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THE USE OF BIOLUMINESCENT BIOREPORTER *Pseudomonas fluorescens* HK44 FOR MEASURMENT OF TOXICITY OF ENVIRONMENTAL POLLUTION

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Key words: bioluminescence, biosensor, toxicity, *Pseudomonas fluorescens* HK44

Introduction

Pseudomonas fluorescens HK44 is a genetically modified bioluminescent bacterium, whose light intensity is increased by numerous chemical compounds (polyaromatic hydrocarbons, naphthalenes, salicylic acids etc.). This predetermines the bacterium for construction of luminescent biosensor for detection of environmental pollution with PAHs. On the other hand toxic compounds cause decrease of the light intensity, which can be used for toxicity assessment. This review summarizes results of measurements and prediction of toxicity to strain HK44.

Pseudomonas fluorescens HK44 is a *lux*-based bioluminescent bioreporter that harbors the pUTK21 plasmid which carries the *nah* genes coding for naphthalene metabolism linked to *luxCDABE* gene cassette coding for bioluminescence (λ_{max}=490 nm). Induction of the *nah* genes by intermediate of naphthalene metabolism salicylic acid results in associated induction of the *luxCDABE* genes and increase in light intensity. Bioluminescence can be induced by any compound that is either structurally similar to salicylic acid or which is metabolized to such compound (such as naphthalene is metabolized to salicylic acid)[2,3]. So far several tens of inducing compounds are known including substituted salicylic acids, salicylic acid-like compounds (e.g. 2-aminobenzoic acid), substituted naphthalenes, naphthalene-like compounds (e.g. quinoline), and polyaromatic hydrocarbons (PAHs)[4–7].

Strain HK44 has been originally designed for simple luminescent monitoring of the polycyclic aromatic hydrocarbons degradation in soils[1]. However inductive bioluminescence enabled other application as a monitoring of cell viability during immobilization process[8]. Major application is seen in biosensing of environmental pollution. Use of free and immobilized cells showed that the bioluminescent response is rapid and it follows the saturation type dependence on inducer concentration[7–9]. The cells responded significantly to naphthalene concentration as low as 0.02 mg l^{-1} (ref. [7,9]). Cells immobilized in silica matrix were reusable for several months and tens of induction cycles[8].

Use of *P. fluorescens* HK44 for toxicity measurement

Use of reusable biosensor for detection of environmental pollution however is not without difficulties. In order to give repeatable data, the cells must be kept under physical and chemical conditions that avoid stresses. However environmental pollution is usually toxic in some way. Although strain HK44 is well adapted for PAH based environmental pollution, a risk of cell damage by toxic compounds must always be taken into account. If possible, toxicity of the sample should be determined before biosensor is applied. As strain HK44 emits significant luminescence even if the inducer is absent measurement of bioluminescence decrease seems to be the right toxicity assay for this purpose[4]. Application of the same bacterial strain for both purposes would ensure no harm is made to the cells integrated in the biosensor.

In our previous study bioluminescence response to 72 compounds was measured in wide concentration range (from $3.2 \cdot 10^{-8}$ mol l^{-1} to saturation); out of them 41 had inhibitory effect on HK44 bioluminescence at elevated concentrations. A five-parameter-equation was proposed for overall description of concentration-bioluminescence dependence, based on the presumption of two independent effects (induction and inhibition) of compound on bioluminescence. Values analogical to EC50 (concentration which causes effect on 50 % of test population) can be calculated in inflection points using non-linear regression[4]:

$$RBL = \{1 + [(L_{max}-1)\, c^n / (c_n + K_L^n)]\}\{1 - [c^m / (c^m + K_I^m)]\} \quad (1)$$

where L_{max} stands for maximum possible bioluminescence, c for concentration of the inducer, n for coefficient of sigmoidity for induction (higher n gives steeper curve), m for coefficient of sigmoidity for inhibition, K_L for concentra-

tion at which the curve reveals the first inflection, K_I for concentration at which the curve reveals the second inflection (corresponding to desired EC50 toxicity value). For compounds that do not induce bioluminescence of the HK44 cells, the K_L value approximates to infinity and equation (*1*) is reduced to:

$$RBL = 1 - [c^m / (c^m + K_I^m)] \quad (2)$$

These results suggest that measurement of inhibition of bioluminescence of HK44 could be also used as a general toxicity assay comparable to standard Microtox® test, which uses luminescent marine bacterium *Vibrio fischeri*[10]. Both strains possess the same lux system thus the same instrumentation is needed. Strain HK44 is a soil bacterium, therefore the test would not require use of saline water as Microtox® does. Also the results for measurement of soil pollution would be more realistic. The main disadvantage of application of strain HK44 is its genetic modification. Although this strain is approved for field application in the USA[11] in Europe manipulation with GMO is allowed only in authorized laboratories.

Prediction of toxicity

So far no quantitative structure-toxicity relationship for strain HK44 was published. However Bundy *et al.* (2001)[5] measured toxicity using related *Pseudomonas fluorescens* strain expressing *lux* genes constitutively. A good correlation of EC80 values to octanol-water partition coefficient (log $P_{O/W}$) was found, however for better correlation the compounds were split into two independent sets. We have used GUHA (General Unary Hypotheses Automaton) data-mining method for automatic formation of structure-toxicity hypotheses[12]. Although the results were only binary (toxic/nontoxic), similar relationship of log $P_{O/W}$ to toxicity was found. Naphthalenes substituted by aliphatic groups (higher log $P_{O/W}$) were usually non toxic as compared to naphthalenes substituted by polar group. Relation of log $P_{O/W}$ to toxicity is a well known fact[13], however for salicylic acids not the only one. Salicylic acids substituted at position 4 induced bioluminescence and were not toxic compared to salicylic acids substituted in positions 3, 5 and 6. This relation among chemical structure, toxicity and bioluminescence corresponds to more common observation of lower the toxicities of compounds which are metabolized by strain HK44.

This work was supported by the Grant Agency of Czech Republic (grant numbers 104/05/2637, 203/06/1244), the Ministry of Education, Youth and Sports (projects ME 892, ME893, MSM 002 162 0822 and MSM 002 162 7501) and the Grant Agency of Academy of Sciences of Czech Republic (research plan AVOZ40320502). The authors would like to thank Dr Miloň Tichý DS for invaluable advice.

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SKIN SENSITIZATION TESTING OF CHEMICALS IN TEXTILE MATERIALS

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Key words: local lymph node assay (LLNA), textile materials, dyes, chemicals, DNCB

Introduction

Chemicals contained in textile materials include dyes, stabilizers, antimicrobial agents and others. They can release from the clothes by various ways (water, sweat, mechanic friction) on the human skin. Skin exposure to some textile materials may lead to allergic dermal reactions or respiratory symptoms in humans[1–4]. Published papers show that possible risk factors of development of allergic dermatitis are textile dyes. Special groups in risk are people with innate predisposition to atopy and small children[1–3].

Allergic reactions on various chemicals in textile materials may represent serious problems to human health and therefore newly developed chemicals need to be adequately tested for sensitizing potential before widespread use. Our study was aimed to examine possible sensitization potential of selected extracts of textile materials with content of dyes, formaldehyde, heavy metals: arsenic, cadmium, lead, chromium (Table I) using local lymph node assay assessing the proliferative activity of lymphocytes[5]. The method is based upon the fact that sensitizing chemicals initiate a primary immunological response in the local lymph nodes, characterized by lymphocyte proliferation.

Material and methods

Animals: Inbred Balb/c strain female mice 7–10 weeks old were obtained from Velaz Praha. The mice were allowed to acclimatize for 10 days prior to first exposure. Animals were maintained at temperature 22 ± 2 °C, relative humidity 40–70 % and with natural light/dark cycle and fed by standard diet and water *ad libitum*.

Positive and negative controls: 0.1 % solution of 1-chloro-2,4-dinitrobenzene (DNCB, Sigma) was used as a positive control and imitation of human sweat as a negative control.

Test extract: Extracts from textile materials were prepared in imitation of human sweat according to STN norm.

In vitro murine auricular lymph node assay: Mice in groups of ten received daily topical application of 25 μl of the test extract on the dorsum of both ears. Control mice were treated with an equal volume of the solution of imitation of sweat alone. The treatment was repeated for twenty-eight consecutive days (excluding weekends). One day following the final exposure, all mice were injected intravenously via the tail vein with 250 μl of PBS containing 20 μCi [^{3}H]-methyl-thymidine (specific activity 2.0 mCi mmol^{-1}, Amersham). Five hours later, mice were sacrificed and draining auricular nodes were excised. A single cell suspension was prepared using the glass homogenizer. Cells were washed once with a PBS and then dissolved in 2.5 ml of Soluene-350 (Packard instruments) for 48 hours. [^{3}H]-TdR incorporation was measured by β-scintillation counting.

The results were expressed as counts per minute (cpm) and index. Index was calculated according to the following formula: proliferative response in exposed auricular lymph node [cpm] / proliferative response in control lymph node [cpm].

Statistical analysis was performed using Student T-test.

Results and discussion

This paper describes testing of skin sensitisation potential of chemicals in textile materials using local lymph node assay (LLNA). In LLNA, mice were epicutaneously administered with extracts of eight textile materials (Table I) in imitation of human sweat. One known allergen – 0.1 % solution of 1-chloro-2,4-dinitrobenzene (DNCB) was used as a positive control and imitation of human sweat as a negative control. Results of the *in vivo* proliferation response of lymphocytes in local auricular lymph nodes of Balb/c mice are summarized in fig. 1. Results are presented as the mean ^{3}H-TdR incorporation for each experimental group in counts per minute and as stimulation indices (SI).

DNCB is known strong allergen[6]. As expected, the maximum proliferation response was observed in lymph nodes of mice exposed to 0.1 % DNCB. Statistical analysis

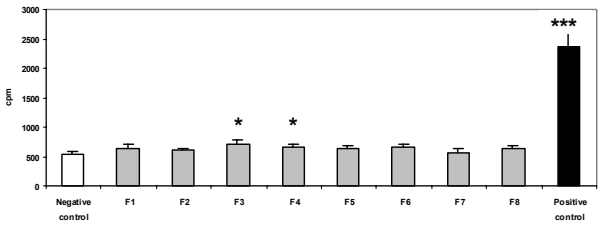

Fig. 1. **Proliferative activity of lymphocytes in the mice lymph node after application of extract from textile materials**

Table I

Identification of the sample	Characterization of textile material / used dyes
F1	Fabric from 100% cotton – shirt – anthracite – melange (content of formaldehyde 101 mg kg^{-1}) direct dyes (azo dyes) and reactive dyes
F2	Knitwear from 100% cotton – T shirt – black (low color stability – water, sweat, saliva) direct dyes (azo dyes) and reactive dyes
F3	Knitwear from 100% cotton – children T shirt – red (low color stability – water, sweat, saliva) direct dyes (azo dyes) and reactive dyes
F4	Knitwear from mixture of cotton/elastan – chidren shorts – red (low color stability – water, sweat, saliva) direct dyes (azo dyes) and reactive dyes
F5	Knitwear from mixture of polyamide/elastan – ladies stocking – black (high content of chromium) acid dyes and metal complex dyes
F6	Knitwear from mixture of polyamide/elastan – ladies underwear claret (low color stability – water, sweat) acid dyes and metal complex dyes
F7	Knitwear from mixture of 44–48% polyester/ 35–49% polyamide/ 3–6% elastan, ladies underwear – black (low color stability – water, sweat) disperse dyes, acid dyes and metal complex dyes
F8	Fabric from mixture of 55% polyester/ 45% polyamide – bed linen blue- violet, (low color stability – water, sweat) disperse dyes, acid dyes and metal complex dyes
Negative Control	Imitation of human sweat
Positive control	DNCB 0.1 % solution of 1-chloro-2,4-dinitrobenzene

revealed that proliferation of lymphocytes was highly significantly increased when compared with proliferation of cells in lymph nodes treated with negative control – imitation of human sweat (P<0.001). The stimulation index (SI) reached 3.41–5.44, mean SI = 4.42. Chemical is regarded as positive – allergen when this ratio is 3 or greater.

Stimulation indices (SI) in test mice administered with extracts of textile materials ranged from 1.06 to 1.34. Proliferation of lymphocytes in local ear lymph node of two groups of mice administered with extracts F3 and F4 of textile materials showed significantly higher values (P<0.05) in comparison with negative control. Results indicate mildly positive allergic potential. Stimulation indices (SI) in those samples were 1.34 and 1.24. Extract F3 was prepared from children red T-shirt made from cotton knitwear. Second extract F4 was done from textile mixture of cotton/elastan colored with red dye. Textile was used for preparation of children shorts. Both materials had poor color stability to water, sweat and saliva. According to the expertise provided by VUTCH-Chemitex, both textile materials were most probably colored with direct dyes (azo-dyes) and reactive dyes.

Treatment of experimental mice with extracts of textile materials resulted in lower proliferation response in local ear lymph nodes when compared to positive control exposed to DNCB. All stimulation indices were below level 3 and samples did not reach criteria of classification to be considered as allergens.

We acknowledge Viera Vachalkova, Helena Turazova and Edita Mrvikova for technical support. This work was supported by Grant Agency of the Slovak Republic APVT-99-010604.

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TOXICOLOGICAL PROFILE OF ALUMINIUM

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Key words: professional exposure, non-occupational exposure, dialysed patients, serum

Introduction

Several deaths have been reported in 1960s after occupational exposure to finely powdered metallic aluminium (Al) used in paints, explosives and fireworks. It should be noted that changes in production technology, the use of breathing masks and controls of the dust levels in factories have resulted in decreased occupational exposures to finely powdered aluminium. Factory workers who breathe large amounts of Al dust can have lung problems, such as coughing. Pulmonary fibrosis is the most frequently reported respiratory effect observed by workers after long-time exposure to fine aluminium dust, aluminium oxide, or bauxite. However, the reports prove the fibrous potential of Al. In some of the cases, the fibrosis was attributed to concomitant exposure to other dusts with content of silicon oxide. For example, pulmonary fibrosis has been observed in a number of bauxite workers. By these workers, it is very likely that there was simultaneous exposure to silica and that this latter was the causative agent rather than the aluminium[1].

In the 1980s dialysed patients (DP) were heavily overloaded with Al. Their daily intake of Al was not only from food and drinking water but also from Al-antacids such as phosphate binders and Al-containing dialysis fluids. At present time the mentioned factors have been eliminated: elimination of Al content in dialysis concentrate by producer, purification of water for preparing dialysis solution, application of antacids on the base of calcium carbonate, not on the base of Al. The removal of Al by dialysis is not easy because almost 85–90 % is protein-bound aluminium, thus only a small amount of Al (10–15 %) is diffusible and ultrafilterable. Elevated aluminium levels have been the cause of various disorders, including dialysis encephalopathia or dementia, Al-induced bone disease and microcytic anaemia[2].

The goal of this study was determination of Al levels in biological material of the aluminium exposed persons (Al industry, patients with chronic renal insufficiency) and in the control groups. With comparison of these values we judged the actual degree of loading from Al.

Materials and methods

The amount of aluminium can be measured in the blood (serum), urine, dialysis fluid or cerebrospinal fluid. These measured values are important for the diagnosis of intoxication, monitoring of Al exposed persons.

We detected the concentration of Al in the blood serum and in the urine of five groups of samples. The detailed characteristics of individual groups are shown in Table I. The data of the biggest group of DP was summarised in 1999–2006. The average concentrations per years indicated the progress of Al levels.

The samples of serum in volume of 1 ml were prepared from 5 ml of native blood. The determination of Al in urine was realised in 20 ml of the morning urine. All samples were stored in refrigerator at 5 °C and analysed within three days. The used analytical method was atomic absorption spectroscopy in graphite furnace (GFAAS) with detection limit of sample 0.1–5 μmol l^{-1} and the atomise temperature 2700 °C. The atomic absorption spectrophotometer AAS Varian SpectrAA 30 P was used with graphite furnace GTA-96. All measurements were performed at the Clinic of Occupational Medicine and Toxicology, Faculty Hospital Martin.

We analysed relationship between age and years of Al exposure to the level of Al in biological material.

Table I
Groups characteristics, the selected biological material, the number of investigated samples of persons with their age and Al exposure in years

	Group 1	Group 2	Group 3	Group 4	Group 5
Characteristic	control children	control adults	smelters	administration	dialysed patients
Biological material	serum	serum, urine	serum, urine	serum, urine	serum
N	24	38	24	15	1110
Age ±S D [years]	5.78±0.57	38.74±10.22	45.96±7.55	44.00±8.73	58.88±14.49
Al exposure ±SD [years]	–	–	16.17±11.06	15.33±7.56	3.64±3.67

Table II
The average group concentrations Al in serum [x ± SD μmol l^{-1}] and in urine [x ± SD μmol l^{-1}; μmol mol cr^{-1}]

Group	Al in serum ± SD [μmol l^{-1}]	Al in urine ± SD [μmol l^{-1}]	Al in urine ± SD [μmol mol cr^{-1}]
G1 – control children	0.320±0.04	–	–
G2 – control adults	0.502±0.18	1.144±0.68	89.75±42.33
G3 – smelters	1.223±0.43	3.268±2.67	341.97±188.81
G4 – administration	1.406±0.58	1.772±1.04	225.60±87.18
G5 – dialysed patients	1.050±1.26	–	–

Results of the examinations were processed by mathematical and statistical methods. Arithmetic mean values (x) and the standard deviations (±SD) were calculated. Groups were compared by using Student´s t-test.

Results

All results of the group average concentrations are illustrated in Table II (cr. = creatinine in urine). We tried to prove that the content of Al in the blood serum increased by age and by Al exposure. Correlation coefficients of all groups were calculated for relation between a.) age and concentration of Al in the serum and b.) exposure and concentration of Al in the serum. These values (from interval <–0.037; 0.421>) do not confirm relation between Al concentration in the serum and the age.

The average concentrations of smelters and administration were increased in comparison to the control group of adults (P<0.001). Therefore, these values do not reached the biological limit 2.224 μmol l^{-1}. These average concentrations of two Al factory groups (smelters and administration) were not significantly different. The highest average concentration of aluminium in urine was measured in group of smelters. But this concentration was smaller than indicative BMH 600 μmol mol cr^{-1}.

In comparison to the control group of adults, dialysed patients did not have increased concentrations of serum aluminium till year 2002. Only 8 samples had higher level than 2.224 μmol l^{-1}, what is EU recommended level for DP. Reason for increased concentrations of serum aluminium in years 2002–2006 was in 20 cases of higher Al level in the same dialysed centre.

Discussion

The recent measurements of aluminium concentrations in the serum of population without occupational exposure revealed the normal background levels 0.320–0.502 μmol l^{-1}. The threshold limit value for aluminium in the blood serum in Slovakia is not defined. The extent of normal values is determined by the concentrations of Al in the serum of the general population and this interval is used as the background. Commission of the European Community (CEC) recommended reference normal value for individuals with normal renal function < 10 μg l^{-1} (= 0.371 μmol l^{-1})[3]. Other authors presented reference Al value in the serum: 0.110 μmol l^{-1}(ref.[4]). Because of the ubiquitous nature of aluminium contamination, we assume that there is a direct relationship between Al in the environment of the Martin Region and the content of Al in the body.

The average Al serum concentration in children was non-significantly lower than in adults. We did not prove that the serum concentration of Al in the population increased with age. Taking into consideration life style, treatments in the past, the eating habits, and some samples of children showed higher concentration of Al in serum than in the adults.

Although aluminium is already not present in the dialysate in the recent ten years we reported increased serum Al concentrations in dialysed patients in 2002–2006 comparing to the control group. But these concentrations were safe. The Spain study analysed the changes in the aluminium content in dialysis fluid and the effect on serum aluminium in 17 dialysis centres in Spain in 8 years. The concentrations of serum were decreasing from year to year onto the value 25.7 μg l^{-1} (= 0.953 μmol l^{-1})[5]. The interval of our average concentrations was from 0.518 μmol l^{-1}in 2002 to 1.309 μmol l^{-1} in 2006. Sulkova described a patient, where aluminium was the cause of various disorders, including dialysis encephalopathy and aluminium-induced bone disease proved by a bone biopsy, and microcytic anaemia. This concentration of Al in the serum before administration of desferrioxamine was 460 μg l^{-1} (= 17.049 μmol l^{-1})[6]. In case of higher Al serum levels we recommended the desferrioxamine test, as well. Commission of the European Community (CEC) recommends reference desirable value by chronic renal failure patients < 60 μg l^{-1} (= 2.224 μmol l^{-1}) and points out the value > 200 μg l^{-1} (= 7.413 μmol l^{-1}) where an urgent action is required, because high risk of toxicity exists in all cases[3].

Aluminium balance in haemodialysis depends mainly on the gradient of diffusible aluminium, on the type of dialysis membranes, on their surface and thickness and also on many other factors, such as the pH of the dialysate. Among all these factors, the most important is the concentration of aluminium in dialysis fluids[7]. Maximum allowed concentration for dialysis fluid is 30 μg l^{-1}(= 1.112 μmol l^{-1}) – CEC recommendation[3].

The increased of values Al in serum of DP in last years is the evidence that the regular annual monitoring of the serum concentration of Al of dialysed patients remains necessary.

The study was the part of Faculty grant 1/2004 of Comenius University Jessenius Faculty of Medicine in Martin, (Slovak Republic).

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DEGRADATION OF HYALURONAN SAMPLES WITH THE ADDITION OF ASCORBIC ACID, Cu(II), Fe(II)

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Key words: hyaluronan, ascorbic acid, hyaluronan degradation, metal ions

Introduction

Hyaluronan (HA) is a high molecular weight biopolysaccharide discovered by Meyer and Palmer in 1934 (ref.[1]) in the vitreous humor of cattle eyes. HA consists of repeating disaccharide units composed of *N*-acetyl D-glucosamine and D-glucuronic acid linked by a β 1-4 glucosidic bonds while the disaccharides are linked by β 1-3 bonds[2]. It plays an important role for both mechanical and transport purposes in the body, e.g. it gives elasticity to the joints and rigidity to the vertebrate disks and it is also a constituent of the major importance in the vitreous body of the eye. Ascorbate is a potent water-soluble antioxidant capable of scavenging various types of reactive oxygen and nitrogen species, however in the presence of redox-active transition metal ions, ascorbate can also act as a pro-oxidant[3].

The aim of this study was to investigate the function of trace concentrations of Fe(II) and Cu(II) ions in the ascorbate autoxidation, in which hyaluronans of various molecular weight are involved as the indicators of the pro- or antioxidative properties of the system.

Material and methods

Six hyaluronan samples used in our study were kindly donated or purchased from the following manufactures: Fidia Farmaceuti S.p.A., Abano Terme, Italy; Genzyme Corp., Cambridge, MA, U.S.A.; Lifecore Biomedical Inc., Chaska, MN, U.S.A.; Sigma-Aldrich Chemical Company, St. Louis, MO, U.S.A.; CPN, Ústí nad Orlicí, Czech Republic. Analytical purity grade NaCl and $CuCl_2 . 2 H_2O$ were from Slavus Ltd., Bratislava, Slovakia; ascorbic acid was from Merck KGaA, Darmstadt, Germany. The water was of Milli-QRG quality (Millipore Corp., Bedford, MA, U.S.A.). $FeCl_2 . 4 H_2O$ was purchased from Penta, Chrudim, Czech Republic. Dynamic viscosity (η) of HA samples (2.5 mg ml^{-1} in 0.15 M-NaCl) was carried out without and with the addition of ascorbate[4]. In the next series of experiments dynamic viscosity (η) of HA samples after the addition of ascorbate (100 μM) followed by Cu(II) or Fe(II) ions, respectively was measured. The tested metal ion concentrations were 5.0 μM $CuCl_2$, 5.0 μM $FeCl_2$ in the system HA-ascorbate. All the measurements were carried out at 25±0.1 °C by using a digital rotational viscometer Brookfield DV-II PRO (Brookfield Engineering Labs., Inc., Middleboro, MA, U.S.A.). Viscosity of the samples was monitored in 3 min intervals for up to 5 h using a rotational spindle at a speed of 180 rpm equipped with a Teflon cup/spindle set of coaxial geometry constructed in our laboratory.

Results

Without exposing HA samples to action of ascorbate and metal ions, the continuous increase of the monitored h value for any of the investigated HA samples was monitored[4]. Rheopectic behavior of the HA solutions could be changed by trace amounts of metal cations. This fact was verified by time-dependent hyaluronan viscometric degradation when applied ascorbate together with Cu(II) or Fe (II) ions, respectively (fig. 1). As can be seen in fig. 1 (right panel), the character of the time dependence of η value upon the addition of $FeCl_2$ can be described as a gradual monotonous concentration-dependent decline, while the addition of $CuCl_2$ (left panel) resulted in a literally "exponential" drop of η value in a very short time interval, after which the decrease of η value continued however at a much lower rate. A possible explanation of this dissimilarity may be most probably in different reaction kinetics of the processes leading to the generation of reactive oxygen species in a system ascorbate *plus* $FeCl_2$ and in that of comprising ascorbate *plus* $CuCl_2$. A plausible explanation and the most simple conclusion might be to state that this HA sample is "heavily contaminated" with metals characterized with pronounced pro-oxidative properties. Another statement could be that the HA macromolecules with large(r) size are more susceptible to chain scission.

Discussion

Hyaluronan is in interest of many researchers due to its high molecular weight, it has anti-angiogenic, anti-inflammatory and immunosupressive properties, while intermediate or low sized fragments act predominantly in an opposite way. Since ascorbate acts as a powerful reducing agent with a standard reduction potential of 0.287 V at pH 7 for the redox couple $Asc^{\bullet -}$/AscH, it is assumed that it reduces traces of transition metals present in the HA samples, particularly Fe(III) and partially also Cu(II). Due to the presence of these metals, the Fe(III)/Fe(II) and Cu(II)/Cu(I), catalyzing ascorbate autoxidation leads to the gen-

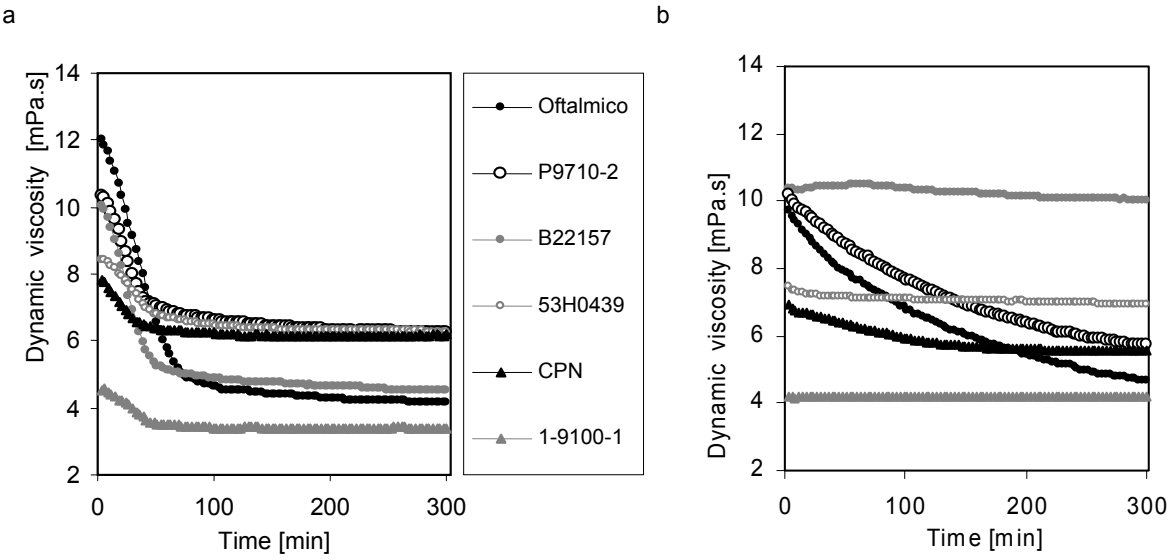

Fig. 1. **Time dependences of dynamic viscosity of HA solutions;** a) solutions of native HA samples with addition of 100 μM ascorbic acid and 5 μM Cu(II), b) solutions of HA samples with addition of 100 μM ascorbic acid and 5 μM Fe(II)

eration of •OH radicals that, after a certain initiation time period, promotes degradation of HA macromolecules, which is manifested by a gradual decrease of the solution dynamic viscosity.

The formation of •OH radicals can occur in several manners, whereas by far the most important *in vivo* mechanism is mediated by hydrogen peroxide. One of the routes of the direct H_2O_2 formation under aerobic conditions involves the system comprising ascorbate plus Cu(II) described already more than half a century ago by Weissberger et al.[5].

In aqueous milieu, hydroxyl radical represents the most reactive species, which virtually reacts with all the compounds containing C-H groups under the abstraction of hydrogen radical (H•) leading to the generation of the corresponding *C*-centered radical. In the case of the hyaluronan macromolecule, the attack of the •OH radical usually occurrs at the C-1 (or C-4) atoms of the D-glucuronate/D-glucuronic acid unit. Hydroxyl radicals may also affect the D-glucuronate/D-glucuronic acid units or *N*-acetylglucosamine moieties of HA leading to the opening of the pyranose ring(s) without cleaving the polymer chain. However, subsequent radical reactions or rearrangement of the generated *C*-centered radicals may produce polymer fragments of lower molecular weight. In a wide variety of biological *in vitro* systems Fe(II) salts and/or non-enzyme complexed ferrous cations (e.g. Fe(II)-EDTA) have been shown to enhance oxygen radical damage by increasing the production of an oxidative species generally believed to be the free hydroxyl radical.

The work was supported by the Grants VEGA 2/5002/5, 2/7028/27 and the Grant APVV-51-017905.

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P60

ANTIHYPERGLYCEMIC AND ANTIOXIDANT EFFECT OF COMPLEX $[Cu_2(H_2O)(sal\text{-}\beta\text{-}ala)_2] \cdot H_2O$ IN ALLOXAN-INDUCED DIABETES IN MICE

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Introduction

Copper(II) complexes of Schiff bases derived from salicyladehyde and various amino acids belong to the class of low molecular antioxidants. The antiradical effect of aqua-bis(*N*-salicylidene-β-alaninato)dicopper monohydrate – $[Cu_2(H_2O)(sal\text{-}\beta\text{-}ala)_2] \cdot H_2O$ was studied under *in vivo* using a model of alloxan-induced diabetes in mice. The development of hyperglycemia and lipid peroxidation in mice stomachs and livers after intravenous administration of alloxan (120 mg kg^{-1}) was evaluated as an index of oxidative damage. The studied copper complex administered intraperitoneally in doses of 40 μmol kg^{-1} significantly suppressed the aloxan-induced hyperglycemia. The antihyperglycemic activity of Cu(sal-β-ala) was comparable with the protective effect of the native enzyme superoxide dismutase. The glucose level of the animal treated did not differ from its initial value. The pre-treatment with the complex 3 hrs before alloxan injection also reduced the mildly increased levels of TBARS and conjugated dienes appeared in the liver and stomach of diabetic mice.

Copper(II) complexes containing a tridentate Schiff base derived from salicylaldehyde and various amino acids have exhibited significant biological activity – antimicrobial[1], anti-inflammatory[2] and radioprotective[3]. Their biological action was suggested to arise from antiradical activity. The superoxide radical scavenging effects were demonstrated in a series of *in vitro* experiments[4,5]. Alloxan is a well-known diabetogenic agent which causes damage to pancreatic cells by producing free radicals and decreasing the level of antioxidative enzyme systems[6]. Thus, the alloxan system was considered adequate for the study of free radical pathology and for screening the properties of antioxidative drugs *in vivo*. From the above mentioned group of copper complexes, the aqua(*N*-salicylidene-β-alaninato)dicopper monohydrate – $[Cu_2(H_2O)(sal\text{-}\beta\text{-}ala)_2] \cdot H_2O$ was chosen to study its efficacy in the attenuation of alloxan-induced diabetes.

Material and methods

Aqua-bis(*N*-salicylidene-β-alaninato)dicopper monohydrate (Cu(sal-β-ala) in abbreviation) was prepared by the method described in[7], alloxan and superoxid dismutase (SOD) were purchased from Sigma. Female ICR mice (25–30 g) were used. Alloxan was dissolved in saline solution and injected into mice in the dose of 120 mg kg^{-1} *via* the tail vein. Cu(sal-β-ala) was administered intraperitoneally in the dose of 40 μmol kg^{-1}, enzyme superoxid dismutase (SOD) in the dose of 150 U g^{-1} according to the time schedule given in Table I. Glucose venous blood concentration was measured using glucoseoxidase assay (Glucotrend®). The animals were fasted for a minimum of 3 hrs before blood collection. The parameters of lipid peroxidation: thiobarbituric acid reactive substance, sulfhy-

Table I
The protective effect of studied copper(II) complex and SOD against alloxan induced hyperglycemia in mice

Treatment		Blood glucose level [nmol l^{-1}]			
		0.day	1.day	2.day	3.day
Saline		7.6 ± 0.4	22.9 ± 2.7	24.8 ± 3.8	25.0 ± 5.4
Cu(sal-β-ala)[a]:					
	6 hr before alloxan[b]	7.1 ± 0.8	10.6 ± 6.5**	12.3 ± 5.8*	11.5 ± 2.9*
	3 hr before alloxan	7.3 ± 0.6	6.9 ± 1.3***	8.3 ± 1.9**	7.3 ± 1.7**
	3 hr after alloxan	7.6 ± 0.7	6.1 ± 2.4***	7.2 ± 1.8***	6.8 ± 0.9**
	6 hr after alloxan	7.5 ± 0.9	6.1 ± 2.2***	10.7 ± 7.4*	8.3 ± 2.1*
SOD	3hr before alloxan	6.0 ± 0.4	6.7 ± 0.6***	7.3 ± 0.1**	7.2 ± 0.3**

[a] Cu(sal-β-ala) intraperitoneal dose 40 μmol kg^{-1}, SOD 150 U g^{-1}, [b] alloxan intravenous dose 120 mg kg^{-1}. Data expressed as mean ± SEM, n=8; statistical significance : $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared control-alloxan group (student´s t-test)

Table II
Lipid peroxidation parameters in mice stomach and liver measured on 3.day after administration of alloxan

Treatment	Stomach		Liver		
	TBARS nmol mg^{-1} protein	SH-groups nmol mg^{-1} protein	TBARS nmol mg^{-1} protein	SH-groups nmol mg^{-1} protein	Conjugated dienes nmol mg^{-1} protein
Saline	0.3 ± 0.02	83 ± 27	0.06 ± 0.02	83 ± 24	20.3 ± 3.9
Alloxan + saline	0.4 ± 0.02	49 ± 21	0.1 ± 0.01	69 ± 20	26.5 ± 7.1
Alloxan + Cu(sal-β-ala):					
6 hr before alloxan	0.3 ± 0.05	71 ± 10*	0.08 ± 0.01	74 ± 27	17.3 ± 6.6
3 hr before alloxan	0.2 ± 0.05*	93 ± 25*	0.06 ± 0.01*	74 ± 20	14.9 ± 4.4*
3hr after alloxan	0.3 ± 0.05	74 ± 35	0.08 ± 0.01	79 ± 39	16.2 ± 7.6
6 hr after alloxan	0.2 ± 0.05	84 ± 20*	0.07 ± 0.02	65 ± 24	16.3 ± 6.0

[a] Cu(sal-β-ala) intraperitoneal dose 40 μmol kg^{-1}, SOD 150 U g^{-1}, [b] alloxan intravenous dose 120 mg kg^{-1}. Data expressed as mean ± SEM, n=8; statistical significance : $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared control-alloxan group (student´s t-test)

dryl groups and conjugated dienes were determined spectrophotometrically by method[8]. All parameters were expressed *per* 1 mg of protein.

Results and discussion

Table I shows that intravenous administration of alloxan resulted in significant hyperglycemia in the mice. Plasma glucose increased to 22.9–25 nmol l^{-1} in comparison to the base level of 7.56 nmol l^{-1}. Hyperglycemia was evaluated until 3 days after injection of alloxan, when maximum β-pancreatic cell should be damaged; subsequently the hyperglycemia did not change. The administration of Cu(sal-β-ala) to animals reduced the elevated blood glucose level with efficiency depending on the application schedule. The cytoprotective effect of Cu(sal-β-ala) was demonstrated not only by pre-treatment but also by administration afterwards of alloxan. Complete significant protection against alloxan induced hyperglycemia was provided by Cu(sal-β-ala) when administered 3 hrs before and after alloxan administration. As shown in Table I, changing the interval between Cu(sal-β-ala) and alloxan to 6 hrs diminished the protection. The antihyperglycemic activity of Cu(sal-β-ala) was comparable with the protective effect of the native enzyme superoxid dismutase. The glucose level of the animal treated did not differ from its initial value.

The role of free oxygen radicals in the mechanism of toxicity of alloxan was declared *in vitro*[9]. Alloxan is reduced by biological reducing agents such as cysteine, gluthatione to dialuric acid; the latter readily autooxidizes, establishing a redox cycle for the generation of superoxide radicals and hydrogen peroxide. Superoxide dismutase and catalase evidently protected β-pancreatic cell of isolated pancreatic islets against alloxan cytotoxicity[9]. The square pyramidal structure of the studied complex is similar to the coordination of the copper(II) in the active centre of the native enzyme SOD. SOD-mimic activity of Cu(sal-β-ala) was demonstrated *in vitro*[4] and could be considered as playing a main role in the antihyperglycemic effect in alloxan induced diabetes. The copper(II) complexes of Schiff bases derived from salicylaldehyde and various amino acids (alanine, valine) also showed antihyperglycemic activity in alloxan-induced diabetic mice[10].

Several studies proved that alloxan produced oxidative stress in other tissues, causing peroxidation of membrane lipids and protein glycation[11,12]. In our experiments, lipid peroxidation was determined by measuring TBARS in liver and stomach, and also conjugated dienes in the liver. The changes in the levels of SH-groups, caused by oxygen radicals were also measured. Lipid peroxidation presented in Table II was increased in alloxan-induced diabetic mice as compared to the control, although these were not statistically significant. Similarly, Yadav et al.[13] have found increased lipid peroxidation in the heart in diabetic rats while no significant changes were observed in the liver and kidney. Treatment with Cu(sal-β-ala) evidently lowered the higher levels of TBARS in both organs and conjugated dienes in the liver when administered 3 hrs before injection of alloxan. The increased SH-groups were found after this treatment in the liver. On the basis of our results chemical reactivity of the studied copper complex in different methods of free radical production was confirmed. Excluding the antiradical activity of copper complexes in the antidibetic effect, the role of copper in insulin metabolism and altering of antioxidative systems should be taken into account[14].

This work was supported by Grant VEGA No. 1/4300/07, 1/3416/06 and APVV 20-030804.

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CYTOTOXICITY OF PLANT EXTRACTS FROM GENUS *Philadelphus* L.

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Keywords: cytotoxicity, *Philadelphus subcanus*, *Philadelphus tenuifolius*, *Philadelphus schrenkii*, A431 cells

Introduction

The great attention has been devoted in previous years to natural plant products, because of their broad spectrum of constituents, which exhibit various biological activities with positive effects on human organism[1]. Some members of the genus *Philadelphus* L. (Hydrangeaceae) are known for their antibacterial, antiradical and immunomodulatory effects[2].

This study describes results of cytotoxic properties screening of ethanol extracts from three decorative shrubs from genus *Philadelphus* L.

Material and methods

Leaves were collected at Arboretum Mlyňany in September 2004. All samples were identified by Ing. Hoťka and voucher specimens are deposited there. Plant material was dried at room temperature. Samples were homogenized and macerated (10.0 g) in 200 ml of 96 % ethanol for 1 week in a dark room at room temperature. Vacuum dried samples were stored at room temperature until experimental utilization. Human skin carcinoma cell line (A431) was used for toxicity studies. Cell suspension was cultured with 20 µl of various doses of individual extracts (dissolved in DMSO – dimethylsulfoxide, its final concentration never exceeded 0.1 % (v/v) in either treated/ control-DMSO samples) for 24 and 72 h. Control cells were incubated in culture medium only. Tested doses of all three plant extracts were in the range of 2.5–50 µg dry material ml^{-1}. The culture medium and tested extracts were changed for fresh ones every 24 h. All extracts were tested in triplicates on the same cell batch. The cell proliferation was evaluated by the colorimetric MTT [3(4,5--dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma, USA) method. The absorbance was measured at 630 nm. The antiproliferative effects of individual extracts were expressed by ED_{50} values calculated from dose response curves by computer program (GraphPad Prism 4.00, GraphPad Software, USA). For statistical analysis Student´s t-test (for time dependence) and One-way ANOVA (for tested extracts) were used.

Results and discussion

The effects of ethanol extracts from leaves of genus *Philadelphus* L. on the proliferation of A431 cells were examined by the MTT assay. Dose response curves constructed in the range 2.5–50 µg ml^{-1} indicate: *a*/ decreasing number of viable cells with increased concentrations of all three extracts, *b*/ time dependent antiproliferative effects (fig. 1, 2 and 3). ED_{50} values (Table I) confirmed graphical

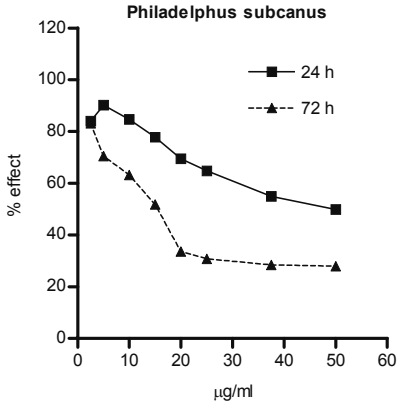

Fig. 1. **MTT cytotoxicity dose response curves of ethanol extract from leaves *Philadelphus subcanus*** after 24 h and 72 h

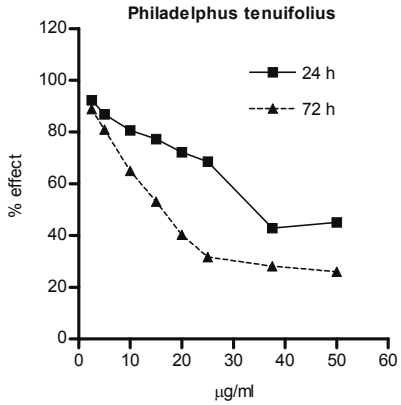

Fig. 2. **MTT cytotoxicity dose response curves of ethanol extract from leaves *Philadelphus tenuifolius*** after 24 h and 72 h

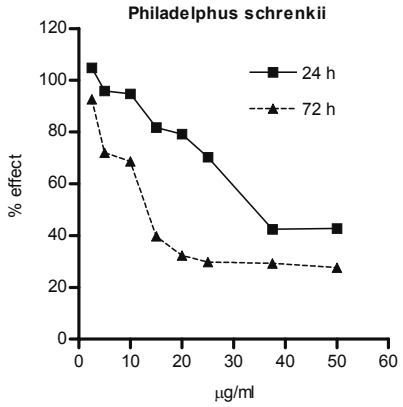

Fig. 3. **MTT cytotoxicity dose response curves of ethanol extract from leaves *Philadelphus schrenkii*** after 24 h and 72 h

Table I
ED_{50} values calculated from dose response curves on figs. 1, 2 and 3. The values are means ± SE of three separate experiments performed in triplicates for each dose

	ED_{50} [μg ml^{-1}]	
	24 h	72 h
Philadelphus subcanus	57.3 ± 2.4 [a]	13.8 ± 1.1 [b]
Philadelphus tenuifolius	44.6 ± 1.9 [a]	14.8 ± 1.4 [b]
Philadelphus schrenkii	32.8 ± 1.7 [a]	13.6 ± 1.3 [b]

[a] $P < 0.001$ (for time dependence), [b] $P<0.01$ (for tested extracts)

results e.g. lower toxicity manifested by all three extracts after acute treatment. After 24h extract from *Philadelphus schrenkii* Rupr. elicited significantly higher toxicity in comparison with other tested plant extracts. The lowest activity displayed the extract from *Philadelphus subcanus* Koehne. Chronic treatment is characterized by the shift of dose response curves to the left. This phenomenon indicates higher toxic efficacy as compared to the short time exposure. This relation was confirmed for all three plant extracts investigated while the effectiveness of individual extracts was comparable. Different acute cytotoxicity of the tested plant extracts on A431 cells may be based on their various chemical composition and relative content of biologically active substances, yet unknown for tested plants. The literature describe the presence of flavonoids, steroids, saccharides, amino acids, terpenes, saponins, phenolic acids and coumarins in genus *Philadelphus* L., while the biological activity is attributed mainly to triterpenes, flavonoids, coumarins or saponins[2,3−7]. This survey provides fundamental data about antiproliferative properties of ethanol extracts from leaves of *Philadelphus subcanus* Koehne, *Philadelphus tenuifolius* Rupr. et Maxim. and *Philadelphus schrenkii* Rupr. (Hydrangeaceae). Further studies are required to identify detailed chemical composition in leaves of the above plants and to investigate mechanism(s) involved in cytotoxicity.

This work was supported by the grant VEGA 1/4289/07 and GUK/10/2007.

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DYSFUNCTION OF HEART AND LIVER MITOCHONDRIA IN RATS EXPOSED TO TRANSIENT ISCHEMIA OF THE BRAIN AND CHRONIC INHALATION OF MINERAL FIBRES

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Key words: brain ischemia-reperfusion, asbestos and wollastonite inhalation, heart and liver mitochondria, toxic products translocation

Introduction

It is generally accepted that mineral fibres such as asbestos and wollastonite can induce inflammatory effects at chronic inhalation[1]. Similarly, inflammation processes are documented in brain due to ischemia-reperfusion injury[2]. However, effects of brain ischemia as well as chronic fiber dust inhalation on extracerebral, resp. extrapulmonal organs are not known. The objective of the present study was tu evaluate the chronic effects of fiber dust inhalation as well as the acute effect of transient cerebral ischemia on the function of heart and liver mitochondrial respiratory chain in rats.

Material and methods

Animals

Male Fisher 344 rats suplied by Charles River Company (Germany) and male Wistar rats supplied by Velaz (Czech Republic) were housed at 22±2 °C, 45 % relative humidity, 12 hour light/dark photoperiodicity in air-conditioned rooms with free access to standard commercial laboratory pellets ST1 (Top Dovo, Slovak Republic) and water ad libitum. All animals received human care in compliance with the Institutional Animal Ethic Committee and with the Guidelines of European Convention for the protection of Vertebrate Animals Used for Experimental Purposes.

The animals were assigned into the four groups (6 animals per group) as follows:

a) Control group without treatments for each experimental group,
b) I/R: acute 50-minute ischemia / 8-days reperfusion of the brain in Wistar rats,
c) A: chronic 6-months exposure to asbestos fibrous dust inhalation in Fisher 344 rats,
d) W: chronic-6 months exposure to wollastonite fibrous dust inhalation in Fisher 344 rats.

Brain ischemia – reperfusion and mineral fiber dusts exposure

Acute cerebral ischemia/reperfusion injury was accomplished by our original surgical procedure for three-vessels occlusion (3-VO) as an vascular model of Alzheimer´s dementia and neuronal degeneration[3]. Minimally invasive transmanubrial approach was used for the occlusion of both, the brachiocephalic trunk (including the right common carotid artery and right vertebral artery) as well as the occlusion of the left common carotid artery (fig. 1).

Animals were exposed to asbestos (amosite) and wollastonite fibrous dust in a nose-only inhalation device (In-Tox, Albuquerque, NM, USA). Dust aerosol was produced at dosage of 60 mg m^{-3} for one hour per exposure. Every exposure was controlled by aerosol withdrawal from the inhalation chamber onto membrane filter (Sartorius, GmgH, Germany) and weighing the dust deposits.

Mitochondrial studies

After 3-VO and after fibrous dust exposures the rats were sacrificed and subsequently the hearts and livers were remowed and placed in an ice-cold isolation solution containing (in mmol l^{-1}) 225 manitol, 75 sucrose and 0.2 EDTA; pH 7.4. Mitochondrial protein concentration was estimated by the method of Lowry[4] using bovine serum albumin as a standard.

Respiratory chain function was measured in a respiratory buffer containing (in mmol l^{-1}) 100 HEPES, 5 KH_2PO_4, 120 KCl, 0.5 EDTA and 2% dextran; pH 7.2 at 30 °C, using Clark-type polarographic oxygen electrode[5]. Sodium glutamate (5 mmol) was used as a NAD substrate for complex I. To iniciate state 3 respiratory activity, 500 nmol of ADP was added to the cuvette. When all the ADP was converted to ATP, state 4 respiration was measured.

The results were evaluated using ANOVA and Student´s t-test for unpaired data, $P<0.05$ were considered as significant.

Results

There was significant decrease of respiratory control ratio RCR in heart and liver ($P<0.001$) mitochondria after the chronic inhalation of asbestos as well as in heart ($P<0.001$) and liver ($P<0.01$) mitochondria after chronic

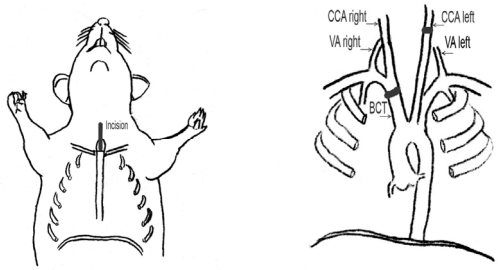

Fig. 1. **Acute cerebral ischemia/reperfusion injury,** ***P*<0.01

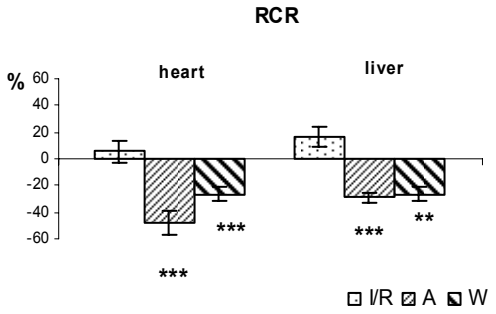

Fig. 2. **Respiratory control ratio**, ***P<0.001, **P<0.01, (change in % vs. adequate control group)

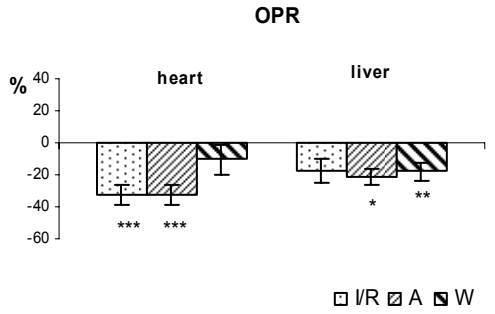

Fig. 4. **Oxidative phosphorylation rate**, ***P<0.001, **P<0.01, *P<0.05 (change in % vs. adequate control group)

inhalation of wollastonite as a consequence of mitochondria membrane damage. In the group of acute I/R injury the RCR was found to be not significantly increased in heart and liver mitochondria (fig. 2).

Significantly decreased oxygen uptake stimulated by ADP QO2(S_3) was found in heart mitochondria after acute I/R injury (P<0.001) and in the liver mitochondria after chronic inhalation of asbestos (P<0.05) as well as after chronic inhalation of wollastonite (P<0.01) (fig. 3).

The rate of ATP production OPR was significantly decreased after acute I/R injury and after chronic inhalation of asbestos in heart mitochondria (P<0.001). In liver mitochondria the rate of ATP production was decreased only after the chronic inhalation of asbestos (P<0.05) and wollastonite (P<0.01), but not after I/R injury of the brain (fig. 4).

Discussion and conclusion

Ischemic and toxic injury lead to mitochondrial dysfunction, with subsequent decrease of ATP generation, inflammation and free radical production[6,7].The lung diseases associated with mineral fibres exposure as well as brain disorders associated with ischemic damage are characterized by inflammation and generation of free radicals[1]. It is known that mediators of inflammation may translocate from the primary target organs through lymphatics into the blood and into the secondary target organs[8,9] Thus, we suppose that reactive oxygen species due to ischemia/ reperfusion injury of the brain as well as microparticles and toxic products of lung inflammation due to chronic inhalation of asbestos and wollastonite fibrous dust, translocate through the regional lymphatics and blood circulation into the secondary target organs leading to dysfunction of heart and liver mitochondria.

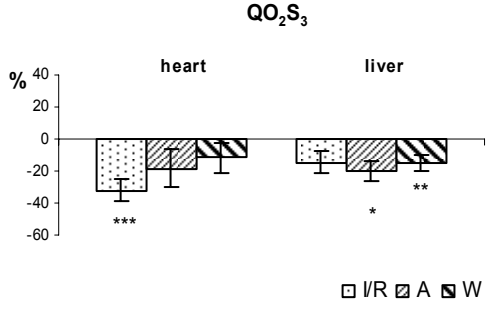

Fig. 3. **ADP stimulated respiration**, ***P<0.001, *P<0.05 (change in % vs. adequate control group)

Supported by Slovak Grant Agency (Project APVV-21-022004), VEGA (project 1/3442/06) and EU (project QLK4-1999-01629). Technical assistance: Ľudmila Butašová.

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CADMIUM AND BLOOD-BRAIN BARRIER

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Key words: cadmium, intratracheal, intragastral, blood-brain barrier

Introduction

Cadmium belongs to metals with dangerous toxic potential to human health. Inhalatory port of entry for cadmium is typical for occupational exposure while oral exposure is usually of non-occupational character. The aim of the study was comparison of cadmium organ retention in conditions of modelled inhalatory or oral experimental administration of this metal to laboratory animal: rat. In the centre of attention was the possibility of cadmium to reach the central nervous system.

Material and Methods

Female Wistar rats, 220–250 g body weight, kept on standard diet, water ad libitum, were used for the study. The rats were administered one dose of 86.4 μg cadmium chloride hemi (pentahydrate) (SIGMA) per kg b.wt. This dose was administered by means of intratracheal (IT) or intragastral (IG) instillation. For lack of adequate information from the Registry of Toxic Effects of Chemical Substances, dose was determined arbitrarily as 1/10 of the lowest toxic dose of cadmium chloride for intravenous administration. Rats were divided in one control (non cadmium) and 2 experimental groups (IT and IG groups). Each group consisted of 8 animals.

After the period of 48 hours the rats were sacrificed and following organs were isolated for cadmium detection: brain, liver, kidney and lung. The cadmium content was determined by means of atomic absorption spectrometry.

At first the isolated organs were stored at –20 °C. Before analysis the samples were de-frozen and weighed into tetrafluormethaxil (TFM) decomposition vessels of microwave oven (Milestone) with rotor MDR 1000/6/100/110. Nitric acid 65 % and hydrogen peroxide 30 % (both of the purity GR, Merck,Germany) were used for the decomposition. Decomposed samples were evaporated to drop, diluted with 0.4 ml HNO_3 (1+1) and transferred with demineralized water (Millipore Q Plus), into volumetric flasks 20 ml. Cadmium standard 1000±2 mg Cd l^{-1} (Merck) was used after sequental dilution for preparation of working standards for measurement by flame AAS (calibration curve range: 0–0.4 μg Cd ml^{-1}) and also for preparation of working standard of 2 ng Cd ml^{-1} for the method of standard additions (electrothermic atomization on GTA 96). As a modifier for cadmium determination ETAAS, the solution of ammoniumdihydrogen phosphate and magnesium nitrate was used.

Then samples of the mineralized organs of rats were analyzed by means of atomic absorption spectrometer SpectrAA30 Varian with graphite tube GTA 96, Zeeman background correction and autosampler for GTA 96 and Data Station DS 15. The sample was applied to pyrolytic platform inside graphite cuvette. Part of samples with higher cadmium content (lung) was processed by means of flame absorption spectrometer Spectr AA 30 with deuterium background correction.

Lung tissues from control rats and rats with IT exposure to cadmium were morphologically examined. The whole project at the start was subdued to the evaluation of the ethical commission for tests on animals.

Results and Discussion

Values for cadmium retention are expressed in the following text as means ± standard deviations (n =8).

Brain cadmium retention

Only the level of cadmium in the brain of rats after IT exposure: 0.8±0.1 ng g^{-1} was higher compared to cadmium untreated control rats brain level or to the brain level of rats with IG exposure method. Because in control or IG rats the cadmium levels were <0.5 ng g^{-1} (at the level of analytical detection by method used), results between groups could not be compared by statistical procedure.

Liver cadmium retention

The level of cadmium in the liver of rats after IT exposure: 930±80 ng g^{-1} was much higher when compared to cadmium liver retention in untreated control rats: 7.67±1.4 ng g^{-1} or to the liver cadmium retention of rats with IG exposure: 23.2±16.8 ng g^{-1}.

Kidney cadmium retention

The level of cadmium in the kidney of rats after IT exposure: 700±110 ng g^{-1} was much higher when compared to cadmium untreated control kidney retention: 15±2.1 ng g^{-1} or to the kidney cadmium retention of rats with IG exposure: 24.9± 8.1 ng g^{-1}.

Lung cadmium retention

The level of cadmium in the lung of rats after IT exposure: 2440±480 ng g^{-1} was excessive when compared to cadmium untreated control rats lung retention: <1 ng g^{-1} (at the level of analytical detection by method used) or to rats with IG exposure: <1 ng g^{-1}.

Intratracheal administration of metal used represents very efficient transport to body compartments because of relatively thin and delicate barrier of alveolar membranes, as documented by dramatic concentration differences in cadmium liver and kidney organ for IT exposure when compared to IG exposure. Advantage of IT exposure method to apply definite dose of cadmium is accompanied by local damage to lung alveolar parenchyma, as was verified morphologicaly.

Rather different is the situation for entry of cadmium in the central nervous system of rats (mammals, man including). Hematoencephalic barrier is effective to prevent excessive damage to neuroglial compartment as demonstrated by cadmium brain retention levels for IG and to a certain degree even IT exposure in presented experiment.

Although one of the tasks of the blood-brain barrier is to prevent the entry of metals, including cadmium, some literary data testify for penetration of cadmium into the central nervous system[1–3]. Character of blood-brain barrier and its role in prevention of entry of potentially toxic substances together with experimental study of the mechanism of metal penetration into the brain were described[4,5]. Interaction of physical factor – electromagnetic field and chemical factor – inorganic manganese compound in relation to manganese entry into the brain in experiment with rats were depicted[6,7]. The problem of organ distribution as well as BBB function in connection with simultaneous exposure of mice to cyanide + Cd/Pb/Mn was studied in conditions of acute or repeated administration[8]. It is obvious that the study of hematoencephalic barrier will necessitate further attention.

Conclusions

Intratracheal administration of single cadmium dose to rats resulted in distinctly higher liver and kidney cadmium retention when compared to intragastral administration of the same cadmium dose. In contrast to these results cadmium entry into the brain of rats was unsuccessful for IG exposure and cadmium was detected in very small quantities in brain of rats with IT exposure approach.

Acknowledgement for the support to MŠMT COST OC 281.004 projct.

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THE INFLUENCE OF METHANANDAMIDE AND SEX ON THE ACTIVITY OF CYTOCHROME P 450 AS A PREDICTIVE FACTOR OF XENOBIOTICS TOXICITY

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Key words: (*R*)-(+)-Methanandamide, cytochrome P 450, isolated perfused rat liver, sex difference

Introduction

Cannabinoids have a long history of consumption for recreational and medicinal reasons. The primary active constituent of the hemp plant Cannabis sativa is Δ^9-tetrahydrocannabinol (THC)[1]. In humans, psychoactive cannabinoids produce euphoria, enhancement of sensory perception, tachycardia, antinociception, difficulties in concentration and impairment of memory. Researchers in the 1970s, 80s, and 90s primarily assessed cannabis ability to temporarily alleviate various disease symptoms, such as the nausea associated with cancer chemotherapy. Of particular interest, scientists are investigating cannabinoid capacity to moderate autoimmune disorders, such as multiple sclerosis, rheumatoid arthritis and inflammatory bowel disease, as well as their role in the treatment of neurological disorders such as Alzheimer's disease and amyotrophic lateral sclerosis. Anandamide, an endogenous ligand for brain cannabinoid CB_1 receptor, produces many behavioural effects similar to those of THC, the main psychoactive ingredient in marijuana[2]. (*R*)-(+)-methanandamide is a synthetic long-lasting arachidonylethanolamide (anandamide) analogue that is metabolically stable and displays higher affinity for the cannabinoid receptor.

The enzymatic system of cytochrome P 450 (CYP450) is part of phase I of enzymatic biotransformation. It consists of many isoenzymes characterized by specific substrates and organ localization. These isoenzymes are most predominant in the liver, but can also be found in the intestine, lungs and other organs[3]. Among the diverse human genes, several have been identified to be particularly important in oxidative metabolism. They are: CYP3A4 (by far the most important), CYP2D6 (is responsible for the metabolism of many psychotherapeutic agents), CYP 2C6 and CYP2C19. CYP450 is involved in metabolism of many endogenous as well as exogenous substrates. Both, the induction and inhibition of specific CYP450 isoenzymes are important in terms of the efficacy or toxicity of drugs that are substrates for this system[3].

Interindividual variability of activity of oxidative and conjugating enzymes, especially the system of CYP450, can be based on many exogenous as well as endogenous influences e.g. sex, age, genetic factors or interactions between simultaneously applied drugs. The tendency to adapt the dosage of drug for the particular patient and to individualize and optimise the therapy to prevent adverse effects, to decrease the duration and costs of therapy is often seen in modern pharmacotherapy. One of the numbers of possible influences of drug metabolism which is frequently missed out is sex. CYP450 metabolizes many drugs including THC, the major psychoactive cannabinoid present in marijuana. The aim of this work was to investigate the effect of repeated administration of (*R*)-(+)-methanandamide on rat liver CYP2D2 isoenzyme using dextromethorphan (DEM) as a specific marker[4]. We have also studied the role of sex on the activity of CYP2D2 isoenzyme.

Materials and methods

The experiment was carried out on male and female Wistar rats (weighing 250±40 g, BioTest, Konarovice, Czech Republic) with free access to food and water, housed under the controlled conditions. After 7 days of adaptation to standard laboratory conditions, rats were randomly divided into 4 groups per 10 animals. Control group (CG) animals were treated with Tocrisolve™ 100 (Tocris Cookson Ltd.) (1 mg kg^{-1} day^{-1} intraperitoneally) and (*R*)-(+)-methanandamide group (R-MG) animals were treated with the drug dissolved in Tocrisolve™ 100 (Tocris Cookson Ltd.) at the dose of 1 mg kg^{-1} day^{-1} intraperitoneally for 7 days.

The rat liver was isolated from donors using a standard surgical technique. Cannulas were introduced into the portal vein and inferior cava vein, the liver was shortly washed out by a tempered (38 °C) saline which was changed for the perfusion medium (120 ml of Williams medium E) equilibrated with a mixture of 95 % O_2 and 5 % CO_2 in a short time. The recirculating perfusion apparatus was constructed according to the principles originated by Hugo Sachs GmbH (Germany). After 20 min preperfusion, a specific marker – dextromethorphan (DEM) (10.0 mg l^{-1}) was added as a bolus into the perfusion medium. Samples of perfusate (1.0 ml) were collected at the 30th, 60th and 120th min of perfusion and were stored at −75 °C until analysis. Quantitative analysis detecting DEM and it's metabolite dextrorphan (DOR) was performed by HPLC method (Shimadzu, Japan). Method by Zimová et all. was used to asses the levels of specific metabolite DEM and it's metabolite DOR in the perfusion medium[5].

For statistical calculations F-test and Student's t-test (Microsoft Excel 2000) were used, $P<0.05$ considered to be statistically significant difference.

Results

In the CG, the levels of CYP2D2 – dependent metabolite DOR were significantly higher compared to the R-MG in both of sexes. The levels of DOR in males were increased in the 30th (42 %), 60th (25 %) and 120th (30 %) minute in CG than in R-MG. In males /R-MG/ level of the parent drug DEM was significantly higher in 30th and 120th min and in females /R-MG/ level of DEM was significantly higher during the whole perfusion (Table I, II). The sex specific changes of the CYP2D2-dependent metabolite DOR concentrations in the perfusate evoked by a 7 day methanandamide treatment are documented in the figs. 1 and 2.

Conclusions

The model used is suitable for investigation of the activity of hepatic CYPs 450 and biotransformation processes is represented by the isolated and perfused liver. The main advantage of this model, in comparison with other methods used for measuring the CYP activity, is that conditions resemble physiological situation in the organism.

As we expected, the activity of CYP2D2 differed due to pretreatment with methanandamide. In our experiments the influence of this compound on rat CYP2D2 was inhibitive and metabolic activity of studied isoenzyme CYP2D2 in male and female rats was significantly higher in the control group than in *(R)*-(+)-methanandamide group. The levels of metabolite DOR in the pre-treated R-MG groups of rats were lower than in controls of both sexes

CYP2D6 (human orthologue of the rat CYP2D2) represents the second most frequent enzyme implicated in the biotransformation of therapeutic drugs (codeine, amitriptiline, clomipramine, imipramine and β-blockers such as propranolol and metoprolol)[6]. The literature data focused on the topic of influence of gender on CYP-450 is scarce and articles describe variable results. Conversely, more recent studies with DEM and metoprolol in extensive metabolizers showed faster clearance in men compared to women[7]. The present study demonstrated that CYP2D2 activity in the rat liver was higher in females than in males. The gender difference was distinguished in the methanandamide-pretreated rats as in the control group, where formation of a CYP 2D2-controlled metabolite DOR was significantly more stimulated in females than in males.

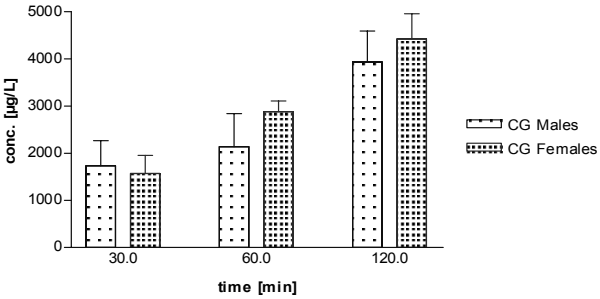

Fig. 1. **Influence of sex in CGs;** columns represent concentrations of DOR in the perfusate in the 30th, 60th and 120th minute of perfusion in males and females (*significant difference between males and females animals $P \leq 0.05$)

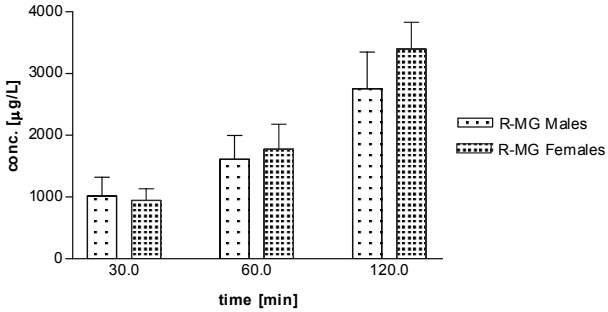

Fig. 2. **Influence of sex in R-MGs;** columns represent concentrations of DOR in the perfusate in the 30th, 60th and 120th minute of perfusion in males and females (*significant difference between males and females animals $P \leq 0.05$, **$P \leq 0.01$)

Table I
Concentrations of marker DEM in CG and R-MG in males and females rat

Time [min]	DEM [µg l^{-1}]			
	CG males	CG females	R-MG males	R-MG females
30	556.34	683.98	767.38*	972.31*
60	282.35	335.86	486,6	542.36**
120	168.33	109.23	350.64*	184.42**

* Significant difference between males and females animals $P \leq 0.05$, **$P \leq 0.01$)

Table II
Concentrations of metabolite DOR in CG and R-MG in males and females rat

Time [min]	DOR [µg l^{-1}]			
	CG males	CG females	R-MG males	R-MG females
30	1729.43	1567.47	1017.42**	945.73**
60	2135.54	2876.66	1612.47***	1776.79***
120	3935.34	4422.66	2755.07**	3399.68**

** Significant difference between males and females animals $P \leq 0.01$, *** $P \leq 0.001$

Financial support by the project MSM 002162240 is gratefully acknowledged. We thank to Květa Sedlářová and Marcela Kučírková for technical assistance.

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P65

THE INFLUENCE OF POLYPHENOLIC EXTRACTS FROM GRAPE BYPRODUCTS ON THE METABOLIC ACTIVITY OF CYP1A2 IN HYPERCHOLESTEROLAEMIC RATS

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Key words: grape byproducts, polyphenols, cytochrome P450

Introduction

Not only the amount, but especially the composition of food is an essential parameter of its positive or negative effect. The modulation of diet composition is suitable for prevention of many serious diseases of cardiovascular system, obesity or diabetes mellitus. Positive or protective effects are mainly caused by non-nutrient components like polyphenols or isothiocyanates. It is believed that they can protect various organs from free radicals damage. Also many other effects are proved by recent studies[1].

Polyphenolic substances are widely distributed in plants namely in fruits and vegetables. Thus their daily intake can be easily modified. The problem is that people are often not willing to change their eating habits and prefer using these protective substances in the form of pills or enriched food products. Great source of polyphenols are plant waste materials such as grape byproducts which are formed after grape pressing in the process of wine production and are composed of grape peels and seeds. Moreover extracts rich in polyphenols can be prepared from this material easily.

Biological effects of polyphenols are mediated by numerous mechanisms. They can interact with specific receptors[2], structures[3], signal pathways[4] or can change the activities of enzymatic systems. One of the most important metabolic systems is cytochrome P450 (CYP450). It is composed of many isoenzymes, which are localized on different sites of organism, but predominantly in liver. The majority of xenobiotics including drugs as well as many of endogenous metabolites are substrates for CYP450 and many of them can also influence its metabolic activity. Thanks to this is knowledge of inductive or inhibitory activity of administrated substances necessary for evaluation of their possible toxicity and interactions with substrates for CYP450. It is known, that flavonoids are inhibitors of CYP450 (ref.[5]) and decrease the activation of some procarcinogens to carcinogens by this enzymatic system. On the other hand some authors showed data indicating inductive activity of polyphenols on CYP450 (ref.[6]).

The aim of our work was to prepare extracts from grape byproducts rich in polyphenols and to investigate their influence on the activity of hepatic CYP450 1A2 (CYP1A2) isoenzyme in hypercholesterolaemic rats.

Materials and methods

Two types of grape byproducts were obtained from Vinné sklepy Lechovice s.r.o., CZ. Mixture of byproducts from varieties of Modrý portugal (Blauer Portugaiser) and Andre, were used for preparation of red extract (RE) and Rulandské šedé (Pinot gris) for preparation of white extract (WE). Fresh byproducts were stored frozen (–20 °C) until drying (20 °C) and homogezation.

Dried and homogenized byproducts were extracted in two steps. First with methanol (Chromservis, CZ) for 60 minutes in the ratio 1 : 1.5 (w/w) and for another 60 minutes in the ratio 1 : 1 (w/w). Deionised water was used as extraction solvent in the second step in the same schema as methanol. Water and methanol parts were mixed and evaporated on the vacuum evaporator (Laborota, Heidolph, IT).

Spectrophotometric method using Folin-Ciocalteu´s reagent for determination of total polyphenolic content and gallic acid as standard was used. Extracts were diluted with deionised water to required concentration. Total polyphenols are shown in gallic acid equivalents (GAE).

HPLC methods were used for partial qualitative analysis. The analysis was performed on HP Agilent 1100 (Agilent Technologies, GER), Luna C18(2) 150 mm, 2 μm column (Phenomenex, USA), UV-VIS detection, $\lambda_1 = 220$ nm, $\lambda_2 = 315$ nm. Mobile phases A – 5 % acetonitrile/water B – 80 % acetonitrile/water, gradient elution 0. min 100 A 40. min 100 % B. Gallic acid, catechin, epicatechin and *trans*-resveratrol were used as standards.

The work was carried out on on male Wistar albino rats (200 ± 40 g, BioTest, CZ) with free access to water and standard or hypercholesterolaemic diet (1.7 % of cholesterol, Sigma, GER). Animals were housed under the controlled conditions (lights on from 6:00 a.m. to 6:00 p.m., temperature 21–22 °C, relative humidity 50–60 %).

Animals were randomly divided into 4 groups per 8 animals. Control group animals (CG) were fed with standard diet S3 (Ing. Máchal, CZ) animals from hypercholesterolaemic group (HG), red extract group (RG) and white extract group (WG) were fed with hypercholesterolaemic diet prepared from standard diet S3 by enriching with cholesterol. Red extract was administered to RG animals, white extract to WG animals by intragastric sond in the dose of 5 mg GAE kg^{-1} day^{-1}. Water was administered to CG and HG animals. After 40 days long premedication

animals were used for liver perfusion.

The rat liver was isolated from donors using a standard surgical technique. Cannula was introduced into the portal vein and liver was shortly washed out by a tempered (38 °C) saline which was changed for the perfusion medium (120 ml of Williams medium E, Sigma GER) equilibrated with a mixture of 95 % O_2 and 5 % CO_2 in a short time. The recirculating perfusion apparatus was constructed according to the principles originated by Hugo Sachs (GER). After 20 min of pre-perfusion, a specific marker – phenacetine (PHEN, Sigma, GER) (10.0 mg l^{-1}) was added as a bolus into the perfusion medium. Samples of perfusate (1.0 ml) were collected at the 30th, 60th, 90th and 120th min of perfusion and were stored at –75 °C until analysis. Quantitative analysis detecting PHEN and its metabolite paracetamol (PAR) was performed on HPLC (Shimadzu, Japan) using method described by Jurica[7].

For statistical calculations F-test and Student´s t-test (Microsoft Excel 2000) were used, $P \leq 0.05$ was considered to be statistically significant difference.

Results

Partial qualitative analysis

We determined epicatechin and catechin as two major polyphenolic compounds of both extracts. Gallic acid was present in both extracts too instead of resveratrol, which was found only in RE.

Activity of CYP1A2

The influence of administered extracts on the activity of CYP1A2 was in general inductive. The levels of PAR were higher during the whole perfusion (WG; $P \leq 0.05$) or in the 30th min of perfusion (RG; $P \leq 0.05$) in comparison to CG animals. We have also observed increased levels of PHEN in the 30th minute of perfusion in RG ($P \leq 0.01$) and WG group ($P \leq 0.05$) compared to CG. Hypercholesterolaemic diet have no influence on the activity of CYP1A2 which was confirmed by similar concentration of PAR and PHEN in CG and HG animals. Data shown in Table I and Table II.

Table I
Concentrations of PHEN in perfusion medium

Time [min]	PHEN [mg l^{-1}]			
	RG	WG	HG	CG
30th	4.96 ± 0.96	5.01±1.92	3.29±0.58	2.81±1.01
60th	3.41±0.63	3.31±1.10	2.42±0.92	2.90±0.82
90th	1.79±0.65	2.07±0.54	1.89±0.86	2.03±0.63
120th	1.21±0.58	1.33±0.6	1.32±0.75	1.45±0.85

Table II
Concentrations of PAR in perfusion medium

Time [min]	PAR [mg l^{-1}]			
	RG	WG	HG	CG
30th	0.17±0.09	0.35±0.26	0.07±0.01	0.06±0.03
60th	0.19±0.13	0.38±0.23	0.11±0.02	0.10±0.03
90th	0.14±0.09	0.37±0.21	0.09±0.04	0.11±0.04
120th	0.15±0.11	0.31±0.18	0.11±0.04	0.10±0.06

Discussion and conclusion

The model of isolated perfused rat liver is suitable for studies focused on the activity of CYP450, because other models which are being used (isolated microsomes or recombinant cytochromes) doesn´t simulate conditions in live organism as well as perfused liver where not only organ integrity, but also system of biochemical links is preserved.

As we predicted, hypercholesterolaemic diet didn´t change metabolism of PHEN. Our opinion is that the only influence of chronic hypercholesterolaemia on the activity of CYP450 can be due to hepatic steatosis and general decreasement of hepatocytes activity. Increasement in the activity of CYP1A2 was manifested as elevated levels of metabolite PAR in the perfusion medium in animals pretreated with both types of extracts when compared to control animals. We observed differences between extracts which were manifested as increasement of PAR levels in RG only in the 30th minute of perfusion instead of elevation during the whole perfusion in animals terated with white extract. Possible explanation is induction of CYP2E1 isoenzyme metabolizing PAR caused by red extract. This can cause lower levels of PAR during the perfusion and similar concentrations of PHEN.

For inductors of CYP450 is characteristic that with increasing levels of metabolite are decreasing levels of specific marker for selected isoenzyme in perfusion medium. Our data showed increase not only in levels of metabolite but also higher levels of marker in the 30th minute. From the concentrations of PHEN and PAR in the 30th minute of perfusion and from the levels in the start of the perfusion is clear, that major part of PHEN is bound in liver and changes in binding capacity of liver for PHEN can be an explanation of observed increase of both marker and metabolite.

We prepared two different extracts from grape by-products with similar composition of major polyphenolic components. Extracts in the dose of 5 mg GAE kg^{-1} day^{-1} acted as inductors of rat CYP1A2 after 40 day long intragastric premedication, which was manifested as increased levels of specific CYP1A2 marker PHEN´s metabolite PAR in the perfusion medium in the model of isolated perfused rat liver when compared to control animals. We have also observed atypical increase of PHEN levels at the

beginning of the perfusion in animals treated with extracts. Hypercholesterolaemic diet did not influence the activity of CYP1A2 when compared to control animals.

Financial support by the Czech Science Foundation project 525/06/1757 is gratefully acknowledged.

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