on OZ stimulated SO generation and MPO release in human PMNL;** values are expressed as percentage of control values obtained after stimulation with OZ. Each value represents mean from 6-8 experiments ±SEM. **P*<0.05, ***P*<0.001 as compared to control values without drug

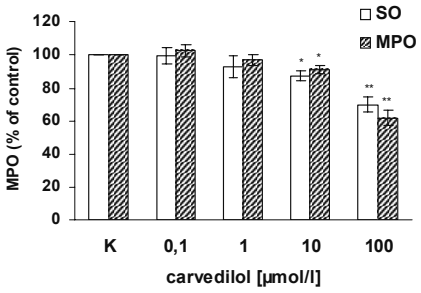

Fig. 2. **Effect of carvedilol on PMA stimulated superoxide generation and myeloperoxidase release in human PMNL;** values are expressed as percentage of control values obtained after stimulation with PMA. Each value represents mean from 6-8 experiments ±SEM. **P*<0.05, ***P*<0.001 as compared to control values without drug

Discussion

Activated PMNL produce ROM and liberate enzymes. Whereas the intracellular ROM and enzymes mediate destruction of phagocytosed material and regulate PMNL activity, the extracellular generation and release results mainly in inflammatory damage of surrounding tissues. ROM and granular enzymes are thought to be involved in the development of a number of pathological conditions and diseases, especially of the cardiovascular system[12].

It has been suggested that CARV may provide greater benefit than traditional β-blockers in chronic heart failure because of its antioxidant actions that synergize with its nonspecific β- and α-blocking effects[5].

The results presented in our study showed *in vitro* inhibitory effect of CARV on activated human PMNL. The two stimuli used are able to activate PMNL to evoke a respiratory burst and enzyme release via various mechanisms. The particulate receptor-operating stimulus OZ and the soluble stimulus PMA, which bypasses receptors and activates NADPH-oxidase via protein kinase C.

In our experimental conditions, CARV decreased SO generation similarly as recorded for PMA-stimulated PMNL[13]. CARV was shown to be a poor scavenger of SO, as proven an a cell-free system[14,7]. In human PMNL, CARV interfered *in vitro* and *ex vivo* with ROM generation as well as with already generated ROM, suggestive of its both „preventive“ and „therapeutic“ effect[15].

MPO is the most abundant enzyme in PMNL and has been implicated directly or through its metabolites, hypochlorus acid and subsequently derived ROM in many life-endangering diseases[16]. CARV inhibited MPO activity of the cell-free system in a concentration-dependent manner[7] and CARV treatment reduced MPO levels at both the area-at-risk and the necrotic zone in the ischemic myocardium[17].

Our results showing that CARV decreased SO generation and MPO release both by membrane-operating stimulus (OZ) as well as membrane bypassing activator (PMA) lead to the conclusion that the inhibitory effect may be attributed to its non-specific action. Since CARV affected SO generation and MPO release only in higher concentrations, and that after both stimuli, each active in a different way, its effect seems to be of non-receptor type. Physicochemical properties of CARV and its antiplatelet activity[18] support this conclusion. The inhibitory effect of CARV on MPO release from stimulated human PMNL indicates the possibility that CARV similarly to other lipophilic β-adrenoceptor/blocking drugs[18,7] interferes with membrane structure, influencing predominantly phospholipid metabolism.

Because CARV effectively participated in the decrease of SO generation in stimulated human PMNL and reduced MPO release, it can be concluded that the toxicity and damage to surrounding tissues caused by MPO, SO itself, and/or by subsequently derived metabolites, would also be diminished.

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P31
OXIDATION OF ELLIPTICINE BY HUMAN AND RAT CYTOCHROMES P450 CORRELATES WITH ITS BINDING TO DNA

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Key words: anticancer drug, ellipticine, metabolism, cytochrome P450, DNA binding

Introduction

Ellipticine (fig. 1), an alkaloid of *Apocyanaceae* plants, exhibits significant antitumor and anti-HIV activities[1–3]. The prevalent mechanisms of its antitumor, mutagenic and cytotoxic activities are intercalation into DNA and inhibition of DNA topoisomerase II[1–3]. We demonstrated that ellipticine covalently binds to DNA after enzymatic activation with cytochrome P450 (CYP) or peroxidases[2–7]. Human and rat CYP1A and 3A are the predominant enzymes catalyzing oxidation of ellipticine either to metabolites that are excreted from organisms (7-hydroxy- and 9-hydroxyellipticine) or form DNA adducts (13-hydroxy- and 12-hydroxyellipticine, the latter formed also spontaneously from another ellipticine metabolite, ellipticine N^2-oxide)[2–6] (fig. 1 and 2). Human cyclooxygenase (COX)-2, ovine COX-1, bovine lactoperoxidases and human myeloperoxidase also generated ellipticine-DNA adducts (fig. 1)[7]. The same DNA adducts were also detected in V79 Chinese hamster lung fibroblasts transfected with human CYP3A4, 1A1 and 1A2 (ref.[8]), in human breast adenocarcinoma MCF-7 cells[9], leukemia HL-60 and CCRF-CEM cells[10] and *in vivo* in rats exposed to this anticancer drug[5]. On the basis of these data, ellipticine might be considered a drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its enzymatic activation in target tissues. Here, we compare the efficiency of human and rat CYP enzymes in Supersomes™ (microsomes from Baculovirus transfected insect cells containing recombinantly expressed human CYPs and NADPH:CYP reductase with or without cytochrome b_5) to oxidize ellipticine and to form DNA adducts. Moreover, we evaluated whether oxidation of ellipticine to metabolites generating DNA adducts correlated with formation of these adducts.

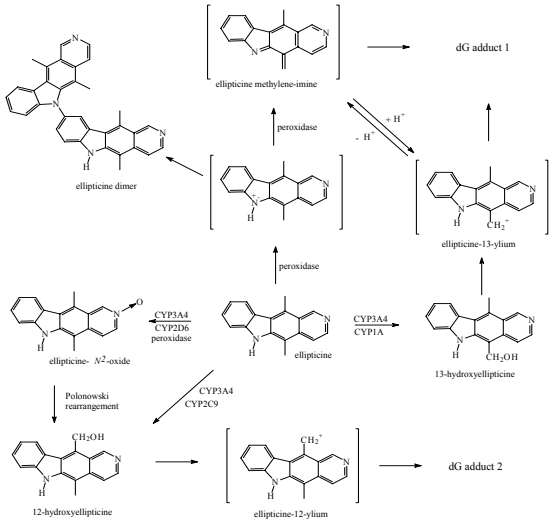

Fig. 1. **Metabolism of ellipticine by human CYPs and peroxidases showing the characterized metabolites found to form DNA adducts**

Material and methods

Incubation mixtures used to study ellipticine metabolism and procedures for separation of the metabolites were as described[6]. Incubation mixtures used for modifying DNA by ellipticine and procedures for analysis of ellipticine-derived DNA adducts by the ^{32}P-postlabeling assay were as shown[2,6].

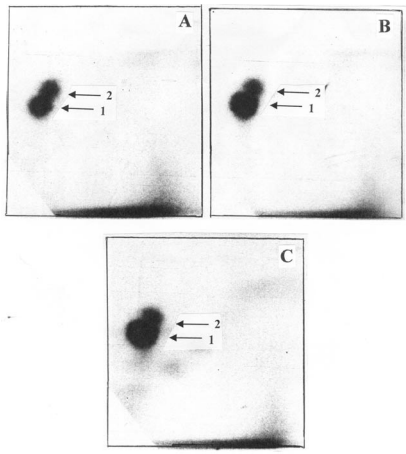

Fig. 2. **Ellipticine-DNA adduct levels in relation to concentration of rat CYP3A1 reconstituted with NADPH:CYP reductase;** 50 pmol (**A**), 100 pmol (**B**) and 250 pmol CYP3A1 (**C**)[2]. Analyses were performed by the nuclease P1 version of the ^{32}P-postlabeling assay

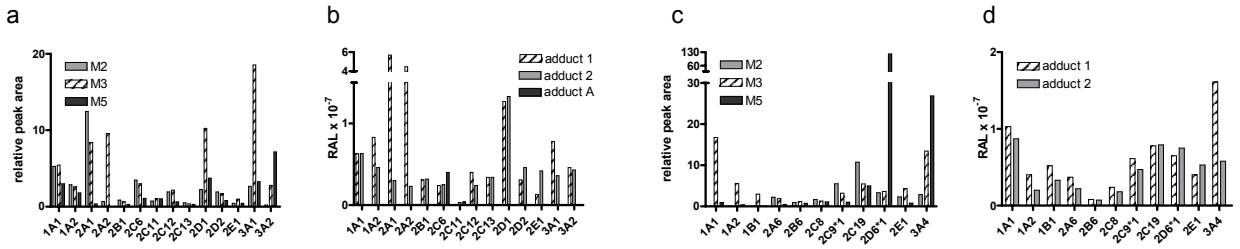

Fig. 3. **Oxidation of ellipticine to 12-hydroxy-, 13-hydroellipticine and ellipticine N^2-oxide by rat (a) and human (c) CYPs in Supersomes™. Ellipticine-DNA adduct formation by rat (b) and human (d) CYPs in Supersomes™ determined by ^{32}P-postlabeling assay;** values are averages and standard deviations of triplicate incubations. RAL - relative adduct labeling

Table I
Correlation coefficients between levels of ellipticine metabolites and those of ellipticine-DNA adduct formation

Metabolite	Human CYPs				Rat CYPs			
	adduct 1		adduct 2		adduct 1		adduct 2	
	r	*P*	r	*P*	r	*P*	r	*P*
M1	0.309	0.356	0.412	0.206	-0.199	0.493	0.206	0.498
M2	0.305	0.462	0.689	0.063	0.651	0.012	0.294	0.329
M3	0.832	0.001	0.644	0.032	0.759	0.003	0.500	0.081
M5	0.203	0.573	0.370	0.256	-0.072	0.825	0.682	0.014

Results and discussion

Oxidation of ellipticine by rat and human CYP enzymes in Supersomes™

To characterize the capability of the *in vitro* CYP systems to oxidize ellipticine several rat and human CYPs in Supersomes™ were used (fig. 3). We focused on ellipticine metabolites responsible for covalent DNA adduct formation. As cytochrome b_5 is known to influence the oxidation of several substrates by some CYPs (ref.[6]), this protein was either co-expressed in Supersomes™, either added into incubations. All CYPs in Supersomes™ oxidized ellipticine to up to five metabolites: 9-hydroxy- (M1), 12-hydroxy-(M2), 13-hydroxy-(M3), 7-hydroxyellipticine (M4) and ellipticine N^2-oxide (M5). 13-Hydroxyellipticine, forming the major ellipticine-deoxyguanosine adduct in DNA, was generated predominantly by CYP3A of both humans and rats, followed by CYP1A1, 1A2, rat 2D1 and human CYP2D6 enzymes. Differences were found in efficiencies of CYPs of a 2A subfamily in both species. While rat CYP2A1 and 2A2 oxidized ellipticine to 13-hydroxyellipticine with efficiency similar to CYP2D1, human CYP2A6 was much less active (fig. 3). 12-Hydroxyellipticine was produced mainly by rat CYP2A1, followed by human CYP2C19 and rat CYP1A1. Human CYP2D6 was the most efficient enzyme generating ellipticine N^2-oxide, followed by human CYP3A4 and rat CYP3A2.

Formation of ellipticine-DNA adducts by recombinant CYP enzymes in Supersomes™

Two major DNA adducts 1 and 2 (fig. 2), formed on deoxyguanosine from 13-hydroxy- and 12-hydroxy-ellipticine[5], were generated from ellipticine by all CYPs. Among the CYPs tested, rat CYP2A were the most efficient in generating adduct 1, which corresponds to the highest levels of 13-hydroxyellipticine formed by these enzymes. Rat and human CYP3A, 2D and 1A also effectively form this adduct (fig. 3). Rat CYP2D1 and human 1A1 were up to four times less efficient than CYP2A, followed by rat CYP1A, 3A and human 2C9, 2C19 and 2D6. Surprisingly, CYP2D6 did not generate such high amounts of adduct 2 as it was expected, because of its high efficiency to form ellipticine N^2-oxide. In contrast to human CYP2D6, rat CYP2D1 was efficient in formation of the adduct 2. This adduct was also effectively produced by both, rat and human CYP1A1, and human CYP2C19 and 2D6 (fig. 3). Rat CYP2C6 and 2C11 also generated another DNA adduct (assigned as adduct A) (not shown), while nor other human CYP2C neither other CYP subfamilies generated this adduct. The levels of 13-hydroxyellipticine correlated with those of the DNA adduct 1 (Table I). In the case of adduct 2, two metabolites, 12-hydroellipticine or ellipticine N^2-oxide, are responsible for its production, thus influencing the correlation coefficients.

Conclusion

The results of this work demonstrate that oxidation of ellipticine correlates with the DNA adduct formation and confirm the results of our former studies, indicating that ellipticine activation to species forming DNA adducts by rat CYPs is similar to that by human enzymes. However, one exception was found; rat CYP2A were significantly more active to form ellipticine-DNA adducts than their human orthologues.

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Abbreviations

COX cyclooxygenase
CYP cytochrome P450

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P32

TOTAL ANTIOXIDANT STATUS – SYSTEMIC MARKER OF OXIDATIVE STRESS IN ADJUVANT ARTHRITIS

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Key words: adjuvant arthritis, oxidative stress, total antioxidant status, glucomannan, Imunoglukan®

Introduction

Several clinical studies as well as preclinical animal model of rheumatoid arthritis have documented an imbalance in the body´s reduction/oxidation (redox) homeostasis to a more pro-oxidative environment, suggesting therapies that restore redox balance may have beneficial effects on the disease process. Different sources of oxygen species (ROS) and reactive nitrogen species (RNS) are produced locally at the site of the inflamed joint[1–3].

Adjuvant arthritis (AA), induced with intradermal administration of mycobacterial adjuvans, is a widely used model of evaluation of potential antirheumatics. ROS and RNS significantly present in this model contribute to systemic and local damage, assessed on the basis of selected clinical and biochemical parameters of AA[4–7]. In the present study, the oxido-redox balance was monitored in the AA model on systemic level by the total antioxidant status (TAS) in plasma.

The aim was: *i*) to compare TAS measured in plasma from the eye retroorbital sinus (RES) and from the heart; *ii*) to monitor TAS during the progress of the disease; *iii*) to compare the clinical parameter hind paw volume (HPV) with TAS.

Materials and methods

AA was induced in Lewis rats by a single intradermal injection of heat-killed *Mycobacterium butyricum* suspended in incomplete Freund´s adjuvant. The experiments included healthy intact animals as reference controls (CO), arthritic animals (AA) without any drug administration, and arthritic animals with administration of glucomannan (AA-GM) in the oral daily dose of 15 mg kg^{-1} b.w. and Imunoglukan® (AA-IMG) in the oral daily dose of 2 mg kg^{-1} b.w. The treatment involved administration of the substances tested from day 0, i.e. the day of immunization, to the experimental day 28. The volume of hind paws (HPV) was measured twice a week. In control, arthritic, and treated animals, TAS[8] was measured in plasma samples collected from the RES on experimental days 14 (AA 14 D), 21 (AA 21 D), and 28 (AA 28 D). At the end of the experiment, on day 28, the rats were sacrificed in anesthesia, and plasma was collected from the heart. To determine the TAS of plasma samples, Randox total antioxidant status manual commercial kit was used. The data were expressed as arithmetic mean and SEM. The arthritis group (AA) was compared to healthy control animals (CO), for significance calculations Student`s t-test was used with – ns ( not significant); * $P$<0.05 (significant); *** $P$<0.001 (extremely significant). The treated arthritis groups (AA-GM, AA-IMG) were compared to untreated arthritis (AA), for the significance calculations Student`s t-test was used with – ns (not significant); + P<0.05 (significant); ++ P<0.01 (very significant).

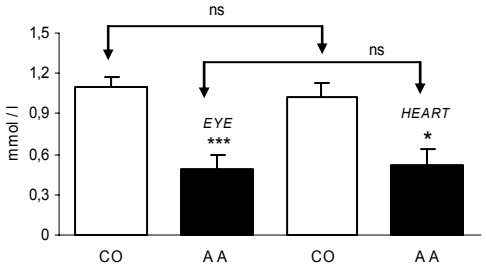

Fig. 1. **Total antioxidant status in plasma of control and arthritic rats;** comparison of heart versus eye levels. Day 28. Mean ±SEM, *P<0.05, ***P<0.001 (CO vs AA)

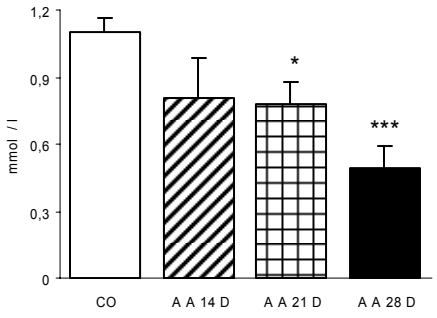

Fig. 2. **Total antioxidant status in plasma of control and arthritic rats in time profile;** mean ± SEM, *P<0.05, ***P<0.001 (CO vs AA)

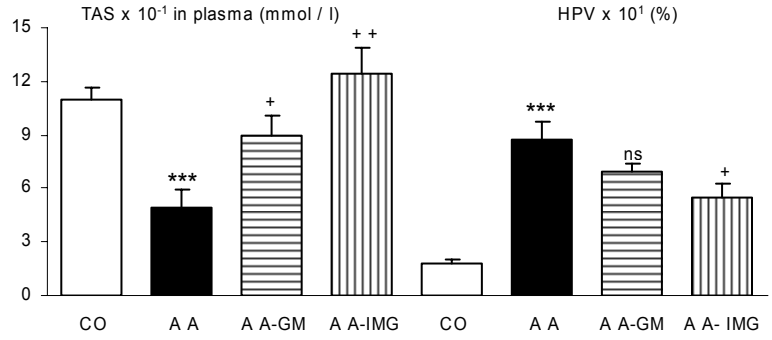

Fig. 3. **Comparison of total antioxidant status in plasma (TAS) with hind paw volume (HPV) in control, arthritic, glucomannan and immunoglukan® treated rats;** day 28. Mean ± SEM, ***P<0.001 (CO vs AA); ns – not significant; + P<0.05, ++ P<0.01 (AA vs AA-GM resp. AA-IMG)

Results and discussion

Oxidative stress is a factor in many human diseases either as cause or effect. Any inflammatory condition inevitably leads to an increased oxidative burden since the release of ROS by macrophages is a part of body´s defense system. It is recommended to use a „battery" of measurements, as no single determination of antioxidant status is sufficient. Various assays for total antioxidant capacity (total radicaltrapping antioxidant potencial, trolox equivalent antioxidant capacity, oxygen radical absorbance capacity, and FRA in plasma measure either radical scavenging or reducing capacity. They were devised, or at least have been used, to assess individual antioxidant status, as reflect in body fluids[9–11]. In this experiment with AA we attempted to clarify the optimal condition for TAS measurements *via* a Randox kit, namely *i*) to compare TAS measured in plasma from the RES and from the heart; *ii*) to monitor TAS during the progress of the disease; *iii*) to compare the clinical parameter HPV with TAS. We found no differences between TAS data measured in plasma from the RES and from heart (fig. 1). The progress of AA was manifest by decreased TAS already on day 14 in comparison with day 0, and the decreasing trend continued to day 28. In AA animals, the plasmatic level of TAS – 0.49 mmol l^{-1} was half of that on day 0, showing an extremely significant drop (fig. 2). The clinical parameter HPV correlated negatively with the measured TAS data both in the arthritic group of animals and the arthritic groups treated with glucomannan and Imunoglukan®. In the latter groups, however, a significant increase in TAS was found. In the AA-IMG group, the HPV was significantly decreased compared to untreated animals, while in the AA-GM group the difference was not significant (fig. 3).

Conclusion

In our further experiments we intend to expand the parameters by measuring not only TAS but also malondialdehyde and its protein adducts. The present study highlighted the importance of TAS as rather helpful parameter for quantification of oxidative stress in AA and for the effect of pharmacological intervention by substances with antioxidant capacity.

This work was supported by the grants :VEGA 2/5051/25,VEGA 1/3442/06, APVV-51-017905, APVV- 21-055205.

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P33
KINETICS OF 3-AMINOBENZANTHRONE OXIDATION BY RAT HEPATIC MICROSOMES

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Key words: 3-aminobenzanthrone, 3-nitrobenzanthrone, oxidation, cytochrome P450, HPLC

Introduction

The aromatic nitroketone 3-nitrobenzanthrone (3--nitro-7*H*-benz[*de*]anthracen-7-one; 3-NBA) is one of the most potent mutagens and a suspected human carcinogen that is found in diesel exhaust and ambient air pollution[1]. We found that 3-NBA is activated by cytosolic and microsomal reductases by simple nitroreduction[1–4]. Previous work indicated that *N*-hydroxy-3-aminobenzanthrone (*N*-OH-ABA) appears that it might be the critical intermediate in 3-NBA-derived DNA adduct formation, which can be further activated by *N*,*O*-acetyltransferases (NATs) and sulfotransferases (SULTs)[2–4]. The main metabolite of 3-NBA, 3-aminobenzanthrone (3-ABA), was recently detected in the urine of smoking and nonsmoking salt mining workers occupationally exposed to diesel emissions[5] demonstrating that exposure to 3-NBA can be significant and is detectable. In addition, 3-ABA was evaluated to be suitable for coloration of microporous polyethylene films, which are widely used for practical purposes such as separation of liquid mixtures, in particular, as separation membranes in chemical batteries[5], or an advantageous fluorescent phospholipid membrane label in the form of its *N*-palmitoyl derivative[6]. This suggests its industrial and/or laboratory utilization. Furthermore, even though the epidemiological study on the toxicity of 3-ABA has not yet been evaluated, formation of DNA adducts by this reductive metabolite of 3-NBA *in vitro* and *in vivo* in rodents indicates its potential genotoxicity[8–11].

We recently showed that 3-ABA is activated by cytochrome P450 (CYP) 1A1 and 1A2 enzymes and human hepatic microsomes forming DNA adduct patterns qualitatively similar to those found *in vivo* in rodents treated with 3-ABA (ref.[9–11]). Moreover, we found that 3-ABA forms the same DNA adducts as 3-NBA *in vitro* and *in vivo* in rodents[3,4,8–11]. Here, we investigated the metabolism of 3-ABA by hepatic microsomes of rats treated with β-naphthoflavone (β-NF), a known inducer of CYP1A enzymes, in order to examine the formed metabolites as well as kinetics of the CYP-mediated reaction.

Materials and methods

3-ABA, 3-NBA and *N*-OH-ABA were synthesized as described[11]. Microsomes were isolated from livers of 10 male Wistar rats pretreated with β-NF inducing CYP1A1/2 as described[9,11]. Incubation mixtures, in a final volume of 500 μl, consisted of 100 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 0.5 mg of microsomal protein, and 5–50 μM 3-ABA (dissolved in DMSO). The reaction was initiated by adding 3-ABA. Incubations with rat microsomes were carried out at 37 °C for 5–180 min. Then, 5 μl of 1 mM phenacetine in methanol was added as an internal standard and the 3-ABA metabolites were extracted twice with ethyl acetate (2 × 1.5 ml). The extracts were evaporated to dryness; residues were dissolved in 30 μl of methanol, and subjected to RP-HPLC to evaluate the amounts of residual 3-ABA and its metabolites. The HPLC was performed with a Dionex HPLC pump P580 with UV/VIS UVD 170S/340S spectrophotometric detector set at 254 nm, and peaks were integrated with a $CHROMELEON_{TM}$ 6.01 integrator. The column used was a Nucleosil 100-5 C_{18} (Macherey-Nagel, Duren, Germany, 25 cm x 4.6 mm, 5 μm) proceeded by a C-18 guard column. Chromatography was under isocratic conditions of 70 % methanol, with a flow rate of 0.6 ml min^{-1}.

Results and discussion

3-Aminobenzanthrone is oxidized by rat hepatic cytochromes P450 in microsomes to three metabolites. These metabolites were separated by HPLC as distinguish product peaks (fig. 1a). Using co-chromatography with synthetic standards (fig. 1b–d), two of them were identified to be oxidative metabolites of 3-ABA, *N*-hydroxy-3-ABA [fig. 1c, the retention times (r.t.) of 6.5 min] and 3-NBA (fig. 1d, r.t. of 25 min). The structure of another metabolite eluted with r.t. of 18 min, assigned as M18 (fig. 1a), remains to be characterized.

Because 3-ABA was found to be the most effectively activated by enzymes of a CYP1A subfamily to species forming DNA adducts[9], kinetics of oxidation of 3-ABA by microsomes isolated from livers of rats treated with β-NF, a known inducer of these CYP enzymes, was analyzed. Oxidation of 3-ABA by hepatic microsomes was dependent on the concentration of this compound (fig. 2) as well as on time of incubation (fig. 3) Time-dependence of 3-ABA oxi-

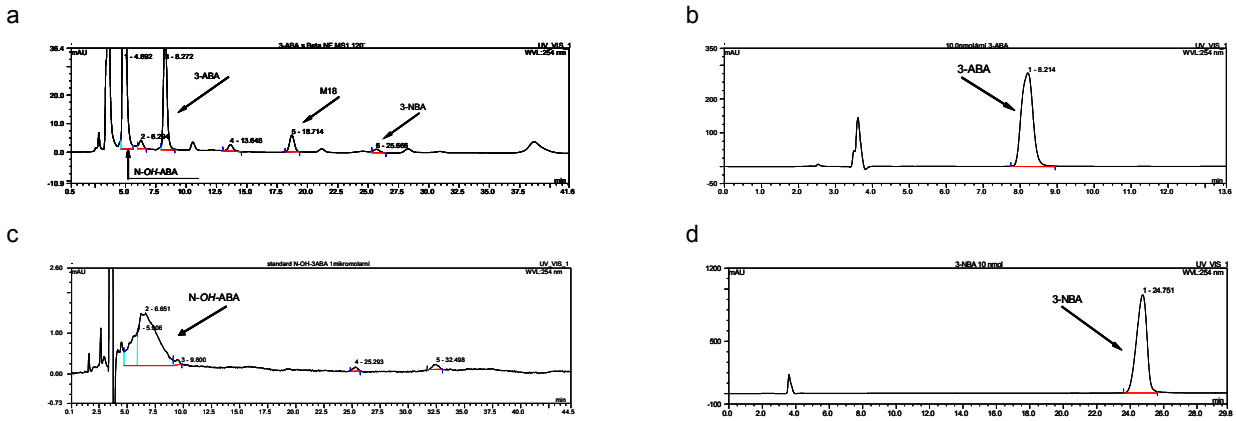

Fig. 1. **HPLC separation of 3-ABA metabolites produced by β-NF microsomes (a), HPLC of 3-ABA (b), *N*-OH-ABA (c) and 3-NBA (d) standards**

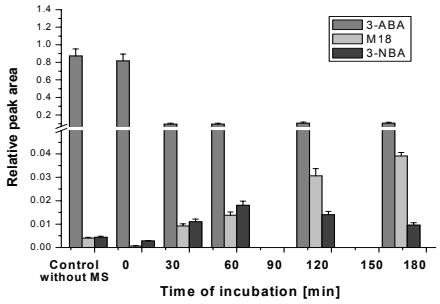

Fig. 2. **Time-dependence of 3-ABA oxidation**

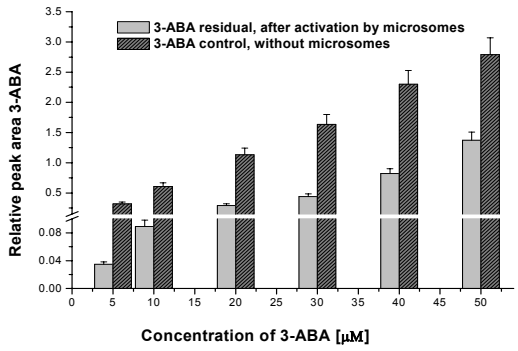

Fig. 3. **Concentration-dependence of 3-ABA oxidation**

dation by microsomes is linear up to 5 minutes of incubation (data not shown). This time interval was therefore, used for kinetical studies. The values of Michealis constant (K_m), maximum reaction velocities (V_{max}) and Hill coefficient (n) for 3-ABA oxidation by the microsomal system used in the study were determined (Table I).

Table I
Kinetic parameters for 3-ABA oxidation by β-NF microsomes

Parameter	
K_m, μM	51.0
V_{max}, nmol 3-ABA min^{-1} mg^{-1}	14.2
n	1.0

Conclusion

The study, showing for the first time identification of two metabolites formed from 3-ABA by CYP-mediated oxidation, suggest the participation of CYP1A enzymes in activation metabolites of 3-ABA forming DNA adducts.

Supported by the GACR (grant 303/05/2195) and the Czech Ministry of Education (grants MSM 0021620808 and 1M4635608802 – Centrum of targeted therapeutics).

Abbreviations

CYP	cytochrome P450
DMSO	dimethylsulfoxide
HPLC	high performance liquid chromatography
NADPH	nicotine amide adenine dinucleotide phosphate reduced
NATs	*N*,*O*-acetyltransferases
SULTs	sulfotransferases
N-OH-ABA	*N*-hydroxy-3-aminobenzanthrone

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P34

CHRONIC EFFECT OF GARDOPRIM PLUS GOLD 500 SC (S-METOLACHLOR, TERBUTHYLAZINE) ON COMMON CARP (*Cyprinus carpio*)

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Key words: bioaccumulation, terbuthylazine, fish, chronic effect

Introduction

Currently used pesticides should be generally less environmentally persistent, that means have shorter half-lives and lower bioaccumulation[1]. For all that, pesticides constitute one of the most hazardous groups of contaminants to human health, fauna and environment in general[2].

s-Metolachlor (chloracetanilides herbicides) and terbuthylazine (chlorotriazine pesticides) are used primarily for control of annual grasses and broadleaf weeds in corn. Monitoring pesticides in agricultural water displays content of terbuthylazine from 0.1 to 4.5 μg l^{-1} (ref.[2]).

Fish are suitable test model for monitoring the effects of contaminated water on the environment.

The aim of our study was to monitor the effect of preparative Gardoprim Plus Gold 500SC containing *s*-metolachlor (312.5 g l^{-1}) and terbuthylazine (187.5 g l^{-1}) on hematological parameters of common carp (*Cyprinus carpio*). Another goal was to determinate the ability of bioaccumulation of terbuthylazine in the tested fish.

Materials and methods

Exposures

Juvenile common carp (initial weight 5.35±1.75 g) were accidentally assigned to five concentrations of tested solutions and a control. Each concentration and control contained 23 fish in 100 l fiberglass aquaria. The test was carried out in a semistatic method, the bath water was exchanged every 48 h. Fish were maintained on a 12 h light: 12 h dark photoperiod. During the test the water temperature varied between 21 to 23 °C, water saturation with oxygen ranged from 60 to 70 % and pH was in range 7.7 to 8.6. The fish were left to adapt to these conditions for three weeks before the beginning the experiment. Then the fish were exposed to concentrations 0,01; 0,1; 1; 5 and 10 mg l^{-1} of Gardoprim Plus Gold 500 SC for 21 days (which corresponds to concentration of terbuthylazine 1.88; 18.8; 188; 935; 1880 μg l^{-1}). The tested concentrations were selected according to LC_{50} (12.3 mg l^{-1}).

The fish were fed by the dose of 1.5 % of the mean weight of the fish. At the end of the test, blood was sampled by cardiac puncture and the dead fish were frozen (−80 °C).

Blood samples were used for determination of red blood count (RBCc), haematocrit (PCV), haemoglobin, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), leukocyte count (WBCc) and leukogramme.

Analysis of chemicals in water

Analysis of *s*-metolachlor was performed on the gas chromatograph Shimadzu GC 2010 (Shimadzu Handelsgesellschaft mbH) equipped with the autoinjector AOC 20i. The electron capture detector (ECD) was used for detection. Helium was the gas carrier. A Equity™-5 Cappilary Columm (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Supelco, Sigma-Aldrich.r.o., CZ) was used. The SPE sorbens Strata C18-E (1000 mg/6 ml) were used for solid phase extraction. Analysis of terbuthylazine was performed on the gas chromatograph Thermo Quest Trace GC with Finnigan Polaris Q detector. The J&W DB-5MS (30 m × 0.25 mm; 0.25 μm) was used for analysis.

Analysis of terbuthylazine in fish homogenate

Analysis of terbuthylazine was performed on the gas chromatograph Thermo Quest Trace GC with Finnigan Polaris Q detector. The J&W DB-5MS (30 m × 0.25 mm; 0.25 μm) was used for analysis. Samples of fish were homogenized and 10 g homogenate was used for analysis. Samples were cleaned with the aid of gel permeation chromatography (GPC) and then analysed on the gas chromatograph with MS detection. The Tukey test was used to compare the values of hematological indices in blood plasma of the control and the experimental groups of fish.

Results and discussion

The Table I shows the effects of Gardoprim Plus Gold 500 SC on hematological indices of common carp. In the two highest concentrations (5 and 10 mg l^{-1}) the fish intook the food badly, that is the reason why the statistical differences were noticed in weights beetwen control and the lowest test concentration (0.01 mg l^{-1}). This influence was showed in the final observed lenght of the fish too. The statistical significant differences were not noticed between groups in haematocrit values and the amount of

Table I
Haematological indices of common carp at the end of the test

Groups of Gardoprim [mg l^{-1}]	Weight [g] mean±SD	Lenght [mm] mean±SD	Haematocrit [PCV] mean±SD	Haemoglobin [g l^{-1}] mean±SD	RBCc [T l^{-1}] mean±SD	MCV [fl] mean±SD	MCH [pg] mean±SD	WBCc [g l^{-1}] mean±SD
control	7.79±1.25^{ac}	63.73±4.42^{a}	0.28±0.01	0.34±0.04^{ac}	1.40±0.25	199.89±40.14	0.25±0.06^{a}	20.0±8.86^{a}
0.01	8.25±1.85^{a}	62.75±5.50^{a}	0.28±0.03	0.35±0.02^{ac}	1.54±0.51	221.94±143.06	0.25±0.10^{a}	16.70±7.19^{ab}
0.1	7.72±1.92^{ad}	62.15±6.11^{a}	0.29±0.04	0.35±0.03^{a}	1.78±0.32	164.04±34.46	0.20±0.04^{ab}	14.50±4.67^{ab}
1.0	6.56±1.50^{bcd}	61.25±7.75^{a}	0.29±0.05	0.27±0.04^{b}	–	–	–	18.00±5.24^{ab}
5.0	6.36±1.19^{bd}	58.50±4.98^{ab}	0.27±0.02	0.27±0.07^{b}	1.43±0.35	195.50±45.45	0.20±0.06^{ab}	12.25±7.08^{ab}
10.0	5.79±1.36^{b}	55.65±6.04^{b}	0.30±0.04	0.29±0.05^{bc}	1.81±0.25	165.35±30.05	0.17±0.05^{b}	11.30±3.86^{b}

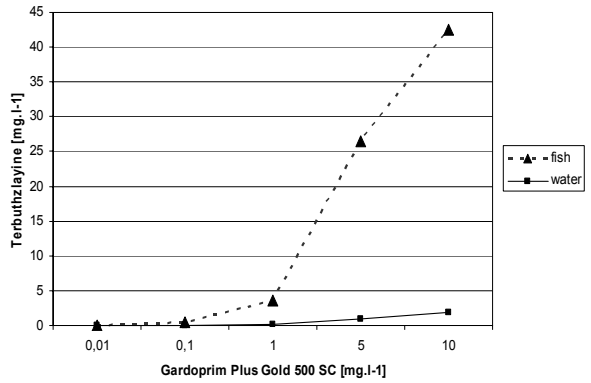

Fig. 1. **The concentration of terbuthylazine in fish** (after 21 days duration the test) and water [mg kg^{-1}]

erytrocytes. In the test group 1 mg l^{-1} the number of erytrocytes were not estimated. On the other hand, the effect of Gardoprim Plus Gold 500 SC caused reduction of leukocytes in all the test concentrations. The statistical significant difference was noticed only between control and the highest concentration (10 mg l^{-1}). The reduction of the amount of haemoglobin was established in concentration 1.5 and 10 mg l^{-1}. Differential leukocyte count has lymphocytic characteristics with lower proportion of mature granulocytes (band and segmentes). The statistical significant differences were not noticed between groups in leukogramme.

Effect of experimental terbuthylazine exposure on the cells of *Dicentrarchus labrax (L.)* was observed[3]. Treated fish in concentration 3.55−7.08 mg l^{-1} displayed cellular and ultrastructural alterations in all the examined organs.

The fig. 1 presents contents of terbuthylazine in fish (compound samples from each group). The concentrations of terbuthylazine in fish were 19−27 times higher than in the test solutions. The amount of residues of atrazine over the 48 hours expozition in the carp's liver was only 2.5 times higher than the atrazine concentration in water[4]. It implies that terbuthylazine has better ability to bioaccumulate in fish tissue in comparison with atrazine. Concentrations of terbuthylazine in wild fish have not been monitored yet, although it seems that fish would be a good indicator of pollution of water environemnt by this substance.

The bioaccumulation of terbutryn increased significantly with increasing water temperature. However, temperature seemed to have no effect on bioaccumulation of terbuthylazine[5].

Conclusions

Gardoprim Plus Gold 500SC, containing *s*-metolachlor (312.5 g l^{-1}) and terbuthylazine (187.5 g l^{-1}), is cumulated in tissue of common carp. Highest effect on change of haematological parameters was significant decrease of leukocyte count.

This study was supported by a grant MSM 621571240.

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P35
THE EFFECT OF CARVEDILOL ON THE OXIDATIVE BURST OF RAT PHAGOCYTES

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Introduction

The production of reactive oxygen species (ROS) by phagocytic cells belongs to basic microbicidal mechanisms of the body. However, extracellularly produced ROS are thought to be involved in the development of a number of pathological conditions and diseases, especially of the cardiovascular system[1]. Therefore, substances reducing production of or quenching extracellularly produced ROS could be used to prevent adverse effects of ROS.

Carvedilol has been in clinical use as an antihypertensive agent for several years and has been shown to possess antiarrhytmic, antiischemic and cerebroprotective effects[2]. These effects are suggested to be mediated through nonselective β-adrenoreceptor and α_1-adrenoreceptor blockade by carvedilol. However, recent studies described carvedilol as a potent antioxidant[3]. The antioxidant potential of carvediol could contribute to the clinically observed positive effects on cardiovascular pathological conditions. For its multifaceted therapeutic potential and for its beneficial antioxidant properties carvedilol itself could substitute the combination of different type of drugs used in the treatment of cardiovascular diseases and decrease the possibility of negative interactions of these agents. Therefore, we studied the potential antioxidant properties of carvedilol. The major aim of the present study was to evaluate the effect of carvedilol on the oxidative burst of blood leukocytes stimulated by various types of activators. Furthermore, direct scavenging effects of carvedilol on various types of ROS generated by cell-free systems were determined.

Materials and methods

Carvedilol [1-[carbazolyl-(4)-oxy]-3-[(2-methoxyphenoxyethyl)amino]-2-propanol] (Zentiva, Czech Republic) was dissolved in tartaric acid solution ($5\cdot10^{-4}$ M) and used in the final concentration range of $1\cdot10^{-4}$ – $1\cdot10^{-7}$ M. Tartaric acid was tested not to have any effects on the CL signal. All other chemicals were obtained from Sigma-Aldrich (Germany) or local distributors in the highest quality.

Heparinized peripheral blood from Wistar rats was obtained by cardiac puncture as described previously[4]. Leukocyte rich plasma was prepared by dextran sedimentation. Obtained buffy-coat was washed and isolated leukocytes were resuspended in HBSS.

Luminol-enhanced chemiluminescence (CL) was employed to determine ROS production by blood phagocytes as described previously[4,5]. The principle of the method is based on luminol interaction with phagocyte-derived ROS, which results in large measurable amounts of light. The CL reaction mixture consisted of isolated leukocytes ($1\cdot10^{-6}$ cells ml^{-1}) with or without carvedilol, 1 mM luminol, and one of the receptor-operating activators: *N*-Formyl-Met-Leu-Phe – fMLP (10^{-7} M), opsonized zymosan particles – OZP (0.1 g l^{-1}) or one of the receptor-bypassing activators: phorbol myristate acetate – PMA (10^{-8} M), calcium ionophore A23187 – CaI ($5\cdot10^{-6}$ M). Spontaneous (non-activated) CL of phagocytes was also measured. The maximum (peak) of the CL response was determined as relative light units (RLU).

The scavenging ability of carvedilol was studied in various chemical systems producing individual ROS: peroxyl-radical produced by thermal decomposition of ABAP [2,2'-azo-bis(2-amidinopropan)], superoxide anion produced by hypoxanthine (1 mg ml^{-1})/xanthine oxidase (0.1 U ml^{-1}) system, hydroxyl radical produced by hydrogen peroxide (2 mM)/ferrous sulphate (1 mM) system and hydrogen peroxide itself (2 mM).

All CL measurements were performed in a microtitre plate luminometer LM-01T (Immunotech, Czech Republic) and in a cuvette luminometer 1251 (Bioorbit, Finland).

Data are expressed as the mean ± standard error of the mean (SEM) of twelve different experiments. The assays were run in duplicates. Results were analyzed by ANOVA, followed by the Student's t-test.

Results

Carvedilol dose-dependently decreased not only the CL of nonactivated leukocytes (data not shown) but also the CL of leukocytes activated by all used activators. The most efficient inhibition of CL by carvedilol was observed in leukocytes stimulated by fMLP and CaI (fig. 1).

Carvedilol dose-dependently scavenged individual ROS. Carvedilol most efficiently inhibited the CL signal generated by hydrogen peroxide and hydroxyl radical and to a lesser extent also by superoxide anion (Table I).

Carvedilol did not exert any scavenging effects on the peroxyl radical (data not shown).

Discussion

All used activators are able to stimulate cells to generate ROS via various mechanisms. fMLP and OZP

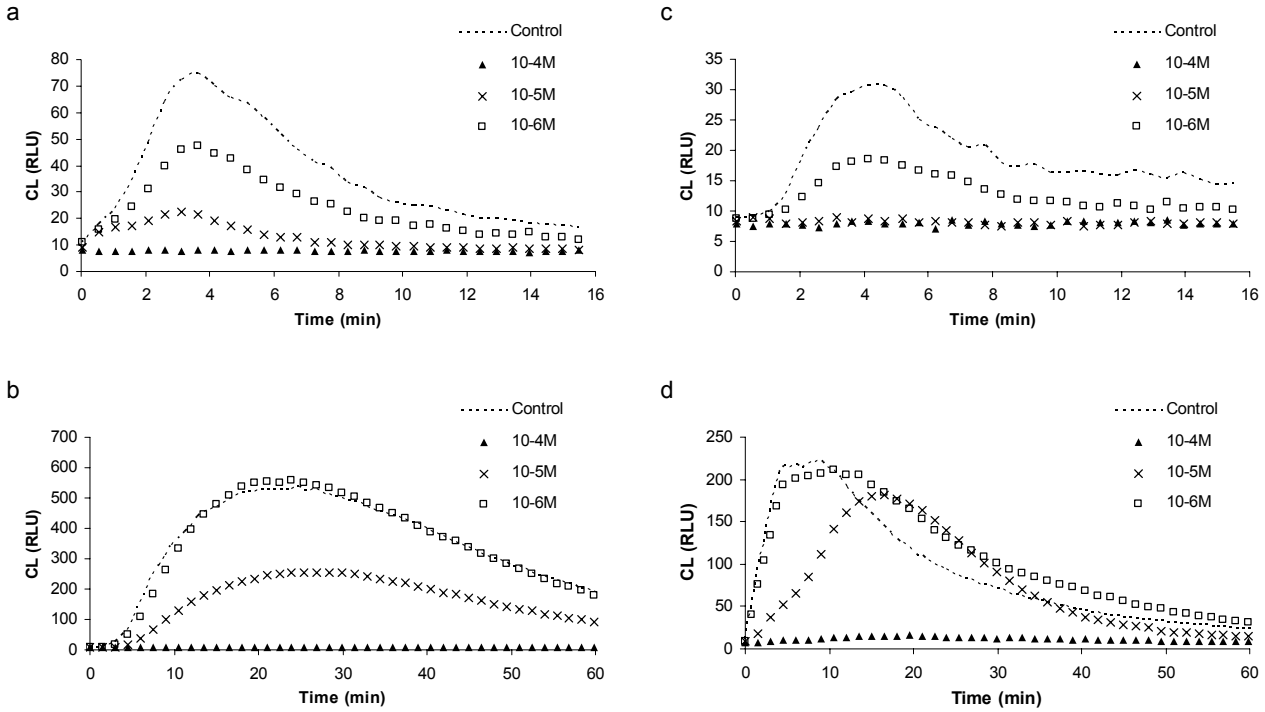

Fig. 1. **The effect of carvedilol on the CL of isolated leukocytes activated by fMLP (a), OZP (b), CaI (c) or PMA (d)**

Table I
Direct scavenging effect of carvedilol against individual ROS. Asterisks show the statistical significance against control at $P \leq 0.05$ (*) and $P \leq 0.01$ (**)

Concentration of carvedilol	Peak CL [% of control]		
	superoxide radical	hydroxyl radical	hydrogen peroxide
Control	100 ± 0.02	100 ± 0.67	100 ± 1.71
10^{-8} M	105 ± 0.77	91 ± 0.27	90 ± 10.30
10^{-7} M	97 ± 0.51	77 ± 0.47	100 ± 0.40
10^{-6} M	100 ± 0.02	100 ± 0.33	91 ± 0.34
10^{-5} M	105 ± 0.19	83 ± 0.13	80 ± 5.12*
10^{-4} M	76 ± 0.12**	46 ± 0.20**	54 ± 8.58**

(zymosan from Saccharomyces cerevisiae) bind to specific receptors, concretely OZP binds to complement and imunoglobulin receptors. On the other hand PMA directly activates proteinkinase C and CaI increases intracelullar level of calcium ions. It is obvious from our results that carvedilol dose-dependently affects the CL of phagocytes stimulated by all used activators in a similar way. It might indicate that carvedilol interferes with already generated ROS rather than with specific receptors for opsonins or chemotactic peptides localized at plasma membrane, with intracellular calcium mobilisation and intracellular activation of protein kinase C[2].

Carvedilol was shown to be a direct dose-dependent scavenger of hydroxyl radical, hydrogen peroxide and superoxide radical in our experiments. The antioxidant properties of carvedilol and some of its metabolites, which are not observed in most of other beta-adrenoceptor antagonists, are attributed to the presence of a carbazole moiety in the drug molecule[3].

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P36
BIOLOGICAL MONITORING OF EXPOSURE TO ETHYLENE OXIDE IN THE STERILIZATION UNITS

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Key words: ethylene oxide, biological monitoring, globin adduct

Introduction

Ethylene oxide (EO, fig. 1), an important industrial intermediate, is also used as a sterilizing agent for heat-sensitive medical equipment and consumables. Due to its electrophilic nature, EO is a directly alkylating agent producing covalent adducts with biological macromolecules including DNA. EO is a rodent carcinogen and was classified as a human carcinogen[1] or a suspect human carcinogen[2]. To reduce the carcinogenic risk in occupationally exposed workers, exposure to EO should be minimized and controlled by adequate measurements. EO has an olfactory detection threshold of ca. 1200 mg m^{-3}, therefore, it cannot be perceived at concentrations normally present at workplaces. Recent studies have shown that exposure to 1.8 mg m^{-3} (1 ppm) EO would not significantly contribute to human cancer risk[3]. Thus, this value was adopted as a threshold limit value by ACGIH[2] or as a technical exposure limit by DFG[4]. The permissible exposure limit (PEL) for EO in the Czech Republic is 1 mg m^{-3}. Currently, the major source of exposure to EO are sterilization units in hospitals or in specialized facilities. These exposures, which are typically intermittent and highly variable, are commonly assessed by environmental monitoring of airborne EO. Biological monitoring based on the determination of EO adducts with blood protein globin is a valuable alternative. The major advantage of globin adducts is their long-term persistence in the organism, allowing molecular dosimetry of EO over the whole lifespan of erythrocytes, i.e., 4 months in humans. The current methodology is based on determination of the EO adduct with *N*-terminal valine of globin (2-hydroxyethylvaline, HEV) using modified Edman degradation procedure[5]. This includes isolation of globin followed by conversion of HEV using pentafluorophenylisothiocyanate reagent to 1-(2-hydroxyethyl)-5-isopropyl-3--pentafluorophenyl-2-thiohydantoin (HE-PFPTH) (fig. 1), which is determined by GC/MS.

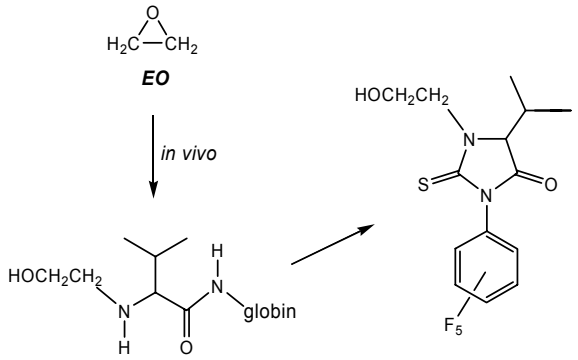

Fig. 1. **Structure of compounds dealt with in the study**

The HEV adduct is present in human globin at physiological levels. In non-smokers with no known exposure to EO, the background levels are about 20–60 pmol g^{-1} globin[6–9]. Smoking increases the HEV level by ca. 10 pmol g^{-1} per cigarette per day[7–9]. From large-scale epidemiological studies it was estimated that long-term occupational exposure to 1 mg m^{-3} EO (8 h/day, 5 days/week, >20 weeks) would result in HEV levels of ca. 3.8 nmol g^{-1} globin[7].

To our knowledge, biomonitoring of EO has not been carried out in the Czech Republic so far. In this study, we adapted the above methodical approach to assess occupational exposure to EO in 3 sterilization units in the South Morava region.

Experimental

Chemicals

N-2-Hydroxyethylvaline-leucine-anilide (calibration standard) and *N*-2-ethoxyethylvaline-alanine-anilide (internal standard) were bought from Bachem. Pentafluorophenylisothiocyanate reagent was from Fluka. Formamide for molecular biology (Merck) was distilled before use. Other chemicals were from various sources.

Subjects

Twenty six subjects (20 men, 6 women; 12 smokers) participated in the study. They were engaged in various operations in two plants sterilizing medical equipment (A, n=14; B, n=8), or performed sterilization of collection items in a museum (C, n=4). Ten subjects (6 men, 4 women, all non-smokers) with no occupational exposures to EO were used as controls. Whole blood was taken from

each person by venipuncture over heparin. Blood samples were stored at 4 °C and processed within 3 days after sampling.

Analytical procedure

Globin was isolated from the whole blood (10 ml) using a standard procedure that included separation, washing and hemolysis of the erythrocytes, followed by precipitation of globin with 2 % hydrochloric acid in acetone, washing and drying[10]. Determination of HEV in globin was carried out as described in[6]. Briefly, globin (100 mg) was dissolved in formamide (3 ml), then 1 M-NaOH (30 μl), solution of the internal standard *N*-2-ethoxyethylvaline-leucine-anilide (10 nmol) in MeOH (100 μl), and pentafluorophenylisothiocyanate (10 μl) were added. The samples were incubated overnight and then extracted with diethylether. The extract was evaporated to dryness and the residue dissolved in toluene. The samples were washed with water and 0.1 M-Na_2CO_3. Then, acetylating mixture (acetonitrile/acetic anhydride/triethylamine 3:1:1, 100 μl) was added and the sample was left at room temperature for 15 min. The sample was brought to dryness and dissolved in toluene (50 μl); 1 μl was analyzed by GC/MS.

GC/MS conditions

The analyses were performed using a DSQ GC/MS instrument (Thermo) on a capillary column DB-5ms (J&W), 30 m × 0.25 mm, film thickness 0.25 μm. Injector was set in a splitless mode (0.75 min). Flow rate of the carrier gas helium: 1.2 ml min^{-1}. Temperature of the column: 90 °C for 0.75 min, 15 °C min^{-1} to 190 °C, then 30 °C min^{-1} to 310 °C. Temperatures of the injector, transfer line, and ion source: 270 °C, 275 °C, and 200 °C, respectively. Detector was operated in the electron impact (EI) ionization mode (70 eV).

Quantitation

Quantitation was based on the ratio of peak areas of acetyl-HE-PFPTH and 2-ethoxyethyl-PFPTH (EOE-PFPTH), both monitored at *m/z* 308. For construction of calibration curve, samples of control globin (100 mg) were spiked with the calibration standard *N*-2-hydroxyethylvaline-leucine-anilide (0–2 nmol). The internal standard *N*-2--ethoxyethylvaline-alanine-anilide (10 nmol) was added and the samples were processed as described above. (On applying the modified Edman degradation procedure, the calibration and internal standards are converted to HE-PFPTH and EOE-PFPTH, respectively.)

Results and discussion

In preliminary GC/MS analyses, performance of the HE-PFPTH determination depended markedly on the cleanliness of the chromatographic system. In routine measurements on a „casual" column, progressive peak widening and tailing was observed. Introduction of an additional derivatization step, namely, conversion of HE-PFPTH to its acetyl derivative, amended the analyses dramatically. The symmetrical peak of acetyl-HE-PFPTH eluted at a retention time close to that of HE-PFPTH and afforded mass spectrum with identical major fragments (*m/z* 308, 350). Acetylation had no effect on the peak of the internal standard. Retention times of acetyl-HE-PFPTH and EOE-PFPTH were 9.20 and 8.78 min, respectively.

The calibration curve was linear over the whole range (0–2 nmol calibration standard) but only the range of 0−0.2 nmol was used for calculation of the calibration curve, y = 0.1581x + 0.0017 (y = peak area ratio of acetyl-HE-PFPTH vs. EOE-PFPTH; x = amount of calibration standard in nmol per sample). The intercept 0.0017 was attributed to the background level of HEV in control globin, employed as a matrix in the calibration samples. This background value, assessed as 0.0017/0.1581 = 0.11 nmol g^{-1}, was then used to correct the calculated HEV levels in unknown samples.

The HEV levels found in the potentially exposed workers and in controls are shown in Table I and fig. 2. Subjects with significantly elevated adduct levels were identified in each of the three facilities A-C, with few values approaching the biological exposure limit of

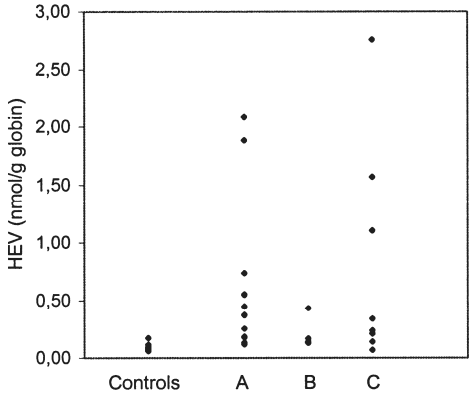

Fig. 2. **Individual values of HEV level in the examined subjects**

Table I
HEV levels in controls and subjects potentially exposed to EO in facilities A-C

	HEV levels [nmol g^{-1} globin]			
	Controls	A	B	C
n	10	14	4	8
Geom. mean	0.09	0.41	0.19	0.41
Max. value	0.17	2.08	0.43	2.76

3.8 nmol g^{-1} globin. All the highest-exposed subjects (>1 nmol g^{-1} globin) were operators of the sterilization chambers. The EO exposures in other workers not directly involved in operating the chambers (stockroom workers, repairmen, cleaning personnel, etc.) resulted in HEV levels <1 nmol g^{-1} globin.

The current study didn´t include simultaneous determination of EO in the workplace air. In the past years, short-term samplings (5−30 min) during the critical operations in the same plants revealed EO concentrations up to 80 mg m^{-3}. However, the personnel is usually wearing protective masks during such operations. The biological monitoring approach presented here indicates that despite the episodes of massive EO leaks, the average long-term exposures to EO were most likely below the level of 1 mg m^{-3}.

In conclusion, operations in the sterilization units using EO are associated with exposures that do not exceed recommended limits but still warrant further attention and support taking suitable protective measures at the workplace.

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P37

KINETICS OF ELLIPTICINE OXIDATION BY CYTOCHROMES P450 1A1 AND 1A2 RECONSTITUTED WITH NADPH:CYTOCHROME P450 REDUCTASE

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Key words: ellipticine, cytochromes P450, NADPH:cytochrome P450 reductase, kinetics

Introduction

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, fig. 1), an alkaloid isolated from *Apocyanaceae* plants, exhibits significant antitumor and anti-HIV activities[1,2]. The main reason for the interest in ellipticine and its derivatives for clinical purposes is their high efficiencies against several types of cancer, their rather limited toxic side effects, and their complete lack of hematological toxicity[1]. Nevertheless, ellipticine is a potent mutagen (for review see[1,2]). The prevalent mechanisms of ellipticine antitumor, mutagenic and cytotoxic activities were suggested to be (*i*) intercalation into DNA (ref.[2,3]) and (*ii*) inhibition of DNA topoisomerase II activity (for review see[1,2]). We demonstrated that ellipticine covalently binds to DNA after being enzymatically activated with cytochrome P450 (CYP) enzymes or peroxidases[2–6]. Human and rat CYPs of 1A and 3A subfamilies are the predominant enzymes catalyzing oxidation of ellipticine either to metabolites that are excreted from organisms (7-hydroxy- and 9-hydroxyellipticine) or form DNA adducts (13-hydroxy- and 12-hydroxyellipticine, the latter formed also spontaneously from another ellipticine metabolite ellipticine N^2-oxide by the Polonowski rearrangement)[2–5] (fig. 1). Of the peroxidases, human cyclooxygenase (COX)-2, ovine COX-1, bovine lactoperoxidases, human myeloperoxidase and horseradish peroxidase efficiently generated ellipticine-derived DNA adducts[6]. The same DNA adducts formed by ellipticine were also detected in V79 Chinese hamster lung fibroblasts transfected with human CYP3A4, 1A1 and 1A2 (ref.[7]), in human breast adenocarcinoma MCF-7 cells[8], leukemia HL-60 and CCRF-CEM cells[9] and *in vivo* in rats exposed to this anticancer drug[4,10]. On the basis of these data, ellipticine might be considered a drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its enzymatic activation in target tissues. Here, we investigated the efficiency of purified CYP1A1 and 1A2 enzymes reconstituted with NADPH:CYP reductase to oxidize ellipticine. In addition, kinetics of ellipticine oxidation by these enzymes was evaluated.

Materials and methods

The rat CYP1A1, rabbit CYP1A2 and rabbit NADPH:CYP reductase were isolated as described[2]. Incubation mixtures used to study ellipticine metabolism contained the following in a final volume of 500 μl: 100 mM potassium phosphate buffer (pH 7.4), 1 mM $NADP^+$, 10 mM D-glucose 6-phosphate, 1 U ml^{-1} D-glucose 6-phosphate dehydrogenase, 0.01–1 μM CYPs reconstituted with NADPH:CYP reductase in liposomes and 10 μM ellipticine dissolved in 10 μl DMSO. The enzyme reconstitution was performed as described[2,3], but different ratios of

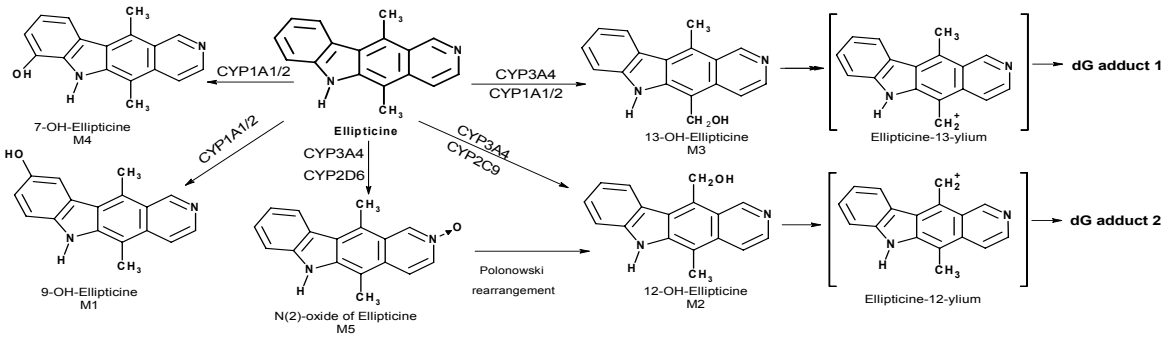

Fig. 1. **Metabolism of ellipticine by human CYPs showing the characterized metabolites found to form DNA adducts**

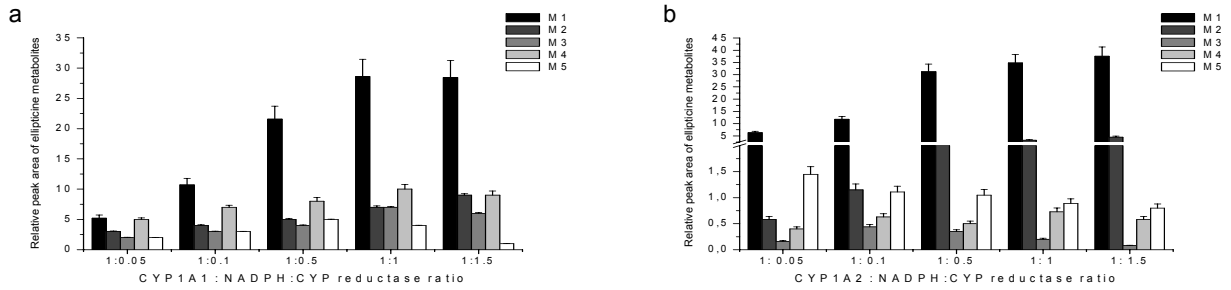

Fig. 2. **The effect of NADPH:CYP reductase on ellipticine oxidation by CYP1A1 (a) and CYP1A2 (b)**

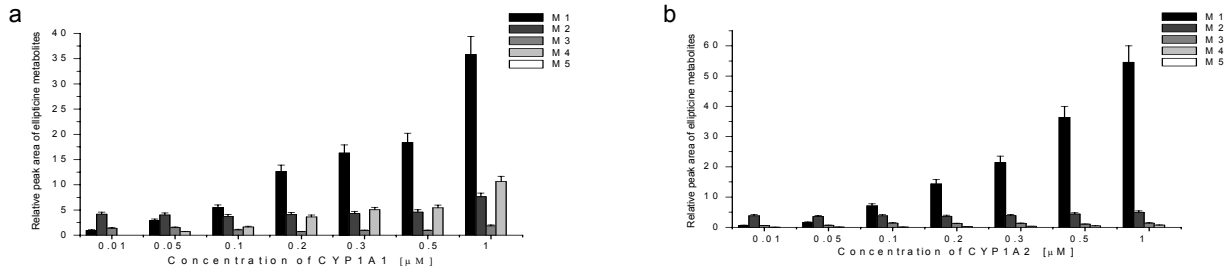

Fig. 3. **The effect of different concentrations of CYP1A1 (a) and CYP1A2 (b) on ellipticine oxidation**

CYP:reductase were utilized (fig. 2 and 3). After incubation (37 °C, 20 min), the reaction was stopped by adding ethylacetate. Thereafter, 5 μl of 1 mM phenacetine in methanol was added as an internal standard and the ellipticine metabolites were extracted twice with ethylacetate (2 × 1 ml). The extracts were evaporated under nitrogen and dissolved in 20 μl of methanol. The ellipticine metabolites were separated by HPLC as described[5]. Five ellipticine metabolites identified previously as 9-hydroxy-, 12-hydroxy-, 13-hydroxy-, 7-hydroxyellipticine and ellipticine N^2-oxide, were eluted at the retention times of 6.3, 6.9, 7.8, 8.5 and 11.2 min, respectively[5].

Results and discussion

The CYP1A1 and 1A2 enzymes reconstituted with NADPH:CYP reductase oxidized ellipticine to five metabolites: 9-hydroxy-, 12-hydroxy-, 13-hydroxy-, 7-hydroxyellipticine and ellipticine N^2-oxide (figs. 2,3), found previously to be formed by human, rat and rabbit hepatic microsomes[5]. The 9-hydroxy- and 7-hydroxyellipticine are the major ellipticine metabolites formed in the enzyme reconstituted systems containing CYP1A1 and 1A2. These results correspond to those found by us in rat, rabbit and human hepatic microsomes utilizing inducers and inhibitors of CYPs[11]. Efficiencies of CYP1A1/2 enzymes reconstituted with its reductase to oxidize ellipticine depends on the CYP:reductase ratios in the reconstitution systems. An

Table I
Kinetics parameters of ellipticine oxidation by CYP1A1 (A) and CYP1A2 (B)

CYP1A1 (A)				CYP1A2 (B)			
Ellipticine metabolites	*n*	V_{max} [min^{-1}]	K_m [μM]	Ellipticine metabolites	*n*	V_{max} [min^{-1}]	K_m [μM]
M1	1.01	1.34	0.10	M1	1.01	1.00	0.23
M2	0.91	0.08	0.82	M2	1.03	0.06	1.63
M3	0.89	0.06	3.50	M3	1.08	0.10	14.00
M4	0.98	0.22	0.51	M4	1.03	0.02	6.93

increase in the NADPH:CYP reductase content in the reconstitution systems resulted in an increase of ellipticine oxidation up to the value of the CYP:reductase ratio of 1:0.5, with negligible or low, insignificant, changes in their efficiencies up to ratios of 1:1 for CYP1A1 and 1:1.5 for CYP1A2 (fig. 2). The ratio of CYP1A1/2:reductase of 1:0.5 was used for evaluation of kinetics of ellipticine oxidation by these CYP enzymes.

An increase in the concentration of CYP1A1 and 1A2 in incubations results in an increase in formation of ellipticine metabolites, predominantly in generation of 9-hydroxyellipticine and 7-hydroxyellipticine, being linear up to CYP concentrations of 0.2 μM (fig. 3). The Michaelis-Menten kinetics was found for oxidation of ellipticine by CYP1A1 and 1A2 (data not shown). The values of Michaelis constant (K_m) and maximum velocity (V_{max}) are shown in Table I.

Conclusion

The results demonstrate that the system of purified CYP1A1 and 1A2 reconstituted with NADPH:CYP reductase oxidizes ellipticine mainly to 9-hydroxy- and 7-hydroxyellipticine, which reflects the situation of the ellipticine oxidation in human, rat and rabbit hepatic microsomes.

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Abbreviations

CYP cytochrome P450
K_m Michaelis constant
n Hill coefficient
V_{max} maximum velocity

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P38
THE EFFECT OF VEGETARIAN DIET ON IMMUNE RESPONSE

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Key words: vegetarian diet, immunity, elderly

Introduction

Several papers document the health benefits of vegetarian dietary practices and the lower incidence of chronic disease, especially heart disease, in vegetarians[1]. Much of the data are derived from investigations in vegetarians, most of them consume a lacto-ovo vegetarian diet. Strict vegetarian or vegan diets, which exclude all foods of animal origin, are increasingly being adopted. The adequacy and nutritional effect of diets based entirely on plant foods is still under investigation. There is a lack of data on possible immunomodulatory effect of diet in vegetarian population. In our study, health status of younger and elderly women habitually consuming a vegetarian diet was evaluated by hematological and immunological measures in comparison with a non-vegetarian group.

Subjects and methods

Study population: Our study population consists of the group of 105 younger women (20–30 years old), (52 non-vegetarians and 53 vegetarians) and group of 69 elderly women (60–70 years old), (35 non-vegetarians and 34 vegetarians).

Design of study: Cross-sectional comparison of vegetarians and age/sex-matched omnivores.

Immunological methods: Phagocytic activity was measured after engulfment of bacteria Staphylococcus aureus marked with fluorescein isotiocyanate (FITC) and respiratory burst of neutrophils was evaluated using hydroxyethidine (HE), simultaneously.

Lymphocyte proliferation was measured by [^{3}H] thymidine incorporation after incubation and stimulation with concanavalin A, phytohemaglutinin, pokeweed mitogen and CD3 antigen.

Natural killer cytolytic activity was determined in peripheral blood mononuclear cells using K562 as target cells. K562 were labeled with fluorescein isothyocyanate (FITC) and propidium iodide (PI) and evaluated by flow cytometry.

Hematological parameters were measured by Sysmex K4500.

Statistical analysis was done using SPSS (SPSS Co., USA). Differences between groups were analyzed using Student T-test.

Results and discussion

The objective of this study was to assess the immune status of vegetarians compared with non-vegetarians in younger and elderly women population. Our results indicate that vegetarian life style resulted in suppression of innate and acquired cellular immune functions. Significantly lower phagocytosis of monocytes and granulocytes was found in elderly vegetarian vs. non-vegetarian population (P<0.05, P<0.001). Similar effect of diet was observed as decreased phagocytic activity of granulocytes in

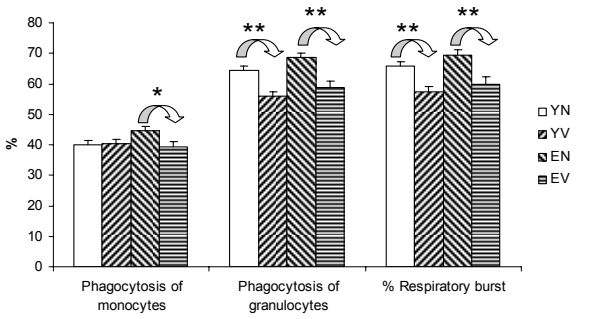

Fig. 1. **Phagocytosis of monocytes, granulocytes and percentage of respiratory burst in vegetarian and non-vegetarian population.** Statistical significance: *P<0.05, **P<0.01. YN – younger non-vegetarians, YV – younger vegetarians, EN – elderly non-vegetarians, EV – elderly vegetarians

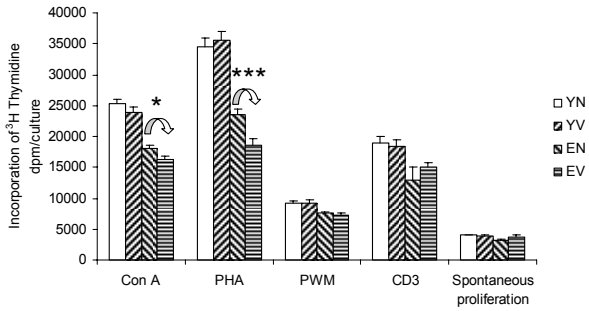

Fig. 2. **Proliferative response of T-lymphocytes and T-dependent B-lymphocytes** and spontaneous proliferation (dpm-disintegrations per minute) in vegetarian and non-vegetarian population (Con A – concanavalin A, PHA – phytohemaglutinin, PWM – pokweed mitogen, CD3 – CD3 antigen). Statistical significance: *P<0.05, ***P<0.001. YN – younger non-vegetarians, YV – younger vegetarians, EN – elderly non-vegetarians, EV – elderly vegetarians

younger vegetarians. Regardless the age, also respiratory burst of phagocytic cells was significantly decreased in vegetarians (fig. 1).

Decreased immune response was found in our elderly vegetarian population also in lymphocyte activity. Suppressive effect of vegetarian diet was markedly expressed in proliferative capacity of T-lymphocytes derived from elderly vegetarians and *in vitro* stimulated with Con A and PHA (fig. 2). Number of lymphocytes was also depressed. Our data are in contrary with findings of Richter et al. who observed identical proliferation of mononuclear cells after stimulation with interleukin-2 (IL-2) and phytohemagglutinin when compared immune function in people consuming two different diets (lacto-ovo vegetarian and meat-rich western diet)[2]. We can hypothesize, that our younger vegetarian population similarly to Richter population of male athletes is able to compensate the gap in specific nutrients. In elderly population suppression became more significant.

Table I
Hematological parameters in younger and elderly non-vegetarians and vegetarians.
Statistical significance: **P*<0.05, ***P*<0.01, ****P*<0.001

		Non-vegetarians	Vegetarians	Sign.
Leukocytes,	younger	6.64	5.84	**
$\cdot 10^3\ \mu l^{-1}$	elderly	7.33	5.25	*
Erythrocytes,	younger	4.58	4.55	
$\cdot 10^6\ \mu l^{-1}$	elderly	4.98	4.65	***
Hemoglobin,	younger	13.9	13.42	*
g dl^{-1}	elderly	15.12	14.33	**
Hematocrit,	younger	44.78	43.34	*
%	elderly	48.58	46.06	**
Platelets,	younger	205.54	209.94	
$\cdot 10^3\ \mu l^{-1}$	elderly	177.97	178.74	
Neutrophils,	younger	3.57	2.93	***
$\cdot 10^3\ \mu l^{-1}$	elderly	3.38	2.96	
Lymphocytes,	younger	2.3	2.23	
$\cdot 10^3\ \mu l^{-1}$	elderly	2.1	1.73	***
Monocytes,	younger	0.56	0.5	*
$\cdot 10^3\ \mu l^{-1}$	elderly	0.52	0.45	
Eosinophils,	younger	0.17	0.16	
$\cdot 10^3\ \mu l^{-1}$	elderly	0.17	0.17	
Basophils,	younger	0.04	0.03	***
$\cdot 10^3\ \mu l^{-1}$	elderly	0.05	0.03	**

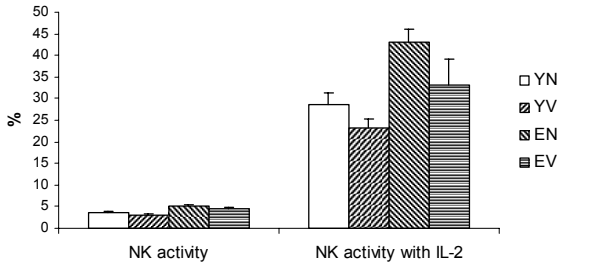

Fig. 3. **Natural killer cell activity (%) (without and with interleukin-2)**. YN – younger non-vegetarians, YV – younger vegetarians, EN – elderly non-vegetarians, EV – elderly vegetarians

Population of natural killer cells seems to be more stable towards different intake of nutrients after eating of various diets. No dramatic differences in natural killer cells activity were found between our young and elderly women populations eating different diets (fig. 3). Even in vegans with substantially lower dietary fat intake, natural killer cell activity did not differ from that of non-vegetarians[1]. Moreover, some published papers described significantly higher cytotoxic activity in vegetarians compared with their omnivorous controls[3].

In our study, elderly and younger population eating vegetarian diet had significant changes in red and white blood cells. All vegetarians had significantly suppressed levels of hemoglobin and hematocrit, older population had also decreased number of erythrocytes. Furthermore, all vegetarians had significantly lower white blood cell count and count of basophiles. Younger population had depressed number of neutrophils and monocytes (Table I). All hematological parameters were in physiological range for our laboratory. Our findings are in agreement with data of Pongstaporn and Bunyaratavej[4] who also found significant alterations in erythrocytes and leukocytes in vegetarian population. Published studies in vegans showed that some blood parameters such as lymphocyte count and mean cell volume was found even changed when compared with lacto- or lacto-ovo vegetarians. Some authors assume that lower lymphocyte and platelet count are accompanied by metabolic evidence that indicated vitamin B12 deficiency[5].

Conclusion

The objective of this pilot study was to assess the immune status of vegetarians compared with non-vegetarians. Analysis of immune functions of vegetarians displayed significantly lower phagocytosis of monocytes and granulocytes and respiratory burst of phagocytic cells. In elderly vegetarians, significantly suppressed proliferative response of T-lymphocytes in response to mitogens was found. Natural killer cell activity in population of vegetarian did not differ from non-vegetarian. Results showed lower count of white blood cells, red blood cells, hemoglobin, hematocrit, as well as neutrophils, lymphocytes, monocytes and basophils in vegetarians compared with non-vegetarians. In conclusion, our data indicate that vegetarian diet might have possible impact on immune response.

We would like to express our gratitude to Viera Vachalková, Helena Turazová and Edita Mrvíková. This

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P39

SUPPRESSION OF OXIDATIVE BURST OF NEUTROPHILS WITH METHOTREXATE IN RAT ADJUVANT ARTHRITIS

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Key words: neutrophil, arthritis, methotrexate, chemiluminescence, reactive oxygen species

Introduction

Neutrophils are the most abundant cells present in the joints of rheumatoid arthritis (RA) producing toxic products such as proteases and reactive oxygen species (ROS). In RA patients the apoptosis of neutrophils is delayed and this may lead to increased tissue damage and failure of the inflammation to resolve[1]. Activated neutrophils emit light from unstable high-energy ROS produced by the plasma-membrane-associated NADPH oxidase and metabolized by cytoplasmic and granule enzymes. The limitation of neutrophil-mediated damage relies in part on the modification of the capacity to generate chemical damage. The light signal from activated neutrophils can be enhanced in the presence of luminol[2]. Low-dose methotrexate (MTX) has become the first-line therapy for treatment of RA. Different mechanisms of action have been suggested for its action[3]. In this study we investigated the effect of low-dose MTX on oxidative burst of blood phagocytes, predominantly neutrophils, in a model of rat adjuvant arthritis (AA).

Methods and materials

Male rats (150–170 g) induced with AA by means of *Mycobacterium butyricum* in Freund's complete adjuvans[4] were pretreated orally with MTX 0.5 mg kg^{-1} two times a week during 28 days. Blood was taken by tail venepuncture in the amount of 10 µl and diluted 200 times with Tyrode solution. Luminol-enhanced emission of light stimulated by singlet oxygen, dependent on both the superoxide and metabolisation of myeloperoxidase released from primary granules. Chemiluminescence (CL) of both spontaneous and phorbol-myristate-acetate (PMA)-stimulated blood was measured in samples containing 50 µl of diluted blood, luminol (250 µmol L^{-1}), horseradish peroxidase (8 U ml^{-1}), PMA and phosphate buffer in 50 µl aliquots. CL was measured in Luminometer Immunotech during 60 min. Neutrophil count was measured in whole blood in Coulter counter by a standard procedure. Mean integral values of CL curves over 3600 s were evaluated. Statistical significance of differences between means was established by Student's t-test and *P* values below 0.05 were considered statistically significant.

Results and discussion

The development of AA in rats was accompanied with an increase in blood neutrophil count when compared with control animals from $0.92.10^4$ to $2.29.10^4$ cells μl^{-1}, as demonstrated in fig. 1. MTX did not alter the absolute neutrophil count in blood, most probably because neutrophils in RA have delayed apoptosis and this inflammatory disease is considered anti-apoptotic. Recent evidence using animal models has shown that neutrophils play a key role in the initiation and progression of AA (ref.[1]). Free radicals, including ROS, play a crucial role in the inflammatory and immunity processes involved in RA (ref.[1,5]). Spontaneous CL of the blood of MTX-treated animals and untreated AA-animals was significantly increased on day 7 of AA development and did not further increase until day 28.

Blood stimulation with PMA resulted in an increase of CL in both groups of animals (MTX-treated, untreated) in a dose-dependent manner.

Fig. 2 demonstrates the reactivity of neutrophils (phagocytes) on day 7,14, 21 and 28 of AA development. Spontaneous CL increased significantly both in the adjuvant arthritis and methotrexate-treated group on day 7 and this difference persisted until day 28 of investigation. MTX significantly decreased CL of whole blood stimulated with PMA (0.005 µmol L^{-1}) by 35 %, as compared with the untreated AA group. A significant inhibitory ef-

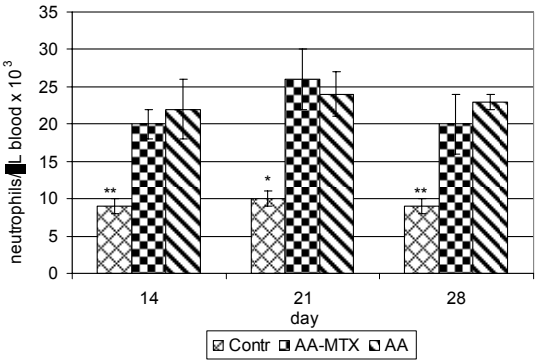

Fig. 1. **Neutrophil count in whole blood;** the values represent the mean from 6 animals as measured in 1 µl of whole blood. Contr – untreated animals, AA – adjuvant arthritis, AA-MTX – animals with adjuvant arthritis pretreated with methotrexate. Mean ± SEM, * $P<0.05$, ** $P< 0.01$ (vs AA)

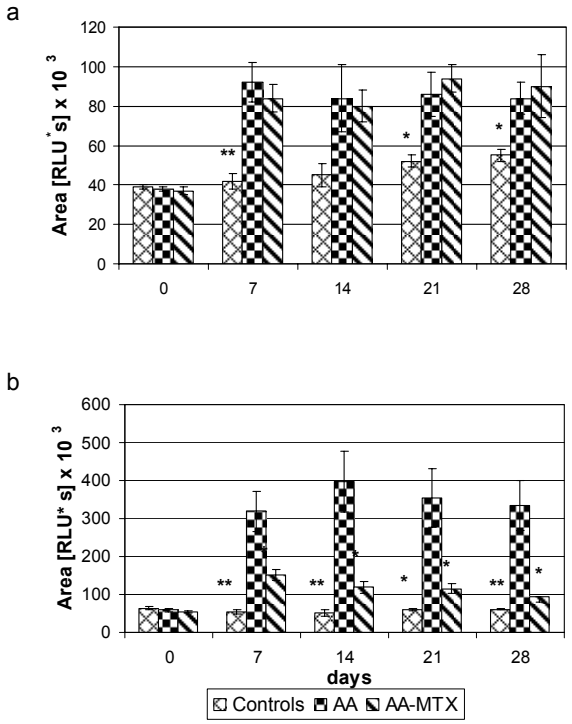

Fig. 2. **Panel a) Spontaneous whole blood chemiluminescence. Panel b) Whole blood chemiluminescence stimulated with 0.005 μmol L^{-1} PMA** (phorbol-myristate-acetate). Values given in relative luminescence units (RLU) are mean from 6 animals. Control-untreated animals, AA-rats with adjuvant arthritis, AA-MTX-rats with adjuvant arthritis treated with methotrexate. Mean ± SEM, **P*<0.05, ** *P*< 0.01 (vs AA)

fect of MTX on oxidative burst of neutrophils in animals with AA was observed on day 7 of investigation.

In monocytic cell lines, the cytotoxic antiproliferative effect of MTX was accompanied by apoptotic manifestation or enhanced ROS generation. The large difference in ROS production between T-lymphocytic and monocytic cell lines was found to be related to the extent of the apoptotic effect of MTX, giving additional support to the suggestion of higher susceptibility of T-lymphocytes to MTX treatment[5]. The suppressive effect of MTX on CL of whole blood stimulated with PMA was significantly increased from day 14 to 28 of AA. As evident from the presented results, the significant increase in CL of whole blood on day 7 precedes the clinical signs of AA, appearing on day 14 of investigation. Increase in neutrophil count correlated with CL of blood in untreated animals. MTX significantly inhibited CL in PMA-stimulated blood of AA animals, presumably due to alterations in the cellular redox state in phagocytes[3]. The antiinflammatory effect of MTX resulting in suppression of oxidative burst of blood phagocytes might be a consequence of its interaction with the cAMP-protein-kinase A-dependent adenosine inhibition of neutrophil oxidative activity via the adenosine A_{2A} receptor[6].

Despite the fact that MTX induced apoptosis of T-cell lines through oxidative stress[5], our results demonstrated that pretreatment of animals with MTX significantly depressed the oxidative burst of stimulated blood phagocytes. The inhibition of neutrophils to produce ROS correlated with the improvement in the overall health state of the animals. The possible mechanism of the protective effect of MTX on oxidative burst of neutrophils in AA will be the subject of further interest.

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P40
MODULATION OF RAT BLOOD PHAGOCYTE ACTIVITY BY SEROTONIN

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Key words: phagocytes, reactive oxygen species, serotonin, serotonin receptors

Introduction

There is now incontrovertible evidence that the nervous and immune systems interact bidirectionally. One potent mediator which plays a role in regulating both the nervous and immune systems is serotonin[1]. Serotonin acts as a neurotransmitter and a neuromodulator but it appears to also have a considerable influence on constituents of the immune system. During inflammatory processes, large amounts of serotonin are released by local mast cell degranulation and aggregated platelets[2]. Serotonin modulates different aspects of both adaptive and innate immunity; however, the available data are rather controversial[3,4]. The increasing use of serotoninergic agents in therapeutics together with the accumulated evidence for a role of serotonin in the immune system emphasizes the need for immunopharmacological studies. Herein, the effect of serotonin on an oxidative burst of rat blood phagocytes was evaluated and the involvement of different serotonin receptors in the effect of serotonin on phagocytes was tested using receptors agonists and antagonists.

The effects of serotonin (serotonin creatinine sulfate salt monohydrate) in a concentration range of 10^{-7}–10^{-3} M on the CL responses of peripheral rat blood leukocytes were evaluated *in vitro*. Four stimuli of phagocyte oxidative metabolism with different mechanisms of activation – opsonized zymosan (OZP), phorbol myristate acetate (PMA), calcium ionophore A23187 (CaI) and *N*-formyl-methionyl-leucyl-phenylalanin (FMLP) were tested.

Methods

The heparinized rat blood obtained via a heart puncture was layered over the separation solution to remove erythrocytes by 1 h sedimentation at room temperature[5]. The rich plasma (buffy coat) of leukocytes was washed twice and leukocyte counts were adjusted to obtain a final concentration $1 \cdot 10^6$ ml^{-1}. The CL response of leukocytes was measured using the microtitre plate luminometer, LM-01T (Immunotech, Czech Republic) and the microtitre plate luminometr, Orion II with injector (Berthold Detection Systems GmbH, Germany) within 1.5 h after blood collection. The principle of the method is based on luminol interaction with the phagocyte-derived free radicals, which results in large measurable amounts of light. Briefly, the reaction mixture consisted of $100 \cdot 10^3$ leukocytes, 1 mM luminol (stock solution of 10 mM luminol in 0.2 M borate buffer) and one of the activators. The final concentrations of activators were selected based on our previous results: 62.5 μg ml^{-1} OZP, 9.55 μM Ca-I, 0.81 μM PMA or 2.85 μM FMLP (ref.[5]). The assays were run in duplicates. Spontaneous CL measurements in samples containing leukocytes and all other substances, but none of the activators, were included in each assay. Light emission, expressed as relative light units (RLU), was recorded continuously at 37 °C for 60 min. Each kinetic curve consisted of 100 points. The intensity of the CL reaction was expressed as the integral of the obtained kinetic curves, which corresponds to the total amount of light produced during the measurements. All data are expressed as the mean of n=6.

Results

The resulting data revealed that serotonin inhibited the CL response of rat blood phagocytes activated by OZP in a dose dependent manner (fig. 1). Similarly, serotonin inhibited the CL response of rat blood phagocytes to PMA, FMLP and Ca-I in a dose dependent manner (data not shown).

Further, the involvement of different serotonin receptors (5-HT$_1$, 5-HT$_2$, 5-HT$_3$, 5-HT$_4$, 5-HT$_5$ and 5-HT$_7$) was evaluated using various agonists and antagonists of these receptors. None of these agonists and antagonists studied exerted any direct antioxidative properties as we showed previously[6].

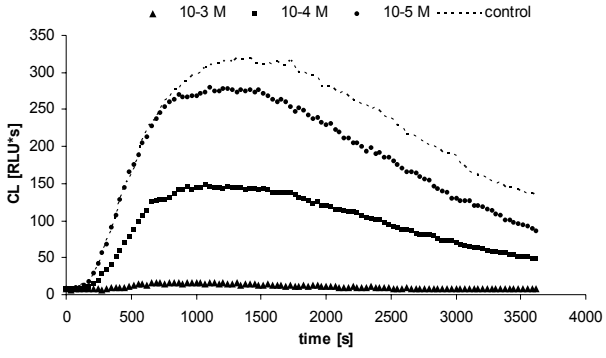

Fig. 1. **The effect of serotonin on the oxidative burst of rat blood phagocytes activated by OZP;** concentrations 10^{-6}–10^{-7} M did not differ from the control

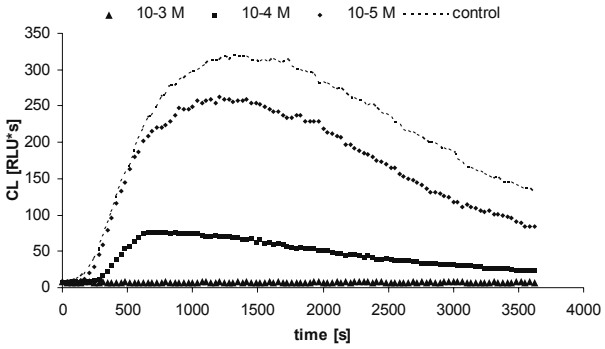

Fig. 2. **The effect of (±)-1-(2,5-dimethoxy-4-iodophenyl)-2--aminopropane hydrochloride on the oxidative burst of rat blood phagocytes activated by OZP;** concentrations 10^{-6}–10^{-7} M did not differ from the control

From all tested agonists, only the selective 5-HT_2 receptor agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI hydrochloride) exerted a similar effect on the respiratory burst as serotonin. In concentrations of 10^{-4} M DOI hydrochloride had an even more potent inhibitory effect on CL response (fig. 2) compared to serotonin despite not having any antioxidative properties[6]. Interestingly, the application of the selective antagonist of this receptor ketanserin did not block effect of serotonin.

Conclusion

The data obtained clearly demonstrated that serotonin was a potent inhibitor of the oxidative burst of rat blood phagocytes. Since the effect of serotonin on phagocytes is complex, our experiments were focused on elucidating the possible individual mechanisms of serotonin activity. It was previously shown that serotonin could act as a true scavenger of reactive oxygen species generated during the respiratory burst of stimulated phagocytes, that it caused aggregation and degranulation of neutrophils, and it inhibited the migration of mononuclear leucocytes[7,8]. Our results suggest that the modulatory effect of serotonin on an oxidative burst of blood phagocytes occurs through the activation of the 5-HT_2 receptor subtype since DOI hydrochloride, a selective 5-HT_2 receptor agonist, had an inhibitory effect on respiratory burst similar to serotonin. However, the application of the antagonist of this receptor did not block the effect of serotonin. Therefore, we could speculate that other mechanisms are involved in the serotonin dependent modulation of rat blood phagocyte activity. Another probable explanation could be the direct scavenging activity of serotonin against free radicals produced during respiratory burst of phagocytes.

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P41
A COMPARISON OF ANTIOXIDANT PROPERTIES OF URIC ACID, ALLANTOIN AND ALLANTOIC ACID

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Key words: uric acid, allantoin, allantoic acid, antioxidant

Introduction

Purines are the heterocyclic aromatic organic compounds, consisting of a pyrimidine ring fused to an imidazole ring. They are components of a number of important biomolecules, such as nucleic acids, adenosine triphosphate, and coenzyme A. Purines are cleared from organism by catabolic pathway, which is common to all animal species. However, during the evolutional process some of the enzymes involved in this catabolic pathway were lost which led to arresting of purine catabolism at different levels. In mammals, purine catabolism ends with allantoin, a product of uric acid degradation by the enzyme urate oxidase. The only exceptions constitute the anthropoid apes and humans in which urate oxidase activity had been completely lost. These species do not express urate oxidase due to nonsense mutations on the gene encoding it[1] and are not able to degrade uric acid to allantoin. This leads to high plasma concentrations of uric acid, which manifold exceed concentrations typical for other mammals. Although the lack of urate oxidase in apes and humans is known for many years, it remains unclear until today why these mutations have been evolutionarily accepted. It was hypothesized, that the loss of urate oxidase might constitute evolutionary advantage over other mammals because of the strong antioxidant properties of uric acid[2]. While the antioxidant properties of uric acid are well described nowadays, little is known about its catabolic products. The aim of this study was therefore to examine the antioxidant properties of allantoin and allantoic acid and to compare them with antioxidant properties of uric acid.

Materials and methods

Scavenging of peroxyl radical was measured luminometrically using the TRAP method[3]. Trolox, a water-soluble analogue of α-tocopherol was used as a standard. The data are expressed as μmol of peroxyl radicals trapped by 1 l of sample. Concentration of tested samples was 300 μM.

Scavenging of hydroxyl radical was measured luminometrically. System Fe^{2+}-EDTA + hydrogen peroxide was used to generate hydroxyl radical. The tested compound (300 μM) was mixed with luminol (1 mM), distilled water and H_2O_2 (2 mM). The reaction was started by adding of 1 mM of Fe^{2+}/1.2 mM of EDTA. The integrals of the resultant kinetic curves were used to evaluate the scavenging activity and expressed as relative light units (RLU).

Scavenging of superoxide was measured colorimetrically using XTT. Xanthine oxidase (0.04 U ml^{-1}) in PBS was mixed with EDTA (100 μM), XTT (40 μM) and sample (300 μM). The reaction was started by addition of xanthine (660 μM). The reaction mixture was incubated at lab temperature for 10 minutes and after incubation absorbance at 470 nm was measured.

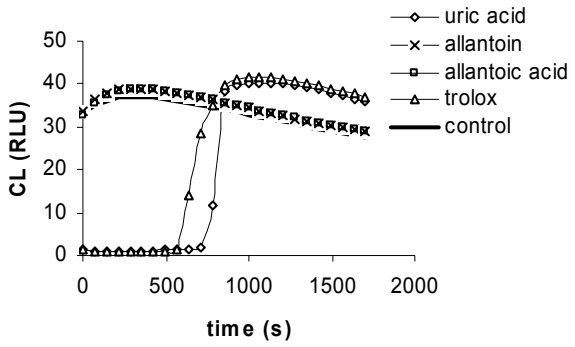

Fig. 1. **Scavenging of peroxyl radical by uric acid, allantoin and allantoic acid measured with TRAP method;** Trolox was used as reference antioxidant

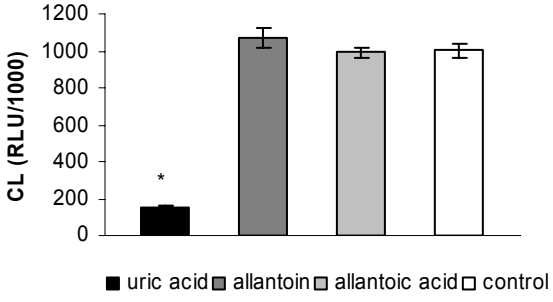

Fig. 2. **Scavenging of hydroxyl radical by uric acid, allantoin and allantoic acid measured luminometrically;** the values represent integrals of resultant kinetic curves expressed as relative light units/1000

Results

Data show that neither allantoin nor allantoic acid scavenged peroxyl radical (fig. 1). Similarly, neither allantoin nor allantoic acid scavenged hydroxyl radical, whereas uric acid was a very effective scavenger of this reactive oxygen species (fig. 2). Uric acid and allantoic acid did not react significantly with superoxide anion. Allantoin reacted significantly with superoxide when compared to control. However, when allantoin was compared to uric acid, none statistically significant difference was observed (fig. 3).

Discussion

Uric acid is known to be one of the most important antioxidants in human body fluids[4,5]. It is an effective scavenger of strong oxidants, as are hydroxyl radical, singlet oxygen[2,6], peroxyl radical, hypochlorous acid[6] and radicals derived from the reaction between peroxynitrite and carbon dioxide[7]. It was also shown to chelate transition metal ions and to inhibit iron ion-catalyzed oxidation of ascorbic acid[8]. Interestingly, at low concentrations typical for most mammals, uric acid was shown to have prooxidant activity. Prooxidant/antioxidant switch occurs approximately at concentrations between 200–400 μM (ref.[9]).

Our results imply, that neither allantoin nor allantoic acid are scavengers of hydroxyl or peroxyl radicals. Conversely, uric acid effectively scavenges these reactive oxygen species. This finding is consistent with observations of other authors[2,6]. As for superoxide scavenging, mild antioxidant action of allantoin was observed but without significant difference compared to uric acid. Skinner et al. observed that allantoin, contrary to uric acid, did not react with peroxynitrite[10]. Whiteman and Halliwell arrived at the same conclusion[11]. Overall, these results are in accordance with the hypothesis, that the arresting of purine catabolism at the level of uric acid could represent an evolutionary advantage for the species involved. However, it still needs to be considered that uric acid, allantoin and allantoic acid were compared only from the aspect of their direct antioxidant properties in our experiments. The question, which remains unanswered, is the other biological effects of tested substances independent of their antioxidant capacity.

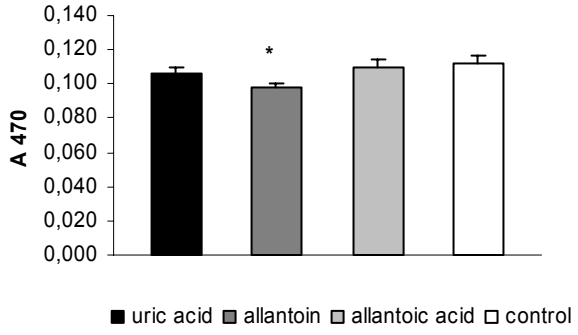

Fig. 3. **Scavenging of superoxide by uric acid, allantoin and allantoic acid measured with XTT test;** the values represent absorbances at λ = 470 nm

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P42
DIAGNOSIS AND ELIMINATION OF THALLIUM AFTER AN INTENTIONAL INTOXICATION

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Key words: Prussian blue, excretion, neurological, alopecia

Introduction

Thallium salts very rarely cause poisoning, as their use as rodenticides has been banned in most countries. The lethal dose is about 10 mg kg^{-1} body weight, i.e. usually less than 1000 mg. Thallium is excreted in the faeces and urine, in a proportion of 2:1. The biological half-life of thallium in documented cases was in a broad range of 1–30 days[1]. Therefore, diagnosis after several months of latency is usually difficult[2–4]. There are no data on the excretion of thallium after the antidote challenge test in the "unexposed" population, where it originates from environmental sources, especially emissions from the manufacturing process of fossil fuels.

In the Czech Republic, two women recently experienced thallium intoxication[5].

First patient

44-year-old woman, developed severe pain with paresthesias in the lower limbs in November 2004. These symptoms disappeared after 3 weeks; however, in March 2005 she suddenly experienced strong muscular pain in the lower extremities, with her gait as painful "as walking on broken glass". Within 5 days she lost her hair. In August 2005, a third period of symptoms in the lower limbs developed, and she also had blurred vision. The diagnosis was not clear. After 3 weeks, mild pain and her vision difficulties still persisted.

Second patient

In December 2005, her 22-year-old daughter developed the same symptoms. Over the following 3 weeks she became unable to walk. In the 4th week she lost all of her hair, developed severely blurred vision, and could only discern fingers at a distance of 0.3 m.

Thallium was found in the urine of the younger woman. Both women suspected they had been poisoned by the father/husband, who had access to a stock of old rodenticides.

In January 2006, both women were hospitalized in our department. Treatment with the antidote Prussian blue[6], ferric hexacyanoferrate $Fe_4[Fe(CN)_6]_3$, was started in the daughter. After the first dose of 6 g, the concentration of thallium in her urine increased twofold from 580 μg l^{-1} to 1170 μg l^{-1}, i.e. to 1760 μg/12 h. Maximum thallium concentration in the faeces was 5220 μg 100^{-1} g, and the daily excretion reached 13,000 μg. Maximum total daily thallium from both urine and faeces can be seen in Table I. Antidotal treatment was continued for 22 days, until the thallium concentration in the urine dropped under 5 μg l^{-1}.

The mother was also admitted, as she was still complaining of blurred vision in the central and upper parts of the visual field and of the inability to read. Therefore, as a challenge test, the mother was given the same dose of 6 g of the antidote. Thallium in the urine, unmeasurable by voltammetry prior to treatment, increased after the antidote to 21 μg/12 h. Maximum total daily excretion in both urine and the faeces is shown in Table I.

A challenge test with 6 g of Prussian blue was performed on a control subject, a woman. After an identical dose of Prussian blue, thallium in the urine increased to only 4 μg/12 h.

By April 2006 the daughter could walk with the aid of a walker for leg support. By August 2006 she walked without support, and she could see fingers from 0.75 m.

Conclusion

Treatment with Prussian blue produced a higher excretion of thallium in both women, compared to the control subject. The challenge test with this antidote can contribute to diagnosis even after 5 months after last ingestion of thallium. The reversibility of the polyneuropathy in the lower extremities and of the vision damage is still questionable.

Table I
Measurement of thallium in biological samples of the patients and a control subject

	Units	Daughter	Mother	Normal values (ref.[1])
Days of treatment with Prussian blue		22	5	
Estimated Latency from Exposure		6 weeks	5 months	
Initial Measurement (Optical Emission Spectrometer-Inductively Coupled Plasma Analysis)				
Blood	[μg l^{-1}]	770	0.3	0.049–0.130
Urine	[μg l^{-1}]	580	8.5	0.018–0.021
Hair	[μg g^{-1}]	6.8	–	0.007–0.650
Peak With Antidote (Voltammetry)				
Urine	[μg l^{-1}]	1170	21	4*
Urine	[μg/12 h]	1750	31	4.2*
Faeces	[mg/100 g]	5.2	0.55	?
Maximum daily thallium elimination	[mg/24 h]	16.0	0.25	?
Measurement After the End of Treatment (Voltammetry)				
Urine	[μg l^{-1}]	2.0	5.4	negative
Urine	[μg/12 h]	2.4	4.0	negative
Faeces	[mg/100 g]	0.10	0.07	negative
Last measurement (Voltammetry)		August 2006	April 2006	
Urine	[μg l^{-1}]	negative	negative	negative
Urine	[μg/12 h]	negative	negative	negative

– not measured, ? not known, *measurement in control subject

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P43

PROGRESS OF PROCESS TO DETERMINATION OF INHALATION ACRYLATE EXPOSURE AS BASEMENT METHOD FOR HUMAN RISK ASSESSMENT

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Introduction

Workers in dental material production, technicians in stomatological laboratories and surgeries are exposed to monomer of methyl methacrylate (MMA)[1,2]. This monomer is the main component of stuffing resins, toothprint material and acrylate teeth. Monomer usage in stomatology is very different from industrial application. There are higher concentration of MMA under laboratory conditions. There is also contact of MMA with worker's skin. MMA is clear, colourless liquid with a acrid odour, extremely volatile organic compound irritated skin, mucosa and eyes, caused chronic conjuctivitis[3,4]. During exposition there is happened skin intrusion[5]. In present there is no human epidemiological study for MMA. Completed workplace studies inform us of appearance of worker's dermatological problems[1,2]. Nobody occupied with concentration monitoring and MMA exposition on workplace. Vast majority of publication is concerned in toxicity tests on animals[6–8].

Measurement of workplace with methyl methacrylate exposure

In first stage of study there were followed out measurements of MMA in company produces acrylic teeth. Monitored workplaces were "matter preparation", "pre-bundle press" and "teeth press". There is three-shift production with working time 7.5 hours. Workers are exposed to MMA through the whole shift. There are totally exposed seventy workers in these workplaces. In room "matter preparation" the monomer is mixed with polymer (resin powder – polymethylmethacrylate) in stirring machine. Monomer is liquid mixture of MMA and 1,4-buthandioldimethylacrylate (BDMA) in mass representation 91 % MMA and 9 % BDMA. Matter is filled into the polyethlyene tube and then kept in cool in refrigerator. In "pre-bundle press" the matter is treated to shape for final teeth press in special devices. Next step is control polymerization of resin matter in presses.

Sample collection was carried out by personal sampling in worker´s expiratory zone. Expiratory zone is semispheric area with average 30 centimeter[8]. In this study there were used SKC pumps type 224-PCXR. These pumps are able to work with constant air flow. Sorptive material was granular activated carbon.

Methyl methacrylate determination in air by gas chromatography method

Methyl methacrylate captured on activated carbon was desorbed by extraction to solvent (CS_2). Its content is determinated by GC-MS method. Gas chromatograph GC-210 with mass detector GCMS-QP 2010S with column DB5-ms (J&W Scientific) was used.

Calibration standard was methyl methacrylate with <99 % purity. Accurate concentration is found by differential weighing to standard flask with carbon sulfide. Calibration standard is injected directly on gas chromatograph column (injection split – 1 µl, injection temperature 250 °C).

Results were calculated from obtained chromatograms by external standard method or by method of standard addition with respected sample dilution by evaluation station "GCMS solution". Retention times are evaluated and peak areas of analyzed compounds are compared. Results are reported in mass concentration mg/sample. Mass concentration mg m^{-3} is calculated from sucked air data.

Measured and limiting concentration comparison

Summary of maximal and all-shifted MMA concentration from chosen workplaces are written in the Table I. Periodic measurements were carried out on workplaces where limiting concentrations were exceeded and always after technical actions contributing to decrease MMA concentration on workplace. Hygienic limits in Czech legislation for MMA on workplace air are: the highest permissible concentration NPK-P 150 mg m^{-3}, permissible exposure limit PEL 50 mg m^{-3}. According table number one the value PEL is still exceeded on workplace "matter preparation". The value is excceded over 88 %.

Discussion

Thanks to the results from measurements and realized treatments on workplaces (increasing of ventilator capacity, fume cupboard installation above the work table), the concentration of MMA were decreased. In spite o fit the MMA values on workplace "matter preparation" of all-shift concentration are still over the limit. One reason is frequent manipulation with liquid monomer MMA in opened vessels outside of fume cupboard. MMA is extremely volatile compound. This workplace in enclosed – without windows. Change of air is happened by ventilators. On the other side their power is limited by microcli-

Table I
Summary of maximal measured concentration and all-shift concentration

Workplace	Maximal measured concentration c_{max} [mg m^{-3}]	All-shift concentration c [mg m^{-3}]
Matter preparation	238.636	224.56
	141.026	107.885
	100.694	94.753
Pre-bundle press	167.119	157.259
	11.407	10.734
Teeth press	30.820	29.0

matic requirements on workplace (circulation, temperature). It is impossible to increase their capacity. It can not be ruled out that MMA vapors diffused through the ventilators to other workplaces. MMA is still released to external environment before final press. Whichever manipulation with this matter brings risk of MMA exposition. These facts obliged company with these productions to discuss what to do. First step will be very large monitoring of MMA concentration. Measurement will be done step by step through the whole production. Before monitoring evolution of device capable of passive sampling of followed compound will be done. This device uses effect of molecular diffusion and is called passive dosimeter. Followed compound diffused to the surface of sorption material is placed to the diffuser according to the gradient of concentration. Passive dosimeter is small and light device which is ease to fix on worker's dress. Usage with it is very simple. In this study there will be also completed biological monitoring of MMA. Biological monitoring will be done by cytogenetical analysis of peripheral lymphocytes. This analysis is used for biological monitoring of genotoxical factors in municipal place and workplace. Presence of genotoxical active compound in place affected human body is probed by frequency of chromosomal aberration in monitoring group.

Conclusion

This article has been trying to introduce basement of problem happened on workplace with liquid MMA manipulation under laboratory conditions. It is not only teeth production but also work of laboratory technicians and dentists. Production is happened under laboratory conditions but MMA amount using there is very dangerous because of its volatility. Epidemiological studies were done mainly on animals. Sampling in workplace with personal pumps is very expensive. Number of parallel samples is limited by number of pumps. Workplace monitoring is suitable to realize in one period under the same conditions (work, climatic and microclimatic conditions, ...). Because of penetration of MMA through the skin, it is suitable not to realize only sampling in air but to do biological monitoring of compound in human organism.

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P44

COMPARISON OF THE CYTOTOXICITY OF AND DNA ADDUCT FORMATION BY THE ANTICANCER DRUG ELLIPTICINE IN HUMAN BREAST ADENOCARCINOMA, LEUKEMIA AND NEUROBLASTOMA CELLS

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Key words: ellipticine, MCF-7, HL-60, CCRF-CEM and neuroblastoma cells, peroxidases, cytochromes P450, DNA adduct

Introduction

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, fig. 1), an alkaloid isolated from *Apocyanaceae* plants, exhibits significant antitumor and anti-HIV activities[1,2]. The main reason for the interest in ellipticine and its derivatives for clinical purposes is their high efficiencies against several types of cancer, their rather limited toxic side effects, and their complete lack of hematological toxicity[1]. Nevertheless, ellipticine is a potent mutagen (for review see[1,2]). Ellipticines are anticancer drugs, whose precise mechanisms of action have not been explained yet. It was suggested that the prevalent mechanisms of their antitumor, mutagenic and cytotoxic activities are (*i*) intercalation into DNA and (*ii*) inhibition of DNA topoisomerase II activity (for review see[1]). Ellipticine and its metabolite 9-hydroxyellipticine also cause selective inhibition of p53 protein phosphorylation in several human cancer cell lines and this correlates with their cytotoxic activity. Ellipticines also uncouple mitochondrial oxidative phosphorylation and thereby disrupt the energy balance of cells (for review see[2]).

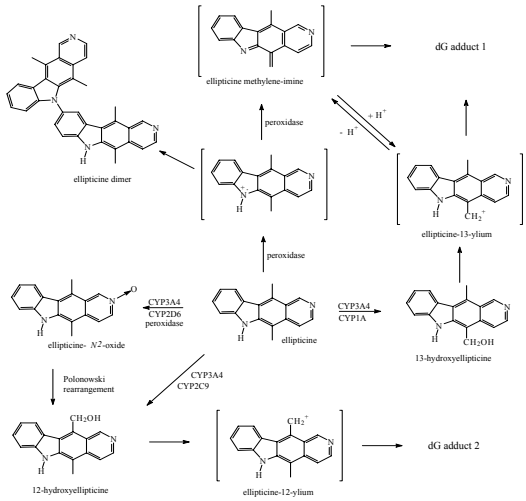

Fig. 1. **Metabolism of ellipticine by human CYPs and peroxidases showing the characterized metabolites found to form DNA adducts**

We demonstrated that ellipticine covalently binds to DNA after being enzymatically activated with cytochrome P450 (CYP) enzymes or peroxidases[2–6]. Human and rat CYPs of 1A and 3A subfamilies are the predominant enzymes catalyzing oxidation of ellipticine either to metabolites that are excreted from organisms (7-hydroxy- and 9-hydroxyellipticine) or form DNA adducts (12-hydroxy- and 13-hydroxyellipticine)[2–5]. Of the peroxidases, human cyclooxygenase (COX)-2, ovine COX-1, bovine lactoperoxidase, human myeloperoxidase and horseradish peroxidase efficiently generated ellipticine-derived DNA adducts (fig. 1)[6]. The same DNA adducts by ellipticine were also detected in V79 Chinese hamster lung fibroblasts transfected with human CYP3A4, 1A1 and 1A2 (ref.[7]) and *in vivo* in rats exposed to this anticancer drug[4,8]. Our recent studies also indicate that ellipticine is toxic to human breast adenocarcinoma MCF-7 cells[9] and leukemia HL-60 and CCRF-CEM cells[10]. On the basis of these data, ellipticine might be considered a drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its enzymatic activation in target tissues.

Here, we investigated the potential of ellipticine to damage another type of cancer cells, human neuroblastoma cells, and examined whether DNA adducts are formed in these cells. In addition, we compare toxic sensitivity of these and MCF-7, HL-60 and CCRF-CEM cells to ellipticine.

Materials and methods

Commercial MCF-7, HL-60 and neuroblastoma IMR-32 cells and the CCRF-CEM, a T lymphoblastoid cell line (from the Department of Pediatric Hematology and Oncology), cultivated in the presence of 0–10 μM ellipticine and tumor cell viability were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide test as described[9,10]. DNA from cells was isolated by the phenol/chloroform extraction method[2]. ^{32}P-postlabeling analyses of ellipticine-derived DNA adducts were performed using nuclease P1 enrichment as described previously[2–10]. Immunoquantitation of CYPs (CYP1A1, 1A2, 2B, 2E1 and 3A), NADPH:CYP reductase and COX-1 and -2 in homoge-

nates of cancer cells was done by Western blot as described[9,10]. Myeloproxidase (MPO) was detected by flow cytometry using anti-human MPO-FITC antibody[10].

Results and discussion

Toxicity of ellipticine to all analyzed cell lines was dose-dependent; ellipticine is the most toxic to neuroblastoma IMR-32 cells [a parent neuroblastoma cell line as well as its daughter line resistant to doxorubicine, IMR-32 (DOXO)], followed by leukemia HL-60 cells, breast adenocarcinoma MCF-7 cells and leukemia CCRF-CEM cells.

Table I
DNA adduct formation by ellipticine in human cancer cell lines and its cytotoxicity to these cell lines

Cells	IC_{50} [μM]	Total DNA adduct levels [relative adduct labeling x 10^{-7}]
IMR-32	0.26	13.1
IMR-32 (DOXO)	0.53	10.0
HL-60	0.64	64.6
MCF-7	1.25	9.3
CCRF-CEM	4.27	7.3

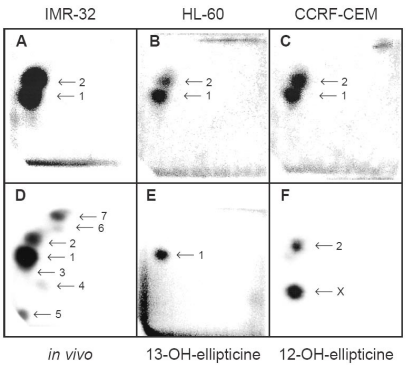

Fig. 2. **Autoradiographs of PEI-cellulose TLC maps of ^{32}P-labeled digests of DNA** isolated from neuroblastoma IMR-32 cells exposed to 10 μM ellipticine (A), HL-60 cells exposed to 1 μM ellipticine (B), CCRF-CEM cells exposed to 5 μM ellipticine (C) for 48 h, of liver DNA of rats treated with 40 mg ellipticine per kilogram body weight (D), from calf thymus DNA reacted with 13-hydroxyellipticine (E) and 12-hydroxyellipticine (F). Analyses were performed by the nuclease P1 version of the ^{32}P-postlabeling assay. (A,B,D) Scans of the plates for 6.5 min from the imager; (C,E,F) autoradiographs of films exposed for 1 h at -80 °C. Origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right)

The IC_{50} value for ellipticine was up to one order of magnitude lower in IMR-32 and HL-60 cells than in MCF-7 and CCRF-CEM cells (Table I).

Using the nuclease P1 version of the ^{32}P-postlabeling assay we found that the ellipticine-derived DNA adducts were generated in all cell lines analyzed in the study (see figure 2A, B and C for IMR-32, HL-60 and CCRF-CEM cells, respectively). This assay yielded a pattern of ellipticine-DNA adducts with two major adducts, similar to the pattern of adducts detected in DNA reacted with ellipticine activated with CYP enzymes or peroxidases *in vitro*[2,5,6] and in DNA *in vivo*, in rats treated with ellipticine (fig. 2D). The two adducts formed in DNA of analyzed cells are identical with those formed by the ellipticine metabolites 13-hydroxy- and 12-hydroxyellipticine with deoxyguanosine in DNA *in vitro* (fig. 2e and f) as confirmed by HPLC of the isolated adducts (not shown). The highest total levels of DNA adducts were generated by ellipticine in HL-60 cells, followed by those of IMR-32, MCF-7 and CCRF-CEM cells (Table I).

Because the two major adducts formed from ellipticine in all tested cell types are identical to adducts derived from 13-hydroxyellipticine or 12-hydroxyellipticine, metabolites of ellipticine formed by CYP enzymes of 3A and 1A subfamilies[2–5] or from metabolites generated by peroxidases such as MPO and COX-1 and -2 (ref.[6]) (fig. 1), we analyzed the expression of these enzymes in studied cell lines. Each of the tested cancer cell lines contains at least one of the enzymes activating ellipticine. Expression of MPO protein in HL-60 cells was proven by flow cytometry using an anti-human MPO-FITC antibody (not shown). HL-60 cells also contain another peroxidase, COX-1, the expression of which was proven by immunoquantitation using an anti-COX-1 antibody (fig. 3). In contrast to this peroxidase, Western blots with polyclonal antibodies raised against COX-2 and various CYPs (CYP1A1, 2B4, 2E1 and 3A4) showed that CYP1A1 only is expressed in HL-60 cells (fig. 3).

No detectable expression of MPO was found in CCRF-CEM cells by FACS analysis. However, the Western blot analyses of other peroxidases (COX-1 and -2) and of CYP (CYP1A1, 2B4, 2E1 and 3A4) enzymes in CCRF-CEM cells revealed that COX-1 and low but detectable

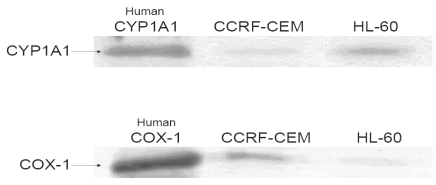

Fig. 3. **Immunoblots of CYP1A1 and COX-1 in HL-60 and CCRF-CEM reacted with antibodies against CYP1A1 and COX-1.** Cell homogenates were subjected to SDS-PAGE, proteins transferred to PVDF membranes and probed with antibodies as described in Material and Methods. Human CYP1A1 and human COX-1 were used as standards

levels of CYP1A1 are expressed in these cells (fig. 3). MCF-7 and neuroblastoma cells express only CYP1A1 (data not shown).

Conclusion

The results presented here demonstrate the formation of covalent DNA adducts with ellipticine in human cancer cell lines (breast adenocarcinoma MCF-7 cells, leukemia HL-60 and CCRF-CEM cells and neuroblastoma IMR-32 cells), and suggest the formation of covalent DNA adducts as a new mode of antitumor action of ellipticine for cancer.

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Abbreviations

CYP	cytochrome P450
MPO	myeloperoxidase
COX	cyclooxygenase
IC_{50}	inhibitor concentration eliciting 50 % inhibition
FITC	fluorescein isothiocyanate
FACS	fluorescence-activated cell sorting

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P45

MITOCHONDRIAL BIOENERGETICS OF SKELETAL MUSCLE STUDIED IN ADJUVANT ARTHRITIS

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Key words: adjuvant arthritis, mitochondrial bioenergetics, oxidative stress, stimulated mitochondrial respiration, oxidative phosphorylation rate

Introduction

Rheumatoid arthritis (RA) is a chronic relapsing immuno-inflammatory multi-system disease with predominant synovial proliferation and destruction of articular cartilage[1]. The exact etiology of RA remains unknown. In chronic persistent synovial inflammation that either a foreign agent or some alteration in control of cellular responses, possibly genetically mediated, has been implicated[2]. Oxidative stress and redox imbalance are considered to contribute to the pathogenesis of chronic inflammatory diseases including RA. Numerous studies have suggested a role for oxidant stress in the pathogenesis of RA (ref.[3,4]). Several reviews have discussed the importance of oxidative stress and redox signaling in vascular inflammation and, in general, have suggested the involvement of NADPH oxidase, xanthine oxidase, mitochondrial respiration, cyclooxygenases and lipoxygenases in the production of vascular reactive oxygen species[5–7]. In general, inflammatory mediators present in inflamed synovial tissue, such as pro-inflammatory cytokines (i.e. TNF-α) and growth factors, activate the intracellular production of reactive oxygen and nitrogen species[8].

Oxidative stress present in rheumatoid arthritis also participates in changes of mitochondrial bioenergetics. These changes lead to a dramatic reduction of physical performance in patients with RA, due to atrophy of skeletal muscles[9].

Aim

In this study we compare the data obtained in different measurements of mitochondrial bioenergetics in skeletal muscle. We selected three independent experiments with different levels of injury in the model of adjuvant arthritis (AA). Clinical parameters (decrease of body weight and hind paw volume) and biochemical parameters, i.e. stimulated mitochondrial respiration (state 3) and the rate of ATP production (OPR − oxidative phosphorylation rate) for complex I (NAD-glutamate) and complex II (FAD-succinate) in mitochondrial respiratory chain, were monitored and compared.

Materials and methods

AA was induced in male Lewis rats (Breeding Farm Dobrá Voda, Slovakia), weighing 150–170 g, by a single intradermal injection of heat-killed *Mycobacterium butyricum* in incomplete Freund´s adjuvant. The three independent experiments[9–12] included healthy animals as reference controls (C) and arthritic animals without any drug administration (AA). We monitored clinical parameters, i.e. change of body weight (CBW) and hind paw volume (HPV). CBW was calculated as the difference of body weight measured on day 28 and body weight measured at the beginning of the experiment. The HPV increase was calculated as the percentage increase of HPV on day 28 in comparison to that at the beginning of the experiment. Mitochondria of the skeletal muscle were isolated by differential centrifugation. Respiratory chain function was measured using Clark oxygen electrode[13]. The data for all parameters were expressed as arithmetic mean and SEM. For significance calculations, Student`s t-test was used with $*P<0.05$ (significant); $**P<0.01$ (very significant); $***P<0.001$ (extremely significant). The arthritic groups were compared to healthy control animals. All parameters were compared as ratios of arthritis to control, excluding the CBW. For this parameter the difference between the control and arthritic animals was used.

Results and dscussion

We selected three independent experiments[9–12] with different levels of injury in the model of adjuvant arthritis. The clinical data are given in Table I. Ratios for HPV and differences of CBW were summarized, and on the basis of their values we classified the three levels of adjuvant arthritis as mild (AA_1)[9], medium (AA_2)[10,11] and severe injury (AA_3)[12]. Stimulated mitochondrial respiration (state 3) and the rate of ATP production (OPR) for complex I (NAD) and complex II (FAD) in mitochondrial respiratory chain are given in Table II and Table III. Mild injury in AA resulted in increase of functional parameters of mitochondrial bioenergetics, whereby the differences between healthy animals and animals with AA were very significant. We explain this finding as a consequence of adaptative mechanisms against oxidative stress caused by AA. Medium injury in AA induced increase in stimulated mitochondrial respiration for complex I. In the other biochemical parameters, only not significant differences were recorded between healthy and arthritic animals. In severe

Table I
Comparison of the severity of adjuvant arthritis using clinical parameters (HPV and CBW)

Groups of animals	HPV [%]	CBW [g]
C_1	22.67 ± 1.71	69.5 ± 2.63
AA_1	65.69 ± 8.63***	37.75 ± 8.76***
AA/C resp. C-AA	2.9	31.75
C_2	14.13 ± 2.11	91.43 ± 4.35
AA_2	56.79 ± 12.92**	31.0 ± 13.93**
AA/C resp. C-AA	4.02	60.43
C_3	17.20 ± 1.81	61.33 ± 10.26
AA_3	80.94 ± 8.7***	-5.11 ± 3.74***
AA/C resp. C-AA	4.71	66.44

HPV – hind paw volume, CBW – change of body weight. Values are mean ± SEM; significance AA compared to control: *P≤0.05, **P≤0.01, ***P≤0.001

Table II
Changes in skeletal muscle mitochondrial function (state 3 and OPR) for complex I (NAD-glutamate) induced by adjuvant arthritis

Groups of animals	State 3 [nAtO/mg prot./min]	OPR [nmol ATP/ mg prot./min]
C_1	92.6 ± 6.16	215.9 ± 19.2
AA_1	151.1 ± 13.4**	354.8 ± 25.9**
AA/C	1.63	1.64
C_2	46.6 ± 9.5	130.0 ± 20.7
AA_2	72.9 ± 11.3	152.0 ± 26.8
AA/C	1.56	1.17
C_3	64.8 ± 4.85	218.8 ± 16.6
AA_3	51.6 ± 4.22	174.3 ± 17.1
AA/C	0.80	0.80

State 3 – stimulated mitochondrial respiration, OPR – oxidative phosphorylation rate. Values are mean ± SEM; significance AA compared to control: *P≤0.05, **P≤0.01, ***P≤0.001

injury of arthritic animals, accompanied by 5-times higher hind paw volumes and decreasing body weight compared with healthy animals, mitochondrial functions tended to be inhibited. This may be explained by exhaustion of adaptative mechanisms of mitochondrial bioenergetics.

Conclusion

Our results indicate a dependence of functional capacity of mitochondria on the severity of induced AA.

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Table III
Changes in skeletal muscle mitochondrial function (state 3 and OPR) for complex II (FAD-succinate) induced by adjuvant arthritis

Groups of animals	State 3 [nAtO/mg prot./min]	OPR [nmol ATP/ mg prot./min]
C_1	111.1 ± 3.81	130.4 ± 7.04
AA_1	186.7 ± 17.2**	245.2 ± 22.2**
AA/C	1.68	1.88
C_2	134.5 ± 22.7	173.6 ± 25.4
AA_2	127.4 ± 17.6	191.2 ± 27.4
AA/C	0.95	1.1
C_3	85.05 ± 8.53	167.9 ± 17.9
AA_3	77.7 ± 4.45	168.5 ± 13.1
AA/C	0.91	1.0

State 3 – stimulated mitochondrial respiration, OPR – oxidative phosphorylation rapidity. Values are mean ± SEM; significance AA compared to control: *P≤0.05, **P≤0.01, ***P≤0.001

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P46

PESTICIDE EXPOSURE ACCORDING TO THE CZECH TOXICOLOGICAL INFORMATION CENTRE FROM 1997 TO 2005

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Key words: Pesticides, Czech Toxicological Information Centre, Rodenticides, Intoxication, Exposure,

Introduction

Pesticides became a part of our households and may represent a danger, especially when they are ingested by children or adults[1,2]. Some exposures lead to life-threatening intoxications[3]. However little has been published about the current situation and frequency of exposures to these substances in Central Europe. The aim of this study was to describe the development and severity of exposures to pesticides, based on the calls to the Toxicological Information Centre, which serves to the Czech population of approximately 10 million.

Methods

Data taken from the Czech Toxicological Information Centre database from periods 1997–2005 were evaluated retrospectively using electronic evidence system.

Results

Overall pesticide poisonings reached 4405 in this period and accounted for 6.3 % of total calls to the Toxicological Information Centre. Forty-eight % calls concerned adults, 40 % children, and 12 % animals.

In human exposures, 59 % of calls involved men, 39 % women; in 2 % the gender was unknown. During the years, the percentage of these calls slowly decreased from 7.8 % in 1997 to 5.0 % in 2005.

Veterinary calls represented only 1.4 % of all calls, but 12% of all calls due pesticides. The course of some intoxication was in life-threatening, with severe symptoms (4.6 %) or lethal (5.0 %).

The exact numbers of inquiries in consecutive years are given in Table I. Among insecticides, the decrease was seen especially in the group of organophosphates and carbamates. More detailed data is shown in Table II. About 79 % of all exposures occurred in the vegetation period from April to October, and only 21 % calls in the other months.

Pesticide poisoning was predominantly an accidental overdose. There were 91 % unintentional exposures, 6 % suicidal, 2.3 % occupational and 0.7 % due to aggressive behavior. Ingestion accounted for 84 %, inhalation for 13 % and skin contamination for 3 %. The symptoms of intoxication were absent in 50.5 % of subjects. About 27.5 % of patients had mild symptoms, 8% medium, 1% severe, in the rest of the cases the symptoms were not known at the time of the call. Death was the reason of phone call in 0.3 % of cases.

In exposures to organophosphates and carbamates, 47.4 % of the subjects had no symptoms; 27.4 % had mild symptoms, 8 % medium and 3 % severe, in other calls they were unknown. Death was known in 1.5 % calls. Only 35 % calls concerned the children.

In exposures to rodenticides 83 % patients were asymptomatic, 5 % had mild symptoms, only 1.8 % medium and 2 % severe symptoms. In other calls the symptoms were not described. About 72 % calls involved children.

In 2005, the hospitalization was found necessary only in 14.4 % of subjects. About 1/3 of them were symptomatic at the time of the call. Antidotal treatment was recommended in 1.6 % of calls only: one patient was given atropine, and the other phytomenadione. In 2005 only one lethal case was registered. It concerned post-mortem consultation due to suicide with pirimicarb.

Table I
Inquiries concerning types of pesticides to the Toxicological Information Centre in the years 1997–2005

	1997	1998	1999	2000	2001	2002	2003	2004	2005
Insecticides	224	291	335	189	198	217	192	183	153
Rodenticides	102	140	105	142	136	150	105	118	164
Herbicides	--	--	--	83	109	117	78	77	89
Other*	--	--	--	110	122	191	117	95	73
Total	326	431	440	524	565	675	492	473	479

* Fungicides, moluscocides, combination or unknown pesticides. -- Data not available, included into Other

Table II
Selected data from the inquiries concerning pesticides in the years 1997–2005

	1997	1998	1999	2000	2001	2002	2003	2004	2005
Female, %	38.7	42.0	37.5	40.8	38.1	37.7	42.8	40.9	41.8
Accidental, %	82.4	85.6	91.3	88.0	93.3	92.4	89.2	92.0	88.3
Ingestion, %	81.7	86.0	82.5	83.8	85.8	85.2	82.1	81.5	86.0
Age (mean, SD)	22.9 (14.4)	21.8 (20.4)	23.0 (19.9)	22.9 (14.4)	25.7 (27.6)	26.6 (30.9)	29.9 (20.3)	27.6 (24.6)	24.6 (27.1)
Time (mean, SD)	15.5 (7.0)	16.4 (5.7)	14.7 (6.8)	13.9 (7.7)	14.1 (8.3)	14.6 (10.7)	14.1 (6.6)	14.1 (6.6)	14.5 (7.9)
Month (mean, SD)	6.9 (4.2)	6.5 (5.3)	6.3 (4.8)	6.8 (6.6)	6.6 (7.7)	6.6 (9.1)	6.4 (5.8)	6.4 (6.1)	6.4 (5.6)

Discussion

The development of inquiries to the Czech Toxicological Information Centre concerning pesticides is relatively favorable. It is true especially for human poisonings, as the most dangerous products had already been replaced by less toxic products. It can be seen in the group of organophosphates insecticides that have been substituted by other substances, i.e., synthetic pyrethroids with low toxicity for the mammals. The situation was even better in the group of rodenticides. In the Czech Republic, solely warfarin or superwarfarin based rodenticides are commercially available, which explains the good course of ingestions of these products. Comparing with the situation in the years 1988–1989 (ref.[1]), the percentage of suicidal attempts with pesticides from all exposures was not different from the recent data. However, the severity of exposures substantially decreased. In the years 1988–1989, total 14 deaths were recorded (7 after suicidal ingestions and 7 after drinking pesticide non-intentionally from a soft-drink bottle).

On the other hand, veterinary calls document a higher danger for exposed animals, which is in agreement with the literature[4,5]. One reason may be ingestion of a higher dose of the pesticide, when the animals are left alone without supervision. Another explanation is the repeated exposure, in some cases even intentional, i.e. by the neighbors, which concerns especially rodenticides poisonings. In some instances improper use of antiparasitic preparations caused the poisoning of cats and dogs.

Conclusions

Acute human pesticide exposure in our country is mainly accidental and has good prognosis in general, due to low toxicity of commercial products used. The number of calls to the Czech Toxicological Information Centre due to pesticides in the past 9 years is relatively stable and shows a mild decrease since the year 2003. More important is the development of the spectrum of pesticides, ingested by the patients either accidentally or intentionally. During the past years, the number of calls concerning toxic substances, such as organophosphates and carbamates insecticides slowly decreases. On the other hand, the number of calls due to rodenticides mildly increased, however no serious sequel has been recorded. Lower number of deaths is the most important difference from the situation in the late 80ies, both after suicidal and non-intentional ingestions.

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P47
ENVIRONMENTAL RISK FACTORS OF ALLERGIC DISEASES IN 5-YEAR-OLD CHILDREN

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Key words: allergic diseases, children, environmental pollution, risk factors, heavy metals

Introduction

Rapid increase in the prevalence of allergic diseases (AD), which includes atopic eczema (AE), asthma respiratory symptoms (ARS), rhinitis allergica (RA) and food allergy (FA), in last decades and particularly in industrialized regions is, besides genetic factors, ascribed to environmental factors.

Subjects and methods

The following methods were applied: follow-up (years 1997–2003) of cohort of children (n=1997) from birth to 5 years of age (n=403) for allergic disease development in environmentally different Slovak regions, annual clinical examination of children by paediatric allergists and administration of maternal questionnaires focused on socio-economic status, life style and risk factors. Child's peripheral blood collection for analyses of toxic metals, analysis of Pb, Cd and Hg by atomic absorption spectrometry (AAS) method, statistical evaluation of associations in EpiInfo v.6 and SPSS v.12.

Four selected Slovak regions were selected according to different predominant environmental pollutants and anthropogenic activities: industrial chemistry (CH) with the main sources of air pollution derived from chemical industry, industrial metallurgy and mining (M), agricultural (A) and rural (R) without any point source of industrial pollution.

Results

Comparison (Chi Square and Mantel-Haenszel tests) of the prevalence of AD in 5-year-old children with relation to risk factors is given in Table I. Influence of parent's positive history of allergic diseases was confirmed. Although we did not find significant influence of concentrations of lead (median 26.5 μg l^{-1}), cadmium (median 0.128 μg l^{-1}) and mercury (median 0.42 μg l^{-1}) in 5-year-old children's blood, we find a negative trend between the Cd levels in blood and incidence of AD in regions with different environmental characteristics (fig. 1).

Table I
Relation of risk factors and prevalence of allergic diseases among Slovak children

Risk Factors	AD	ARS	RA	AE	FA
Family history of AD	**		**	*	
Maternal history of AD	***		**	*	
Paternal history of AD	*			*	*
Region type	**	*	***		
Type of delivery				*	
Male gender	**	*			
Mother smoked in past		*			
Exclusive breast	*				*

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Prevalence of allergic diseases, asthma respiratory symptoms and rhinitis allergica among children differed with respect to environmental characteristics of the regions. Statistically significant differences were found in the frequency of allergic diseases (fig. 2) between regions: M/R and M/CH (P=0.01). Prevalence of asthma respiratory symptoms (fig. 3) differed between regions: CH/A (P=0.05) and CH/M (P=0.01). Prevalence of allergic rhinitis (fig. 4) was different between regions: R/M (P=0.001), R/CH, R/A and CH/M (P=0.01).

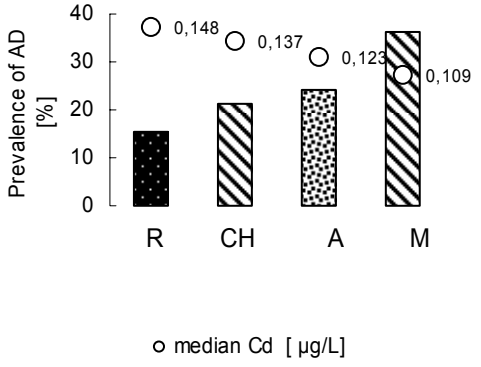

Fig. 1. **Prevalence of Allergic Diseases in 5-year-old children and Cd concentrations in blood**

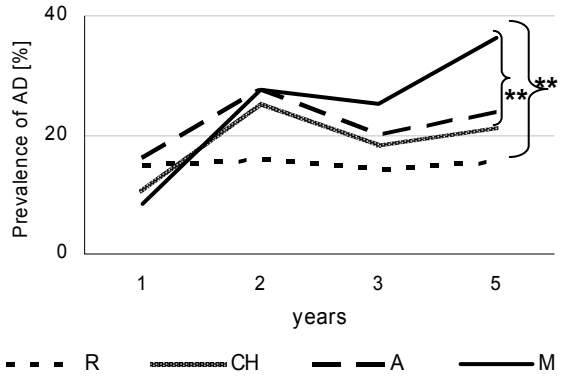

Fig. 2. **Development of Allergic Diseases**

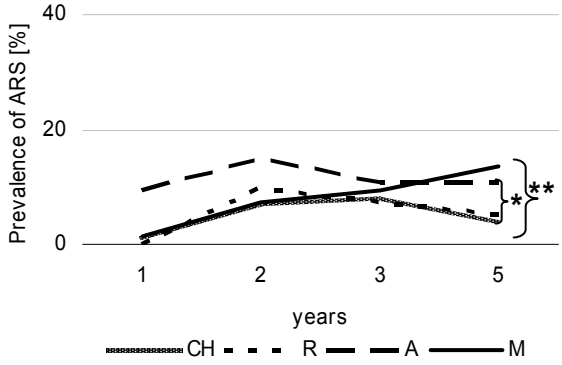

Fig. 3. **Development of Asthma Respiratory Symptoms**

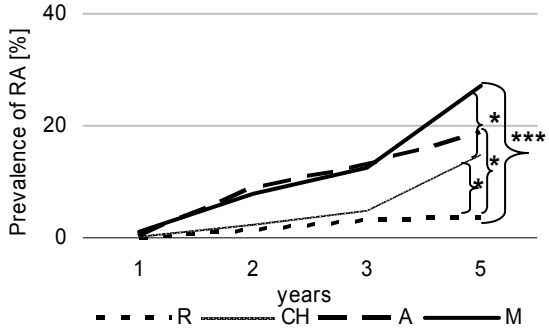

Fig. 4. **Development of Rhinitis Allergica**

Conclusions

Negative trend of AD prevalence with respect to Cd blood levels support the knowledge of immunotoxic effects of Cd. In our previous work, negative correlation was found between the concentrations of Cd and IgE levels in cord blood[1]. Differences in AD prevalence among regions may result from different environmental exposures.

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P48
HAZARD IDENTIFICATION OF BINARY CHEMICALS MIXTURES BY QSAR TECHNIQUES

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Introduction

QSAR and QAAR techniques applying knowledge of data on *Tubifex tubifex* test[1,2] (EC50(T.t.)), R-analysis[3] (analysis of plot of mixed EC50(T.t.) against molar fraction of a binary mixture) and partition coefficient of compounds between *n*-octanol and water (log P) were used to identify additivity or non-additivity of acute toxicity in mixtures of chemicals and to study their nature. The *Tubifex tubifex* test takes 3 minutes exposure and was verified by correlation with both log K_{ow} and acute toxicity indices measured with fish and ciliates[1].

Materials and method

Following chemicals were used: 2-nitroanisole (Aldrich, 99.9+ %), phenol (Aldrich, 99.9+ %), *n*-octanol (Aldrich, 99%), water (GORO AQUA 200, deionized, filtered through 0.22 mm membrane).

Determination of K_{ow}

The studied compound is added to a system of *n*-octanol and water where the volume of both phases is adjusted according to the expected value of K_{ow}. For each pair of tested compounds a series of binary mixtures with molar ratio of benzene 1.0, 0.9, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, and 0.0 was prepared. Each series was then tested at a sum concentration of both compound to be 0.005, 0.02 and 0.2 mol l^{-1}.

The system was shaken gently until equilibrium was achieved (5 to 10 hr) and then centrifuged to separate the two phases, especially if an emulsion has formed. Both phases, i.e. the *n*-octanol and the aqueous phase, were directly analyzed for both studied compounds using gas chromatography or liquid chromatography. The GC system used consisted of Agilent 6890N gas chromatograph with Agilent 7683 series injector and FID detector. The column used was J&W scientific capillary column (30 m × 0.25 mm × 0.25 m). Nitrogen (99.99 %) was used as a carrying gas. Injection volume was 1 ml.

The HPLC system used consisted of two ECOM LCP 4020 pumps, a Knauer dynamic mixing chamber, and an ECOM LCD 2083 UV/VIS detector. The column used was a Chromolith™ Performance RP-18e (4.6 × 100 mm) with Chromolith™ Guard Cartrige RP-18e (4.6 × 5 mm) guard column (Merck, Prague, CR). Mobile phase for pump A consisted of degassed water and for pump B 4 : 1 methanol/water mixture. Injection volume was 20 μl and the flow rate was 1.2 ml min^{-1}. For analysis of samples generated in experiments was used isocratic mode 40 % A and 60 % B. The wavelength of detection was 254 nm.

Determination of K_{mix}

Coefficient of distribution K_{mix} was determined according to[4], using following equation:

$$K^1_{mix} = \frac{W}{V} \times \frac{\sum_{i=1}^{n} \frac{Q_i}{1+\frac{W}{VK_i}}}{\sum_{i=1}^{n} Q_i - \sum_{i=1}^{n} \frac{Q_i}{1+\frac{W}{VK_i}}} \qquad (1)$$

where W is a volume of the aqueous phase, V is a volume of the octanol phase, n is a number of compounds in the mixture, Q_i is total amount of the compound i in the system and K_i is partition coefficient of compound i. The value of K_{mix} is normalized, i.e. in case of additivity the resulting K_{mix} is equal 1.

Scientific graphic and analyzing software ORIGIN® was used for statistical calculations. The additivity was tested using chi-square test and t-test with Bonferonis correction.

Determination of EC50

The inhibition of the movement of oligochaeta *Tubifex* was measured as the effective concentration that causes 50 % of maximal response. Batches of six worms were immersed in aqueous solutions of the binary mixtures and the concentration – response curve was determined. The number of immobilized worms was counted in each batch precisely three minutes after their immersion. The measurement was repeated three times with each concentration on various days and in triplicate on each day. The reproducibility of the measurements was checked by the parallel determination of the EC50 for aqueous $MnCl_2$ solution[1].

Results and discussion

Acute toxicity EC50 (normalized value) of binary mixture phenol-2-nitroanisole (fig. 1) is expressed as the inhibition of movement of oligochaeta *Tubifex tubifex*. The dashed line shows EC50 in case of additivity (normalized value 1). The normalization was used for purpose of a better understandable explanation of toxic effect of mixture and for mathematical modeling. Values of normalized EC50 above dashed line indicate inhibition of the toxic effect and values below dashed line indicate synergistic toxic effect.

Fig. 2 represented partition coeficient K_{ow} of 2-nitroanisole, fig. 3 partition coeficient K_{ow} of phenol and fig. 4 normalized coefficient of distribution K_{mix} of the

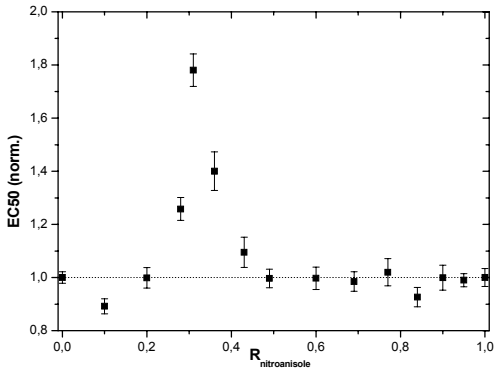

Fig. 1. **EC50 of the phenol-2-nitroanisole mixture**

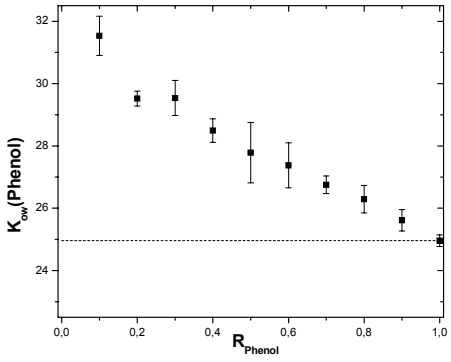

Fig. 3. K_{ow} **of the phenol in the phenol-2-nitroanisole mixture**

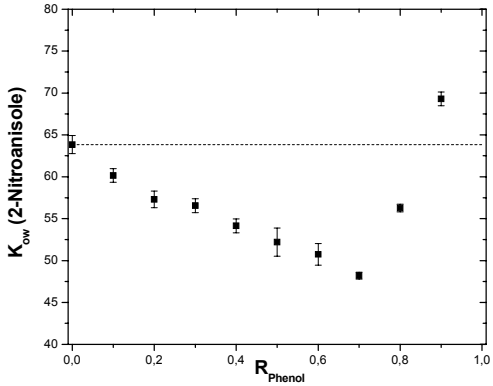

Fig. 2. K_{ow} **of the 2-nitroanisole in the phenol-2-nitroanisole mixture**

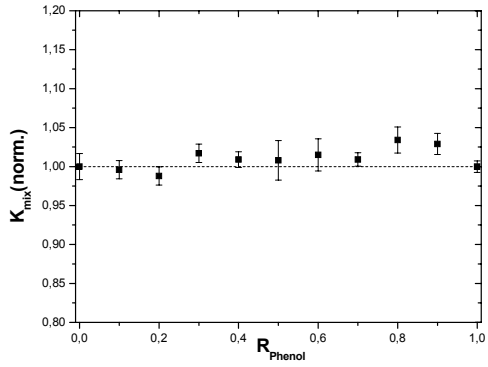

Fig. 4. K_{mix} **of the phenol-2-nitroanisole mixture**

mixture phenol-2-nitroanisole, total concentration of the compounds were 0.02 mol l^{-1}.

In the real life, the exposure to chemicals in mixture is more common than exposure to single compounds. Resulting activity should be different from additivity approach, widely used in studies on mixture toxicity[5,6]. The analysis indicates that the mixtures of industrial solvents phenol and 2-nitroanisole indicates clear „mixture interaction“, i.e. inhibition or potentiation depending on ratio of components in the mixture. An attempt to simulate the relationship between EC50 and molar ratio of mixture with log K_{ow} is shown.

Using of K_{mix} for QSAR modeling seems to be not fully sufficient. As is shown at fig. 4, there are no big changes of the K_{mix} for whole range of the mixture. On the other hand, the log K_{ow} of phenol and 2-nitroanisole show significant differences from log K_{ow} for both pure compounds. Especially the log K_{ow} of 2-nitroanisole could explain the acute toxicity of phenol – 2-nitro-anisole. On fig. 2 is shown decrease of K_{ow} for 2-nitro-anisole. It is suggested that lower value of K_{ow} of compound in the mixture lead to inhibition of toxicity of this mixture due to the less accumulation of 2-nitro-anisole, which is more toxic than the phenol, in an organic tissue of worms.

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P49

THIODIGLYCOLIC ACID – INDICATOR OF METABOLIC UNEQUILIBRIUM

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Key words: thiodiglycolic acid, TDGA, metabolism, creatine, glutathione

Introduction

Thiodiglycolic, also thiodiacetic, mercaptodiacetic acid or dicarboxydimethyl sulfide, $S(CH_2COOH)_2$, TDGA, is one of the normal products of human metabolism and it occurs at low concentrations in urine[1,2]. It is believed to be formed in a number of natural metabolic pathways however, the most frequently studied pathway involves the formation of TDGA from two carbon (2C) released from carcinogenic xenobiotics (e.g., vinylchloride monomer, ethylene oxide, 1,2-dichloroethane, chloroalkyl ethers[1–8]). These 2C units are oxidized via cytochrome P450 to chloroacetaldehyde, which reacts with reduced GSH[3,8]. From this point is this metabolic pathway common with that of natural formation of TDGA from glycine over glyoxylic and glycolic acids[3,9]. The process in presence of transaminases and vitamin B_6 continues over *S*-carboxymethyl-L-cysteine and *S*-carboxymethyl lactic acid to TDGA which in turn gets oxidized to thiodiglycol sulfoxide[3]. A significant increase is observed when the organism is exposed to the above mentioned 2C units, to the cytostatics ifosfamide and thiotepa[2,3], or to the *S*-carboxymethyl-L-cysteine[10,11], or to antihistamine Zyrtec (cetirizine dihydrochloride)[2].

TDGA is also one of the final products of the degradative pathway of thiodiglycol (TDG), which is produced by alkaline hydrolysis of mustard gas (bis(2-chlorethylsulphide)) (MG)[12,13]. In accordance with the Chemical Weapons Convention a concept of chemical detoxification and complete biodegradation of organic products by a biotechnological method using bacterias utilizing TDG was studied by Ermakova et al. and Lee et al.[12–14]. TDGA is one of the final products. Some bacterial strains can therefore be used for bioremediation of MG contaminated soils. A considerations of metabolic formation and fate of TDGA, structurally an analogue of MG, the microbiological studies on MG detoxification[13] seem to offer interesting aspects. Therefore, TDGA was found in urine of Iranian victims of an alleged attack with MG[15].

Experimental

The analysis of TDGA was carried out by the computer-controlled Eco-Tribo Polarograph using the software Polar 5.1 version for Windows on hanging mercury drop electrode (HMDE) (all Polaro-Sensors, Czech Republic). Other experiments were performed on mercury meniscus modified or polished silver solid amalgam electrodes[16–21] and on solid composite electrodes[22–29] to compare[30] the results with those obtained on HMDE. The process of TDGA reduction on the mercury electrode surface was studied in detail using Elimination Voltammetry with Linear Scan[31]. For characterization of body composition and of pending parameters the multi-frequency impedance analyzer "In Body 3.0" was used.

TDGA was analyzed, after pre-separation from urine on a column of powdered PVC and after elution by 0.2 M perchloric acid, by the D.C. voltammetric procedure described in[1,3]. The common laboratory methods were used for determination of other compounds determined parallel with TDGA, which were useful for elucidation of metabolic pathways (in blood: uric acid, folates, vitamin B_{12}, cholesterol, homocysteine (HoCySH), testosterone, cortisone; in urine: creatine, creatinine, pH, total proteins content). Their corrections for specific gravity were calculated[14].

Results and discussion

The natural level of TDGA in urine of healthy volunteers[2] varies between 10 and 20 mg L^{-1}. Its abnormally increase was found in the morning urine of individuals, who suffered from certain metabolic disorders[14]. The TDGA levels decreased substantially during the day. The time dependences of TDGA levels can be explained as the result of fluctuations of the daily rhythm of thiolic substances[14]. Creatine, consumed regularly as a food supplement, increases the TDGA level in the morning urine. Creatine supplemented to groups of randomly selected individuals, in any time of day, increased excretion of TDGA rapidly within 4–8 hours following per oral intake of creatine. Normal ranges of urinary TDGA levels were usually reached within the following 1–2 hours. The supplemented creatine mostly decreased the amount of normally excreted creatinine and increased the pH value of urine in the time of maximal TDGA excretion. The amount of excreted TDGA is specific for different individuals. It can be supposed that supplemented creatine reduces its endogenous production in humans. In accordance with previous findings[2,14], vitamin B_{12}, p.o., increased the TDGA level independently of creatine supple-

mentation, and did not affect the pH-value of urine. The stimulation of TDGA excretion after vitamin B_{12} application is caused by disturbance of redox equilibria induced by an increased supply of thiolic substances. Supplementation of creatine increases the input of 1C and 2C units into metabolic pathways, participated by TDGA. An increased level of TDGA in urine indicates a disturbance of redox equilibria in human body. Creatine supplementation affects metabolism of thiocompounds and excretion of some compounds into blood and urine, the metabolism of which is connected with the synthesis of creatine or with its usage in the body. Therefore, the levels of folates, vitamin B_{12}, homocysteine in blood, the levels of creatine, creatinine, TDGA, and pH in urine were followed and evaluated. The results were accomplished by data of bioimpedance measurements. According to the changes in creatine, folates and vitamin B_{12} levels before and after creatine administration in course of one month, it was possible to divide the volunteers under study into 4 groups. Each of them is characterized by typical changes in levels of the above mentioned substances. The results confirmed that creatine given as food supplement is used in human body not only for creatine phosphate formation, but that it affects the whole metabolic transformation and body constitution (mass, fat, and proteins).

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P50
STUDY OF PEROXIDASE-MEDIATED OXIDATION OF CARCINOGENIC AZO DYE SUDAN I

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Key words: chemical carcinogenesis, Sudan I, peroxidase, chromatography, mass-spectroscopy

Introduction

Sudan I [1-(phenylazo)-2-naphthalene, C.I. Solvent Yellow 14] was used as a food colorant in several countries[1], but it has been recommended as unsafe, because it causes tumors in the liver or urinary bladder in rats, mice, and rabbits, and is considered a possible carcinogen and mutagen for man[1,2]. Nevertheless, it is widely used to color materials such as hydrocarbon solvents, oils, fats, waxes, plastics, printing inks, and shoe and floor polishes[1,2]. While the metabolism of Sudan I is not understood in humans, its metabolism has been characterized in rabbits[1], where it is metabolized primarily in the liver by oxidative or reductive reactions. Oxidation of Sudan I catalyzed by cytochrome P450 (CYP) enzymes and peroxidases, was investigated and helped us to resolve its carcinogenic mechanism (for a review see[3]). C-Hydroxylated metabolites 4'-OH-Sudan I and 6-OH-Sudan I were found to be the major products of Sudan I oxidation *in vivo* and excreted in urine[1], and of its oxidation by rat and human hepatic microsomal CYP *in vitro*[3,4]. Besides the C-hydroxylated metabolites, which are considered detoxication products, the benzenediazonium ion (BDI) formed by enzymatic splitting of the azo group of Sudan I was found to react with DNA *in vitro*[3–6] (fig. 1). The major DNA adduct formed in this reaction has been characterized and identified as the 8-(phenylazo)guanine adduct[6]. This adduct was also found in liver DNA of rats exposed to Sudan I (ref.[7]). Sudan I and its C-hydroxylated metabolites are also oxidized by peroxidases, as a consequence DNA, RNA and protein adducts are formed[5,8–12] (fig. 1). While CYPs were found to be responsible for the activation of Sudan I in human or animal liver, their limited role in the *in vivo* metabolic activation of Sudan I in the urinary bladder should be taken into account. This organ has little or no detectable CYP; peroxidases are, however, present at relatively high levels in this tissue. It the case of peroxidase, the metabolites formed by this enzyme have, however, not been identified as yet. Therefore, the present work was undertaken to isolate two major Sudan I peroxidase-mediated metabolites and characterize them partially by mass spectroscopy.

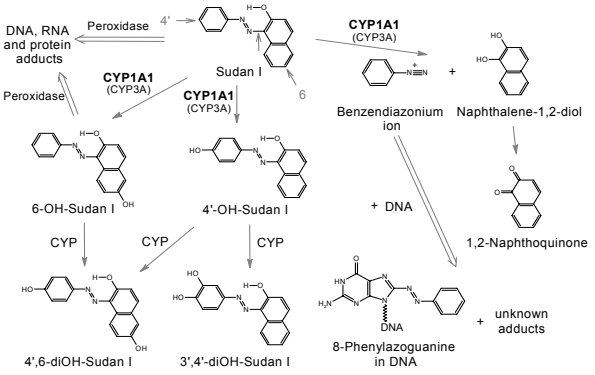

Fig. 1. **Scheme of Sudan I metabolism**

Materials and methods

Incubation mixtures contained the following in a final volume of 70 ml: 10 mM sodium phosphate buffer (pH 8.4), 0.5 μmol horseradish peroxidase (HRP), 100 μmol Sudan I dissolved in methanol and 200 μmol H_2O_2. After incubation (37 °C, 20 min) the mixtures were extracted with ethyl acetate (2 × 15 ml). The extracts were evaporated, dissolved in a methanol, and separated on HPLC or TLC. Silica-gel TLC plates were developed in hexane-diethyl ether (1:3, v/v). Spots of Sudan I metabolites M_1 and M_2 with R_f of 2.14 and 1.93, respectively, were extracted with methanol. Alternatively, the products were separated by HPLC on a Tessek Separon Hema S 1000 (8.0 × 250 mm) C-18 column. Gradient elution (75–100 % methanol) with a flow rate of 0.3–1.5 ml min^{-1} was used. Sudan I metabolites were detected at 215, 254, 333 and 480 nm. Two product peaks with r.t. of 32 and 46 min (peaks 1 and 2 in fig. 1) were collected. Sudan I metabolites were analyzed by mass spectroscopy. Spectra were measured using Esquire 3000 Bruker Daltonics (APCI, ESI – positive ionization).

Results and discussion

The absorption spectrum of the reaction mixture containing Sudan I and peroxidase significantly changes during the Sudan I oxidation (fig. 2). During the reaction, the absorption maximum at 480 nm (due to Sudan I) decreases

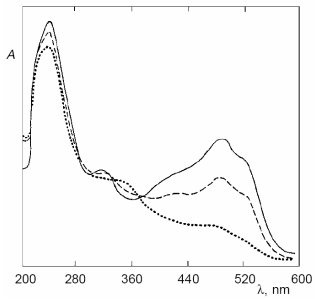

Fig. 2. **Oxidation of Sudan I by the peroxidase/H_2O_2 system**. The samples (1 ml) contained 50 mM Na phospahte buffer pH 8.4, 0.2 mg horseradish peroxidase, 0.15 mM Sudan I, and 0.5 mM H_2O_2. The spectra were recorded sequentially at 0 min (——), 2.5 min (– – –) and 10 min (. . . .)

whiles the absorbance at about 340 nm increases slightly (fig. 2). Sudan I contains a free hydroxyl group in its molecule. Many phenolic compounds can serve as substrates for peroxidases, being oxidized to phenoxyl radicals which will undergo secondary reactions in dependence on their individual free radicals chemistries. This mechanism was also found by us previously for Sudan I as a substrate; peroxidase oxidizes this carcinogen, giving rise to an oxygen-centered radical (naphthoxyl radical)[11,12]. The products formed during peroxidase-mediated Sudan I oxidation includes BDI and C-hydroxy derivatives [6-OH-Sudan I and 4',6-di(OH)-Sudan I][5]. But major metabolites are unstable (sensitive to light and elevated temperature)[5] and their structures have not been elucidated yet. In the present paper we used two separation procedures (TLC and HPLC) to obtain individual Sudan I metabolites in purity sufficient for their partial characterization. HPLC utilizing a Tessek Separon Hema S 1000 C-18 column was originally developed here and used for purification of the two major Sudan I oxidation products (M_1 and M_2, fig. 3). These products were characterized by their mass spectra (fig. 4).

In all cases the products decomposed during mass spectroscopy. The fragmentation peak at *m/z* 232 in the metabolite M_1 indicate the presence of the Sudan I molecule without a hydroxyl group. The peak at *m/z* 405 suggests the presence of the ion composed from hydroxylated Sudan I and another molecule of Sudan I, but without the benzene ring and nitrogen atoms (fig. 4). The fragment peak at *m/z* 495 in the metabolite M_2 indicates the ion composed of two Sudan I molecules (fig. 4). Moreover, fragmentation peaks at *m/z* 159, 172, 247, 389 (391) and 417 (fig. 4) seem to correspond to decomposition of the Sudan I dimer molecule. Nevertheless, the real structure of the both metabolites will be evaluated in detail using NMR spectroscopy. This work is under way in our laboratory.

Conclusion

The results of the present and previous studies[9,11,12] suggest that one-electron oxidation products (radicals) are the primary intermediates in the peroxidase-mediated oxidation of Sudan I. The fate of the primary free radical depends on the environment in which it exists. We found that

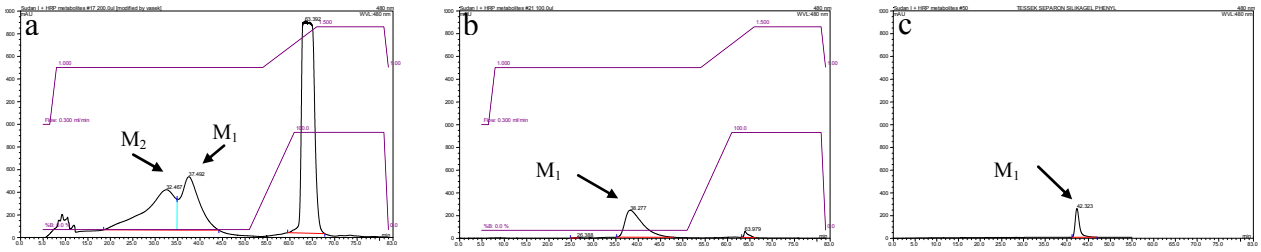

Fig. 3. **HPLC of Sudan I metabolites formed by peroxidase (a) and their HPLC re-chromatography (b,c)**

Fig. 4. **Mass spectra (APCI) of Sudan I metabolite M_1 (a) and M_2 (b)**

the Sudan I reactive free radicals: (*i*) form additional products; (*ii*) react with other compounds of potential physiological interest (reaction with NADH, ascorbate)[8,9,11,12], (*iii*) react with SH groups of glutathione (reducing Sudan I radicals with the formation of a thiyl radical[12] and (*iv*) react with macromolecules (DNA, RNA proteins) to form potentially toxic adducts *in vitro* and *in vivo*[5,8–12]. Structure elucidation of the Sudan I-(deoxy)guanosine-adducts[8,11] will be the objective of a future study.

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Abbreviations

APCI	atmospheric pressure chemical ionization
BDI	benzendiazonium ion
CYP	cytochrome P450
ESI	electrospray-ionization
HRP	horseradish peroxidase
TLC	thin layer chromatography

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P51
CANCER MORBIDITY AND MORTALITY IN WORKERS EXPOSED TO VINYL CHLORIDE MONOMER

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Key words: vinyl chloride-monomer (VCM), demonstrated chemical carcinogen, standardised mortality ratio

Introduction

The production of the most popular plastic material – polyvinyl chloride (PVC) was started in 1933 and during the past 60 years has been applied in almost every industrial activity.

According to IARC Lyon Agency monograph of 1987 VCM is classified I. group as a demonstrated carcinogen[3] with target effects on the liver[2]. Exposed workers showed significantly increased incidence of liver tumours, mostly angiosarcoma (ASL), insignificant mortality rise for tumours in general, in particular higher incidence lung[1], brain cancers, and lymphosarcoma[4].

Materials and methods

Data about workers employed in the vinylchloride manufacturing plant from 1974 until the end of 2003 were obtained from the factory card file. The health status study of this manufacturing plant was designed as a retrospective cohort study exploiting data on the Czech population health status provided by Institute of Health Information and Statistics of the Czech Republic (IHIS) and National Cancer Register (NCR) also administered by IHIS. Mortality and morbidity of exposed workers was compared with an external control group, which constituted total Czech population. Data on the incidence of monitored phenomena specific for sex, five-year age groups, and calendar years were used in calculations. Indirect standardization was used to control potentially confounding effects of sex and age; changes in the morbidity a mortality trends in particular calendar periods were also taken into consideration.

Results

VCM production in Czech Republic

VCM, the polymerization of which produces polyvinylchloride is in the Czech Republic produced by a single plant. At the time when this production was launched, the effects of VCM on human organism have already been known, including the chronic and acute effects and carcinogenic hazards. All available information and hygienic instructions for the staff of the district hygienic station of the Central Bohemian locality that were in charge of the plant's hygienic supervision ensure efficient environmental health protection of the workers.

Characteristics of the production and the workplace conditions

VCM is produced in hermetically sealed columns in the open. The initial substance is ethylene and chlorine. The maximum acceptable concentration (MAC) mean is 10 mg m^{-3}, MAC limit is 30 mg m^{-3} – until June 2001 and 20 mg m^{-3} afterwards. Values measured at workplaces were usually under the MAC value limits; only exceptionally they were higher but never exceeded 100 mg m^{-3}.

Malignant tumours

Vinyl chloride pilot plant production was started in 1974 full run production in 1977. Since 1974 until the end of 2003, a total of 908 employees worked in the PVC production plant with an average exposure of 9.4 years (SD 9.09). There were a total of 208 women with an average exposure of 6.7 (0.1–28.8) years and 700 men with an average exposure of 9.9 (0.1–29) years. Comparison of the cohort data with the National Cancer Register revealed 44 workers (15 women and 29 men) who were diagnosed with tumour disease by the end of 2003. Twenty seven of them died (6 women and 21 men). Angiosarcoma was not diagnosed, hepatoma was diagnosed twice, carcinoma of pancreas three times, gall bladder twice, and lung and bronchi nine times (data about smoking were not available). Analysis of malignant tumours morbidity and mortality (MT) has shown that the observed MT mortality (27) is statistically significantly different from the expected one (10.7) in men – 21 observed deaths vs. 9.37 expected (SMR 1.43; 95 % CI 1.43–3.36) as well as in women – 6 observed deaths vs. 1.35 expected (SMR 4.44; 95 % CI 2.18–8.26). Similar results were found also for total mortality and MT incidence (Table I).

Discussion

Analysis results indicate statistically significantly increased deviation of the health status of observed/ followed up cohort of workers exposed to vinylchloride in comparison with the Czech population health status regarding total mortality as well as tumour mortality and morbidity. We are very careful in their interpretation as

Table I
Standardized cancer mortality ratios according to calendar years

	CANCER OCCURENCE												
	EXPECTED			OBSERVED			FEMALE				MALE		
Year	Female	Male	F+M	Female	Male	SMR	95% Cl		Sig.	SMR	95% Cl		
1980	0.022	0.147	0.169	1	0	45.26	4.10	211.02	0.012	0.00			0.809
1981	0.020	0.152	0.172	0	0	0.00			0.254	0.00			0.823
1982	0.028	0.166	0.194	0	0	0.00			0.399	0.00			0.857
1983	0.030	0.181	0.211	0	0	0.00			0.411	0.00			0.890
1984	0.026	0.223	0.249	0	0	0.00			0.391	0.00			0.977
1985	0.031	0.243	0.274	1	2	32.33	2.93	150.73	0.017	8.23	1.64	26.37	0.018
1986	0.039	0.276	0.316	0	0	0.00			0.456	0.00			0.927
1987	0.048	0.320	0.367	0	1	0.00			0.496	3.13	0.28	12.17	0.227
1988	0.054	0.331	0.385	0	3	0.00			0.523	9.07	2.51	24.19	0.004
1989	0.059	0.366	0.425	0	2	0.00			0.543	5.47	1.09	17.53	0.042
1990	0.069	0.415	0.484	0	2	0.00			0.579	4.82	0.96	13.31	0.054
1991	0.078	0.441	0.519	1	1	12.90	1.17	60.15	0.042	2.27	0.21	10.57	0.377
1992	0.074	0.442	0.516	0	1	0.00			0.597	2.26	0.21	10.54	0.338
1993	0.087	0.442	0.529	0	1	0.00			0.642	2.26	0.21	10.55	0.338
1994	0.073	0.481	0.554	1	0	13.74	1.24	64.04	0.039	0.00			0.656
1995	0.079	0.474	0.553	0	0	0.00			0.615	0.00			0.663
1996	0.064	0.495	0.559	0	0	0.00			0.562	0.00			0.672
1997	0.069	0.528	0.597	0	1	0.00			0.579	1.90	0.17	8.84	0.418
1998	0.063	0.530	0.593	0	1	0.00			0.558	1.89	0.17	8.80	0.420
1999	0.061	0.427	0.487	1	0	16.50	1.50	76.94	0.032	0.00			0.716
2000	0.060	0.431	0.491	0	1	0.00			0.547	2.32	0.21	10.81	0.328
2001	0.058	0.441	0.499	0	3	0.00			0.537	6.80	1.88	18.13	0.009
2002	0.071	0.482	0.553	0	2	0.00			0.585	4.15	0.83	13.30	0.073
2003	0.029	0.426	0.455	1	0	35.04			0.015	0.00			0.717
Total	1.353	9.372	10.725	6	21	4.44	2.18	8.26	0.003	2.24	1.43	3.36	0.001

evaluating the exposure of workers is very complicated because the individual length and intensity of exposures are very different.

All employees were included into the study even those not expected to have been exposed to carcinogens or those who worked at the worksite for one month only. We did not exclude these employees from the sample due to the total sample size. We have not calculated standardized indexes for particular types of tumour diseases due to their small numbers.

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P52
FREQUENCY OF TPMT FUNCTIONAL POLYMORPHISMS IN THE CZECH POPULATION

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Key words: pharmacogenetics, single nucleotide polymorphism, thiopurine S-methyltransferase

Introduction

Azathioprine and its metabolite 6-mercaptopurine are thiopurines with the latter acting as a purine antimetabolite which can inhibit purine biosynthesis by both de novo and salvage pathways. Metabolically, 6-mercaptopurine is converted by hypoxanthine phosphoribosyltransferase to activated 6-thioguanine nucleosides. These latter intermediates are ultimately incorporated into DNA as false bases. Inactivation of 6-mercaptopurine and 6-mercaptopurine nucleosides is mediated by methylation with thiopurine S-methyltransferase (TPMT) to inactive metabolites methylmercaptopurine or methylmercaptopurine nucleosides, respectively. TPMT is well characterized cytosolic enzyme, with several functionally important genetic polymorphisms leading to decreased enzyme activity.

There is substantial amount of evidence, which shows that individual activity of TPMT is one of the major factors for wide variation in the metabolism, the drug efficacy, and mainly severe toxicity of thiopurine drugs[1–5]. The patients, who have low or no detectable enzyme activity, suffer more frequently from myelotoxicity when treated with standard doses of azathioprine. On the other hand, subjects with high activity of the enzyme could have reduced clinical effect of the treatment. Genetic basis for such a variation in TPMT activity is known only for deficient states while no corresponding factors for ultra-rapid type of TPMT metabolism were found so far[6].

Methods

Two hundred and sixty-seven young healthy volunteers (161 men and 106 women, aged 18–56 years) were included in the study after obtaining their written informed consent. All subjects participating in the study were unrelated subjects of Czech nationality.

Peripheral blood samples were collected from all volunteers in 7 ml collecting tubes with ethylenediaminetetraacetic acid (EDTA). Genomic DNA was subsequently isolated by a standard phenol-chlorophorm method and stored at 4 °C until analysis.

PCR amplification was run in a MyCycler (Bio-Rad, USA) using primers as described previously and specified in Table I. Subsequent RFLP analysis produced fragments specified in Table I, allele specific PCR was run for TPMT*2. The fragments were separated in 3 % agarose gel and visualized by staining with ethidium bromide. The primers were ordered at Sigma-Aldrich (St. Louis, USA), all other components of PCR reaction mix and Top Vision agarose were purchased from Fermentas (Lithuania).

The study was approved by the Ethics Committee of the General Teaching Hospital in Prague.

Expected genotype frequencies were calculated using Hardy–Weinberg equilibrium from the observed allelic frequencies. Prevalence was compared by the chi-square test. Microsoft Excel 8.0 (Microsoft, USA) and Statgraphics Plus 3.1 (StatPoint, Inc., USA) were used for data handling.

Results

Table II shows observed genotypes and respective allelic frequencies in our study. The most frequent allele was TPMT*3A, while no subject carrying TPMT*3B allele was found. Totally 26 (9.7 %) subjects were found to be heterozygous carriers of one of the variant alleles and 1 (0.4 %) volunteer was homozygous poor metabolizer. The distribution of genotypes was similar to the predicted numbers.

Table I
Sequences of the primers used, restriction enzymes and discrimative lenghths of fragments [bp]

Polymorphism	Primer name - sequence	Restriction enzyme	Fragment size
G460A	P460F 5'-ATAACAGAGTGGGGAGGCTGC		
	P460R 5'-CTAGAACCCAGAAAA AGTATAG	Acc I	293/207
A719G	P719R 5'-TGTTGGGATTACAGGTGTGAGCCAC		
	P719F 5'-CAGGCTTTAG CATAATTTTCAATTCCTC	Mwo I	365/267
G238C	P2W 5'-GTATGATTTTAT GCAGGTTTG		
	P2M 5'-GTATGATTTTATGCAGGTTTC		
	P2C 5'-TAAATAGGAACCATCGGACAC	–	254

Table II
Number of subjects with specific TPMT genotypes and respective allelic frequencies

Polymorphism	Observed genotypes			Alelic frequencies [%]	
	w/w	w/v	v/v	w	v
TPMT*2	266	1	0	99.82	0.18
TPMT*3A	244	22	1	95.52	4.48
TPMT*3B	267	0	0	100.00	0.00
TPMT*3C	264	3	0	99.44	0.56

w – wild-type allele, v – variant allele

Discussion

Similarly to our findings, variant allele TPMT*3A was reported as the most prevalent deficient allele in other Caucasian populations, whereas TPMT*3C is the most frequent variant in Asian populations[7,8].

A good correlation between high concentrations of activated 6-thioguanine nucleosides and severe forms of bone marrow suppression in TPMT homozygous poor metabolizers treated with usual doses of azathioprine has been well established. Some studies revealed similar pattern of relationship between high levels of 6-thioguanine nucleosides and clinical effects in heterozygous intermediate metabolizers, but other authors did not report such findings. Although there exists such a discrepancy between the data a screening for TPMT deficient patients prior to beginning of azathioprine therapy seems to be cost effective approach[9,10]. Our data provide necessary basis, for transferring the observed individual, social and economic consequences of TPMT deficiency from other Caucasians into the Czech population.

Conclusions

Our results show similar distribution of the most frequent variant TPMT alleles in comparison to other Caucasian populations. Therefore the consequences of azathioprine therapy arising from TPMT deficiency can be expected similar proportions as described in some other European countries. These results also provide basal information for future pharmacogenetic and pharmacoeconomic studies in the Czech population.

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P53

OXIDATIVE DAMAGE AND ANTIOXIDANT DEFENCE IN THE PLASMA OF PEOPLE EXPOSED TO MINERAL FIBRES

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Keywords: Rockwool, oxidative DNA damage, DNA repair; antioxidative enzymes

Introduction

Oxidative damage of biomolecules (DNA, proteins, lipids) caused by free radicals is involved in the pathogenesis of different diseases such as cancer, atherosclerosis, inflammation, etc.[1] Cells respond to toxic levels of reactive oxygen species (ROS) by activating a diverse arrays of protective responses. This includes a complex range of enzymatic antioxidants such as catalase (CAT), glutathione peroxidase (GPX), superoxide dismutase (SOD), glutathione-S-transferase (GST) and non-enzymatic antioxidants such as glutathione, tocopherols and carotenoids[2]. Oxidative repair mechanisms, includes DNA repair, protein and lipid repair, is degradation pathways[3].

The adverse effect that arises from exposure to asbestos has stimulated an extensive research into the development of substitute materials. One such substitute is rockwool, made from natural basic rock material, which is used mainly as isulation for thermal, acoustic and fire protection of roofs, walls and floors. However, little is known about the health effects of these fibres. The potentially harmful effects of all types of respirable fibres are at present one of the most important fields of interest in industrial hygiene[4].

Subjects and methods

In order to study the effect of rockwool exposure on oxidative DNA damage and lipid peroxidation, an epidemiological study was conducted in a rockwool factory in Slovakia.

To examine the interactions between fibre- and cigarette smoke- induced effects, the cohorts examined included non-smokers as well as individuals smoking different numbers of cigarettes. Altogether 141 subjects were investigated (21 to 58 years old), 43 controls (20 men, 23 women and 27 non-smokers, 16 smokers) and 98 exposed (75 men, 23 women and 61 non-smokers, 37 smokers). The rock wool exposure lasted at least 5 years.

All subjects contributed a single blood donation in autumn. A urine sample was used for measurement of cotinine to determine smoking status. Blood was collected by venipuncture from fasted subjects, using an anticoagulant as EDTA used for isolation of lymphocytes and erythrocytes. The samples of plasma, lymphocytes and lymphocyte extracts were stored on ice until assayed or were frozen at −80 °C for various biochemical measurements.

Measurement of DNA damage and repair

The comet assay (single cell alkaline gel electrophoresis)[5] was applied to freshly isolated lymphocytes (Lymphoprep, Nycomed, Oslo, Norway) for measurement of DNA strand breaks, oxidized bases and DNA alkylation[6,7]. The assay for OGG1 activity[5] was used to measure the ability of a cell-free lymphocyte extract to incise substrate DNA containing 8-oxoGua[6,7].

Measurement of activities of antioxidant enzymes

Erythrocytes were washed three times with isotonic saline. Isolated erythrocytes were used for measurement of activities of antioxidant enzymes. The activity of glutathione peroxidase (GPx) was determined indirectly by oxidation of NADPH to $NADP^+$ measured by the kinetic method according to Paglia and Valentine[8]. Catalase (CAT) was measured spectrophotometrically by a modified method of Cavarocchi et al.[9] and glutathione-S--transferase (GST) by a kinetic method according to Habig et al[10]. The activity of superoxide dismutase (SOD) was estimated by a commercial test (Randox lab, Ltd., U.K.).

The index of the combined non-enzymatic antioxidant capacity of plasma (FRAP) was measured spectrophotometrically according to Benzie and Strain[11] ferric to ferrous ion reduction at low pH causes formation of a coloured ferrous–tripyridyltriazine complex.

Ceruloplasmin oxidase activity in plasma was assayed with the use of *o*-dianisidine dihydrochloride according to the method of Schosinsky et al.[12]

Plasma vitamin C was detected by HPLC[13]. Lipid peroxidation was determined by the levels of malondialdehyde (MDA) using a modified HPLC method in plasma[14].

Results and discussion

Rockwool exposure induced elevation of DNA strand breaks in the lymphocytes of investigated subjects (*P*=

0.05). When analysed according to sex and smoking habit, this effect was apparent only in the group of non smokers. DNA strand breaks were higher in exposed subjects compared to controls (P=0.004). The effect of occupational exposure to rockwool on oxidative DNA damage and repair was already published by Dusinska et. al.[15]. DNA repair of oxidative damage in lymphocytes of exposed subjects was higher in group of men (P=0.02) compared to women, and controls (P=0.07).

We found higher MDA levels in the group of all exposed workers (P=0.025) and in exposed non-smokers (p=0.003) possibly as the consequence of significantly suppressed activity of CPL-oxidase (P=0.02, 0.016 respectively) and CAT in these groups (P=0.04, 0.012 respectively). The activity of GST was affected by exposure to rockwool; the GST levels were significantly lower in the all exposed subjects (P=0.04), in the exposed non-smokers (P=0.03), and exposed men (P=0.007). Concentration of vitamin C in plasma and the FRAP were not affected by the rockwool exposure.

There was a significant negative correlation between the activity of GPX and MDA in the whole group (P=0.007) and in the exposed group, and between CAT activity and MDA in the all subjects (P=0.009). We found inverse correlations between activities of several antioxidant enzymes (GPX, GST, and CAT) and DNA damage. GST activity correlated inversely with oxidised purines measured as Endonuclease sensitive sites almost in all subgroups: in all subjects (P=0.002), in exposed (0.014), men (0.038), women (0.004), and exposed men (0.041). We found a significant negative correlations between DNA repair and GPX in all subjects as well as in control men (P=0.03, 0.028 respectively) and CAT in all control subjects and control men (P=0.019, 0.009 respectively). Interestingly we found the positive correlations between DNA repair and MDA in all subjects and in all exposed (P=0.008, 0.026 respectively).

Conclusion

The presented results indicate that rockwool exposure induces an increase in the oxidative damage of biomolecules especially in the group of male non-smokers; however, the optimal levels of antioxidants could have a protective effect.

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P54
TLC PROOF OF INTOXICATION BY TAXUS BACCATA

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Key words: Taxus, Taxine, TLC, intoxication

Introduction

The toxicity of yew (Taxus baccata) has been known since antiquity. It was believed that as "a tree of death" it endangered lives of those who stayed in its shade for a longer time. Yew was used in homicide, suicide, and as an abortive. All parts of yew, except for the red epicarp of the berries, are toxic. The toxicity of yew is attributed to taxin, a mixture of several pseudoalkaloids, in which the evidently dominant components are taxin B and isotaxin B. These taxin alkaloids are cardiotoxic. Their effect can be determined as soon as 1/2 hour after ingestion. Death after 2 and 1/2 hours after ingestion of two handfuls of yew leaves was reported[1].

Description of toxicological proofs of yew poisoning is not very frequent in literature. There is a description of intoxication evidence by thin layer chromatography[2,3], of the evidence achieved by chromatogram similarities between yew extract and extract of stomach content[4] (GC/FID), of the finding of 3,5-dimethoxyphenol by gas chromatography[5] (3,5-dimethoxyphenol is considered as a marker of yew ingestion) and the evidence of taxin B and isotaxin B determined in biological material by liquid chromatography with mass detector[6,7].

In the following paragraphs the authors describe a case of lethal intoxication caused by the decoction of yew leaves, and the proof of it by thin layer chromatography (TLC).

Case report

A 28-year-old man was found dead in the family house. For toxicological analysis the police supplied a brown liquid (pH 9) found in a bottle and a glass in the living room. Forensic autopsy stated the following: acute catarrhal esophagitis, acute gastritis, a mash of plant material in the stomach and duodenum (in dry form 3.6 g), epicranial, pleural and epicardial pete-chiae, brain swelling, haemorrhagical swelling of the lungs, acute venostasis of the parenchymatous organs, and liquid status of the blood. The following was supplied for toxicological analysis: blood, urine, samples of the brain and lung tissue, samples of the liver and kidney tissue and stomach and duodenum content (pH 8). No blood alcohol was detected. Nor any volatile toxic compounds in the brain and lung tissue were found. Toxicological analysis using GC/MS detected carboxy-tetrahydrocannabinol, caffeine and nicotine in the blood. Morphological and anatomical evaluation of the mash plant material found in the stomach content detected the presence of Taxus baccata leaves (evaluation performed by RNDr. Dagmar Nová).

Materials and methods

Material used

Mixture of taxin B and isotaxin B prepared in the Institute of Legal Medicine, University Hospital Münster. Chromatographic plates Kieselgel 60 F254 Merck, Fast Blue B reagent (FBB), Ehrlich reagent, Drägendorff reagent, ethanol 96 %, organic solvents used in extraction and preparation of mobile phases were p.a. purity, ammonium hydroxide solution 26 % p.a., hydrochloric acid conc. p.a.

Extraction of comparative plant material

100 ml of 3.7 % hydrochloric acid was added to 50 g of Taxus baccata leaves, the mixture was homogenized and macerated for 1 hour, then filtered. The filtrate was extracted twice with 250 ml diethyl ether and then aIkalized to pH 10 with sodium hydroxide and extracted twice with 250 ml diethyl ether. The acidic and basic extract was evaporated and the evaporates dissolved in 1.5 ml ethanol.

Extraction of section material

Samples of liver and kidney tissue (50 g) were homogenized, 24 hours soaked in acetone and ethanol mixture (450 ml, 7:3) and then the macerate was filtered and evaporated to 50 ml. The stomach content was diluted with distilled water and filtered. Urine, the macerate of the liver and kidney tissue, the filtrate of the stomach content and the found brown liquid were acidified with hydrochloric acid to pH 3 and extracted 2 times with 250 ml diethyl ether. The water fractions were subsequently alkalized to pH 10 with the solution of sodium hydroxid and extracted with 250 ml diethyl ether and 250 ml chloroform. Acidic and basic extracts were vaporized and consequently dissolved in 1.5 ml ethanol. Further, urine was hydrolyzed and after neutralization extracted with 250 ml diethyl ether.

Mobile phase

A ethyl acetate : ethanol : ammonium hydroxide solution 26 % = -36:2:2
B chloroform : ethanol : ammonium hydroxide solution 26 % = 38:2:1 / paper

Abbreviations

RfA retention factor in mobile phase A
RfB retention factor in mobile phase B

Results

Fast BlueB detection

Some substances from acidic extract of yew show a very prominent colour reaction with the FBB reagent. In acidic extracts of stomach content, urine and the found brown liquid FBB reagent proved a presence of substances of corresponding character with substances contained in the extract of Taxus baccata. A bright orange-red spot in the position RfA=0.8; RfB=0.4 was particularly persuasive. A presence of substances contained in Taxus baccata extract was not determined in the liver and kidney tissue nor in the urine hydrolysate. FBB reagent showed no colour reaction with taxin B / isotaxin B standard.

Ehrlich reagent detection

With the Ehrlich reagent some substances from acidic and basic extracts of yew show a prominent colour reaction after heating the chromatographic plate. In acidic and basic extracts of the stomach content and urine Ehrlich reagent proved a presence of substances of corresponding character with substances contained in the extract of Taxus baccata. Particularly persuasive was a pink spot on the chromatogram of the acidic extract in position RfA=0.8; RfB=0.4. After heating the plate, intensified spots of blue-violet colour showed on the chromatogram of basic extract of yew, and two of them also appeared in the basic extract of stomach content (RfAl/A2=0.7/0.6; RfBl/B2=0.5/0.4). A pre-sence of substances contained in Taxis baccata extract was not determined in the liver and kidney tissue nor in the urine hydrolysate. Ehrlich reagent showed no colour reaction with taxin B / isotaxin B standard.

Drägendorff reagent detection

Substances contained in basic and acidic extracts of yew react with the Drägendorff reagent. Some spots on the chromatogram of basic extract of yew correspond with spots on the chromatogram of basic extract of stomach content.. Because of multiple spots the chromatogram is less clear. A presence of substances contained in Taxus baccata extract was not determined in the liver and kidney tissue nor in the urine.

The Drägendorff reagent reacts in colour with the taxin B / isotaxin B standard. In chromatographic conditions mentioned above both the compounds do not separate and only one spot on the chromatogram appears (RfA=0.6; RfB=0.4). A presence of taxin B / isotaxin B was proved in the basic extract of stomach content. In the liver and kidney tissue extracts, as well as in the urine and urine hydrolysate a presence of taxin B / isotaxin B was not detected.

Conclusion

On the basis of the described results, the authors assume that the intoxication with Taxus baccata can be proved analytically by the TLC method. With detection of Fast Blue B and Ehrlich reagent, compounds of corresponding character with substances contained in Taxus baccata were identified. The presence of these substances was proved in the stomach and duodenum content and urine, whereas it was not detected in the liver and kidney tissue and urine hydrolysate. The Drägendorff reagent reacts with a number of substances present in the yew leaves extract, particularly with substances contained in the extract from basic water environment. Some spots on the chromatogram of the yew basic extract correspond with the spots on the chromatogram of the stomach and duodenum content. The chromatogram is not clear enough because of multiple spots. The comparision of biological material extracts with the taxin B / isotaxin B standard proved that one of the spots reacting positively with the Drägendorff reagent on the chromatogram of the basic extract of stomach and duodenum content is taxin B / isotaxin B. Under the stated chromatographic conditions there was no separation of taxin B from isotaxin B.

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P55

REDUCTIVE ACTIVATION OF ENVIRONMENTAL POLLUTANTS 3-NITROBENZANTHRONE AND 2-NITROBENZANTHRONE

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Key words: 3-nitrobenzanthrone, 2-nitrobenzanthrone, reduction, NAD(P)H:quinone oxidoreductase, HPLC

Introduction

3-Nitrobenzanthrone (3-nitro-7*H*-benz[*de*]anthracen--7-one, 3-NBA, fig. 1), occurs in diesel exhaust and in airborne particulate matter[1]. 3-NBA might originate both from incomplete combustion of fossil fuels and from reaction of the parent aromatic hydrocarbon with nitrogen oxides in the atmosphere. 3-NBA can spontaneously isomerize to 2-nitrobenzanthrone (2-nitro-7*H*-benz[*de*]anthracen--7-one, 2-NBA), which can become more than 70-fold higher in concentration in ambient air[1,2]. 3-Aminobenzanthrone (3-ABA, fig. 1), suggested to be the main reductive metabolite of 3-NBA, has been found in urine samples of salt mine workers occupationally exposed to diesel emissions[3], demonstrating that human exposure to 3-NBA in diesel emissions can be significant and is detectable. 3-NBA is carcinogenic in rats, causing lung tumours after intratracheal instillation[4]. It is also an exceptionally potent mutagen[1]. Its genotoxicity has been further documented by the detection of specific DNA adducts formed *in vitro* as well as *in vivo* in rodents[1,4–7]. Most of the metabolic activation of 3-NBA *in vitro* is attributable to human and rat cytosolic NAD(P)H:quinone oxidoreductase (NQO1), while human *N*,*O*-acetyltransferase (NAT), NAT2, followed by NAT1, sulfotransferase (SULT), SULT1A1 and, to a lesser extent, SULT1A2 are the major phase II enzymes activating 3-NBA (ref.[7]). Microsomal NADPH:cytochrome P450 (CYP) reductase is also effective in the activation of 3-NBA (ref.[6]), but in a model organism, mice, 3-NBA is predominately activated by cytosolic nitroreductases such as NQO1 rather than microsomal NADPH:CYP reductase[7] (fig. 1).

While 3-ABA was suggested to be the main reductive metabolite of 3-NBA (ref.[7]), the reactions of 3-NBA to 3-ABA by enzymatic systems *in vitro* and *in vivo* has not been investigated as yet. Reductive metabolism of 3-NBA is, therefore, investigated in this work. In addition, reduction of 3-NBA is compared with that of its isomer 2-NBA.

Materials and methods

3-NBA, 2-NBA, 3-ABA and *N*-OH-ABA were synthesized as described[1,2,8]. Incubations with human recombinant NQO1 or rabbit NADPH:CYP reductase, in a final volume of 500 μl, consisted of 50 mM Tris-HCl buffer (pH 7.4), containing 0.4 % Tween 20, 1 mM NADPH, from 5 to 50 μM 3-NBA or 2-NBA (in DMSO) and from 10 to 100 μg ml^{-1} of NQO1 or NADPH:CYP reductase. In incubations testing the time-dependent formation of 3-NBA-DNA adducts mediated by human recombinant NQO1 (20 μg ml^{-1}), incubation times varied between 15 and 60 minutes and 20 mM 3-NBA were used. The incubation mixtures were extracted with ethyl acetate (2 × 1 ml) and 5 μl of 1 mM phenacetine was added as an internal standard. The extracts were evaporated to dryness;

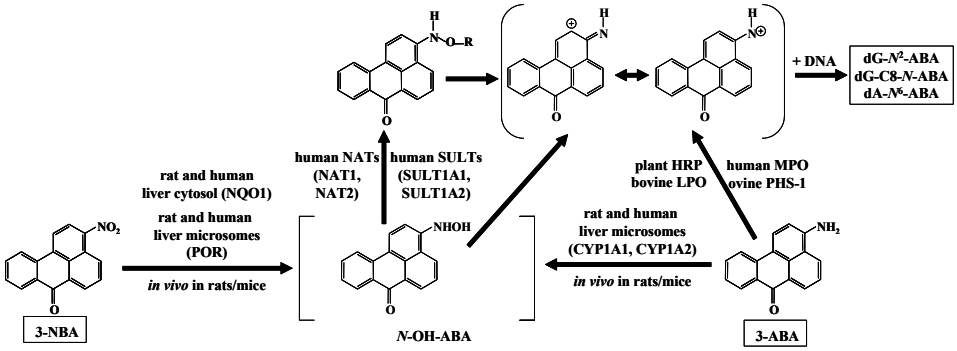

Fig. 1. **Pathways of metabolic activation and DNA adduct formation of 3-nitrobenzanthrone and 3-aminobenzanthrone;** see text for details. R = -$COCH_3$ or -SO_3H; dA-N^6-ABA, 2-(2'-deoxyadenosin-N^6-yl)-3-aminobenzanthrone; dG-N^2-ABA, *N*-(2'-deoxyguanosin-N^2-yl)-3-aminobenzanthrone; dG-C8-*N*-ABA, *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone

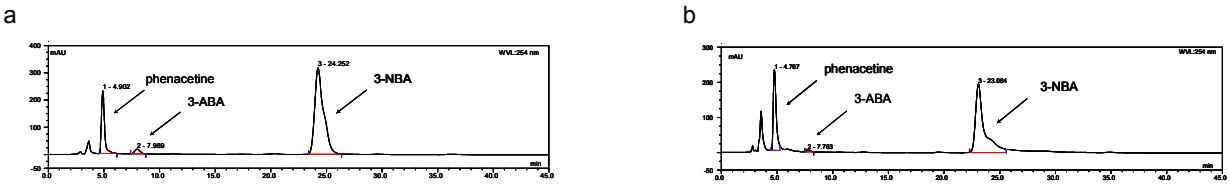

Fig. 2. **HPLC of incubations of 3-NBA with NQO1 (a) and NADPH:cytochrome P450 reductase (b)**

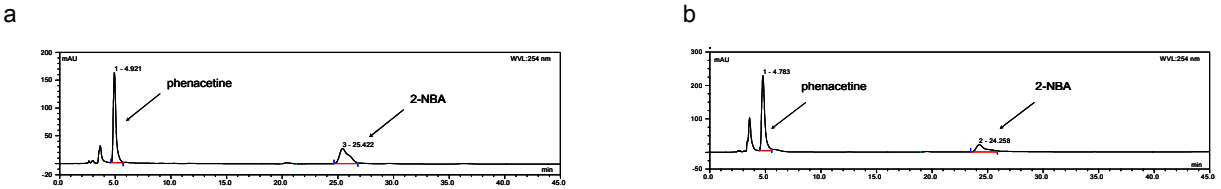

Fig. 3. **HPLC of incubations of 2-NBA with NQO1 (a) and NADPH:cytochrome P450 reductase (b)**

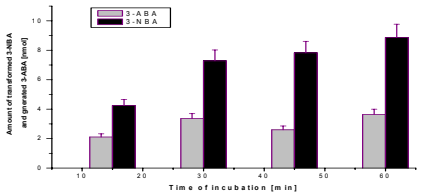

Fig. 4. **Time-dependence of 3-NBA reduction by NQO1**

residues dissolved in 30 µl of methanol, and subjected to RP-HPLC to evaluate the amounts of products of 3-NBA and 2-NBA reduction. HPLC was under isocratic conditions of 70 % methanol, with a flow rate of 0.6 ml min^{-1}. The modeling of the binding of 3-NBA to the active site of NQO1 was performed with the program Autodock 3.0.3. and Sybyl 6.6.5 (Tripos GmbH, Germany) by the procedure described[7].

Results and discussion

Human NQO1 metabolizes 3-NBA to one major product identified as its reductive metabolite found in urine samples of humans exposed to diesel emissions[3], 3-ABA. Both compounds were separated by HPLC as two distinguish peaks (fig. 2). NQO1 is the more effective enzyme to form this product than NADPH:CYP reductase (fig. 2a,b). In contrast to 3-NBA, no 2-NBA metabolites were generated by both enzymatic systems, which indicate that this compound is a much worse substrate (if any) for these enzymes than 3-NBA (fig. 3a,b). The metabolism of 3-NBA by human recombinant NQO1 was studied in detail. We investigated the time-dependence of 3-NBA reduction (fig. 4), dependence of converted 3-NBA and produced 3-ABA on NQO1 (fig. 5) and 3-NBA concentrations (fig. 6). A time-dependent decrease in 3-NBA concentrations in incubations correlated with an increase in 3-ABA formation (fig. 4). Reduction of 3-NBA to 3-ABA by human NQO1 in the presence of NADPH exhibits the Michaelis-Menten kinetics (data not shown). The values of Michaelis constant K_m of human NQO1, measured for 3-NBA reduction and 3-ABA production, were 10.3 and 7.6 µM, respectively.

In order to examine the molecular basis of the potent reduction of 3-NBA by human NQO1, the binding of 3-NBA to the active centre of NQO1 was modeled (fig. 7). The model structures for the human NQO1-3-NBA-complex were calculated. It is evident that 3-NBA fits well into the active site of human and rat NQO1, being bound near the isoalloxazine ring of the flavin prosthetic group of the enzyme. This allows an electron transfer during the reduction of 3-NBA. The value of the apparent dissociation constant for the human NQO1-3-NBA-complex was calculated to be 0.26 µM, respectively.

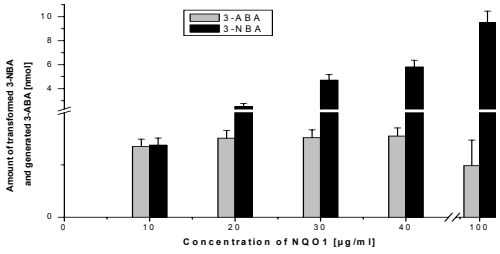

Fig. 5. **Dependence of 3-NBA reduction by NQO1 on the enzyme concentration**

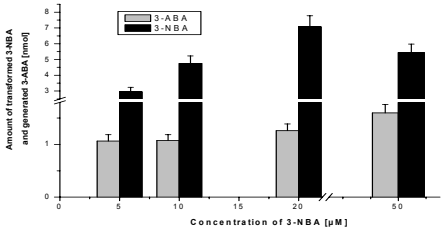

Fig. 6. **Dependence of 3-NBA reduction by NQO1 on 3-NBA concentration**

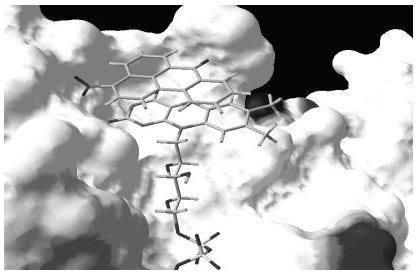

Fig. 7. **3-NBA docked to the active site of human NQO1**

Conclusion

The results demonstrate for the first time that 3-NBA is reduced by NQO1 and NADPH:CYP reductase to 3-ABA. The results also explain a strong mutagenicity and carcinogenicity of 3-NBA in contrast to 2-NBA. Much higher mutagenicity and carcinogenicity of 3-NBA evolve from its potential to be easily reduced to reactive species binding to DNA.

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Abbreviations

CYP	cytochrome P450
NQO1	NAD(P)H:quinone oxidoreductase
NATs	*N*,*O*-acetyltransferases
SULTs	sulfotransferases
N-OH-ABA	*N*-hydroxy-3-aminobenzanthrone

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P56

THE USE OF BIOLUMINESCENT BIOREPORTER *Pseudomonas fluorescens* HK44 FOR MEASURMENT OF TOXICITY OF ENVIRONMENTAL POLLUTION

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Key words: bioluminescence, biosensor, toxicity, *Pseudomonas fluorescens* HK44

Introduction

Pseudomonas fluorescens HK44 is a genetically modified bioluminescent bacterium, whose light intensity is increased by numerous chemical compounds (polyaromatic hydrocarbons, naphthalenes, salicylic acids etc.). This predetermines the bacterium for construction of luminescent biosensor for detection of environmental pollution with PAHs. On the other hand toxic compounds cause decrease of the light intensity, which can be used for toxicity assessment. This review summarizes results of measurements and prediction of toxicity to strain HK44.

Pseudomonas fluorescens HK44 is a *lux*-based bioluminescent bioreporter that harbors the pUTK21 plasmid which carries the *nah* genes coding for naphthalene metabolism linked to *luxCDABE* gene cassette coding for bioluminescence (λ_{max}=490 nm). Induction of the *nah* genes by intermediate of naphthalene metabolism salicylic acid results in associated induction of the *luxCDABE* genes and increase in light intensity. Bioluminescence can be induced by any compound that is either structurally similar to salicylic acid or which is metabolized to such compound (such as naphthalene is metabolized to salicylic acid)[2,3]. So far several tens of inducing compounds are known including substituted salicylic acids, salicylic acid-like compounds (e.g. 2-aminobenzoic acid), substituted naphthalenes, naphthalene-like compounds (e.g. quinoline), and polyaromatic hydrocarbons (PAHs)[4–7].

Strain HK44 has been originally designed for simple luminescent monitoring of the polycyclic aromatic hydrocarbons degradation in soils[1]. However inductive bioluminescence enabled other application as a monitoring of cell viability during immobilization process[8]. Major application is seen in biosensing of environmental pollution. Use of free and immobilized cells showed that the bioluminescent response is rapid and it follows the saturation type dependence on inducer concentration[7–9]. The cells responded significantly to naphthalene concentration as low as 0.02 mg l^{-1} (ref. [7,9]). Cells immobilized in silica matrix were reusable for several months and tens of induction cycles[8].

Use of *P. fluorescens* HK44 for toxicity measurement

Use of reusable biosensor for detection of environmental pollution however is not without difficulties. In order to give repeatable data, the cells must be kept under physical and chemical conditions that avoid stresses. However environmental pollution is usually toxic in some way. Although strain HK44 is well adapted for PAH based environmental pollution, a risk of cell damage by toxic compounds must always be taken into account. If possible, toxicity of the sample should be determined before biosensor is applied. As strain HK44 emits significant luminescence even if the inducer is absent measurement of bioluminescence decrease seems to be the right toxicity assay for this purpose[4]. Application of the same bacterial strain for both purposes would ensure no harm is made to the cells integrated in the biosensor.

In our previous study bioluminescence response to 72 compounds was measured in wide concentration range (from $3.2 \cdot 10^{-8}$ mol l^{-1} to saturation); out of them 41 had inhibitory effect on HK44 bioluminescence at elevated concentrations. A five-parameter-equation was proposed for overall description of concentration-bioluminescence dependence, based on the presumption of two independent effects (induction and inhibition) of compound on bioluminescence. Values analogical to EC50 (concentration which causes effect on 50 % of test population) can be calculated in inflection points using non-linear regression[4]:

$$RBL = \{1 + [(L_{max}-1)\, c^n / (c_n + K_L^n)]\}\{1 - [c^m / (c^m + K_I^m)]\} \quad (1)$$

where L_{max} stands for maximum possible bioluminescence, c for concentration of the inducer, n for coefficient of sigmoidity for induction (higher n gives steeper curve), m for coefficient of sigmoidity for inhibition, K_L for concentra-

tion at which the curve reveals the first inflection, K_I for concentration at which the curve reveals the second inflection (corresponding to desired EC50 toxicity value). For compounds that do not induce bioluminescence of the HK44 cells, the K_L value approximates to infinity and equation (*1*) is reduced to:

$$RBL = 1 - [c^m / (c^m + K_I^m)] \quad (2)$$

These results suggest that measurement of inhibition of bioluminescence of HK44 could be also used as a general toxicity assay comparable to standard Microtox® test, which uses luminescent marine bacterium *Vibrio fischeri*[10]. Both strains possess the same lux system thus the same instrumentation is needed. Strain HK44 is a soil bacterium, therefore the test would not require use of saline water as Microtox® does. Also the results for measurement of soil pollution would be more realistic. The main disadvantage of application of strain HK44 is its genetic modification. Although this strain is approved for field application in the USA[11] in Europe manipulation with GMO is allowed only in authorized laboratories.

Prediction of toxicity

So far no quantitative structure-toxicity relationship for strain HK44 was published. However Bundy *et al.* (2001)[5] measured toxicity using related *Pseudomonas fluorescens* strain expressing *lux* genes constitutively. A good correlation of EC80 values to octanol-water partition coefficient (log $P_{O/W}$) was found, however for better correlation the compounds were split into two independent sets. We have used GUHA (General Unary Hypotheses Automaton) data-mining method for automatic formation of structure-toxicity hypotheses[12]. Although the results were only binary (toxic/nontoxic), similar relationship of log $P_{O/W}$ to toxicity was found. Naphthalenes substituted by aliphatic groups (higher log $P_{O/W}$) were usually non toxic as compared to naphthalenes substituted by polar group. Relation of log $P_{O/W}$ to toxicity is a well known fact[13], however for salicylic acids not the only one. Salicylic acids substituted at position 4 induced bioluminescence and were not toxic compared to salicylic acids substituted in positions 3, 5 and 6. This relation among chemical structure, toxicity and bioluminescence corresponds to more common observation of lower the toxicities of compounds which are metabolized by strain HK44.

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P57
SKIN SENSITIZATION TESTING OF CHEMICALS IN TEXTILE MATERIALS

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Key words: local lymph node assay (LLNA), textile materials, dyes, chemicals, DNCB

Introduction

Chemicals contained in textile materials include dyes, stabilizers, antimicrobial agents and others. They can release from the clothes by various ways (water, sweat, mechanic friction) on the human skin. Skin exposure to some textile materials may lead to allergic dermal reactions or respiratory symptoms in humans[1–4]. Published papers show that possible risk factors of development of allergic dermatitis are textile dyes. Special groups in risk are people with innate predisposition to atopy and small children[1–3].

Allergic reactions on various chemicals in textile materials may represent serious problems to human health and therefore newly developed chemicals need to be adequately tested for sensitizing potential before widespread use. Our study was aimed to examine possible sensitization potential of selected extracts of textile materials with content of dyes, formaldehyde, heavy metals: arsenic, cadmium, lead, chromium (Table I) using local lymph node assay assessing the proliferative activity of lymphocytes[5]. The method is based upon the fact that sensitizing chemicals initiate a primary immunological response in the local lymph nodes, characterized by lymphocyte proliferation.

Material and methods

Animals: Inbred Balb/c strain female mice 7–10 weeks old were obtained from Velaz Praha. The mice were allowed to acclimatize for 10 days prior to first exposure. Animals were maintained at temperature 22 ± 2 °C, relative humidity 40–70 % and with natural light/dark cycle and fed by standard diet and water *ad libitum*.

Positive and negative controls: 0.1 % solution of 1-chloro-2,4-dinitrobenzene (DNCB, Sigma) was used as a positive control and imitation of human sweat as a negative control.

Test extract: Extracts from textile materials were prepared in imitation of human sweat according to STN norm.

In vitro murine auricular lymph node assay: Mice in groups of ten received daily topical application of 25 μl of the test extract on the dorsum of both ears. Control mice were treated with an equal volume of the solution of imitation of sweat alone. The treatment was repeated for twenty-eight consecutive days (excluding weekends). One day following the final exposure, all mice were injected intravenously via the tail vein with 250 μl of PBS containing 20 μCi [^{3}H]-methyl-thymidine (specific activity 2.0 mCi mmol^{-1}, Amersham). Five hours later, mice were sacrificed and draining auricular nodes were excised. A single cell suspension was prepared using the glass homogenizer. Cells were washed once with a PBS and then dissolved in 2.5 ml of Soluene-350 (Packard instruments) for 48 hours. [^{3}H]-TdR incorporation was measured by β-scintillation counting.

The results were expressed as counts per minute (cpm) and index. Index was calculated according to the following formula: proliferative response in exposed auricular lymph node [cpm] / proliferative response in control lymph node [cpm].

Statistical analysis was performed using Student T-test.

Results and discussion

This paper describes testing of skin sensitisation potential of chemicals in textile materials using local lymph node assay (LLNA). In LLNA, mice were epicutaneously administered with extracts of eight textile materials (Table I) in imitation of human sweat. One known allergen – 0.1 % solution of 1-chloro-2,4-dinitrobenzene (DNCB) was used as a positive control and imitation of human sweat as a negative control. Results of the *in vivo* proliferation response of lymphocytes in local auricular lymph nodes of Balb/c mice are summarized in fig. 1. Results are presented as the mean ^{3}H-TdR incorporation for each experimental group in counts per minute and as stimulation indices (SI).

DNCB is known strong allergen[6]. As expected, the maximum proliferation response was observed in lymph nodes of mice exposed to 0.1 % DNCB. Statistical analysis

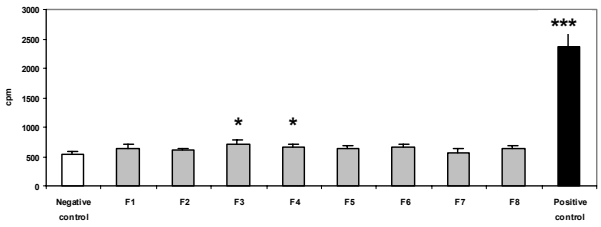

Fig. 1. **Proliferative activity of lymphocytes in the mice lymph node after application of extract from textile materials**

Table I

Identification of the sample	Characterization of textile material / used dyes
F1	Fabric from 100% cotton – shirt – anthracite – melange (content of formaldehyde 101 mg kg^{-1}) direct dyes (azo dyes) and reactive dyes
F2	Knitwear from 100% cotton – T shirt – black (low color stability – water, sweat, saliva) direct dyes (azo dyes) and reactive dyes
F3	Knitwear from 100% cotton – children T shirt – red (low color stability – water, sweat, saliva) direct dyes (azo dyes) and reactive dyes
F4	Knitwear from mixture of cotton/elastan – chidren shorts – red (low color stability – water, sweat, saliva) direct dyes (azo dyes) and reactive dyes
F5	Knitwear from mixture of polyamide/elastan – ladies stocking – black (high content of chromium) acid dyes and metal complex dyes
F6	Knitwear from mixture of polyamide/elastan – ladies underwear claret (low color stability – water, sweat) acid dyes and metal complex dyes
F7	Knitwear from mixture of 44–48% polyester/ 35–49% polyamide/ 3–6% elastan, ladies underwear – black (low color stability – water, sweat) disperse dyes, acid dyes and metal complex dyes
F8	Fabric from mixture of 55% polyester/ 45% polyamide – bed linen blue- violet, (low color stability – water, sweat) disperse dyes, acid dyes and metal complex dyes
Negative Control	Imitation of human sweat
Positive control	DNCB 0.1 % solution of 1-chloro-2,4-dinitrobenzene

revealed that proliferation of lymphocytes was highly significantly increased when compared with proliferation of cells in lymph nodes treated with negative control – imitation of human sweat (P<0.001). The stimulation index (SI) reached 3.41–5.44, mean SI = 4.42. Chemical is regarded as positive – allergen when this ratio is 3 or greater.

Stimulation indices (SI) in test mice administered with extracts of textile materials ranged from 1.06 to 1.34. Proliferation of lymphocytes in local ear lymph node of two groups of mice administered with extracts F3 and F4 of textile materials showed significantly higher values (P<0.05) in comparison with negative control. Results indicate mildly positive allergic potential. Stimulation indices (SI) in those samples were 1.34 and 1.24. Extract F3 was prepared from children red T-shirt made from cotton knitwear. Second extract F4 was done from textile mixture of cotton/elastan colored with red dye. Textile was used for preparation of children shorts. Both materials had poor color stability to water, sweat and saliva. According to the expertise provided by VUTCH-Chemitex, both textile materials were most probably colored with direct dyes (azo-dyes) and reactive dyes.

Treatment of experimental mice with extracts of textile materials resulted in lower proliferation response in local ear lymph nodes when compared to positive control exposed to DNCB. All stimulation indices were below level 3 and samples did not reach criteria of classification to be considered as allergens.

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P58
TOXICOLOGICAL PROFILE OF ALUMINIUM

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Key words: professional exposure, non-occupational exposure, dialysed patients, serum

Introduction

Several deaths have been reported in 1960s after occupational exposure to finely powdered metallic aluminium (Al) used in paints, explosives and fireworks. It should be noted that changes in production technology, the use of breathing masks and controls of the dust levels in factories have resulted in decreased occupational exposures to finely powdered aluminium. Factory workers who breathe large amounts of Al dust can have lung problems, such as coughing. Pulmonary fibrosis is the most frequently reported respiratory effect observed by workers after long-time exposure to fine aluminium dust, aluminium oxide, or bauxite. However, the reports prove the fibrous potential of Al. In some of the cases, the fibrosis was attributed to concomitant exposure to other dusts with content of silicon oxide. For example, pulmonary fibrosis has been observed in a number of bauxite workers. By these workers, it is very likely that there was simultaneous exposure to silica and that this latter was the causative agent rather than the aluminium[1].

In the 1980s dialysed patients (DP) were heavily overloaded with Al. Their daily intake of Al was not only from food and drinking water but also from Al-antacids such as phosphate binders and Al-containing dialysis fluids. At present time the mentioned factors have been eliminated: elimination of Al content in dialysis concentrate by producer, purification of water for preparing dialysis solution, application of antacids on the base of calcium carbonate, not on the base of Al. The removal of Al by dialysis is not easy because almost 85–90 % is protein-bound aluminium, thus only a small amount of Al (10–15 %) is diffusible and ultrafilterable. Elevated aluminium levels have been the cause of various disorders, including dialysis encephalopathia or dementia, Al-induced bone disease and microcytic anaemia[2].

The goal of this study was determination of Al levels in biological material of the aluminium exposed persons (Al industry, patients with chronic renal insufficiency) and in the control groups. With comparison of these values we judged the actual degree of loading from Al.

Materials and methods

The amount of aluminium can be measured in the blood (serum), urine, dialysis fluid or cerebrospinal fluid. These measured values are important for the diagnosis of intoxication, monitoring of Al exposed persons.

We detected the concentration of Al in the blood serum and in the urine of five groups of samples. The detailed characteristics of individual groups are shown in Table I. The data of the biggest group of DP was summarised in 1999–2006. The average concentrations per years indicated the progress of Al levels.

The samples of serum in volume of 1 ml were prepared from 5 ml of native blood. The determination of Al in urine was realised in 20 ml of the morning urine. All samples were stored in refrigerator at 5 °C and analysed within three days. The used analytical method was atomic absorption spectroscopy in graphite furnace (GFAAS) with detection limit of sample 0.1–5 μmol l^{-1} and the atomise temperature 2700 °C. The atomic absorption spectrophotometer AAS Varian SpectrAA 30 P was used with graphite furnace GTA-96. All measurements were performed at the Clinic of Occupational Medicine and Toxicology, Faculty Hospital Martin.

We analysed relationship between age and years of Al exposure to the level of Al in biological material.

Table I
Groups characteristics, the selected biological material, the number of investigated samples of persons with their age and Al exposure in years

	Group 1	Group 2	Group 3	Group 4	Group 5
Characteristic	control children	control adults	smelters	administration	dialysed patients
Biological material	serum	serum, urine	serum, urine	serum, urine	serum
N	24	38	24	15	1110
Age ±S D [years]	5.78±0.57	38.74±10.22	45.96±7.55	44.00±8.73	58.88±14.49
Al exposure ±SD [years]	–	–	16.17±11.06	15.33±7.56	3.64±3.67

Table II
The average group concentrations Al in serum [x ± SD μmol l^{-1}] and in urine [x ± SD μmol l^{-1}; μmol mol cr^{-1}]

Group	Al in serum ± SD [μmol l^{-1}]	Al in urine ± SD [μmol l^{-1}]	Al in urine ± SD [μmol mol cr^{-1}]
G1 – control children	0.320±0.04	–	–
G2 – control adults	0.502±0.18	1.144±0.68	89.75±42.33
G3 – smelters	1.223±0.43	3.268±2.67	341.97±188.81
G4 – administration	1.406±0.58	1.772±1.04	225.60±87.18
G5 – dialysed patients	1.050±1.26	–	–

Results of the examinations were processed by mathematical and statistical methods. Arithmetic mean values (x) and the standard deviations (±SD) were calculated. Groups were compared by using Student´s t-test.

Results

All results of the group average concentrations are illustrated in Table II (cr. = creatinine in urine). We tried to prove that the content of Al in the blood serum increased by age and by Al exposure. Correlation coefficients of all groups were calculated for relation between a.) age and concentration of Al in the serum and b.) exposure and concentration of Al in the serum. These values (from interval <–0.037; 0.421>) do not confirm relation between Al concentration in the serum and the age.

The average concentrations of smelters and administration were increased in comparison to the control group of adults (P<0.001). Therefore, these values do not reached the biological limit 2.224 μmol l^{-1}. These average concentrations of two Al factory groups (smelters and administration) were not significantly different. The highest average concentration of aluminium in urine was measured in group of smelters. But this concentration was smaller than indicative BMH 600 μmol mol cr^{-1}.

In comparison to the control group of adults, dialysed patients did not have increased concentrations of serum aluminium till year 2002. Only 8 samples had higher level than 2.224 μmol l^{-1}, what is EU recommended level for DP. Reason for increased concentrations of serum aluminium in years 2002–2006 was in 20 cases of higher Al level in the same dialysed centre.

Discussion

The recent measurements of aluminium concentrations in the serum of population without occupational exposure revealed the normal background levels 0.320–0.502 μmol l^{-1}. The threshold limit value for aluminium in the blood serum in Slovakia is not defined. The extent of normal values is determined by the concentrations of Al in the serum of the general population and this interval is used as the background. Commission of the European Community (CEC) recommended reference normal value for individuals with normal renal function < 10 μg l^{-1} (= 0.371 μmol l^{-1})[3]. Other authors presented reference Al value in the serum: 0.110 μmol l^{-1}(ref.[4]). Because of the ubiquitous nature of aluminium contamination, we assume that there is a direct relationship between Al in the environment of the Martin Region and the content of Al in the body.

The average Al serum concentration in children was non-significantly lower than in adults. We did not prove that the serum concentration of Al in the population increased with age. Taking into consideration life style, treatments in the past, the eating habits, and some samples of children showed higher concentration of Al in serum than in the adults.

Although aluminium is already not present in the dialysate in the recent ten years we reported increased serum Al concentrations in dialysed patients in 2002–2006 comparing to the control group. But these concentrations were safe. The Spain study analysed the changes in the aluminium content in dialysis fluid and the effect on serum aluminium in 17 dialysis centres in Spain in 8 years. The concentrations of serum were decreasing from year to year onto the value 25.7 μg l^{-1} (= 0.953 μmol l^{-1})[5]. The interval of our average concentrations was from 0.518 μmol l^{-1}in 2002 to 1.309 μmol l^{-1} in 2006. Sulkova described a patient, where aluminium was the cause of various disorders, including dialysis encephalopathy and aluminium-induced bone disease proved by a bone biopsy, and microcytic anaemia. This concentration of Al in the serum before administration of desferrioxamine was 460 μg l^{-1} (= 17.049 μmol l^{-1})[6]. In case of higher Al serum levels we recommended the desferrioxamine test, as well. Commission of the European Community (CEC) recommends reference desirable value by chronic renal failure patients < 60 μg l^{-1} (= 2.224 μmol l^{-1}) and points out the value > 200 μg l^{-1} (= 7.413 μmol l^{-1}) where an urgent action is required, because high risk of toxicity exists in all cases[3].

Aluminium balance in haemodialysis depends mainly on the gradient of diffusible aluminium, on the type of dialysis membranes, on their surface and thickness and also on many other factors, such as the pH of the dialysate. Among all these factors, the most important is the concentration of aluminium in dialysis fluids[7]. Maximum allowed concentration for dialysis fluid is 30 μg l^{-1}(= 1.112 μmol l^{-1}) – CEC recommendation[3].

The increased of values Al in serum of DP in last years is the evidence that the regular annual monitoring of the serum concentration of Al of dialysed patients remains necessary.

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P59

DEGRADATION OF HYALURONAN SAMPLES WITH THE ADDITION OF ASCORBIC ACID, Cu(II), Fe(II)

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Key words: hyaluronan, ascorbic acid, hyaluronan degradation, metal ions

Introduction

Hyaluronan (HA) is a high molecular weight biopolysaccharide discovered by Meyer and Palmer in 1934 (ref.[1]) in the vitreous humor of cattle eyes. HA consists of repeating disaccharide units composed of *N*-acetyl D-glucosamine and D-glucuronic acid linked by a β 1-4 glucosidic bonds while the disaccharides are linked by β 1-3 bonds[2]. It plays an important role for both mechanical and transport purposes in the body, e.g. it gives elasticity to the joints and rigidity to the vertebrate disks and it is also a constituent of the major importance in the vitreous body of the eye. Ascorbate is a potent water-soluble antioxidant capable of scavenging various types of reactive oxygen and nitrogen species, however in the presence of redox-active transition metal ions, ascorbate can also act as a pro-oxidant[3].

The aim of this study was to investigate the function of trace concentrations of Fe(II) and Cu(II) ions in the ascorbate autoxidation, in which hyaluronans of various molecular weight are involved as the indicators of the pro- or antioxidative properties of the system.

Material and methods

Six hyaluronan samples used in our study were kindly donated or purchased from the following manufactures: Fidia Farmaceuti S.p.A., Abano Terme, Italy; Genzyme Corp., Cambridge, MA, U.S.A.; Lifecore Biomedical Inc., Chaska, MN, U.S.A.; Sigma-Aldrich Chemical Company, St. Louis, MO, U.S.A.; CPN, Ústí nad Orlicí, Czech Republic. Analytical purity grade NaCl and $CuCl_2 . 2 H_2O$ were from Slavus Ltd., Bratislava, Slovakia; ascorbic acid was from Merck KGaA, Darmstadt, Germany. The water was of Milli-QRG quality (Millipore Corp., Bedford, MA, U.S.A.). $FeCl_2 . 4 H_2O$ was purchased from Penta, Chrudim, Czech Republic. Dynamic viscosity (η) of HA samples (2.5 mg ml^{-1} in 0.15 M-NaCl) was carried out without and with the addition of ascorbate[4]. In the next series of experiments dynamic viscosity (η) of HA samples after the addition of ascorbate (100 μM) followed by Cu(II) or Fe(II) ions, respectively was measured. The tested metal ion concentrations were 5.0 μM $CuCl_2$, 5.0 μM $FeCl_2$ in the system HA-ascorbate. All the measurements were carried out at 25±0.1 °C by using a digital rotational viscometer Brookfield DV-II PRO (Brookfield Engineering Labs., Inc., Middleboro, MA, U.S.A.). Viscosity of the samples was monitored in 3 min intervals for up to 5 h using a rotational spindle at a speed of 180 rpm equipped with a Teflon cup/spindle set of coaxial geometry constructed in our laboratory.

Results

Without exposing HA samples to action of ascorbate and metal ions, the continuous increase of the monitored h value for any of the investigated HA samples was monitored[4]. Rheopectic behavior of the HA solutions could be changed by trace amounts of metal cations. This fact was verified by time-dependent hyaluronan viscometric degradation when applied ascorbate together with Cu(II) or Fe (II) ions, respectively (fig. 1). As can be seen in fig. 1 (right panel), the character of the time dependence of η value upon the addition of $FeCl_2$ can be described as a gradual monotonous concentration-dependent decline, while the addition of $CuCl_2$ (left panel) resulted in a literally "exponential" drop of η value in a very short time interval, after which the decrease of η value continued however at a much lower rate. A possible explanation of this dissimilarity may be most probably in different reaction kinetics of the processes leading to the generation of reactive oxygen species in a system ascorbate *plus* $FeCl_2$ and in that of comprising ascorbate *plus* $CuCl_2$. A plausible explanation and the most simple conclusion might be to state that this HA sample is "heavily contaminated" with metals characterized with pronounced pro-oxidative properties. Another statement could be that the HA macromolecules with large(r) size are more susceptible to chain scission.

Discussion

Hyaluronan is in interest of many researchers due to its high molecular weight, it has anti-angiogenic, anti-inflammatory and immunosupressive properties, while intermediate or low sized fragments act predominantly in an opposite way. Since ascorbate acts as a powerful reducing agent with a standard reduction potential of 0.287 V at pH 7 for the redox couple $Asc^{\bullet -}$/AscH, it is assumed that it reduces traces of transition metals present in the HA samples, particularly Fe(III) and partially also Cu(II). Due to the presence of these metals, the Fe(III)/Fe(II) and Cu(II)/Cu(I), catalyzing ascorbate autoxidation leads to the gen-

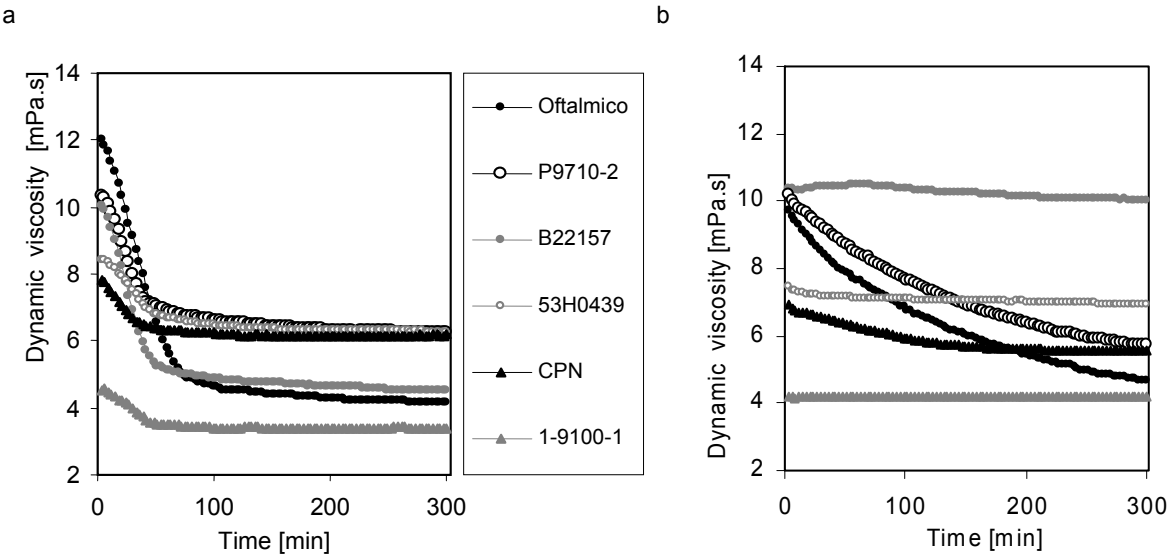

Fig. 1. **Time dependences of dynamic viscosity of HA solutions;** a) solutions of native HA samples with addition of 100 μM ascorbic acid and 5 μM Cu(II), b) solutions of HA samples with addition of 100 μM ascorbic acid and 5 μM Fe(II)

eration of •OH radicals that, after a certain initiation time period, promotes degradation of HA macromolecules, which is manifested by a gradual decrease of the solution dynamic viscosity.

The formation of •OH radicals can occur in several manners, whereas by far the most important *in vivo* mechanism is mediated by hydrogen peroxide. One of the routes of the direct H_2O_2 formation under aerobic conditions involves the system comprising ascorbate plus Cu(II) described already more than half a century ago by Weissberger et al.[5].

In aqueous milieu, hydroxyl radical represents the most reactive species, which virtually reacts with all the compounds containing C-H groups under the abstraction of hydrogen radical (H•) leading to the generation of the corresponding *C*-centered radical. In the case of the hyaluronan macromolecule, the attack of the •OH radical usually occurrs at the C-1 (or C-4) atoms of the D-glucuronate/D-glucuronic acid unit. Hydroxyl radicals may also affect the D-glucuronate/D-glucuronic acid units or *N*-acetylglucosamine moieties of HA leading to the opening of the pyranose ring(s) without cleaving the polymer chain. However, subsequent radical reactions or rearrangement of the generated *C*-centered radicals may produce polymer fragments of lower molecular weight. In a wide variety of biological *in vitro* systems Fe(II) salts and/or non-enzyme complexed ferrous cations (e.g. Fe(II)-EDTA) have been shown to enhance oxygen radical damage by increasing the production of an oxidative species generally believed to be the free hydroxyl radical.

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P60

ANTIHYPERGLYCEMIC AND ANTIOXIDANT EFFECT OF COMPLEX $[Cu_2(H_2O)(sal\text{-}\beta\text{-}ala)_2] \cdot H_2O$ IN ALLOXAN-INDUCED DIABETES IN MICE

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Introduction

Copper(II) complexes of Schiff bases derived from salicyladehyde and various amino acids belong to the class of low molecular antioxidants. The antiradical effect of aqua-bis(*N*-salicylidene-β-alaninato)dicopper monohydrate – $[Cu_2(H_2O)(sal\text{-}\beta\text{-}ala)_2] \cdot H_2O$ was studied under *in vivo* using a model of alloxan-induced diabetes in mice. The development of hyperglycemia and lipid peroxidation in mice stomachs and livers after intravenous administration of alloxan (120 mg kg^{-1}) was evaluated as an index of oxidative damage. The studied copper complex administered intraperitoneally in doses of 40 μmol kg^{-1} significantly suppressed the aloxan-induced hyperglycemia. The antihyperglycemic activity of Cu(sal-β-ala) was comparable with the protective effect of the native enzyme superoxide dismutase. The glucose level of the animal treated did not differ from its initial value. The pre-treatment with the complex 3 hrs before alloxan injection also reduced the mildly increased levels of TBARS and conjugated dienes appeared in the liver and stomach of diabetic mice.

Copper(II) complexes containing a tridentate Schiff base derived from salicylaldehyde and various amino acids have exhibited significant biological activity – antimicrobial[1], anti-inflammatory[2] and radioprotective[3]. Their biological action was suggested to arise from antiradical activity. The superoxide radical scavenging effects were demonstrated in a series of *in vitro* experiments[4,5]. Alloxan is a well-known diabetogenic agent which causes damage to pancreatic cells by producing free radicals and decreasing the level of antioxidative enzyme systems[6]. Thus, the alloxan system was considered adequate for the study of free radical pathology and for screening the properties of antioxidative drugs *in vivo*. From the above mentioned group of copper complexes, the aqua(*N*-salicylidene-β-alaninato)dicopper monohydrate – $[Cu_2(H_2O)(sal\text{-}\beta\text{-}ala)_2] \cdot H_2O$ was chosen to study its efficacy in the attenuation of alloxan-induced diabetes.

Material and methods

Aqua-bis(*N*-salicylidene-β-alaninato)dicopper monohydrate (Cu(sal-β-ala) in abbreviation) was prepared by the method described in[7], alloxan and superoxid dismutase (SOD) were purchased from Sigma. Female ICR mice (25–30 g) were used. Alloxan was dissolved in saline solution and injected into mice in the dose of 120 mg kg^{-1} *via* the tail vein. Cu(sal-β-ala) was administered intraperitoneally in the dose of 40 μmol kg^{-1}, enzyme superoxid dismutase (SOD) in the dose of 150 U g^{-1} according to the time schedule given in Table I. Glucose venous blood concentration was measured using glucoseoxidase assay (Glucotrend®). The animals were fasted for a minimum of 3 hrs before blood collection. The parameters of lipid peroxidation: thiobarbituric acid reactive substance, sulfhy-

Table I
The protective effect of studied copper(II) complex and SOD against alloxan induced hyperglycemia in mice

Treatment		Blood glucose level [nmol l^{-1}]			
		0.day	1.day	2.day	3.day
Saline		7.6 ± 0.4	22.9 ± 2.7	24.8 ± 3.8	25.0 ± 5.4
Cu(sal-β-ala)[a]:					
	6 hr before alloxan[b]	7.1 ± 0.8	10.6 ± 6.5**	12.3 ± 5.8*	11.5 ± 2.9*
	3 hr before alloxan	7.3 ± 0.6	6.9 ± 1.3***	8.3 ± 1.9**	7.3 ± 1.7**
	3 hr after alloxan	7.6 ± 0.7	6.1 ± 2.4***	7.2 ± 1.8***	6.8 ± 0.9**
	6 hr after alloxan	7.5 ± 0.9	6.1 ± 2.2***	10.7 ± 7.4*	8.3 ± 2.1*
SOD	3hr before alloxan	6.0 ± 0.4	6.7 ± 0.6***	7.3 ± 0.1**	7.2 ± 0.3**

[a] Cu(sal-β-ala) intraperitoneal dose 40 μmol kg^{-1}, SOD 150 U g^{-1}, [b] alloxan intravenous dose 120 mg kg^{-1}. Data expressed as mean ± SEM, n=8; statistical significance : $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared control-alloxan group (student´s t-test)

Table II
Lipid peroxidation parameters in mice stomach and liver measured on 3.day after administration of alloxan

Treatment	Stomach		Liver		
	TBARS nmol mg^{-1} protein	SH-groups nmol mg^{-1} protein	TBARS nmol mg^{-1} protein	SH-groups nmol mg^{-1} protein	Conjugated dienes nmol mg^{-1} protein
Saline	0.3 ± 0.02	83 ± 27	0.06 ± 0.02	83 ± 24	20.3 ± 3.9
Alloxan + saline	0.4 ± 0.02	49 ± 21	0.1 ± 0.01	69 ± 20	26.5 ± 7.1
Alloxan + Cu(sal-β-ala):					
6 hr before alloxan	0.3 ± 0.05	71 ± 10*	0.08 ± 0.01	74 ± 27	17.3 ± 6.6
3 hr before alloxan	0.2 ± 0.05*	93 ± 25*	0.06 ± 0.01*	74 ± 20	14.9 ± 4.4*
3hr after alloxan	0.3 ± 0.05	74 ± 35	0.08 ± 0.01	79 ± 39	16.2 ± 7.6
6 hr after alloxan	0.2 ± 0.05	84 ± 20*	0.07 ± 0.02	65 ± 24	16.3 ± 6.0

[a] Cu(sal-β-ala) intraperitoneal dose 40 μmol kg^{-1}, SOD 150 U g^{-1}, [b] alloxan intravenous dose 120 mg kg^{-1}. Data expressed as mean ± SEM, n=8; statistical significance : $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared control-alloxan group (student´s t-test)

dryl groups and conjugated dienes were determined spectrophotometrically by method[8]. All parameters were expressed *per* 1 mg of protein.

Results and discussion

Table I shows that intravenous administration of alloxan resulted in significant hyperglycemia in the mice. Plasma glucose increased to 22.9–25 nmol l^{-1} in comparison to the base level of 7.56 nmol l^{-1}. Hyperglycemia was evaluated until 3 days after injection of alloxan, when maximum β-pancreatic cell should be damaged; subsequently the hyperglycemia did not change. The administration of Cu(sal-β-ala) to animals reduced the elevated blood glucose level with efficiency depending on the application schedule. The cytoprotective effect of Cu(sal-β-ala) was demonstrated not only by pre-treatment but also by administration afterwards of alloxan. Complete significant protection against alloxan induced hyperglycemia was provided by Cu(sal-β-ala) when administered 3 hrs before and after alloxan administration. As shown in Table I, changing the interval between Cu(sal-β-ala) and alloxan to 6 hrs diminished the protection. The antihyperglycemic activity of Cu(sal-β-ala) was comparable with the protective effect of the native enzyme superoxid dismutase. The glucose level of the animal treated did not differ from its initial value.

The role of free oxygen radicals in the mechanism of toxicity of alloxan was declared *in vitro*[9]. Alloxan is reduced by biological reducing agents such as cysteine, gluthatione to dialuric acid; the latter readily autooxidizes, establishing a redox cycle for the generation of superoxide radicals and hydrogen peroxide. Superoxide dismutase and catalase evidently protected β-pancreatic cell of isolated pancreatic islets against alloxan cytotoxicity[9]. The square pyramidal structure of the studied complex is similar to the coordination of the copper(II) in the active centre of the native enzyme SOD. SOD-mimic activity of Cu(sal-β-ala) was demonstrated *in vitro*[4] and could be considered as playing a main role in the antihyperglycemic effect in alloxan induced diabetes. The copper(II) complexes of Schiff bases derived from salicylaldehyde and various amino acids (alanine, valine) also showed antihyperglycemic activity in alloxan-induced diabetic mice[10].

Several studies proved that alloxan produced oxidative stress in other tissues, causing peroxidation of membrane lipids and protein glycation[11,12]. In our experiments, lipid peroxidation was determined by measuring TBARS in liver and stomach, and also conjugated dienes in the liver. The changes in the levels of SH-groups, caused by oxygen radicals were also measured. Lipid peroxidation presented in Table II was increased in alloxan-induced diabetic mice as compared to the control, although these were not statistically significant. Similarly, Yadav et al.[13] have found increased lipid peroxidation in the heart in diabetic rats while no significant changes were observed in the liver and kidney. Treatment with Cu(sal-β-ala) evidently lowered the higher levels of TBARS in both organs and conjugated dienes in the liver when administered 3 hrs before injection of alloxan. The increased SH-groups were found after this treatment in the liver. On the basis of our results chemical reactivity of the studied copper complex in different methods of free radical production was confirmed. Excluding the antiradical activity of copper complexes in the antidibetic effect, the role of copper in insulin metabolism and altering of antioxidative systems should be taken into account[14].

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P61
CYTOTOXICITY OF PLANT EXTRACTS FROM GENUS *Philadelphus* L.

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Keywords: cytotoxicity, *Philadelphus subcanus*, *Philadelphus tenuifolius*, *Philadelphus schrenkii*, A431 cells

Introduction

The great attention has been devoted in previous years to natural plant products, because of their broad spectrum of constituents, which exhibit various biological activities with positive effects on human organism[1]. Some members of the genus *Philadelphus* L. (Hydrangeaceae) are known for their antibacterial, antiradical and immunomodulatory effects[2].

This study describes results of cytotoxic properties screening of ethanol extracts from three decorative shrubs from genus *Philadelphus* L.

Material and methods

Leaves were collected at Arboretum Mlyňany in September 2004. All samples were identified by Ing. Hoťka and voucher specimens are deposited there. Plant material was dried at room temperature. Samples were homogenized and macerated (10.0 g) in 200 ml of 96 % ethanol for 1 week in a dark room at room temperature. Vacuum dried samples were stored at room temperature until experimental utilization. Human skin carcinoma cell line (A431) was used for toxicity studies. Cell suspension was cultured with 20 µl of various doses of individual extracts (dissolved in DMSO – dimethylsulfoxide, its final concentration never exceeded 0.1 % (v/v) in either treated/ control-DMSO samples) for 24 and 72 h. Control cells were incubated in culture medium only. Tested doses of all three plant extracts were in the range of 2.5–50 µg dry material ml^{-1}. The culture medium and tested extracts were changed for fresh ones every 24 h. All extracts were tested in triplicates on the same cell batch. The cell proliferation was evaluated by the colorimetric MTT [3(4,5--dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma, USA) method. The absorbance was measured at 630 nm. The antiproliferative effects of individual extracts were expressed by ED_{50} values calculated from dose response curves by computer program (GraphPad Prism 4.00, GraphPad Software, USA). For statistical analysis Student´s t-test (for time dependence) and One-way ANOVA (for tested extracts) were used.

Results and discussion

The effects of ethanol extracts from leaves of genus *Philadelphus* L. on the proliferation of A431 cells were examined by the MTT assay. Dose response curves constructed in the range 2.5–50 µg ml^{-1} indicate: *a*/ decreasing number of viable cells with increased concentrations of all three extracts, *b*/ time dependent antiproliferative effects (fig. 1, 2 and 3). ED_{50} values (Table I) confirmed graphical

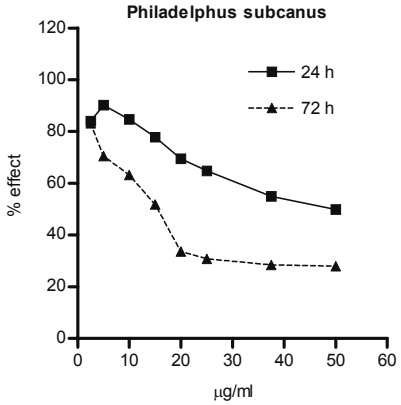

Fig. 1. **MTT cytotoxicity dose response curves of ethanol extract from leaves *Philadelphus subcanus*** after 24 h and 72 h

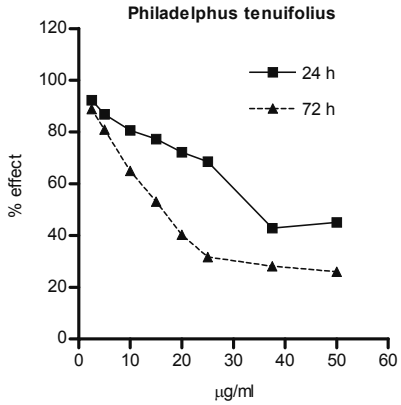

Fig. 2. **MTT cytotoxicity dose response curves of ethanol extract from leaves *Philadelphus tenuifolius*** after 24 h and 72 h

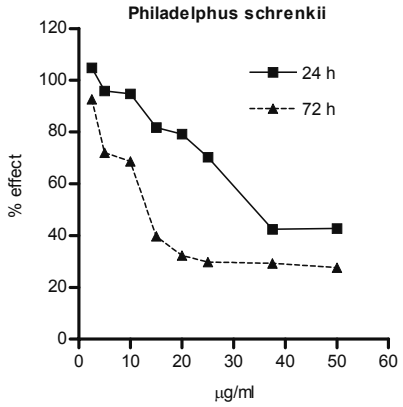

Fig. 3. **MTT cytotoxicity dose response curves of ethanol extract from leaves *Philadelphus schrenkii*** after 24 h and 72 h

Table I
ED_{50} values calculated from dose response curves on figs. 1, 2 and 3. The values are means ± SE of three separate experiments performed in triplicates for each dose

	ED_{50} [μg ml^{-1}]	
	24 h	72 h
Philadelphus subcanus	57.3 ± 2.4 [a]	13.8 ± 1.1 [b]
Philadelphus tenuifolius	44.6 ± 1.9 [a]	14.8 ± 1.4 [b]
Philadelphus schrenkii	32.8 ± 1.7 [a]	13.6 ± 1.3 [b]

[a] $P < 0.001$ (for time dependence), [b] $P<0.01$ (for tested extracts)

results e.g. lower toxicity manifested by all three extracts after acute treatment. After 24h extract from *Philadelphus schrenkii* Rupr. elicited significantly higher toxicity in comparison with other tested plant extracts. The lowest activity displayed the extract from *Philadelphus subcanus* Koehne. Chronic treatment is characterized by the shift of dose response curves to the left. This phenomenon indicates higher toxic efficacy as compared to the short time exposure. This relation was confirmed for all three plant extracts investigated while the effectiveness of individual extracts was comparable. Different acute cytotoxicity of the tested plant extracts on A431 cells may be based on their various chemical composition and relative content of biologically active substances, yet unknown for tested plants. The literature describe the presence of flavonoids, steroids, saccharides, amino acids, terpenes, saponins, phenolic acids and coumarins in genus *Philadelphus* L., while the biological activity is attributed mainly to triterpenes, flavonoids, coumarins or saponins[2,3−7]. This survey provides fundamental data about antiproliferative properties of ethanol extracts from leaves of *Philadelphus subcanus* Koehne, *Philadelphus tenuifolius* Rupr. et Maxim. and *Philadelphus schrenkii* Rupr. (Hydrangeaceae). Further studies are required to identify detailed chemical composition in leaves of the above plants and to investigate mechanism(s) involved in cytotoxicity.

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P62

DYSFUNCTION OF HEART AND LIVER MITOCHONDRIA IN RATS EXPOSED TO TRANSIENT ISCHEMIA OF THE BRAIN AND CHRONIC INHALATION OF MINERAL FIBRES

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Key words: brain ischemia-reperfusion, asbestos and wollastonite inhalation, heart and liver mitochondria, toxic products translocation

Introduction

It is generally accepted that mineral fibres such as asbestos and wollastonite can induce inflammatory effects at chronic inhalation[1]. Similarly, inflammation processes are documented in brain due to ischemia-reperfusion injury[2]. However, effects of brain ischemia as well as chronic fiber dust inhalation on extracerebral, resp. extrapulmonal organs are not known. The objective of the present study was tu evaluate the chronic effects of fiber dust inhalation as well as the acute effect of transient cerebral ischemia on the function of heart and liver mitochondrial respiratory chain in rats.

Material and methods

Animals

Male Fisher 344 rats suplied by Charles River Company (Germany) and male Wistar rats supplied by Velaz (Czech Republic) were housed at 22±2 °C, 45 % relative humidity, 12 hour light/dark photoperiodicity in air-conditioned rooms with free access to standard commercial laboratory pellets ST1 (Top Dovo, Slovak Republic) and water ad libitum. All animals received human care in compliance with the Institutional Animal Ethic Committee and with the Guidelines of European Convention for the protection of Vertebrate Animals Used for Experimental Purposes.

The animals were assigned into the four groups (6 animals per group) as follows:

a) Control group without treatments for each experimental group,
b) I/R: acute 50-minute ischemia / 8-days reperfusion of the brain in Wistar rats,
c) A: chronic 6-months exposure to asbestos fibrous dust inhalation in Fisher 344 rats,
d) W: chronic-6 months exposure to wollastonite fibrous dust inhalation in Fisher 344 rats.

Brain ischemia – reperfusion and mineral fiber dusts exposure

Acute cerebral ischemia/reperfusion injury was accomplished by our original surgical procedure for three-vessels occlusion (3-VO) as an vascular model of Alzheimer´s dementia and neuronal degeneration[3]. Minimally invasive transmanubrial approach was used for the occlusion of both, the brachiocephalic trunk (including the right common carotid artery and right vertebral artery) as well as the occlusion of the left common carotid artery (fig. 1).

Animals were exposed to asbestos (amosite) and wollastonite fibrous dust in a nose-only inhalation device (In-Tox, Albuquerque, NM, USA). Dust aerosol was produced at dosage of 60 mg m^{-3} for one hour per exposure. Every exposure was controlled by aerosol withdrawal from the inhalation chamber onto membrane filter (Sartorius, GmgH, Germany) and weighing the dust deposits.

Mitochondrial studies

After 3-VO and after fibrous dust exposures the rats were sacrificed and subsequently the hearts and livers were remowed and placed in an ice-cold isolation solution containing (in mmol l^{-1}) 225 manitol, 75 sucrose and 0.2 EDTA; pH 7.4. Mitochondrial protein concentration was estimated by the method of Lowry[4] using bovine serum albumin as a standard.

Respiratory chain function was measured in a respiratory buffer containing (in mmol l^{-1}) 100 HEPES, 5 KH_2PO_4, 120 KCl, 0.5 EDTA and 2% dextran; pH 7.2 at 30 °C, using Clark-type polarographic oxygen electrode[5]. Sodium glutamate (5 mmol) was used as a NAD substrate for complex I. To iniciate state 3 respiratory activity, 500 nmol of ADP was added to the cuvette. When all the ADP was converted to ATP, state 4 respiration was measured.

The results were evaluated using ANOVA and Student´s t-test for unpaired data, $P<0.05$ were considered as significant.

Results

There was significant decrease of respiratory control ratio RCR in heart and liver ($P<0.001$) mitochondria after the chronic inhalation of asbestos as well as in heart ($P<0.001$) and liver ($P<0.01$) mitochondria after chronic

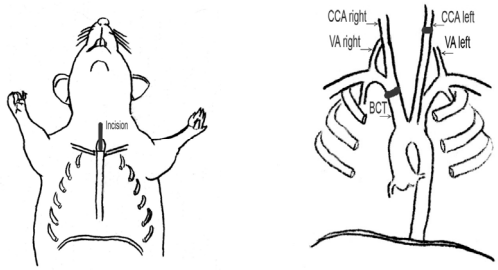

Fig. 1. **Acute cerebral ischemia/reperfusion injury,** ***P*<0.01

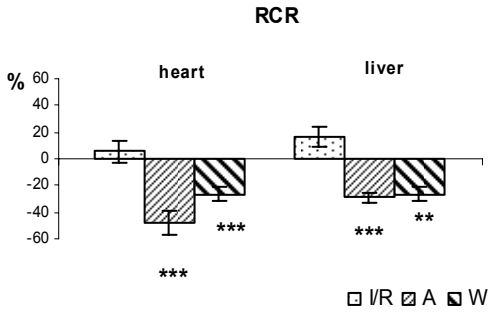

Fig. 2. **Respiratory control ratio**, ***P<0.001, **P<0.01, (change in % vs. adequate control group)

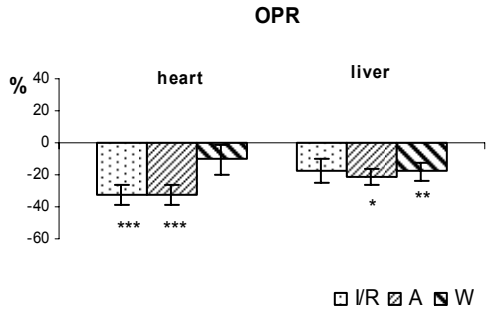

Fig. 4. **Oxidative phosphorylation rate**, ***P<0.001, **P<0.01, *P<0.05 (change in % vs. adequate control group)

inhalation of wollastonite as a consequence of mitochondria membrane damage. In the group of acute I/R injury the RCR was found to be not significantly increased in heart and liver mitochondria (fig. 2).

Significantly decreased oxygen uptake stimulated by ADP QO2(S_3) was found in heart mitochondria after acute I/R injury (P<0.001) and in the liver mitochondria after chronic inhalation of asbestos (P<0.05) as well as after chronic inhalation of wollastonite (P<0.01) (fig. 3).

The rate of ATP production OPR was significantly decreased after acute I/R injury and after chronic inhalation of asbestos in heart mitochondria (P<0.001). In liver mitochondria the rate of ATP production was decreased only after the chronic inhalation of asbestos (P<0.05) and wollastonite (P<0.01), but not after I/R injury of the brain (fig. 4).

Discussion and conclusion

Ischemic and toxic injury lead to mitochondrial dysfunction, with subsequent decrease of ATP generation, inflammation and free radical production[6,7].The lung diseases associated with mineral fibres exposure as well as brain disorders associated with ischemic damage are characterized by inflammation and generation of free radicals[1]. It is known that mediators of inflammation may translocate from the primary target organs through lymphatics into the blood and into the secondary target organs[8,9] Thus, we suppose that reactive oxygen species due to ischemia/ reperfusion injury of the brain as well as microparticles and toxic products of lung inflammation due to chronic inhalation of asbestos and wollastonite fibrous dust, translocate through the regional lymphatics and blood circulation into the secondary target organs leading to dysfunction of heart and liver mitochondria.

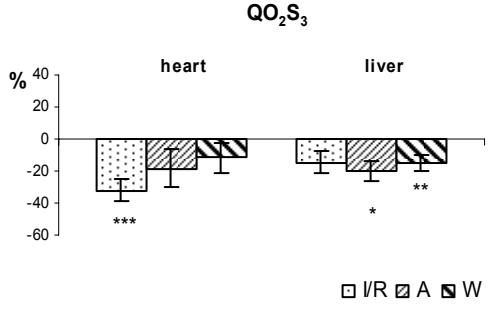

Fig. 3. **ADP stimulated respiration**, ***P<0.001, *P<0.05 (change in % vs. adequate control group)

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P63
CADMIUM AND BLOOD-BRAIN BARRIER

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Key words: cadmium, intratracheal, intragastral, blood-brain barrier

Introduction

Cadmium belongs to metals with dangerous toxic potential to human health. Inhalatory port of entry for cadmium is typical for occupational exposure while oral exposure is usually of non-occupational character. The aim of the study was comparison of cadmium organ retention in conditions of modelled inhalatory or oral experimental administration of this metal to laboratory animal: rat. In the centre of attention was the possibility of cadmium to reach the central nervous system.

Material and Methods

Female Wistar rats, 220–250 g body weight, kept on standard diet, water ad libitum, were used for the study. The rats were administered one dose of 86.4 μg cadmium chloride hemi (pentahydrate) (SIGMA) per kg b.wt. This dose was administered by means of intratracheal (IT) or intragastral (IG) instillation. For lack of adequate information from the Registry of Toxic Effects of Chemical Substances, dose was determined arbitrarily as 1/10 of the lowest toxic dose of cadmium chloride for intravenous administration. Rats were divided in one control (non cadmium) and 2 experimental groups (IT and IG groups). Each group consisted of 8 animals.

After the period of 48 hours the rats were sacrificed and following organs were isolated for cadmium detection: brain, liver, kidney and lung. The cadmium content was determined by means of atomic absorption spectrometry.

At first the isolated organs were stored at –20 °C. Before analysis the samples were de-frozen and weighed into tetrafluormethaxil (TFM) decomposition vessels of microwave oven (Milestone) with rotor MDR 1000/6/100/110. Nitric acid 65 % and hydrogen peroxide 30 % (both of the purity GR, Merck,Germany) were used for the decomposition. Decomposed samples were evaporated to drop, diluted with 0.4 ml HNO_3 (1+1) and transferred with demineralized water (Millipore Q Plus), into volumetric flasks 20 ml. Cadmium standard 1000±2 mg Cd l^{-1} (Merck) was used after sequental dilution for preparation of working standards for measurement by flame AAS (calibration curve range: 0–0.4 μg Cd ml^{-1}) and also for preparation of working standard of 2 ng Cd ml^{-1} for the method of standard additions (electrothermic atomization on GTA 96). As a modifier for cadmium determination ETAAS, the solution of ammoniumdihydrogen phosphate and magnesium nitrate was used.

Then samples of the mineralized organs of rats were analyzed by means of atomic absorption spectrometer SpectrAA30 Varian with graphite tube GTA 96, Zeeman background correction and autosampler for GTA 96 and Data Station DS 15. The sample was applied to pyrolytic platform inside graphite cuvette. Part of samples with higher cadmium content (lung) was processed by means of flame absorption spectrometer Spectr AA 30 with deuterium background correction.

Lung tissues from control rats and rats with IT exposure to cadmium were morphologically examined. The whole project at the start was subdued to the evaluation of the ethical commission for tests on animals.

Results and Discussion

Values for cadmium retention are expressed in the following text as means ± standard deviations (n =8).

Brain cadmium retention

Only the level of cadmium in the brain of rats after IT exposure: 0.8±0.1 ng g^{-1} was higher compared to cadmium untreated control rats brain level or to the brain level of rats with IG exposure method. Because in control or IG rats the cadmium levels were <0.5 ng g^{-1} (at the level of analytical detection by method used), results between groups could not be compared by statistical procedure.

Liver cadmium retention

The level of cadmium in the liver of rats after IT exposure: 930±80 ng g^{-1} was much higher when compared to cadmium liver retention in untreated control rats: 7.67±1.4 ng g^{-1} or to the liver cadmium retention of rats with IG exposure: 23.2±16.8 ng g^{-1}.

Kidney cadmium retention

The level of cadmium in the kidney of rats after IT exposure: 700±110 ng g^{-1} was much higher when compared to cadmium untreated control kidney retention: 15±2.1 ng g^{-1} or to the kidney cadmium retention of rats with IG exposure: 24.9± 8.1 ng g^{-1}.

Lung cadmium retention

The level of cadmium in the lung of rats after IT exposure: 2440±480 ng g^{-1} was excessive when compared to cadmium untreated control rats lung retention: <1 ng g^{-1} (at the level of analytical detection by method used) or to rats with IG exposure: <1 ng g^{-1}.

Intratracheal administration of metal used represents very efficient transport to body compartments because of relatively thin and delicate barrier of alveolar membranes, as documented by dramatic concentration differences in cadmium liver and kidney organ for IT exposure when compared to IG exposure. Advantage of IT exposure method to apply definite dose of cadmium is accompanied by local damage to lung alveolar parenchyma, as was verified morphologicaly.

Rather different is the situation for entry of cadmium in the central nervous system of rats (mammals, man including). Hematoencephalic barrier is effective to prevent excessive damage to neuroglial compartment as demonstrated by cadmium brain retention levels for IG and to a certain degree even IT exposure in presented experiment.

Although one of the tasks of the blood-brain barrier is to prevent the entry of metals, including cadmium, some literary data testify for penetration of cadmium into the central nervous system[1–3]. Character of blood-brain barrier and its role in prevention of entry of potentially toxic substances together with experimental study of the mechanism of metal penetration into the brain were described[4,5]. Interaction of physical factor – electromagnetic field and chemical factor – inorganic manganese compound in relation to manganese entry into the brain in experiment with rats were depicted[6,7]. The problem of organ distribution as well as BBB function in connection with simultaneous exposure of mice to cyanide + Cd/Pb/Mn was studied in conditions of acute or repeated administration[8]. It is obvious that the study of hematoencephalic barrier will necessitate further attention.

Conclusions

Intratracheal administration of single cadmium dose to rats resulted in distinctly higher liver and kidney cadmium retention when compared to intragastral administration of the same cadmium dose. In contrast to these results cadmium entry into the brain of rats was unsuccessful for IG exposure and cadmium was detected in very small quantities in brain of rats with IT exposure approach.

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P64

THE INFLUENCE OF METHANANDAMIDE AND SEX ON THE ACTIVITY OF CYTOCHROME P 450 AS A PREDICTIVE FACTOR OF XENOBIOTICS TOXICITY

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Key words: (*R*)-(+)-Methanandamide, cytochrome P 450, isolated perfused rat liver, sex difference

Introduction

Cannabinoids have a long history of consumption for recreational and medicinal reasons. The primary active constituent of the hemp plant Cannabis sativa is Δ^9-tetrahydrocannabinol (THC)[1]. In humans, psychoactive cannabinoids produce euphoria, enhancement of sensory perception, tachycardia, antinociception, difficulties in concentration and impairment of memory. Researchers in the 1970s, 80s, and 90s primarily assessed cannabis ability to temporarily alleviate various disease symptoms, such as the nausea associated with cancer chemotherapy. Of particular interest, scientists are investigating cannabinoid capacity to moderate autoimmune disorders, such as multiple sclerosis, rheumatoid arthritis and inflammatory bowel disease, as well as their role in the treatment of neurological disorders such as Alzheimer's disease and amyotrophic lateral sclerosis. Anandamide, an endogenous ligand for brain cannabinoid CB_1 receptor, produces many behavioural effects similar to those of THC, the main psychoactive ingredient in marijuana[2]. (*R*)-(+)-methanandamide is a synthetic long-lasting arachidonylethanolamide (anandamide) analogue that is metabolically stable and displays higher affinity for the cannabinoid receptor.

The enzymatic system of cytochrome P 450 (CYP450) is part of phase I of enzymatic biotransformation. It consists of many isoenzymes characterized by specific substrates and organ localization. These isoenzymes are most predominant in the liver, but can also be found in the intestine, lungs and other organs[3]. Among the diverse human genes, several have been identified to be particularly important in oxidative metabolism. They are: CYP3A4 (by far the most important), CYP2D6 (is responsible for the metabolism of many psychotherapeutic agents), CYP 2C6 and CYP2C19. CYP450 is involved in metabolism of many endogenous as well as exogenous substrates. Both, the induction and inhibition of specific CYP450 isoenzymes are important in terms of the efficacy or toxicity of drugs that are substrates for this system[3].

Interindividual variability of activity of oxidative and conjugating enzymes, especially the system of CYP450, can be based on many exogenous as well as endogenous influences e.g. sex, age, genetic factors or interactions between simultaneously applied drugs. The tendency to adapt the dosage of drug for the particular patient and to individualize and optimise the therapy to prevent adverse effects, to decrease the duration and costs of therapy is often seen in modern pharmacotherapy. One of the numbers of possible influences of drug metabolism which is frequently missed out is sex. CYP450 metabolizes many drugs including THC, the major psychoactive cannabinoid present in marijuana. The aim of this work was to investigate the effect of repeated administration of (*R*)-(+)-methanandamide on rat liver CYP2D2 isoenzyme using dextromethorphan (DEM) as a specific marker[4]. We have also studied the role of sex on the activity of CYP2D2 isoenzyme.

Materials and methods

The experiment was carried out on male and female Wistar rats (weighing 250±40 g, BioTest, Konarovice, Czech Republic) with free access to food and water, housed under the controlled conditions. After 7 days of adaptation to standard laboratory conditions, rats were randomly divided into 4 groups per 10 animals. Control group (CG) animals were treated with Tocrisolve™ 100 (Tocris Cookson Ltd.) (1 mg kg^{-1} day^{-1} intraperitoneally) and (*R*)-(+)-methanandamide group (R-MG) animals were treated with the drug dissolved in Tocrisolve™ 100 (Tocris Cookson Ltd.) at the dose of 1 mg kg^{-1} day^{-1} intraperitoneally for 7 days.

The rat liver was isolated from donors using a standard surgical technique. Cannulas were introduced into the portal vein and inferior cava vein, the liver was shortly washed out by a tempered (38 °C) saline which was changed for the perfusion medium (120 ml of Williams medium E) equilibrated with a mixture of 95 % O_2 and 5 % CO_2 in a short time. The recirculating perfusion apparatus was constructed according to the principles originated by Hugo Sachs GmbH (Germany). After 20 min preperfusion, a specific marker – dextromethorphan (DEM) (10.0 mg l^{-1}) was added as a bolus into the perfusion medium. Samples of perfusate (1.0 ml) were collected at the 30th, 60th and 120th min of perfusion and were stored at −75 °C until analysis. Quantitative analysis detecting DEM and it's metabolite dextrorphan (DOR) was performed by HPLC method (Shimadzu, Japan). Method by Zimová et all. was used to asses the levels of specific metabolite DEM and it's metabolite DOR in the perfusion medium[5].

For statistical calculations F-test and Student's t-test (Microsoft Excel 2000) were used, $P<0.05$ considered to be statistically significant difference.

Results

In the CG, the levels of CYP2D2 – dependent metabolite DOR were significantly higher compared to the R-MG in both of sexes. The levels of DOR in males were increased in the 30th (42 %), 60th (25 %) and 120th (30 %) minute in CG than in R-MG. In males /R-MG/ level of the parent drug DEM was significantly higher in 30th and 120th min and in females /R-MG/ level of DEM was significantly higher during the whole perfusion (Table I, II). The sex specific changes of the CYP2D2-dependent metabolite DOR concentrations in the perfusate evoked by a 7 day methanandamide treatment are documented in the figs. 1 and 2.

Conclusions

The model used is suitable for investigation of the activity of hepatic CYPs 450 and biotransformation processes is represented by the isolated and perfused liver. The main advantage of this model, in comparison with other methods used for measuring the CYP activity, is that conditions resemble physiological situation in the organism.

As we expected, the activity of CYP2D2 differed due to pretreatment with methanandamide. In our experiments the influence of this compound on rat CYP2D2 was inhibitive and metabolic activity of studied isoenzyme CYP2D2 in male and female rats was significantly higher in the control group than in *(R)*-(+)-methanandamide group. The levels of metabolite DOR in the pre-treated R-MG groups of rats were lower than in controls of both sexes

CYP2D6 (human orthologue of the rat CYP2D2) represents the second most frequent enzyme implicated in the biotransformation of therapeutic drugs (codeine, amitriptiline, clomipramine, imipramine and β-blockers such as propranolol and metoprolol)[6]. The literature data focused on the topic of influence of gender on CYP-450 is scarce and articles describe variable results. Conversely, more recent studies with DEM and metoprolol in extensive metabolizers showed faster clearance in men compared to women[7]. The present study demonstrated that CYP2D2 activity in the rat liver was higher in females than in males. The gender difference was distinguished in the methanandamide-pretreated rats as in the control group, where formation of a CYP 2D2-controlled metabolite DOR was significantly more stimulated in females than in males.

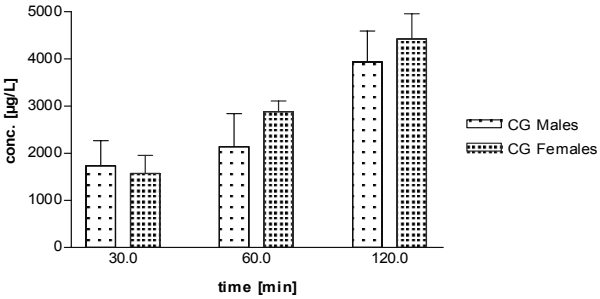

Fig. 1. **Influence of sex in CGs;** columns represent concentrations of DOR in the perfusate in the 30th, 60th and 120th minute of perfusion in males and females (*significant difference between males and females animals $P \leq 0.05$)

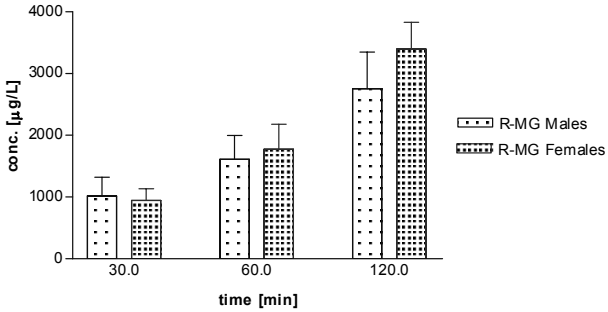

Fig. 2. **Influence of sex in R-MGs;** columns represent concentrations of DOR in the perfusate in the 30th, 60th and 120th minute of perfusion in males and females (*significant difference between males and females animals $P \leq 0.05$, **$P \leq 0.01$)

Table I
Concentrations of marker DEM in CG and R-MG in males and females rat

Time [min]	DEM [µg l^{-1}]			
	CG males	CG females	R-MG males	R-MG females
30	556.34	683.98	767.38*	972.31*
60	282.35	335.86	486,6	542.36**
120	168.33	109.23	350.64*	184.42**

* Significant difference between males and females animals $P \leq 0.05$, **$P \leq 0.01$)

Table II
Concentrations of metabolite DOR in CG and R-MG in males and females rat

Time [min]	DOR [µg l^{-1}]			
	CG males	CG females	R-MG males	R-MG females
30	1729.43	1567.47	1017.42**	945.73**
60	2135.54	2876.66	1612.47***	1776.79***
120	3935.34	4422.66	2755.07**	3399.68**

** Significant difference between males and females animals $P \leq 0.01$, *** $P \leq 0.001$

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P65

THE INFLUENCE OF POLYPHENOLIC EXTRACTS FROM GRAPE BYPRODUCTS ON THE METABOLIC ACTIVITY OF CYP1A2 IN HYPERCHOLESTEROLAEMIC RATS

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Key words: grape byproducts, polyphenols, cytochrome P450

Introduction

Not only the amount, but especially the composition of food is an essential parameter of its positive or negative effect. The modulation of diet composition is suitable for prevention of many serious diseases of cardiovascular system, obesity or diabetes mellitus. Positive or protective effects are mainly caused by non-nutrient components like polyphenols or isothiocyanates. It is believed that they can protect various organs from free radicals damage. Also many other effects are proved by recent studies[1].

Polyphenolic substances are widely distributed in plants namely in fruits and vegetables. Thus their daily intake can be easily modified. The problem is that people are often not willing to change their eating habits and prefer using these protective substances in the form of pills or enriched food products. Great source of polyphenols are plant waste materials such as grape byproducts which are formed after grape pressing in the process of wine production and are composed of grape peels and seeds. Moreover extracts rich in polyphenols can be prepared from this material easily.

Biological effects of polyphenols are mediated by numerous mechanisms. They can interact with specific receptors[2], structures[3], signal pathways[4] or can change the activities of enzymatic systems. One of the most important metabolic systems is cytochrome P450 (CYP450). It is composed of many isoenzymes, which are localized on different sites of organism, but predominantly in liver. The majority of xenobiotics including drugs as well as many of endogenous metabolites are substrates for CYP450 and many of them can also influence its metabolic activity. Thanks to this is knowledge of inductive or inhibitory activity of administrated substances necessary for evaluation of their possible toxicity and interactions with substrates for CYP450. It is known, that flavonoids are inhibitors of CYP450 (ref.[5]) and decrease the activation of some procarcinogens to carcinogens by this enzymatic system. On the other hand some authors showed data indicating inductive activity of polyphenols on CYP450 (ref.[6]).

The aim of our work was to prepare extracts from grape byproducts rich in polyphenols and to investigate their influence on the activity of hepatic CYP450 1A2 (CYP1A2) isoenzyme in hypercholesterolaemic rats.

Materials and methods

Two types of grape byproducts were obtained from Vinné sklepy Lechovice s.r.o., CZ. Mixture of byproducts from varieties of Modrý portugal (Blauer Portugaiser) and Andre, were used for preparation of red extract (RE) and Rulandské šedé (Pinot gris) for preparation of white extract (WE). Fresh byproducts were stored frozen (–20 °C) until drying (20 °C) and homogezation.

Dried and homogenized byproducts were extracted in two steps. First with methanol (Chromservis, CZ) for 60 minutes in the ratio 1 : 1.5 (w/w) and for another 60 minutes in the ratio 1 : 1 (w/w). Deionised water was used as extraction solvent in the second step in the same schema as methanol. Water and methanol parts were mixed and evaporated on the vacuum evaporator (Laborota, Heidolph, IT).

Spectrophotometric method using Folin-Ciocalteu´s reagent for determination of total polyphenolic content and gallic acid as standard was used. Extracts were diluted with deionised water to required concentration. Total polyphenols are shown in gallic acid equivalents (GAE).

HPLC methods were used for partial qualitative analysis. The analysis was performed on HP Agilent 1100 (Agilent Technologies, GER), Luna C18(2) 150 mm, 2 μm column (Phenomenex, USA), UV-VIS detection, $\lambda_1 = 220$ nm, $\lambda_2 = 315$ nm. Mobile phases A – 5 % acetonitrile/water B – 80 % acetonitrile/water, gradient elution 0. min 100 A 40. min 100 % B. Gallic acid, catechin, epicatechin and *trans*-resveratrol were used as standards.

The work was carried out on on male Wistar albino rats (200 ± 40 g, BioTest, CZ) with free access to water and standard or hypercholesterolaemic diet (1.7 % of cholesterol, Sigma, GER). Animals were housed under the controlled conditions (lights on from 6:00 a.m. to 6:00 p.m., temperature 21–22 °C, relative humidity 50–60 %).

Animals were randomly divided into 4 groups per 8 animals. Control group animals (CG) were fed with standard diet S3 (Ing. Máchal, CZ) animals from hypercholesterolaemic group (HG), red extract group (RG) and white extract group (WG) were fed with hypercholesterolaemic diet prepared from standard diet S3 by enriching with cholesterol. Red extract was administered to RG animals, white extract to WG animals by intragastric sond in the dose of 5 mg GAE kg^{-1} day^{-1}. Water was administered to CG and HG animals. After 40 days long premedication

animals were used for liver perfusion.

The rat liver was isolated from donors using a standard surgical technique. Cannula was introduced into the portal vein and liver was shortly washed out by a tempered (38 °C) saline which was changed for the perfusion medium (120 ml of Williams medium E, Sigma GER) equilibrated with a mixture of 95 % O_2 and 5 % CO_2 in a short time. The recirculating perfusion apparatus was constructed according to the principles originated by Hugo Sachs (GER). After 20 min of pre-perfusion, a specific marker – phenacetine (PHEN, Sigma, GER) (10.0 mg l^{-1}) was added as a bolus into the perfusion medium. Samples of perfusate (1.0 ml) were collected at the 30th, 60th, 90th and 120th min of perfusion and were stored at –75 °C until analysis. Quantitative analysis detecting PHEN and its metabolite paracetamol (PAR) was performed on HPLC (Shimadzu, Japan) using method described by Jurica[7].

For statistical calculations F-test and Student´s t-test (Microsoft Excel 2000) were used, $P \leq 0.05$ was considered to be statistically significant difference.

Results

Partial qualitative analysis

We determined epicatechin and catechin as two major polyphenolic compounds of both extracts. Gallic acid was present in both extracts too instead of resveratrol, which was found only in RE.

Activity of CYP1A2

The influence of administered extracts on the activity of CYP1A2 was in general inductive. The levels of PAR were higher during the whole perfusion (WG; $P \leq 0.05$) or in the 30th min of perfusion (RG; $P \leq 0.05$) in comparison to CG animals. We have also observed increased levels of PHEN in the 30th minute of perfusion in RG ($P \leq 0.01$) and WG group ($P \leq 0.05$) compared to CG. Hypercholesterolaemic diet have no influence on the activity of CYP1A2 which was confirmed by similar concentration of PAR and PHEN in CG and HG animals. Data shown in Table I and Table II.

Table I
Concentrations of PHEN in perfusion medium

Time [min]	PHEN [mg l^{-1}]			
	RG	WG	HG	CG
30th	4.96 ± 0.96	5.01±1.92	3.29±0.58	2.81±1.01
60th	3.41±0.63	3.31±1.10	2.42±0.92	2.90±0.82
90th	1.79±0.65	2.07±0.54	1.89±0.86	2.03±0.63
120th	1.21±0.58	1.33±0.6	1.32±0.75	1.45±0.85

Table II
Concentrations of PAR in perfusion medium

Time [min]	PAR [mg l^{-1}]			
	RG	WG	HG	CG
30th	0.17±0.09	0.35±0.26	0.07±0.01	0.06±0.03
60th	0.19±0.13	0.38±0.23	0.11±0.02	0.10±0.03
90th	0.14±0.09	0.37±0.21	0.09±0.04	0.11±0.04
120th	0.15±0.11	0.31±0.18	0.11±0.04	0.10±0.06

Discussion and conclusion

The model of isolated perfused rat liver is suitable for studies focused on the activity of CYP450, because other models which are being used (isolated microsomes or recombinant cytochromes) doesn´t simulate conditions in live organism as well as perfused liver where not only organ integrity, but also system of biochemical links is preserved.

As we predicted, hypercholesterolaemic diet didn´t change metabolism of PHEN. Our opinion is that the only influence of chronic hypercholesterolaemia on the activity of CYP450 can be due to hepatic steatosis and general decreasement of hepatocytes activity. Increasement in the activity of CYP1A2 was manifested as elevated levels of metabolite PAR in the perfusion medium in animals pretreated with both types of extracts when compared to control animals. We observed differences between extracts which were manifested as increasement of PAR levels in RG only in the 30th minute of perfusion instead of elevation during the whole perfusion in animals terated with white extract. Possible explanation is induction of CYP2E1 isoenzyme metabolizing PAR caused by red extract. This can cause lower levels of PAR during the perfusion and similar concentrations of PHEN.

For inductors of CYP450 is characteristic that with increasing levels of metabolite are decreasing levels of specific marker for selected isoenzyme in perfusion medium. Our data showed increase not only in levels of metabolite but also higher levels of marker in the 30th minute. From the concentrations of PHEN and PAR in the 30th minute of perfusion and from the levels in the start of the perfusion is clear, that major part of PHEN is bound in liver and changes in binding capacity of liver for PHEN can be an explanation of observed increase of both marker and metabolite.

We prepared two different extracts from grape by-products with similar composition of major polyphenolic components. Extracts in the dose of 5 mg GAE kg^{-1} day^{-1} acted as inductors of rat CYP1A2 after 40 day long intragastric premedication, which was manifested as increased levels of specific CYP1A2 marker PHEN´s metabolite PAR in the perfusion medium in the model of isolated perfused rat liver when compared to control animals. We have also observed atypical increase of PHEN levels at the

beginning of the perfusion in animals treated with extracts. Hypercholesterolaemic diet did not influence the activity of CYP1A2 when compared to control animals.

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CONTENTS

Lectures

Posters

P01 *D. Aimová, J. Poljaková, R. Kubík, V. M. Arlt, C. J. Henderson, C. R. Wolf, D. H. Phillips, E. Frei, M. Stiborová* Eeffect of benzo[a]pyrene treatment on hepatic cytochrome p450 and nadph:cytochrome p450 reductase (por) levels in mice with liver-specific deletion of the por gene

P02 *M. Barančoková, A. Kažimírová, Z. Kováčiková, M. Hurbánková, S. Černá, M. Beňo, S. Wimmerová* Frequency of micronuclei in alveolar macrophages of rats after exposure to industrial mineral fibres dusts and in combination with cigarette smoke

P03 *L. Bartošíková, J. Nečas, M. Chromý, T. Bartošík, M. Pavlík, P. Fráňa, J. Fráňová* Incidence of fatal benzodiazepine intoxications in brno region

P04 *L. Bartosova, L. Brzobohaty, F. Novak, T. Parak, R. Opatrilova, V. Brunclik, M. Frydrych, M. Bebarova, P. Suchy* Effect of the newly synthesized compound 44bu on aconitine induced cardiotoxicity – compared with lidocaine and propaphenone

P05 *K. Bauerová, E. Paulovičová, D. Mihalová, D. Komendová, S. Poništ* Monitoring of pro-inflammatory cytokines in the development of adjuvant arthritis

P06 *J. Blahová, I. Slatinská, K. Kružíková, J. Velíšek, Z. Svobodová* The effect of deltamethrin on activity of glutathione S-transferase of common carp (*Cyprinus carpio)*

P07 *R. Buňková, I. Papežíková, M. Podborská, A. Lojek* Effect of uric acid on oxidative damage

P08 *S. Černá, M. Hurbánková, Z. Kováčiková, M. Beňo, S. Wimmerová, T. Kiss, A. Bohumelová, I. Iľanovská* Changes in the cytotoxic parameters of bronchoalveolar lavage of rats after 6 months exposure to refractory ceramic fibres, amosite asbestos and cigarette smoke

P09 *P. Černá, D. Kotyzová, V. Eybl* Interaction of natural antioxidants with indium in vivo

P10 *D. Černý, Z. Stiaková, E. Kmoníčková, Z. Zídek, N. Kutinová Canová, H. Farghali* Caspase-3 and other markers in hepatocyte apoptotic and necrotic studies

P11 *V. Dostálová, P. Dostál, P. Višňovský* Delayed toxic encephalopathy after two month toluene exposure – case report

P12 *K. Drábiková, R. Nosáľ, K. Bauerová, D. Mihalová, V. Jančinová, M. Petríková, D. Košťálová* Chemiluminescence as a measure of oxidative stress in paws and spleen of rats with adjuvant arthritis

P13 *V. Hájková, A. Moravcová, M. Číž, V. Velebný, A. Lojek, L. Kubala* Glucomannan isolated from *candida utilis* modulates the activity of human phagocytes

P14 *I. Holcátová, A. Slámová, M. Schejbalová* Risk of chemical compounds in indoor environment

P15 *M. Hurbánková, S. Černá, M. Beňo, T. Kiss, S. Wimmerová* Influence of combined exposure to cigarette smoke and refractory ceramic fibres on the selected bronchoalveolar lavage parameters in experiment

P16 *V. Jančinová, M. Petríková, T. Perečko, K. Drábiková, R. Nosáľ, K. Bauerová, S. Poništ, D. Košťálová* Inhibition of neutrophil oxidative burst with arbutin. effects *in vitro* and in adjuvant arthritis

P17 *A. Kažimírová, M. Barančoková, L. Wsólová, M. Dušinská* Cytogenetic analysis of lymphocytes of workers occupationally exposed to rockwool and glass fibres. molecular-epidemiologic study

P18 *H. Klusoňová, J. Kučeravcová, E. Švajdlenka, L. Spáčilová, P. Višňovský* Blood morphine quantity connected with natural opium and poppy heads products abuse

P19 *M. Kopáni, M. Weis, T. Málek, J. Jakubovský* Protection effect of vitamin c on alcohol binding to phospholipid monolayers

P20 *D. Kotyzová, P. Černá, J. Koutenský, O. Hes, V. Eybl* Effect of curcumin and quercetin on oxidative tissue damage induced by ferric nitrilotriacetate (Fe-NTA)

P21 *Z. Kováčiková, M. Hurbánková, S. Černá, M. Beňo, S. Wimmerová, E. Tátrai* Antioxidant status in lung of rats exposed to fibrous dust or cigarette smoke

P22 *D. Krejčová, K. Okénková, R. Konopka, A. Lojek, L. Kubala* Inhibitors of nadph oxidase decrease endotoxin mediated induction of inducible nitric oxide expression in mouse macrophages

P23 *M. Křenová, D. Pelclová* Consequences following intoxication with hepatotoxic and nephrotoxic substances

P24 *J. Křížková, K. Burdová, P. Hodek, R. Kizek, M. Stiborová* Effects of a flavonoid structure on cytochromes P450 induction

P25 *M. Kuneš, J. Květina, P. Kubant, J. Maláková, V. Herout, Z. Svoboda* Influence of methotrexate and L-carnitine on transintestlnal transport of model substances (the rat small intestine in situ perfusion)

P26 *I. Kurcova, H. Rakovcova* Buprenorphine – traditional drug with a new potential risk of abuse

P27 *M. Kuricová, J. Tulinská, A. Líšková, E. Neubauerová, Z. Kováčiková, E. Tátrai* The effect of nio nanoparticles on immune system of rats

P28 *J. Kůsová, H. Miturová, J. Burdová, H. Tomášková* Ames test as an alternative method: mutagenicity of ostravian air (year 2006)

P29 *S. Mácová, V. Pištěková, Z. Svobodová, I. Bedáňová, E. Voslářová* Comparison of acute toxicity of potassium

permanganate to juvenile and embryonic stages of *danio rerio*

P30 *T. Mačičková, J. Pečivová, R. Nosáľ, D. Holomáňová* Superoxide generation and myeloperoxidase release in human polymorphonuclear leukocytes treated with carvedilol

P31 *E. Martínková, P. Hodek, J. Hudeček, E. Frei, M. Dontenwill, M. Stiborová* Oxidation of ellipticine by human and rat cytochromes P450 correlates with its binding to DNA

P32 *D. Mihalová, S. Poništ, J. Kucharská, D. Komendová, K. Bauerová* Total antioxidant status – systemic marker of oxidative stress in adjuvant arthritis

P33 *J. Mizerovská, H. Dračínská, M. Martínková, J. Hudeček, P. Hodek, Heinz H. Schmeiser, V. M. Arlt, E. Frei, M. Stiborová* Kinetics of 3-aminobenzanthrone oxidation by rat hepatic microsomes

P34 *H. Modrá, K. Kružíková, R. Dobšíková, Z. Svobodová, M. Lickova* Chronic effect of gardoprim plus gold 500 SC (*s*-metolachlor, terbuthylazine) on common carp (*Cyprinus carpio*)

P35 *A. Moravcová, A. Lojek, M. Číž, J. Pečivová, V. Jančinová, R. Nosáľ* The effect of carvedilol on the oxidative burst of rat phagocytes

P36 *J. Mráz, Š. Dušková, P. Šimek, J. Marečková, H. Nohová, V. Stránský* Biological monitoring of exposure to ethylene oxide in the sterilization units

P37 *B. Mrázová, V. Kotrbová, M. Kořínková, L. Svobodová, J. Hudeček, P. Hodek, R. Kizek, E. Frei, M. Stiborová* Kinetics of ellipticine oxidation by cytochromes P450 1A1 and 1A2 reconstituted with nadph:cytochrome P450 reductase

P38 *E. Neubauerova, J. Tulinska, M. Kuricova, A. Liskova, E. Jahnova, K. Volkovova, M. Kudlackova, M. Dusinska* The effect of vegetarian diet on immune response

P39 *R. Nosál, V. Jančinová, M. Petríková, S. Poništ, K. Bauerová* Suppression of oxidative burst of neutrophils with methotrexate in rat adjuvant arthritis

P40 *K. Okénková, A. Lojek, L. Kubala, M. Číž* Modulation of rat blood phagocyte activity by serotonin

P41 *I. Papežíková, A. Lojek, M. Číž* A comparison of antioxidant properties of uric acid, allantoin and allantoic acid

P42 *D. Pelclová, Z. Šenholdová, E. Lukáš, P. Urban, P. Ridzoň, K. Vlček, T. Navrátil* Diagnosis and elimination of thallium after an intentional intoxication

P43 *Z. Podzimkova, D. Pelikanova, M. Spacek* Progress of process to determination of inhalation acrylate exposure as basement method for human risk assessment

P44 *J. Poljaková, E. Frei, T. Eckschlager, J. Hraběta, K. Figová, R. Kizek, J. Hudeček, P. Hodek, M. Stiborová* Comparison of the cytotoxicity of and dna adduct formation by the anticancer drug ellipticine in human breast adenocarcinoma, leukemia and neuroblastoma cells

P45 *S. Poništ, J. Kucharská, A. Gvozdjaková, D. Komendová, D. Mihalová, K. Bauerová* Mitochondrial bioenergetics of skeletal muscle studied in adjuvant arthritis

P46 *H. Rakovcová, D. Pelclová, B. Řičařová, T. Navrátil* Pesticide exposure according to the czech toxicological information centre from 1997 to 2005

P47 *K. Rausová, Ľ. Palkovičová, M. Ursínyová, V. Mašánová, E. Reichrtová, P. Čižnár, L. Wsólová* Environmental risk factors of allergic diseases in 5-year-old children

P48 *M. Rucki, M. Tichý, I. Hanzlíková, R. Uzlová* Hazard identification of binary chemicals mixtures by QSAR techniques

P49 *B. Řičařová, L. Černá* Thiodiglycolic acid – indicator of metabolic unequilibrium

P50 *M. Semanská, V. Martínek, J. Hudeček, P. Hodek, M. Dračínský, E. Frei, M. Stiborová* Study of peroxidase-mediated oxidation of carcinogenic azo dye sudan i

P51 *A. Slámová, Z. Šmerhovský, E. Kudlová* Cancer morbidity and mortality in workers exposed to vinyl chloride monomer

P52 *O. Slanař, H. Buzková, K. Pechandová, J. Marek, F. Perlík* Frequency of tpmt functional polymorphisms in the czech population

P53 *M. Staruchová, K. Volkovová, C. Mišľanová, Z. Kováčiková, L. Wsólová, A. R. Collins, L. Staruch, M. Dušinská* Oxidative damage and antioxidant defence in the plasma of people exposed to mineral fibres

P54 *J. Stříbrný, M. Dogoši, Z. Šňupárek, I. Černá, M. Sokol* TLC proof of intoxication by taxus baccata

P55 *M. Svobodová, J. Šístková, H. Dračínská, J. Hudeček, P. Hodek, H. H. Schmeiser, V. M. Arlt, E. Frei, M. Stiborová* Reductive activation of environmental pollutants 3-nitrobenzanthrone and 2-nitrobenzanthrone

P56 *J. Trögl, J. Hálová, G. Kuncová, L. Kubicová, P. Pařík, K. Demnerová, S. Ripp, G. S. Sayler* The use of bioluminescent bioreporter pseudomonas fluorescens HK44 for measurment of toxicity of environmental pollution

P57 *J. Tulinska, A. Liskova, M. Kuricova, E. Neubauerova, M. Pollak, J. Szokolayova* Skin sensitization testing of chemicals in textile materials

P58 *J. Valachova, E. Jakubovičová, J. Buchancova* Toxicological profile of aluminium
P59 *K. Valachová, P. Gemeiner, E. Hrabárová, K. Bauerová, L. Šoltés* Degradation of hyaluronan samples with the addition of ascorbic acid, Cu(II), Fe(II)
P60 *J. Valentová, L. Šolcová, J. Sokolík, M. Žemlička* Antihyperglycemic and antioxidant effect of complex $[Cu_2(H_2O)(sal\text{-}b\text{-}ala)_2] \times H_2O$ in alloxan-induced diabetes in mice
P61 *V. Vaľko, E. Pravdová, M. Nagy, D. Grančai, M. Ficková* Cytotoxicity of plant extracts from genus *Philadelphus* L.
P62 *O. Vančová, M. Beňo, M. Dušinská, M. Hurbánková, J. Horecký* Dysfunction of heart and liver mitochondria in rats exposed to transient ischemia of the brain and chronic inhalation of mineral fibres
P63 *M. Vojtíšek, M. Krmenčíková, J. Knotková, L. Kašparová, E. Švandová, R. Holuša, J. Putta, Z. Tejnorová, K. Chotašová* Cadmium and blood-brain barrier
P64 *L. Zahradníková, O. Zendulka, J. Juřica, E. Hadašová* The influence of methanandamide and sex on the activity of cytochrome P450 as a predictive factor of xenobiotics toxicity
P65 *O. Zendulka, L. Zahradníková, J. Juřica, J. Totušek* The influence of polyphenolic extracts from grape byproducts on the metabolic activity of cyp1a2 in hypercholesterolaemic rats

AUTHOR INDEX