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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Key words: benzo[*a*]pyrene, induction, cytochromes P450, NADPH:cytochrome P450 reductase, HRN™ mice

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,

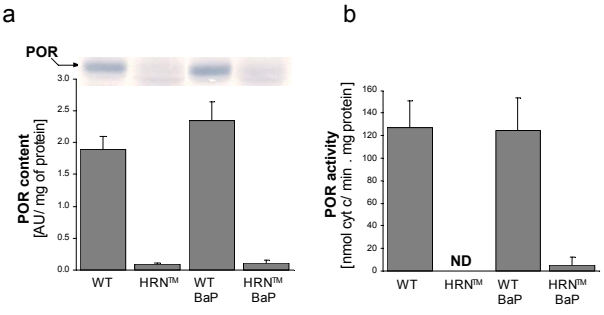

Fig. 1. **POR protein levels (a) and activity (b) in hepatic microsomes of HRN™ and WT mice, untreated or treated with BaP.** Inset in a): immunoblots of microsomal POR from all mice groups, stained with chicken polyclonal antibody against rabbit POR. Hepatic microsomal samples were pooled from 3 animals,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

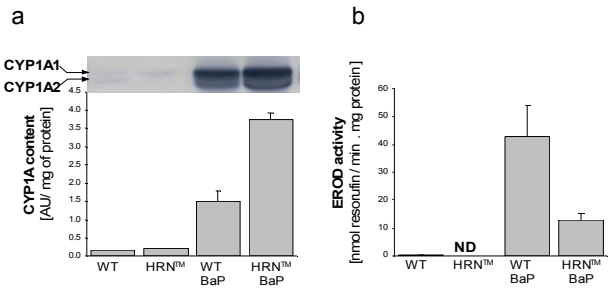

Fig. 2. **CYP1A protein levels (a) and EROD activity (b) in hepatic microsomes of HRN™ and WT mice, untreated or treated with BaP.** Inset in a): immunoblot of microsomal CYP1A from all mice groups, stained with chicken polyclonal antibody against rat recombinant CYP1A1. Hepatic microsomal samples were 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

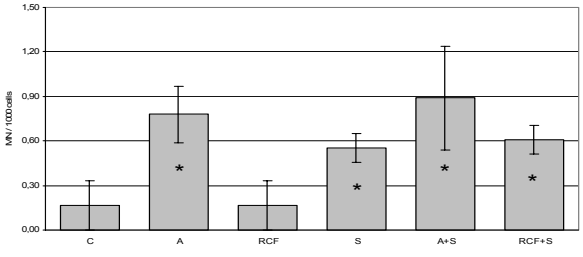

Fig. 1. **The influence of combined exposure on MN frequency;** **P*=0.05 compared to the control. The error bars represent S.D.

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

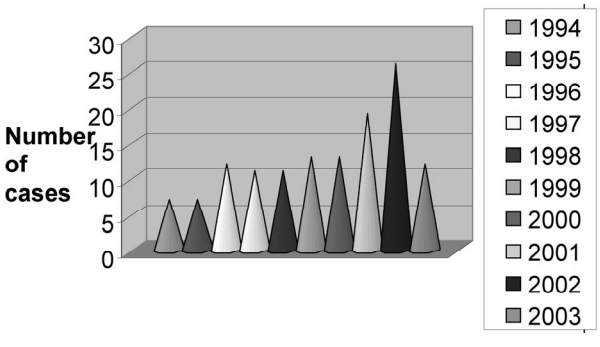

Fig. 1. **Number of fatal benzodiazepine intoxications in 1994–2003;** in 1994 – 7 cases; in 1995 – 7 cases, in 1996 – 12 cases, in 1997 – 11 cases, in 1998 – 11 cases, in 1999 – 13 cases, in 2000 – 13 cases, in 2001 – 19 cases, in 2002 – 26 cases, and in 2003 – 12 cases

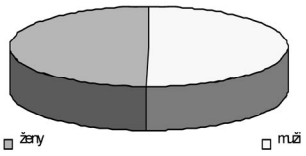

Fig. 2. **The proportion of men and women in the incidence of fatal benzodiazepine intoxications in 1994–2003;** 1 – men: 66 cases, 2 – women: 65 cases

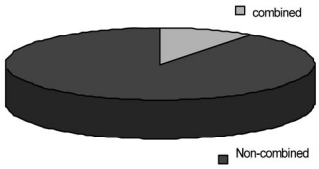

Fig. 3. **Proportion of combined and non-combined benzodiazepine intoxications in 1994–2003;** 1– non-combined intoxications – 13 cases, 2 – combined intoxications – 118 cases

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].

This study was supported by grants GA CR No. 305/06/0863 and IGA MZ CR No. NR9126-3/2006.

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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

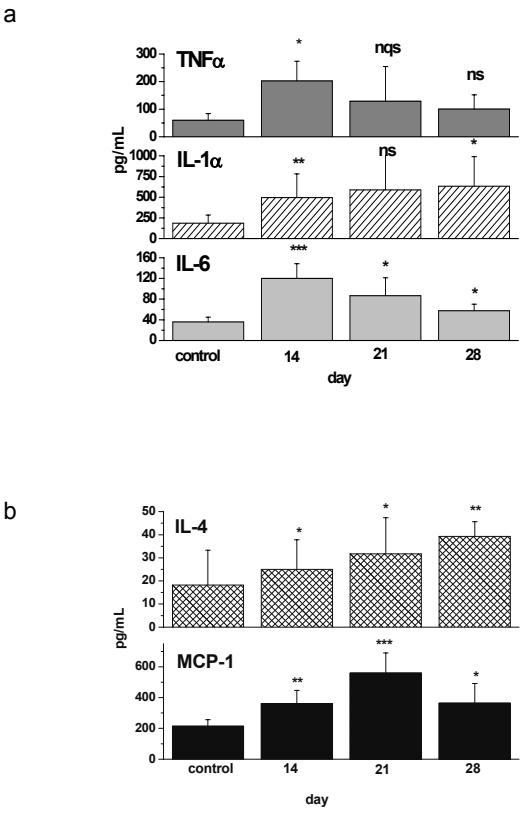
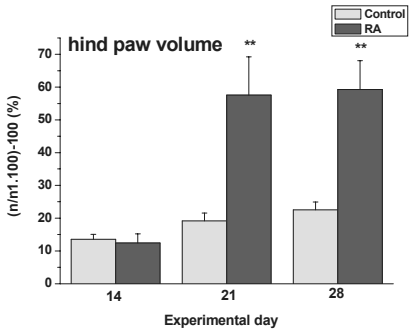


Fig. 1. **Time profiles of measured cytokines;** (mean ± standard deviation)

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.

This work was supported by the grants: VEGA 2/5051/25, APVV- 51- 017905, APVV- 21- 055205.

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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].

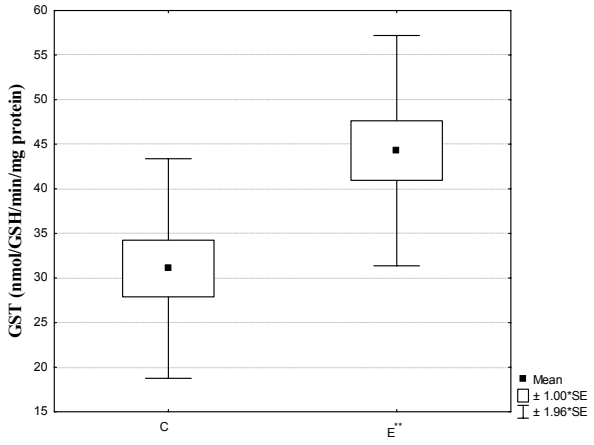

Fig. 1. **Effect of acute exposure to Decis flow (0.13 mg l^{-1}) on activity of GST in common carp;** C – control group, E – experimental group; $^{**}P< 0.01$

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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-

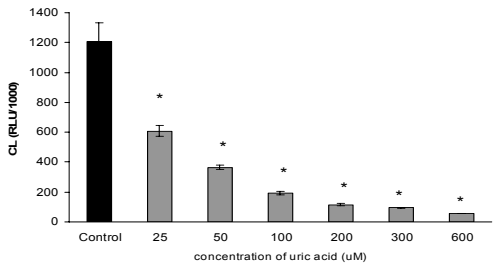

Fig. 1. **Scavenging of radicals formed in Fenton reaction by uric acid measured luminometrically;** the values represent integrals of resultant kinetic curves expressed as relative lights units/1000

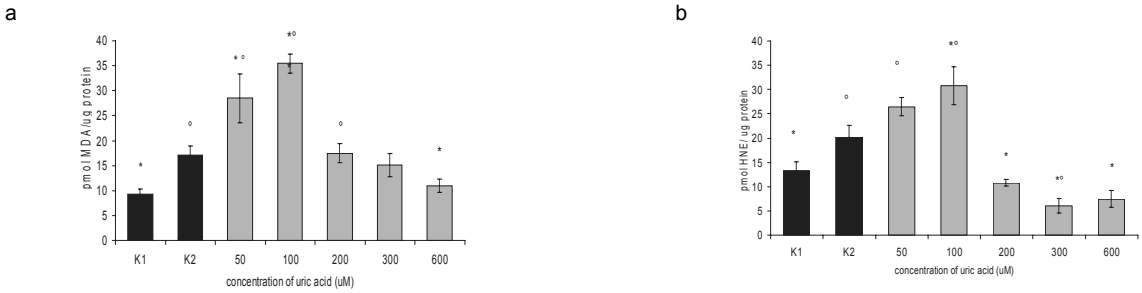

Fig. 2. **Effect of uric acid on lipid peroxidation expressed as MDA concentration (a) and HNE concentration (b);** values are the mean±S.E.M. of four different experiments and are expressed as pmol mg^{-1} protein. Data were analyzed with Student t-test, **P*<0.05 vs. control 2(K2); °*P*<0.05 vs. control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

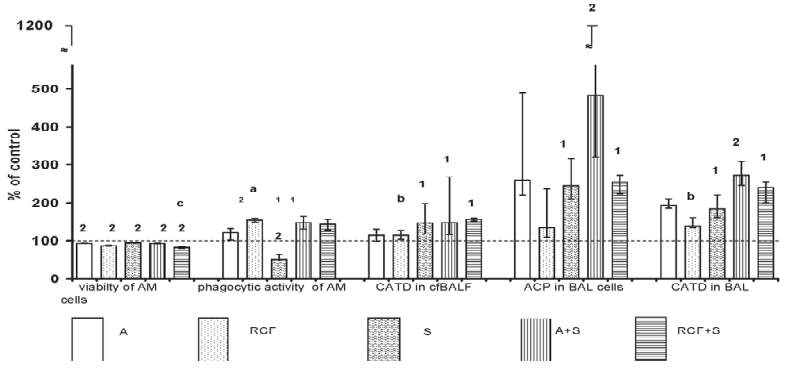

Fig. 1. **Viability of alveolar macrophages, phagocytic activity of alveolar macrophages, activity of cathepsin D** in cell-free bronchoalveolar lavage fluid, activity of acid phosphatase and cathepsin D in bronchoalveolar lavage cells after 6 month exposure to amosite, refractory ceramic fibres, cigarette 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.

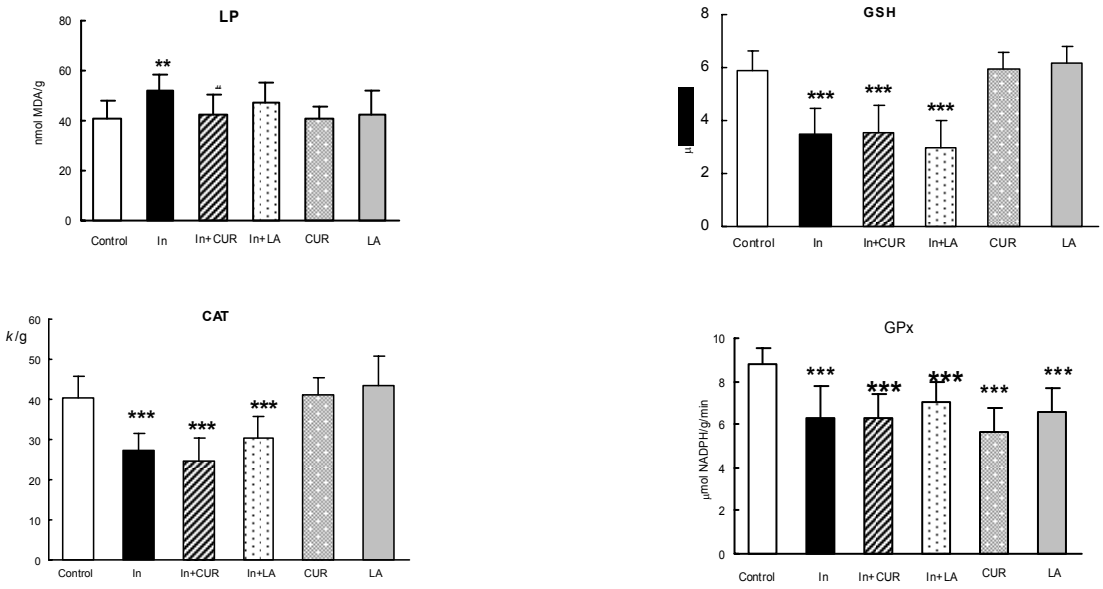

Fig. **The effect of curcumin (CUR) and alpha-lipoic acid pretreatment on hepatic lipid peroxidation (LP) and glutathione (GSH) levels and activities of catalase (CAT) and glutathione peroxidase (GPx) in In-treated mice;** significant difference: *** P<0.001, ** P<0.01 vs. Control group, # P< 0.05 vs.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

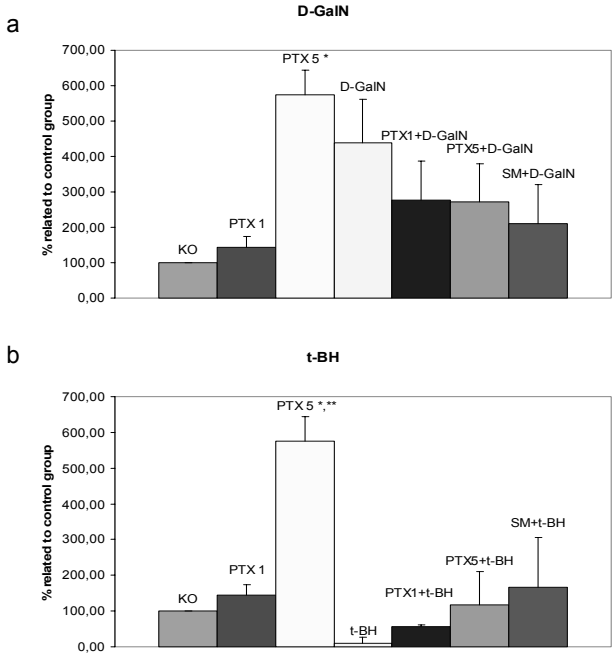

Fig. 1. **Caspase-3 like,** KO – control group – complete medium, D-GalN – D-galactosamine 5 mM in monotherapy, t-BH – *tert*-butylhydroperoxide 1 mM in monotherapy, PTX 1 – pentoxifylline 1 mM in monotherapy, PTX 5 – pentoxifylline 5 mM in monotherapy, PTX 1+ D-GalN – D-GalN 5 mM + 30 minutes pretreatment 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

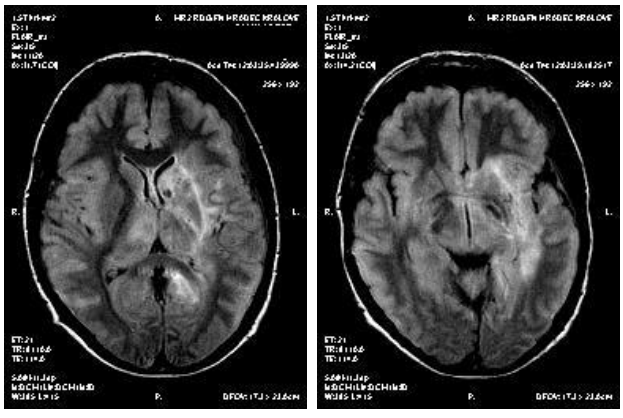

Fig. **MRI (T2-weighted images) showed reduced signal intensity in thalamus**; symmetrical hyperintensity lesions in the corticospinal tracts and ventral part of the pons, in deep cerebral white matter including the centrum semiovale, periventricular white matter, posterior limb of the internal capsule,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

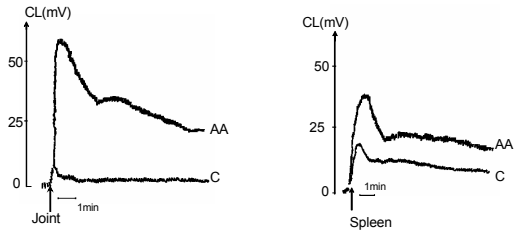

Fig. 1. **Chemiluminescence curves representative of 14 -15 separate samples of tissue**

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}).

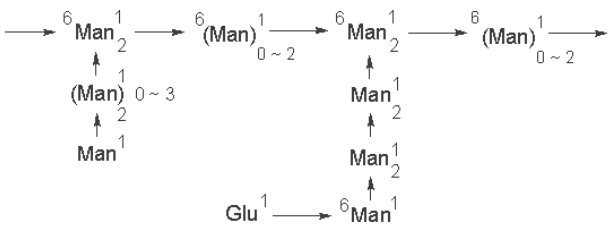

Fig. 1. **Structure of tested GM. α-(1→6)-D-mannopyranosyl backbone carrying mannooligosaccharidic side chains** containing α-(1→2) linkages, some of the side chains are terminated with non-reducing D-glucopyranosyl residues

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].

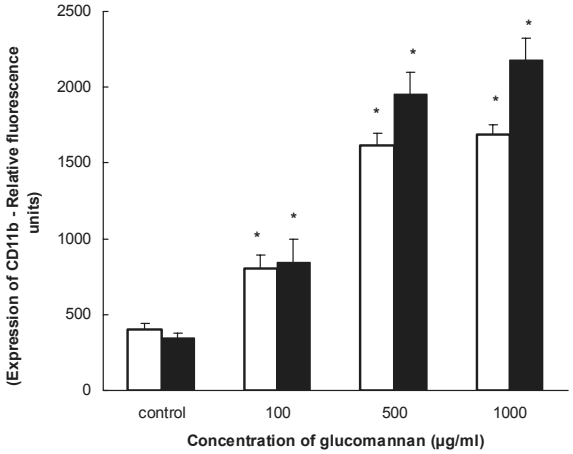

Fig. 2. **Flow cytometric detection of CD11b expression both on monocytes (white bars) and PMNL (black bars)** incubated with GM (100, 500 and 1000 μg ml^{-1}) for 3 hours; data are expressed as relative fluorescence units (mean ±SEM, n=8). Asterisks indicate statistically significant differences (P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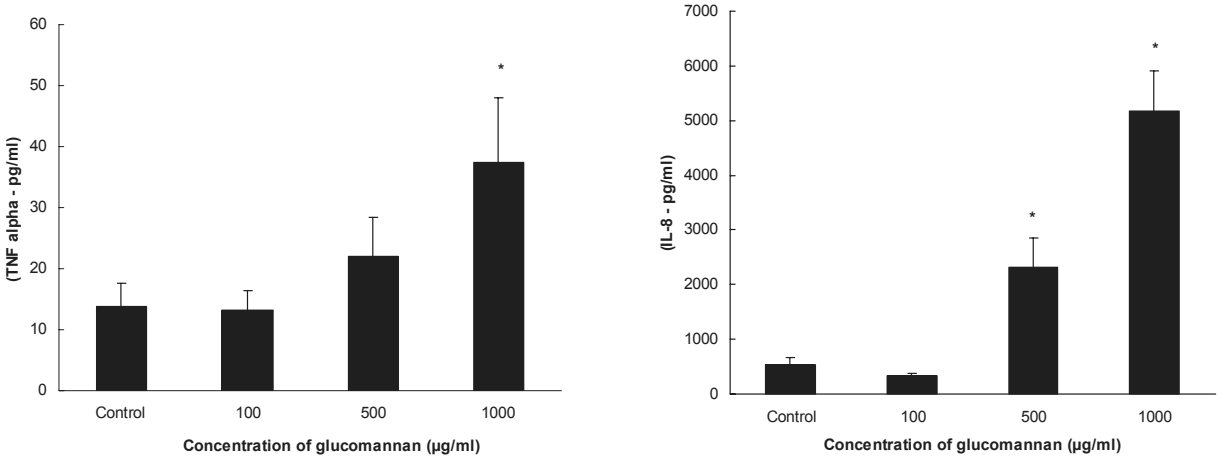

Fig. 3. **Production of TNF-α and IL-8 in the whole blood incubated with GM (100, 500 and 1000 μg ml^{-1}) for 24 hours;** data are expressed as concentration of cytokines (pg ml^{-1}) in plasma (mean ±SEM, n=8). Asterisks indicate statistically significant differences (P<0.05) compared with control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