VOJENSKÉ ZDRAVOTNICKÉ LISTY

ROČNÍK LXVI PROSINEC 1997 SUPLEMENTUM

IInd Czech-Slovak Toxicological Meeting

Purkyně Military Medical Academy, Hradec Králové

May 23, 1997

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Program

The opening of the conference: Prof. J. Fusek and Prof. J. Květina

Introductory lectures (8.30 - 9.30 h)

- **Benešová, O.:** The importance of adequate experimental models in the preclinical research of the drug's effect (25 min)
- **Patočka, J., Koupilová, M., Křížová, R.:** Ecotoxicology of aluminum (5 min)
- **Tichý, M.:** Information of ECVAM (European Committe for Validation of Alternative Methods) (10 min)

Part I. Alternative methods in toxicology (9.45-11.00 h)

Lectures:

- **Tichý, M., Rucki, M., Dohalský, V., Feltl, L.:** Akute toxicity indices: from tubifex worms to computers (15 min)
- **Kubová, J., Tulinská, J., Lišková, A., Horáková, K.:** In vivo and in vitro testing of immunotoxicity of xenobiotics (10 min)
- **Navarová, J., Horáková, K., Ujházy, E., Dubovický, M.:** In vivo and in vitro effects of cyclophosphamide (10 min)
- **Štětina, R., Štětinová, V., Grossmann, V.:** Oxidative DNA damage in lymphocytes and endothelial cells of aorta induced by cholesterol diet in rats (10 min)
- **Maloňová, H., Koupil, S.:** Toxicity of biologically active preparations atonik and racine (10 min)

Posters:

- **Bozsakyová, E., Chalupa, I., Blaško, M.:** Comparison of genotoxic effect of pentoxifylline and theophylline in *in vitro* cultivated V79 cells
- **Chalupa, I., Bozsakyová, E., Slameňová, D., Šebová, L.:** Frequency of structural chromosomal aberrations and micronuclei induced by pentoxifylline in *in vitro* cultivated human lymphocytes
- **Blaško, M., Lonská, H., Bozsakyová, E., Chalupa, I.:** The influence of cytochalazine B on the human malignant melanoma cells in vitro
- **Urbančíková, M.:** The effect of CU^{2+} complex induces partial restoration of actin filamentous structures in transformed cells
- **Babáčková, L., Varga-Martínez, G., Havel, J., Patočka, J.:** The estimation of benactyzine, biperiden, and benzhexol by capillary electrophoresis

A discussion to take place by the posters: 13.30-14.30 h

Part II. Varia (11.45-13.30 h)

Lectures:

- **Yamamoto, A., Starec, M., Rokyta, R., Kršiak, M., Rašková, M.:** Repeated sress and pathological changes in organs (10 min)
- **Eybl, V., Topolčan, O., Koutenský, J.:** The influence of some metals on the level of TSH and hormones of thyroid gland (10 min)
- **Ujházy, E., Dubovický, M., Kovačovský, P., Rychlík, I., Navarová, J.:** Effect of long-term administration of stobadine to pregnant rats and postnatal development of their offspring (10 min)
- **Uher, M., Pisarčíková, M., Roland, R., Filka, J., Hulíková, M., Adamová, H., Podracká, Ľ., Šašinka, M., Mocák, I.:** The organophosphate intoxication in children (10 min)
- **Sadloňová, I., Koller, J., Bakoš, D.:** Implantation tests of a new dermal substitute on mice and rats (10 min)
- **Vopršalová, M., Žáčková, P., Jahodář, L., Višňovský, P.:** Some problems in education of toxicology on the Faculty of Pharmacy UK in Hradec Králové

Posters:

- **Kuchta, M., Uher, M., Filka, J., Lovašová, D., Majlingová, S., Pisarčíková, M., Yakuciová, M.:** Possibilities of complication of drug's dependency in teenegers
- **Kadrabová,J., Maďarič, A., Kováčiková, E., Ginter, E.:** Selenium status and glutathione peroxidase activity in the blood of asthma patients
- **Hadašová, E., Nerušilová, K., Charvátová, Z.:** Influence of NO synthase inhibition on the LPS-evoked changes in the pharmacokinetics of procainamide in the rat
- **Suchánková, J., Geršl, V., Višňovský, P., Fiala, Z., Palička, V., Mazurová, Y., Vávrová, J., Voglová, J.:** The effect of acute exposure to sulphur dioxide (SO^2) on biochemical, hematological and histological parameters in guinea pigs
- **Suchánková, J., Geršl, V., Višňovský, P., Fiala, Z., Tatár, M., Karcolová, D.:** The effect of acute exposure to sulphur dioxide (SO_2) on non-invasive parameters of cardiac function in guinea pigs
- **Chorvatovičová, D., Kováčiková, Z.:** Protective effects of stobadine on blood reticulocytes and heart of Co^{60} irradiated mice
- **Křížová, R., Patočka, J.:** Cooking in aluminum-cookware as a secondary source of dietary aluminum
- **Kopecká, J., Štaud, F.:** High-performance liquid chromatography determination of dipotassium clorazepate
- **Fiala, Z., Kura, E. H., Andreassen, A.:** Biological monitoring of expositions to polycyclic aromatic hydrocarbons

A discussion to take place by the posters: 13.30-14.30 h

ECOTOXICOLOGY OF ALUMINUM

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Aluminum (Al) is the most abundant metal of the earth's litosphere[1]. Although Al is ubiquitously distributed in the environment, it has no known biological function, and there is abundant evidence that accumulation of Al in body tissues is harmful [2, 3]. Al being implicated as interfering with a variety of cellular metabolic processes in the nervous system and in other systems [4]. The toxicity of Al is usually limited by a combination of poor intestinal absorption and effective renal clearance. However, in situations such as dialysis [5] and total parenteral nutrition [6] this intestinal barrier is bypassed. Under these circumstances, the abundance of Al in foods, drugs, and water supplies can pose a serious threat. Al has been associated with several neurodegenerative disorders such as amyotrophic lateral sclerosis [7], dialysis encephalopathy [8], Parkinson dementia of Guam [7] and a number of studies have implicated Al as a possible factor in the pathogenesis of Alzheimer's disease [9, 10].

Al is ubiquitously distributed in the environment and also in all dietary sources. The diet is the main source of Al for man. Normal oral daily intake from food and drinking water is estimated in Europe to range between 2 - 10 mg per person, in the USA 20 - 25 mg per person [11]. Only a small proportion of the dietary Al is absorbed [3], but this absorbed Al must be excreted to prevent tissue accumulation. Numerous factors are involved in the control of intestinal Al absorption. Chronic renal failure has been recognized as an enhancing condition [12].

The chemistry of Al in biological systems is very complex [13]. The relative stabilities of different Al complexes, as for instance with citrate, phosphate, silicate, fluorid, transferrin, etc., are extraordinarily dependent on pH, expecially in the physiological range [14]. Thus, Al may undergo stepwise ligand-exchange as it is transported in the body. From the gut Al may be taken up as Al citrate, whereas in the blood it binds to transferrin [15]. It is very probably, that different ligands in food or water may bind Al so strongly that the resultant complex is transported unchanged through the different compartments of the body, and at the same time is able to cross the blood-brain barrier. A ligand with such properties may be citrate [16] or maltol [17]. An important role has been suggested for silicon [14].

Al compounds may occur in food and drinking water, either as natural or added ingredients. The amount of Al in different foods and beverages is varied in wide range. High concentrations of Al were search in brewed teas [18]. More important than primary sources of dietary Al may be different secondary additional ones. The most common sources of additional dietary Al are specific food additives, Al-containing water purifying agents, medical purposes used as antacids, buffered aspirins, and Al from cookware and containers. Some studies shown that naturally-occured organic acids may leach large amount of Al from Al cookware [19].

In 1976 a neurological disease was described among patients with renal deficiency on long-term hemodialysis [8]. This disease, which became known as dialysis encephalopathy, was characterized by speech disturbances, twitching, myoclonic jerks, motor apraxia, and seizures. Different psychiatric abnormalities occured with visual and auditory hallucinations the EEG patterns were altered and dementia manifested itself gradually in the course of disease. Death within a year was common. After intensive research Al was found to be the cause of dialysis or aluminum encephalopathy [20]. Al originated from the media used for dialysis of the patients. Low concentration of Al in dialysis media reduced the incidence of this disease.

Al as a possible risk factor for Alzheimer's disease (AD) has long been studied and discussed [21]. A higher than normal level of Al has been found in the core of senile plaque and this observation has raised the question of whether Al may be contribute to the development of AD. The cause of AD remains unknown, but three factors are under consideration and Al toxicity is one of them [22].

The ability of Al to induced massive degenerative accumulation of neurofilaments in neurons of cultured animal as well as human neurons [23], appears to be one of the major arguments raised to support a dismetabolism of naturally induced Al in humans as a possible cause of AD, as well as a positive correlation between the incidence of AD and concentrations of Al in drinking water [24]. A recent report suggests that the chelating agent desferrioxamine slows the rate of cognitive decline in AD patients [25]. Al from drinking water forms only a small part of the total daily intake but it may make a disproportionate contribution to the total amount absobed from the gastrointestinal tract. Because the total amount of Al found in AD brains is only 1 mg or less [18], the

consumption as a matter of fact negligible amount of Al in diet may be a consequential risk factor in the development of AD in special situations.

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ACUTE TOXICITY INDICES: FROM *TUBIFEX TUBIFEX* WORMS TO COMPUTERS

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Experiments and tests with experimental animals are integral part of protection of human and environmental health against toxic and undesired effects of chemicals. It includes studies on mechanisms of the effects and biotransformation of the xenobiotics in a body. They are inevitable in a search of medical treatments. During time, however, people have abtained more and more experiences nad knowledge on the experimental animals and their reactions on the chemicals. More and more sensitive and responsible scientists are pointing out that we have today enough information to make the testing using other principles with other experimental objects excluding animals. There are ethical rules and directives how to deal with animals experiments. Alternative methods are searched for. It is necessary to prove firstly that these alternative methods yield adequate information as the methods in vivo using higher animals.

One often forgets, however, that the searching for alternative methods have still another reason: an economical one and a desire to have quickly some, at least rough approximative, information about a hazard of a new, still unknown, substance. For these reasons, alternative methods have already a long tradition of decades. The ethical problems are a new dimension of them, only.

ECVAM (European Centre of the Validation of Alternative Methods) was established in 1991 by a communication from the European Commision to the Council and the European Parliament, responding to a requirement in the directive on the protection of animals used for experimental and other scientific purposes (Directive 86/609/EEC). It states that the Commision and Member States should encourage research into the development and validation of alternative techniques which could provide **the same level of information** as that obtained in experiments using animals, but which involve fewer or none animals or which entail less paintful procedures. Main goal of ECVAM, as defined in 1993 by the Scientific Advisory Committee, is, thus, to promote the scientific and regulatory acceptance of alternative methods which are of importance to the biosciences and which reduce, refine or replace the use of laboratory animals. One of the first priorities set by ECVAM was the implementation of procedures which would enable it to become well-informed about the state-of-art of non-animal test development and validation, and the potential for the possible incorporation of alternative tests into regulatory procedures.

Validation is the process whereby the reliability and relevance of a procedure is established for a particular purpose. This involves a pre-validation study to develop an optimised protocol and to establish its transferability, followed by an interlaboratory blind trial with coded test materials and an independent, unbiased evolution of the outcome.

ECVAM organized a number of workshops on specific topics, at which small groups of invited experts would review the current status of various types of tests and their potential uses, as well as to identify the best way forward. The workshops initiated a formation of documents published as ECVAM Workshop Reports in ATLA (28 up today). The topics include the various computer models. One of the last workshops was organized (October 1996) on the development and validation of expert systems (computerized) for predicting toxicity and metabolism.

The alternative methods and tests include the work with not only lower organisms and plants or their parts or *in vitro* experiments with isolated cells and/or tissue cultures, but also computer modelling of relationships between chemical structure of compounds and their biological activities or toxicokinetic rules.

Acute toxicity is one of a relatively quickly obtainable information about a hazard of a chemical. Determination of its indices needs long time and a large financial support and it is necessary to destroy a large number of animals. In order to develop a rapid and cheap testing of acute toxicity of compounds soluble in water, we have chosen in our laboratory, the determination of effective concentration for the inhibition of movements of the worms *Tubifex tubifex* and their lethal concentration at the same worms, respectively. The method has been described (1). For the inner correctness of the determination (QA), the use of a solution of manganese (II) chloride dihydrate was suggested, whose acute toxicity indices have been determined in a long-term observation:

logEC50 = -0.845±0.0326 (mol/L)
logLC50 = -0.726±0.0390 (mol/L).

The determination of EC50, LC50, NOEL and EC100 for one chemical lasts about three hours including preparation of the solutions and a verification of their concentrations, a pilot testing and triplicate determination of the toxicity of the tested and reference chemicals.

The Figure 1 demonstrates a correlation between logEC50 determined with the worms *Tubifex tubifex* and logLD50 determined with mice after iv. application for a series of alcohols. Although it is only a simple series of compounds and not possible to be generalized, number of such exaples has increased. It promises a hope that at least in some groups of compounds it would be valid.

The alternative methods as that abovementioned make it possible to from the data bases large enough for a development and validation of predictive computer models. They can be developed, however, even using the data obtained by a classical way, eg. with mice or other higher animals. The generalization to the predictive models is done by the techniques of QSAR (Quantitative Structure-Activity Relationships) analysis. The toxicological predictive models are also formed as expert systems based on the knowledge bases of rules and toxic fragments.

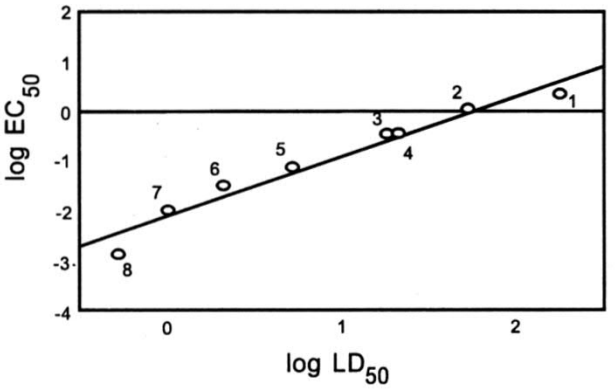

Figure 1. Correlation between logEC50 (Tubifex tubifex) and logLD50 (mice, iv.) for a series of alcohols (1).

It is already possible to use a set of predictive computer programs - models in toxicology, some of them commercially obtainable: HAZARDEXPERT SYSTEM, METABOLEXPETT SYSTEM or PROLOGD by CompuDrug Chemistry Ltd. (Budapest), ECOSAR by EPA US DERREK by LHASA (University of Leeds, UK, Harvard University, Boston, USA), TOXALERT, CASE or CASETOX by Multicase, Inc. (Cleveland, Uhio), COMPACT by University of Surrey, School of Biological Sciences, UK), ONCOLOGIC by LogiChem, Inc. (Boyertown, Pennsylvania), CLEMENTINE by Integral Solutions Ltd. (Basingstoke, Hampshire, UK), TOPKAT by Health Desing Inc. (Rochester, N. Y.) or SKIN PENETRATION, QSAR Programmes by National Institute of Public Health (Praha). Especially carcinogenicity and mutageicity are the effects of the concern. Most of them were discussed during the ECVAM workshop for development and validation of expert systems for toxicology (2).

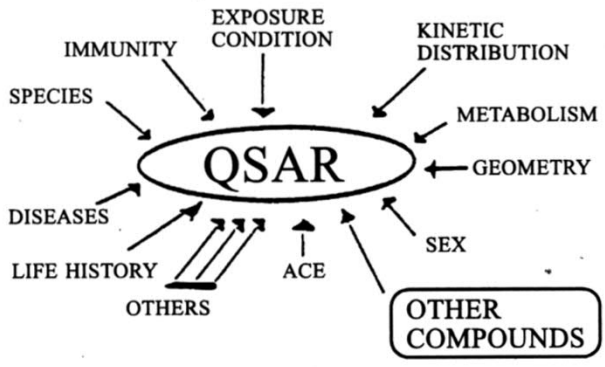

Figure 2. Factors which may influence estimates by predictive models of the QSAR type.

Although predictions by the predictive computer programs often contain an indication of their variation, many inner and outer factors can influence a value of the toxic indices predicted (Figure 2).

Validated express alternative methods make it possible not only to save laboratory animals but also to obtain a sufficient number of data in a reasonable time to study the influence of inner and outer factors on the estimates by models.

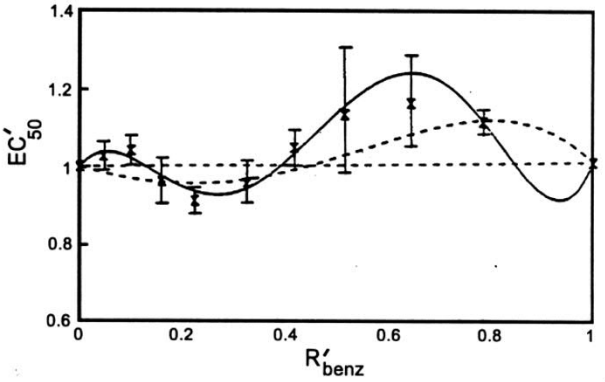

Figure 3. Dependence of acute toxicity measured as EC50 (inhibition of movement of the worms Tubifex tubifex) for the binary mixture benzene-phenol (3).

Eg. the Figure 3 demonstrates how acute toxicity index EC50 *(Tubifex tubifex)* can change if a compound is a component of a mixture. A sufficient amount of validated data for such a study is impossible to obtain by a classical testing like with mice.Thus, the alternative methods are promissing also in a research where a huge number of experimental data are needed. An extrapolation of the conclusions is another story.

And, which need not be forgoten, the final result must be proved by *in vivo* experiments if asked for a health safety. Secondly, the hazard or individual toxic activities are predicted, not the risk. All the models, including the alternative methods, are only approximations and simplifications. But for a human the mice or rats as well.

Acknowledgement

We acknowledge the support of the projects by the grants no. 203/94/1534 and 20397/1027 of the Grant Agency of Czech Republic, no. 1851-3 of the Internal Grant Agency of the Ministry of Health of Czech Republic and by the National Intitute of Public Health, Praha.

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IN VIVO AND IN VITRO TESTING OF IMMUNOTOXICITY OF XENOBIOTICS

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Introduction

In interaction between xenobiotics and immune system a variety of effects is considered - induction of immunosuppression (possibly causing deficient resistance to opportunistic infections and neoplastic disorders or sensitivity to vaccination), or immunostimulation (possibly resulting in autoimmunity or hypersensitivity).

The frequent methods for evaluation of the xenobiotics are experiments on laboratory animals. The key role in these experiments have standard and valid methods. A number of different approaches for evaluating the immune system of experimental laboratory animals as a target for immunotoxicity have been published. In 1988 Luster published the results of the US National Toxicology Program (NTP), interlaboratory program, whose purpose was to show that selected immunological assays were valid in the mouse. These assays are divided in two tiers. The first includes the weight and histology of spleen and thymus, spleen cellularity, haematology and functional assays, including the antibody response to

sheep red blood cells. The second include enumeration of the cell types including T and B cells and four functional assays for the evaluating of the cell mediated immunity: spleen lymphocyte responses to the mitogen stimulation in vitro, the mixed lymphocyte culture response, the cytotoxic T lymphocyte assay and the delayed hypersensitivity test (1).

The principal commissions of contemporary immunotoxicology are:

A. Development of **battery of tests** for detection of changes in immune system, which will be sufficiently valid as international standard in immunotoxicity testing of xenobiotics.
B. **Epidemiological studies** of immunological parameters in human exposed to xenobiotics according to chart proposed of WHO. Results of these epidemiological studies should be correlated with results of studies in laboratory animals. Parallely use of these epidemiological and experimental model of asses is very important for extrapolation of data from animals to man and risk assessment of xenobiotics.
C. The mechanisms of immunotoxic action of xenobiotics in humans should be elucidated by a combination of studies in laboratory animals in vivo and **experiments** with human and animal tissues and cell lines **in vitro**.

„Cell toxicology" is defined as the discipline aimed at studying the general principles of chemical interference with cellular structures and/or functions. Cell toxicology is relatively new, challenging sub-discipline of toxicology as the scientific basis for the development of in vitro toxicity testing strategies.

To characterize cytotoxic effect of tested xenobiotic import need to answer following questions:

1. Which concentrations are necessery to inhibit cell proliferation?
2. Which concentrations are cytolethal for non-dividing cells?
3. What are the possible targets for cytotoxic action?
4. Are there indications of an influence of biotransformation on cytotoxic potency?
5. Are there indications of differing sensitivities of differentiated cells, i.e. selective cytotoxicity? (2)

Material and Methods

The purpose of this study was to appreciate the possibility of using selected *in vitro* methods in testing schemes for evaluating xenobiotic-induced effects.

The most common used *in vitro* methods in HeLa cell line were selected for the evaluation of metabolic and functional activity of subcellular organels (mitochondria) and the determination of cell number and protein content: direct method of counting of cells, indirect method of total cell protein content assay, MTT - reductase - assay (3) as indicator of activity of mitochondries, NR (neutral red) - assay (4) based on accumulation of colouring matter in lysosomes and KB (Kenacid blue R) - assay (5) based on tinge of cell proteins.

In vivo effect was investigated in subacute immunotoxicity study in Wistar rats. Rat is the most commonly employed species in routine toxicity testing. Consequently, a great deal of data exists for any number of chemicals, in regard to their toxicity, metabolism and pharmacokinetics in the rat.

N-cyclohexyl-2-benzothiazolesulfenamide CBS) was chosen as commonly use xenobiotic in rubber industry as accelerator for rubber vulcanization.

Results and Discussion

The oral administration of CBS during 28 days resulted in significant decrease of the PFC response per spleen at a dosage of 1000 and 2000 mg/kg/day CBS. This effect probably reflects the decrease of spleen cellularity. PFC response per 10^6 spleen cells was slightly increased in rats treated with 400 mg/kg/day and slightly reduced in other tested doses. These two phase activity has much in common with two phase activity of drugs that influenced of the immune system, for example cyclophosphamide.

Proliferation activity of lymphocytes in response to STM was statistically significant increased in both lower concentrations of CBS.

No significant decrease of phagocytic activity in all tested doses was observed.

Our results indicated expressive, dose dependent and statistically significant decrease of spleen, thymus and bone marrow cellularity in the middle and the highest doses.

The data presented here has implications pertaining to the cytotoxic potential of CBS.

To characterize closely cytotoxic effect of tested xenobiotic we used in vitro methods.

Cell proliferation in response to 25-400 μmol/l CBS was inhibited dose dependently. Total inhibition was observed in HeLa cell line when 400 μmol/l CBS was added. According to ID_{50} and ID_{100} classification CBS was classified as weak toxic xenobiotic. Moreover results obtained by direct method of counting of cells and indirect method of total cell protein content assay indicated no signs of induction of unbalanced cell growth. Evidence was provided by results of total cell protein content expressed per 10^6 cells. No significant differences between any CBS treated HeLa cell line and control cells were recorded.

In the second phase of in vitro experiments MTT, NR and KB assays were used to characterize closely cytotoxic effect of CBS. MTT assay is

based on reduction of MTT dye superoxide and MTT reductase and follows mitochondrial activity. NR assay measures accumulation of neutral red in lysosomes. In KB assay cell proteins are stained by Kenacid Blue R dye. Generally lower sensitivity of indirect methods were observed in the range of cell proliferation is visible after comparison of toxicity curves.

Coincidence of methods was observed in the range of inactive concentrations of CBS.

Differences between direct method of counting of cells and MTT, NR and KB assay but also among the indirect methods were observed.

Course of toxicity curves of NR and KB assay was in mutual correlations. Cytotoxic effect evaluated by MTT assay was significantly decreased, reduction was particularly expressed in the highest concentrations. Possible explanation for lower sensitivity of MTT assay was increased production of formazan with longer crystals in comparison with control confirmed by microphotography of vitally stained HeLa cells. There were statistically significant differences in percentage of cytotoxicity determined by MTT and NR assay, MTT and KB assay respectively.

Our results indicated that CBS affects energetic metabolism of cells and induces oxygen radicals production.

In vitro methods are sufficient to identify a mechanism of effect of xenobiotic in cell and detect consequence of very low concentrations of tested chemical compound. Cell lines are homogenous and do not contain large numbers a diverse populations. Cell lines are stably available and quality controlled by an established cell bank. HeLa cell line as tumor cell line has lower activity of SOD than „normal" non-tumor cells. They are suitable and sufficiently sensitive for the detection of the induction of oxygen radicals by xenobiotics. Further advantages of these methods are low cost and rapidity. They allow to test a big number of xenobiotics in various concentrations simultaneously in multiplets and get results quickly.

There are some limitations of *in vitro* methods. First example is fact that the results from any cellular in vitro systems are not only dependent on the properties cell type used but on experimental conditions such as exposure time, medium composition etc.

Second limitations of *in vitro* systems is that exogenous metabolic activation are often required and uncertainties of extrapolating the results of experimental studies to living organism.

Findings of the present investigation suggest that *in vitro* methods should be added as the second tier after in vivo assays in toxicity testing of xenobiotics. Subsequent using of both these testing strategies could counterbalance disadvantages in vitro assays.

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IN VIVO AND IN VITRO EFFECTS OF CYCLOPHOSHAMIDE

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Cyclophosphamide (CP) is one of the most widely used antitumour and immunosuppressant drug. CP is metabolised *in vivo* and *in vitro* to reactive intermediates, phosphoramide mustard and acrolein, which are believed to be responsible for its cytostatic and toxic effect, respectively (1). The present study compared the *in vivo* effects of CP in ICR non pregnant and pregnant mice with its *in vitro* effect in HeLa cells.

In vivo experiments

ICR/DV (23-25g, 8-10 weeks old) non pregnant mice (n=10) were treated twice (24-hours interval) with CP in a dose of 80mg/kg body weight intraperitoneally (i.p.). The animals were sacrificed 24 h after the second CP injection. Activity of the lysosomal enzymes acid phosphatase (APH) and N-acetyl-β-D-glucosaminidase (NAGA), and levels

of DNA, RNA and protein were determined in tissue homogenates of spleen, liver and kidney as markers of cell damage (2, 3).

CP induced significant increase in APH and NAGA activity and decrease of DNA level in the spleen and kidney. In liver, the lysosomal enzyme activities as well as DNA and RNA content were unchanged. No changes in protein levels were recorded.

CP produced a broad spectrum of malformations in a variety of mamalian species dependent on dose and gestation time of exposure (4).

In our teratological experiment, CP in the dose of 10 mg/kg b.w. (i.p.) was administered daily from day 11 to 17 of gestation to pregnant ICR mice. On day 18 of pregnancy the animals were sacrificed and biochemical and teratogenic variables were determined. APH and NAGA activities were assayed in homogenates of maternal spleen, kidney and liver and in foetal liver.

CP induced toxic damage was associated with an increase in maternal APH and NAGA activities in the liver and spleen and with an increase in foetal APH liver activity. Protein levels remained unchanged in all maternal and foetal organs tested.

Administration of CP decreased the foetal weight and induced exophthalmos, cleft palate, limb malformations, brachycaudia, tail haematoma, hydrops abdomen.

In vitro experiments

HeLa cells were placed into test tubes and on the second day of cultivation CP was added (5) in the concentration range of 0.1-100 mmol/l. After 48 h the effect of CP on cell number (direct method) and on total protein level (3) was determined. HeLa cells in 96-well microtiter plates were exposed to the same concentrations of CP. The degree of cytotoxicity by MTT- (6), NR- (7) and KB-assay (8) was observed 24 h later (indirect methods). Cytotoxicity was characterised by ID_{20}, ID_{50} and ID_{80} values calculated as a mean of three parallel determinations.

The cytotoxic effect of CP was dose dependent. After exposure to 100 μmol/l of CP total inhibition of HeLa cell proliferation was recorded. There were no significant differences between ID_{20}, ID_{50} and ID_{80} as determined by the five methods used in the study. It is noteworthy that none of the CP concentration tested did achieve 100% cytotoxicity. According to the ID_{50} (5.9-7.4 mmol/l), CP belongs to toxic drugs in HeLa cells.

Conclusions

The results of *in vivo* experiments confirmed the embryotoxic and teratogenic effects of CP on organogenesis. CP negatively influenced also biochemical variables in the organs examined in both non pregnant and pregnant mice.

In vitro experiments using colorimetric methods showed that at the concentrations used a part of the CP treated cell population was still viable although they had lost their ability to divide.

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TOXICITY OF BIOLOGICALLY ACTIVE PREPARATIONS ATONIK AND RACINE

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ATONIK is a plant stimulant, containing 0,1% sodium-5-nitro-guajacolate (5NG), 0,2% sodium-ortho-nitrophenolate (ONP) and 0,3% sodium-para-nitrophenolate (PNP). RACINE contains in addition to that 0,1% 1-naphtaleneacetic acid sodium salt (NaNAA). The producer of Atonik and Racine is fa. ASAHI CHEMICAL MFG.CO., LTD., Osaka, Japan.

ATONIK - according to allegation of the producer - accelerates cytoplasmic streaming and metabolism of plant cells, increases the uptake of minerals, increases yields and growth of many crops etc. (6, 7).

RACINE is recommended to control the pre-harvest drop of apples (4). In our country it is registered for improving rooting of ornamental plants (5).

The field trials were conducted at 4 apple varieties in randomized blocks in 1993-1996. Both preparations were applied with handgun sprayer directly to the leaves as dilute sprays at concentration from 0,04 to 0,06%. In the frame of trials there were evaluated: fruit set, yield, level of alternate bearing, fruit quality, shoot growth, mineral leaf composition and hygienic aspects. Partial results of trials were published (1, 2, 3).

The subject of this paper is the evaluation of residues of 3 effective substances. The samples were examined by means of gas chromatography. For the identification the ECD and NPD detector was used. The sensibility for 3 examined substances was 0,01 $mg.kg^{-1}$. The analyses were conducted in the year 1994 and 1995. The residue of NaNAA was not determined. The results are summed up in the table 1.

From the table 1 it is obvious, that residues of all 3 effective substances are on or below the limit of detection. On the basis of these results it is possible to propose MLR for all 3 effectives substances 0,2 $mg.kg^{-1}$. day^{-1}. RACINE, which is applied to the end of vegetation, can leave - in contrast to ATONIK - relatively higher residues, which means that its application can be tied with certain risk.

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Key words: Plant stimulant; Atonik; Racine; Apples; Residues.

Table 1

Residues in the fruits ($mg.kg^{-1}$)

VARIETY	VARIANT	1994			1995		
		ONP	PNP	5NG	ONP	PNP	5NG
GLOSTER	treat	0,01	0,01	< 0,01	< 0,01	< 0,01	< 0,01
GLOSTER	contr	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01
IDARED	treat	< 0,01	0,02	< 0,01	< 0,01	< 0,01	< 0,01
IDARED R.	treat	0,06	0,04	< 0,01	0,04	< 0,01	0,02
IDARED	contr	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,02
MELROSE	treat	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01
MELROSE	contr	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01
STARKRIM	treat	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01
STARKRIM	contr	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01

treat = treated, contr = control, R = RACINE, STARKRIM = STARKRIMSON

COMPARISON OF GENOTOXIC EFFECT OF PENTOXIFYLLINE AND THEOPHYLLINE IN *IN VITRO* CULTIVATED V79 CELLS

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The aim of this study was to compare the genotoxic effects of pentoxifylline and theophylline in *in vitro* cultivated V79 Chinese hamster cells by cytogenetic methods. In exposed V79 cells two cytogenetic parameters were investigated - chromosomal aberrations (CA) and sister chromatid exchanges (SCEs). In both cases, after pentoxifylline and theophylline treatment increased values of the followed cytogenetic parameters were found. As to the difference in effect of the two methylxanthines, teophylline showed stronger genotoxic effect than pentoxifylline.

Pentoxifylline, a dimethyl derivative of xanthine, is an oxohexyl-substituted analogue of theobromine. The clinical use of pentoxifylline is more preferable than that of, for example, caffeine, because it is less cardiotoxic and neurotoxic. Pentoxifylline, like other methylxanthines, enhances the antitumour effects of alkylating agents *in vitro* and *in vivo* (2, 3).

Theophylline is a methylated purine derivative and a monohydrate of 2,3-dimethylxanthine. It belongs to the most important group of naturally occurring methylxanthines. It is used in clinical practice as a diuretic, in the treatment of asthma and bronchitis, as a relaxant of smooth muscle, and in ischaemic disease of the heart.

In this study we compare the genotoxic effect of pentoxifylline and theophylline on mammalian cells in vitro. Two cytogenetic endpoints -CA and SCEs - were investigated in V79 Chinese hamster cells exposed to the two above-mentioned methylxanthines.

V79 Chinese hamster cells were seeded into Petri dishes and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

In the case of **chromosomal aberrations (CA) assay** the cells were cultivated 24 h before and 18 h after pentoxifylline and theophylline exposure, respectively. Pentoxifylline and theophylline concentrations (10^{-4}, 10^{-3}, $2x10^{-3}$, $5x10^{-3}$ mol) used in this assay were selected on the basis of preliminary cytotoxicity tests. Theophylline was dissolved directly before use in dimethyl sulphoxide (DMSO). Therefore DMSO was used as a solvent control, its volume did not exceed 1% of the volume of cultivation medium. As a positive control MMC (0.5 μg/ml) was added to the cultures. Slides were prepared by the standard air drying method and were stained with 10% Giemsa for 10 min. To detect the chromosomal aberrations in V79 cells, 100 well-spread metaphases containing the modal number of chromosomes (22) ± 2 were analysed by microscopic examination. The results were statistically evaluated using the test of difference of two relative values.

In the **sister chromatid exchange (SCE) assay** V79 cells were grown in Petri dishes for 12 h. Then the tested agents and 5-bromo-2-deoxyuridine (BrdU) were added and the cultures were incubated for further 34 h in dark. Because this assay is more sensitive than the CA assay, also lower concentrations (10^{-5}, $5x10^{-5}$, 10^{-4}, $5x10^{-4}$, 10^{-3}, $2x10^{-3}$ mol) of the two chemicals were used. As a solvent control DMSO (1% of the volume of the cultivation medium), and as a positive control MMC (0.01 μg/ml) were used. Slides were prepared using the standard air drying procedure and were stained by the fluorescence-plus Giemsa (FPG) technique. Whenever possible, 100 second-division mitoses per dose were scored to determine mean SCE frequency and the number of chromosomes per cell. The frequency of SCEs per chromosome was calculated. The data were statistically analysed with the Student's t-test.

The frequency of structural chromosomal aberrations, aberrant metaphases and mean SCE frequencies in V79 Chinese hamster cells after pentoxifylline and theophylline treatment are summarized in Table 1. The data show a clear dose-related increase of all investigated cytogenetic parameters. After pentoxifylline exposure, the lowest concentration which yielded a statistically significant difference from negative control in the total number of structural CA was 10^{-3} mol, in the frequency of aberrant metaphases $2x10^{-3}$ mol and in the mean number of SCEs per cell and SCEs per chromosome $5x10^{-4}$ mol. After theophylline treatment the statistical significance of frequencies of structural CA and aberrant metaphases as compared to solvent control first appeared at the concentration of 10^{-4} mol and that of mean SCEs per cell and SCEs per chromosome at the concentration as low as $5x10^{-5}$ mol.

As our results show the both investigated methylxanthines to have genotoxic effects, while theophylline seems to be a stronger genotoxic agent than pentoxifylline.

In the research of methylxanthines the efforts are focused on caffeine. As to the other methylxanthines (such as the pentoxifylline and theophylline), despite their current application in medicine less information is available. In general, on the basis of literary data and our results we can conclude that pentoxifylline and theophylline induced CA and SCEs (1, 4, 6), so they have a clastogenic effect. Slameňová et al. (5) found that theophylline had no genotoxic effect on bacteria nor two mammalian

	Pentoxifylline			Theophylline		
Conc. (mol)	Total No. of CA	% of aberrant metaphases	SCE/cell±S.E.	Total No. of CA	% of aberrant metaphases	SCE/cell±S.E.
NC	4	4	5,46±2,2909	2	2	4,96±1,9996
SC	NT	NT	NT	1	1	5,15±2,1834
10^{-5}	NT	NT	5,96±2,5334	NT	NT	5,58±2,3545
$5x10^{-5}$	NT	NT	5,90±2,5632	NT	NT	6,26±1,9164 ***
10^{-4}	8	6	5,97±2,5708	7 *	6 *	7,97±2,4918 ***
$5x10^{-4}$	NT	NT	[a] 6,54±2,9447 *	NT	NT	9,71±2,7579 ***
10^{-3}	13 *	11	6,27±2,9928 *	10 **	10 **	9,99±3,0512 ***
$2x10^{-3}$	29 ***	24 ***	7,17±3,1750 ***	31 ***	24 ***	12,15±2,7327***
$5x10^{-3}$	50 ***	35 ***	NE	35 ***	28 ***	NE
PC	97 ***	50 ***	34,85±8,2260 ***	68 ***	51 ***	33,81±7,9230 ***

Table 1: **Frequency of chromosomal aberrations, aberrant metaphases and SCE/cell induced by pentoxifylline and theophylline in cultured V79 Chinese hamster cells. (100 metaphases in each sample were scored.)**

Abbreviations: CA - chromosomal aberrations, NE - not evaluated, NT - not tested, NC - negative control, SC - solvent control, PC - positive control (0,5 µg/ml of MMC was used in the CA assay and 0,01 µg/ml in the SCE assay)
[a] - number of evaluted metaphases is 52
Values marked with asterisks are significantly different from the negative resp. solvent control value
(* 0,01< P< 0,05; **0,001< P< 0,01; *** P< 0,001)

cell lines. These literary data are difficult to compare, because in different laboratories different concentrations of the investigated agents, different length of treatment, different biological material (lymphocytes, bone marrow, Chinese hamster V79 cells, etc.) were used. Also the mode of expressing the concentration values is problematic. In literature the concentrations of the chemicals tested are expressed mostly as mg/ml, molar (mol) values are given only rarely. Therefore the data obtained in our experiments were statistically compared in two ways: either for concentrations expressed in mol, or for concentrations recalculated in mg/ml. This statistical analysis showed that the comparison of data is more reliable when concentrations are expressed in mol.

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FREQUENCY OF STRUCTURAL CHROMOSOMAL ABERRATIONS AND MICRONUCLEI INDUCED BY PENTOXIFYLLINE IN *IN VITRO* CULTIVATED HUMAN LYMPHOCYTES

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We tested the possible genotoxic effects of pentoxifylline on human lymphocytes cultured *in vitro* using the chromosome aberration test and micronucleus test. Statistically significant elevation in the number of cells with aberrations, total number of aberrations and micronuclei was found. We conclude that pentoxifylline had genotoxic effects on human lymphocytes cultured *in vitro*.

Pentoxifylline, a derivative of xanthine, is an oxohexyl-substituted analogue of theobromine. It has become widely used clinically for the treatment of arterial disease. Pentoxifylline, like other methylxanthines, enhances the antitumour effects of alkylating agents *in vitro* and *in vivo*, and radiation

damage in tumours, a phenomenon attributed to the prevention of DNA repair in the late S-G_2 phases of the cell cycle (1, 2 ,3, 4).

As this drug is frequently used, it is desirable that its genotoxic activity be accurately tested. The aim of this study was to obtain information on the possible genotoxic effects of pentoxifylline using the chromosome aberration and micronucleus tests *in vitro*. For both assays we used human lymphocytes (from one man and one woman) cultured in the presence of pentoxifylline in the culture medium. The tested concentrations of pentoxifylline were 0.02, 0.1, 0.2, 1.0 and 2.0 mg/ml of medium. Mitomycin C (0.5 μg/ml) was used as a positive control. In the case of chromosome aberration test slides were prepared using the standard air drying procedure after 24 h of treatment of lymphocytes with pentoxifylline. In the case of micronucleus test lymphocytes were exposed 31 h and then harvested.

Chromosomal aberrations and micronuclei were scored in conventional Giemsa-stained preparations. To detect the presence of chromosomal aberrations, 100 well-spread metaphases from the male and 100 well-spread metaphases from the female, both containing 46 centromeres were analysed for each sample by microscopic examination. The number of cells analysed for the presence of micronuclei was 10,000 (5,000 from the man and 5,000 from the woman) for each concentration used. In addition, the mitotic index (mitosis/100 cells) was recorded for each treatment point. The results were statistically evaluated using the test of difference of two relative values. The induction of chromosomal aberrations and micronuclei in human peripheral blood lymphocytes by pentoxifylline is summarized in Tables 1 and 2. The data show a clear dose-related increase of all investigated cytogenetic parameters. Statistically significant elevation of the number of aberrant metaphases as well as the total number of structural chromosomal aberration was found after treatment with the concentration as low as 0.1 mg/ml of pentoxifylline in the culture medium. Statistically significant elevation in the number of micronuclei was observed from the concentration 0.2 mg/ml. Thus the results of this study show that pentoxifylline has genotoxic effects and that the chromosomal aberration test proved to be more sensitive than the micronucleus test.

Table 1

Chromosomal aberrations induced by pentoxifylline in cultured human peripheral blood lymphocytes. 200 cells in each sample were analysed.

Sample	% of aberrant metaphases	Number of chromosomal aberrations / 100 metaphases								
		Chromatid		Isochromatid		Exchange				Total no. of CA
		g	b/f	g	b/f	dic	dmin	qr	tr	
NC	0,5	7	0,5	2,5	-	-	-	-	-	0,5
P 0.02	2,5	8,5	2,5	-	-	-	-	-	-	2,5
P 0.1	6,5***	11	6,5***	5	-	-	-	0,5	-	7***
P 0.2	7,5***	17	7,5***	2,5	-	-	-	-	-	7,5***
P 1.0	42 ***	17	54 ***	1	2,5**	-	1,5*	-	-	58***
P 2.0	61 ***	44	121 ***	1	3***	0,5	0,5	1*	1*	127***
PC	73 ***	32	86,5***	13	4***	-	-	12***	18***	120,5***

Abbreviations: NC - negative control,
P - pentoxifylline (0.02-2.0 mg/ml),
PC - positive control (0.5 μg/ml mitomycin C),
CA - chromosomal aberrations
g - gap
b/f - break and/or fragment
dic - dicentric
dmin - double minute
qr - quadriradial
tr - triradial

Values with asterisks are significantly different from the negative control value (*0.01<p<0.05, ** 0.001<p<0.01, *** p<0.001).
The numbers of gaps were not evaluated statistically.

Table 2

Frequency of micronuclei induced by pentoxifyllin e in human peripheral blood lymphocytes. The micronuclei were analysed in 10,000 cells.

Sample	No. of micronuclei	% of micronuclei	mitotic index
NC	20	0.2	5.88
P 0.02	20	0.2	6.23
P 0.1	25	0.25	4.92
P 0.2	35 *	0.35	3.81
P 1.0	53 ***	0.53	1.85
P 2.0	48 ***	0.48	0.63
PC	436 ***	4.36	1.71

Abbreviations: NC - negative control
P - pentoxifylline (0.02-2.0 mg/ml)
PC - positive control (0.5 μg/ml mitomycin C)
Values with asterisks are significantly different from the negative control value (*0.01<p<0.05, ** 0.001<p<0.01, *** p<0.001).

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THE EFFECT OF Cu^{2+} COMPLEX INDUCES PARTIAL RESTORATION OF ACTIN FILAMENTOUS STRUCTURES IN TRANSFORMED CELLS

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Cytoskeleton plays an important role in most cellular processes associated with different types of movement, including cell migration, cell division, transport of organelles and molecules, secretion, phagocytosis, etc. A tetrahydroaminobenzaldehyde copper complex ($CuTAABCl_2$) exhibits a large structural similarity with a superoxid- dismutase molecule. Both molecules have four donor nitrogen atoms associated with Cu^{2+}, their macrocyclic ring system consists of sixteen atoms that make the chromophore flexible (Ďuračková and Labuda, 1995). The structural similarity is accompanied by a similar catalytic activity; they both catalyse superoxide anion to a molecular oxygen and hydrogen peroxide. To know if some cell functions which are known to be associated with actin cytoskeletal structures are affected by Cu-complex we investigated actin microfilament organisation and its changes in culture cells exposed to the Cu-complex.

Cell Culture

Human hepatoma cell line HepG2 was cultured in Minimum Essential Medium (Biocom, Brno, Czech Republic) supplemented with 10% fetal calf serum (Biocom, Brno, Czech Republic) at 37 °C in humidified 5% CO_2 atmosphere. For visualization of cytoskeletal structures cells were seeded at 4 x 10^4 cells per ml on cover slips a day before treatment. Cells were incubated in six different concentrations of $CuTAABCl_2$, i.e. 0.125 μ/ml, 0.25 μg/ml, 0.5 μg/ml, 1.0 μg/ml, 2.0 μg/ml and 4.0 μg/ml in medium without fetal calf serum for 1 hr at 37 °C.

F-actin Labeling

Visualization of actin microfilament bundles was done according to Urbančíková (Urbančíková and Grófová, 1990) with some modifications. After 1 hr incubation with $CuTAABCl_2$ cells were washed three times with PBS and fixed with 4% paraformaldehyde for 20 min. Then cells were permeabilized with 0.2% Triton X-100 for 3 min. Cells were stained with FITC-phalloidin (Sigma) at 1:1000 dilution in PBS for 30 min. Then cover slips were mounted on microscope slides with elvanol (polyvinylalcohol, Serva, Germany). Fluorescence was evaluated and photographed using Opton Axiophot microscope. For calculation of percentage of cells with filamentous structure 100 cells per sample were scored.

One criterion of cell damage is disintegration of cytoskeletal organization (Marinovich, 1990). To know if Cu $TAABCl_2$-complex affects cell architecture, actin microfilament bundles were stained with phalloidin conjugated with fluorescein and analysed by fluorescent microscopy. We found that presence of filamentous structures in the HepG2 cells after $CuTAABCl_2$ treatment was Concentration dependent. Untreated cells of

HepG2 hepatoma tissue culture exhibit heterogeneity of actin cytoskeleton pattern. Most of the cells do not possess actin bundles, so called stress fibers. Some of the cells have in cytoplasm lot of patches which represent short thick actin filaments. Many cells show perinuclear difuse staining. Only small portion of the cell population contained thick irregular bundles of actin microfilaments. HepG2 cells exposed to the lowest concentration tested (0.125 µg/ml) of $CuTAABCl_2$ for 1 h were very similar to untreated cells, but we did not find cells with very thick irregular bundles. There were some cells with at least two times greater volume then we observed in control population. Exposure to 0.25 µg/ml $CuTAABCl_2$ resulted in creation of a very fine net of filaments in some cells. The number of cells with filamentous structures as well as the thickness of filaments increased up to 2 µ/ml $CuTAABCl_2$. Four µg/ml $CuTAABCl_2$ caused depolymerization of filamentous structures in comparison to lower concentrations. Based on cell proliferation experiments (IC_{50} = 0.05 µg/ml for 24 h of exposure) we assume that partial restoration of actin filamentous structures in transformed cells by $CuTAABCl_2$ leads into partial restoration of regulation mechanism of cell proliferation which was manifested by inhibition of cell proliferation.

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THE ESTIMATION OF BENACTYZINE, BIPERIDEN, AND BENZHEXOL BY CAPILLARY ELECTROPHORESIS

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Benactyzine, biperiden and benzhexol are centrally effective cholinolytics. Benactyzine has spasmolytic, antihistaminic and anticholinergic effect and in the therapy found use at anxiety, stammer treatment and stage-fright repressing. Biperiden has anticholinergic and antiparkinsonic effect. Biperiden is suitable for the treatment of all forms of Parkinsonism and to keep down neuroleptic-induced extrapyramidal side effects. Benzhexol, known also as trihexyphenidyl, is the drug with anticholinergic, spasmolytic and antiparkinsonic effects. Its pharmacological profile is similar to a biperiden, but with higher centrally stimulating effect (1,2).

All these three cholinolytics are able to antagonize the muscarinic receptor-associated effects of acetylcholine in organophosphate nerve agent intoxication and give a good account of effective antidote therapy (3, 4).

In recent years a number of new techniques such as capillary zone electrophoresis (CZE) have been developed. These techniques involve miniaturization to a significant degree, thus reducing costs and being much friendlich to the environment. CZE has been established as an alternative method to GC and HPLC for studies of the metabolism and pharmacokinetics of different drugs (5, 6). The utilization of CZE for separation of some central active cholinolytics we studied earlier (7).

The possibilities of estimation of three centrally active cholinolytics by CZE, i.e. benactyzine, biperiden and benzhexol, and thereby using this for therapeutic drug monitoring have been studied in this paper.

The CZE separations were performed on a Beckman apparatus CZE, model P/ACE system 5500, equipped with diode array detector. The fused-silica capillary of 75 µm I.D. was 36.6 cm of the total length and 29.0 length to detector. Electrokinetic sampling for 10 s at 5 kV and separation voltage 15 kV has been used. UV absorbance was detected at 195 nm.

The electropherograms of benactyzine, biperiden and benzhexol showed that the separation and detection is possible in less than seven minutes, using very low sample volume 30 µL. The separation and reproducibility of the migration times were very good. The calibration curves were linear in the range from 0.1 to 10 µg/mL. The detection limits defined as S/N = 3 were 0.1 µg/mL for benactyzine, 0.2 µg/mL for biperiden and 0.3 µg/mL for benzhexol. We found that CZE is very useful for determination of these compounds in different drug application forms. The pilot studies

with CZE estimation of all three cholinolytics in urine and blood serum were also performed but the optimization of method will be necessary.

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REPEATED STRESS AND PATHOLOGICAL CHANGES IN ORGANS

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Introduction

Many studies of stress tried to find a common denominator of this phenomenon with the hope to understand its complex nature, and on the other hand to disclose any simple markers that could be applied in medical practice. Different methodological approaches, different models of stress, different functions, and different strains studied brought the controversial results. The effort is therefore concentrated on the maximal reduction of many intervening variables where besides others the reactivity pattern of the autonomic nervous system can play a central role. In this context the using of the physiologically defined inbred strains seems to be highly recommended.

In our experiments, two inbred Wistar rat strains with different sensitivity to isoprenaline were used. The isoprenaline sensitive rats (IS) react on the injection of isoprenaline with large myocardial lesions while the rats resistant to isoprenaline (IR) have significantly smaller myocardial defects (Mráz et al., 1995). Besides physiological differences substantial behavioral differences between IS and IR rats were described (Starec et al., 1996).

The acute stress evokes in both strains the heart pathology of the same extent as isoprenaline. Moreover, the gastrointestinal system represents another target influenced by stress, however, the opposite effect in these two strains was observed. The greatest gastric lesions occurred in IR rats. The inverse results were obtained when animals were prestressed with mild stressors. The degree of ulceration was greater in IS rats (Rašková et al., 1996).

The aim of our study was to examine different intensity of stressors and the time scale of their application in order to reveal any quantitative dynamic changes in organopathology in these two strains.

Methods and results

In the first experiment the different repeated stressors were applied within the 5-7 days intervals during 14 days (repeated tail-flicks measurements before and after the 3 minutes swim stress, light ether anaesthesia during ECG measurement, and 4 hours water immersion restrained stress). In the second experiment the animals of both sexes were tested in two consecutive days. The 4 hour water immersion stress performed during the second day was preceded and followed by the measurement of tail-flick latency as a criterion of stress induced analgesia. This experimental procedure was repeated again with the second group of animals with one day shift, so the tail-flick prestress in this second group were overlapped with the immobilisation stress and with the sacrifying of the first group performed in the parallel room. This experimental arrangement simulated psychological or anticipation stress. The weights of adrenals, spleens, hearts, and the extent of gastric ulcers were compared in IS and IR rats in both types of experiments.

The experiment with repeated stressors showed that the adrenals weights and the glucose level were significantly higher in IS rats while the heart and the gastric ulcers were more frequent in IR rats.

The results of ANOVA from the second experiment revealed substantial differences between the effect of stress itself (factor STRESS) and the effect of stress anticipation (factor ANTICIPATION) on the weights of organs. The greatest sensitivity to anticipation stress was observed in the relative weights of adrenals. The decrease was found both in stressed and control animals. The acute stress influenced mostly the weight of spleens that were lower in experimental groups than in controls independently on the anticipation.

The changes of adrenals weights with the increasing stress intensity were confirmed with the significant positive correlation found between the relative weights of adrenals and the degree of stress induced analgesia.

The gastric ulcers occurred more frequently in IR

rats but significant differences were observed only in the group influenced by anticipation. The stress anticipation more homogenised the data that resulted in the markedly decreased variability.

Conclusions and discussion

From results of all experiments it can be concluded that with respect to stress induced gastric lesions, IR rats are more sensitive to acute stress, while the repeated stresses attenuate this effect. The similar picture was described by Mráz on the myocardial lesions in IS rats when repeated injection of isoprenaline reduced the toxic effect of a single dose of it (Mráz et al., 1995). Therefore we hypothesised that IS rats are more catecholaminergic while IR rats are more cholinergic.The acute stress acts in IS rats on the dominant sympathetic system, and the prestress accumulation effect reduces probably catecholamine synthesis and/or reduced receptor density. The antagonistic - parasympathetic system then will prevail and result in the organopathology redirected from the cardiovascular system to the gastrointestinal system. This can explain the originally paradoxical results.

Repeated prestress serves as adaptive preconditioning of the dominant autonomic system, however, the relative input of complementary system will increase with all positive and/or negative consequences.

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EFFECT OF LONG-TERM ADMINISTRATION OF STOBADINE TO PREGNANT RATS AND POSTNATAL DEVELOPMENT OF THEIR OFFSPRING

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Introduction

Stobadine (stb, (-)-cis-2,8-dimethyl-2,3,4,4a,5, 9b-hexahydro-1h-pyrido-[4,3-b]indole) is an effective cardio- and neuroprotective drug with antioxidative stress properties (1). Long-term administration of STB may be considered in the therapy of cardiovascular and cerebral diseases also during gestation, yet little information is available concerning its effects on prenatal and especially on postnatal development. The present study was conducted to assess the effects long-term STB administration on pregnant rats and postnatal development of their offspring.

Material and Methods

The dams (108) Wistar rats were treated by oral gavage with STB (in the form of dipalmitate salt DP 1031 m. w. 715.2, 99.5% purity) in doses of 5, 15 and 50 mg/kg/d from the 6th day of gestation up to weaning of pups (day 21 post partum, pp.). The control group received 0.5% Methocell suspension as vehicle. A part of pregnant rats (73) were killed on day 20 of gestation to detect embryo-foetal toxicity. The remaining females (35) were allowed to deliver in term to observe selected variables of their offsprings´ postnatal development during lactation: duration of gestation, litter weight and size, physical growth and maturation (unfolding of external ear, incisor eruption, development of fur, ear and eye opening, testes descent and vaginal opening) and neuromotor and reflex development (righting reflex - the rat´s ability to turn over from supine position, tested on day 5 pp., negative geotaxia - the rat´s ability to turn 180^0 on a 25^0 incline placed head down, tested on day 8 pp., forelimb grip strength - ability to hold on to a thin wire, tested on day 13 pp.).

Results and Discussion

Dailly oral administration of DP 1031 during gestation period was well tolerated and no female

aborted or died. No adverse effects on embryo-foetal development were found (Table 1). There were no significant differences between the DP 1031 and control groups in litter weight and size assessed from birth through weaning (data not shown). Physical growth and maturation of pups were not influenced by DP 1031 treatment (Table 2). Concerning the neuromotor and reflex development, we found a significant decrease in time latency of the righting reflex and negative geotaxia in offspring of mothers treated with DP 1031 (Figure 1).

The presented study did not reveal adverse effects of DP 1031 on pregnancy and its results are compatible with those of reproductive toxicity and teratological studies performed in rats (2, 3). Decrease in time latency of the righting reflex and negative geotaxia can not be considered as an unfavourable effect, on the contrary, the offspring exposed to DP 1031 via their mothers seem to be more skillful and mobile than controls. Explanation of this phenomenon might relate to the nonsignificantly lower foetal weight before parturition and also with significantly increased exploratory behaviour, reported in our previous behavioural studies in young rats whose mothers were treated chronically with DP 1031 during pregnancy and lactation (4, 5).

Table 1

Influence of oral administration of DP 1031 on embryo-foetal development in rats

	Dose (mg/kg)			
	C	5	15	50
No. of mothers	18	16	17	22
Gestation period (days)	22.2	22.5	22.1	22.2
Corpora lutea[1]	12.4	13.3	12.9	13.7
Implantations[1]	11.5	11.4	11.3	12.1
Viable foetuses[1]	11.2	10.1	11.0	11.4
Dead foetuses[1]	0	0	0	0
Resorptions total[1]	0.33	0.75	0.29	0.68
Preimplantation loss (%)[2]	7.4	13.0	12.1	11.8
Postimplantation loss (%)[3]	2.8	6.7	2.3	6.2
Foetal weight (g)	3.6	3.4	3.2	3.5

C - control
[1] - mean number
[2] - corpora lutea - implant. sites/corpora lutea (x100)
[3] - implant. sites - viable foetuses/implant. sites (x100)

Conclusion

Long-term administration of DP 1031 in the doses of 5, 15 and 50 mg/kg to rats during gestation and lactation had no adverse effects on the course of pregnancy, embryo-foetal and postnatal development of the offspring.

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Table 2

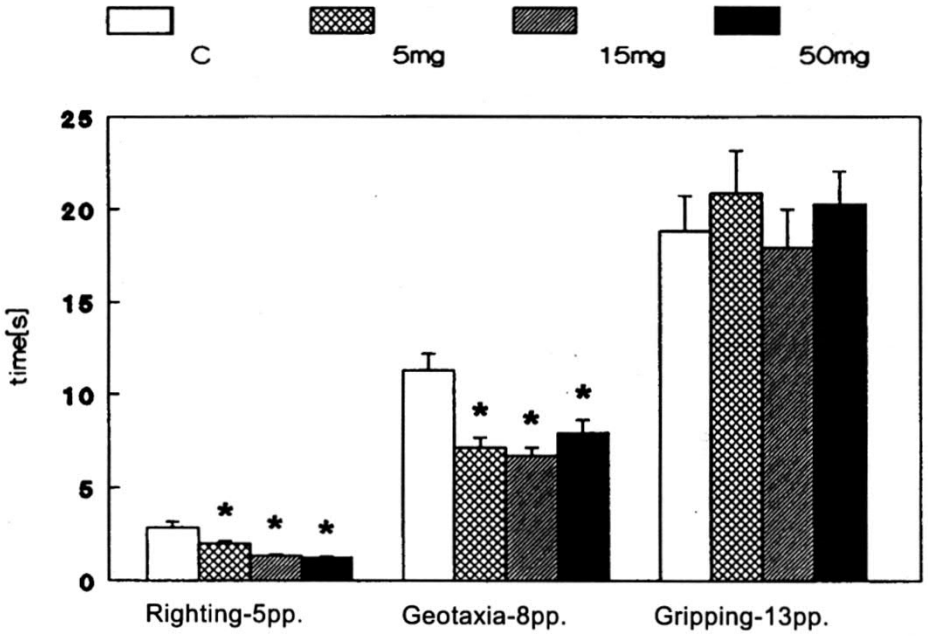
Postnatal development of rat offspring

	Dose (mg/kg)			
	C	5	15	50
No. of pups	100	65	97	100
Unfolding of external ear[d]	2-3	2	2-3	2-3
Incisor eruption[d] - lower - upper	8 10-11	8 9-12	8-9 10-12	8-9 9-12
Development fur [d]	10-11	9-12	10-12	11-12
Ear opening[d]	12	12-13	12-13	11-13
Eye opening[d]	14-15	13-15	14-15	13-16
Testes descent[d]	23-32	22-32	23-32	23-33
Vaginal opening[d]	32-38	31-38	31-39	30-39

C - control
[d] - time of appearance (days)

Figure 1

Neuromotor and reflexive development of rat offspring



*p < 0.05 significant differences from control (C)
pp. - post partum

IMPLANTATION TESTS OF A NEW DERMAL SUBSTITUTE ON MICE AND RATS

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In extensive skin lesions, in particular those arising as a consequence of burns, restoration of skin is very important and is very often a factor of survival. Along with traditional substitutes, donated by humans or animals, much effort has been invested in development of synthetic dermal substitutes. Prior to application in patients, any materials, designed for this purpose, should pass in vivo tests. An appropriate - and compulsory - test, suiting this purpose, is an implantation test on laboratory animals, since it provides information on overall reaction of host tissue on the implant. In the present study, we tested a new synthetic dermal substitute to answer two principal questions: 1. What modifications appear in the subcutaneous tissue of laboratory animals in consequence of material implantation, and 2. What is the resorption time of the material to the tissue. We used five ICR strain mice and five Wistar strain rats. The animals were staged in conventional laboratory animal quarters at optimal conditions for the rodents used (temperature 20-24 °C, relative humidity 40-60%, controlled 12 hours light/12 hours dark regime). Under ether anesthesia, two types of synthetic material were implanted subcutaneously on the right side of dorsum in pieces of 1 cm × 1 cm × 1 mm. After 3, 5, 15, 22 and 29 days of implantation, samples of tested material and host tissue were taken for histological examination. The samples were processed using a common formol-

paraffine method. Slices were stained with haematoxyline and eosin according to Mason and Van Gieson with emphasis on collagen staining. We found that an inflammation reaction of host tissue prevails during the first 15 days, manifesting by an initially intense leukocytic infiltration in the close vicinity of the implant. The leukocytes gradually penetrated the tested material. Since the 15th day, cellular infiltration changed, and macrophages and lymphocytes prevailed in samples. By this time we also noted presence of fibroblasts. A pronounced vascularization was observed in implants. The original structure of implants became less apparent and implants were partially resorbed in the tissue. On day 22, only residuals of implants were found in the tissue and on day 29, the structure of implanted materials became indistinguishable from that of the skin. No serious damage of tissue following an implantation was found. The inflammation reactions, observed in the initial phase, corresponded, as to their extent, to a normal tissue reaction. No difference in reactions across species was found for the two materials tested. Conclusions of the present study were used as a starting point for a design of a more extensive implantation study, for which laboratory animal species (rat) was selected and histological sampling points were set to 1, 4, and 12 weeks of implantation. The proposed study shall become a part of pre-clinic documentation and is one of the necessary conditions of a successful application of dermal substitutes in their ultimate recipients - human patients.

Key words: Dermal substitute; Implant; Tissue reaction; Histological examination.

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SELENIUM STATUS AND GLUTATHIONE PEROXIDASE ACTIVITY IN THE BLOOD OF ASTHMA PATIENTS

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In asthma reversible airflow obstruction and bronchial hyperresponsiveness are accompanied by chronic inflammation and the influx of inflammatory cells into the airways. Activation of these cells by immunological or non-immunological stimuli induces a respiratory burst which results in the generation of reactive oxygen species such as superoxide anion, hydrogen peroxide an hydroxyl radicals. These oxygen free radicals may be involved in many of the pathophysiological processes associated with asthma (Misso et al.,1996).

Selenium is an essential component of the glutathione peroxidase which have a major role in the defense against oxidant stress. GSH-Px has an important influence on arachidonic acid metabolism and leukotriene formation and elevated blood levels of these mediators have been associated with increased asthma risk (Beasley et al., 1991).

The demonstration of Bibi et al. (1988) that erytrocyte GSH-Px levels were lowest during the acute asthmatic attack strongly suggest that oxidant stress as a consequence of the inflammatory response during the acute phase may lead to GSH-Px depletion. This further suggests that individuals with lower levels of Se intake have diminished GSH-Px activity and are more likely to experience GSH-Px depletion and uncompensated oxidant stress, with subsequent precipatation of acute asthmatic symptoms.

A linear relationship exists between blood Se level and dietary Se intake in humans (Schrauzer, White, 1978). Slovakia belongs to the countries with the lowest Se status in Europe (Maďarič et al., 1994). Since asthmatics have even lower Se status, they are at risk of Se deficiency. Se deficiency might enhance the development or progression of asthma.

In our study 22 intrinsic asthma patients (6 men and 16 women) were examined. Control group consisted of 33 apparently healthy subjects (12 men and 21 women). Venous blood was collected into heparinized trace element-free tubes after overnight fast. Plasme and erythrocytes were separated and stored in freezer until analyzed for Se and GSH-Px. Se was analyzed by the atomic absorption spectrophotometry (Maďarič et al., 1994) and the GSH-PX was measured according to the method of Paglia and Valentine (1967). The results are summarized in the table.

	Intrinsic asthma patients	Controls
Number of subjects	22	33
Age (years)	51.6 ± 2.9	50.8 ± 1.9
Plasma Se (μg/l)	50.1 ± 1.6 [x]	58.4 ± 1.2
Erythrocyte Se (ng/g Hb)	250.9 ± 11.6 [x]	297.2 ± 7.2
Erythrocyte GSH-Px (U/g Hb)	26.8 ± 1.7 [x]	33.6 ± 1.2

[x] $P<0.01$

Plasma and erytrocyte Se concentrations and the activity of GSH-PX were found to be decreased in the group of intrinsic asthma patients. A positive correlation was found between plasma and erythrocyte Se concentration ($r = 0.829$, $p = 0.00000$).

Lowered level of Se, an essential part of the enzyme glutathione peroxidase, in asthmatics decreases the protection against reactive oxygen radicals released by inflammantory cells. Se supplementation of asthma patients may inhance the antioxidant and antiinflammatory status of the organism and should be of great importance.

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INFLUENCE OF NO SYNTHASE INHIBITION ON THE LPS-EVOKED CHANGES IN THE PHARMACOKINETICS OF PROCAINAMIDE IN THE RAT

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Introduction

Nitric oxide (NO) produced in mammalian cells is involved in the bioregulation of numerous physiological processes. NO produced in macrophages was shown to be a potent endogenous suppressor of certain immunological processes, probably helping to prevent inadequate and harmful activation of the immune system (Mills, 1991; Medeiros et al. 1995). It also plays an important mediatory role in the immunologically induced changes in the drug metabolism, particularly in the suppression of the hepatic oxidative metabolism *via* the cytochrome P450 system (Khatsenko et al., 1993). But, there is still limited information about the role of NO in the conjugative drug metabolic reactions including N-acetylation.

In the present study, the pharmacokinetic behaviour of procainamide (PA) and its metabolic conversion to N-acetylprocainamide (NAPA) in the rat was followed after pretreatment with immunostimulator LPS E. coli or its combination with the NO synthase (NOS) inhibitors, N^{ω}-nitro-L-arginine methyl ester (L-NAME) or aminoguanidine (AG).

Materials and Methods

Male Wistar albino rats (180-230 g, Konárovice, CR) were housed under standard laboratory conditions (temperature 24 °C, 12/12 h light/dark rhythm) with free access to standard food and water. Rats were randomly divided into 6 groups ($N = 24$) and treated according to the following design: controls, 0.9 % NaCl ip (2 mL/kg), L-NAME (5 mg/kg ip) and AG (50 mg/kg ip) three consecutive days before PA; LPS, 4 mg/kg ip (2mL/kg), a single dose 12 h before PA; in combinations of LPS + L-NAME or LPS + AG, the dosages were identical to those given alone. All agents were purchased from Sigma Chem. Co.

Following iv injection of procainamide (50 mg/kg, 1 mL/kg) the blood samples were withdrawn from the retrobulbar venous plexus for GC analysis of PA and NAPA. Pharmacokinetic evaluation was performed using TOPFIT 2.0. (HEINZEL et al., Fischer Verl., Stuttgart 1993). Statistical calculations were performed using WILCOXON rank test, $P< 0.05$ was accepted as a limit of significance.

Results

Pretreatment of rats with NOS inhibitors L-NAME or AG did not evoke any changes in the pharmacokinetics of PA and NAPA. LPS increased the NAPA concentration in serum (AUC_{NAPA} = 202 % of controls) and slightly decreased PA elimination. Combined administration of LPS with L-NAME, but not with AG, resulted in enormously elevated serum concentration of NAPA (AUC_{NAPA} = 294 % of controls) while PA kinetics was not substantially modified. Results of pharmacokinetic analysis are given in the Table 1.

Discussion and Conclusions

N-acetylation reaction belongs to the most important conjugative pathways of biodegradation of drugs and xenobiotics in the organism. It is catalyzed by a non-inducible enzyme, hepatic cytosolic arylamine N-acetyltransferase (NAT2). There has been evidenced that some immunostimulatory agents increase the acetylation rate in animal experiments (Notter and Roland, 1978; Zídek and Janku, 1981; Hadašová et al., 1991). In our recent studies, pretreatment of rats with LPS *in vivo* increased the activity of NAT2 in the cytosolic fraction of the rat liver; the stimulatory effect of LPS was still enhanced by its co-administration with L-NAME (Walter et al., 1995). The present results proved, besides other changes in the PA pharmacokinetics, the marked enhancement of the LPS-induced increase in the NAPA concentration in serum after pretreatment with a non-selective NOS inhibitor L-NAME. Thus, this study indicated that NO has probably been involved in regulation of the pharmacokinetic behaviour of procainamide including its metabolic conversion to N-acetylmetabolite.

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Podporováno grantem IGA MZ ČR č. 3430-3.

Table 1

Pharmacokinetic parameters of PA after administration of 50 mg/kg iv to rats pretreated with NaCl (control), LPS, L-NAME, AG, LPS + L-NAME and LPS + AG.

Parameter:	Group:	control	LPS	L-NAME	AG	LPS + L-NAME	LPS + AG
k_{el}	[min^{-1}]	0.0082	0.012	0.0011	0.0096	0.0085	0.011
k_{met}	[min^{-1}]	0.011	0.0088	0.010	0.012	0.0094	0.010
AUC_{PA}	[mg.min.l^{-1}]	3860	4850	3650	3620	4750*	4820
AUC_{NAPA}	[mg.min.l^{-1}]	3087	6250*	2900	3210	9070*	6050
CL_{met}	[$ml.min^{-1}.kg^{-1}$]	6.19	6.11	6.22	6.45	5.64	5.96
CL_{ren}	[$ml.min^{-1}.kg^{-1}$]	6.99	4.67*	5.96	7.01	5.54*	4.25

Values are means calculated from eight kinetic curves of PA and NAPA in each group.

* $p < 0.05$ (Wilcoxon rank test)

THE EFFECT OF ACUTE EXPOSURE TO SULPHUR DIOXIDE (SO_2) ON BIOCHEMICAL, HEMATOLOGICAL AND HISTOLOGICAL PARAMETERS IN GUINEA PIGS

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Environmental pollution is one of the most overwhelming contemporary problems. Sulphur dioxide is one of the most important atmospheric pollutants. Although its ecological importance has decreased in many countries of the world, in our region it still ranks among the major factors producing dangerous pollution. Sulphur dioxide and various particles are emitted into the atmosphere by the burning of fossil fuels and smelting of metals. Many of these particles can promote the conversion of SO_2 to the more irritant sulphuric acid. Inversion, fog, and cold temperatures also enhance the harmful consequences of exposure to SO_2. (1,2,3)

The effects of SO_2 on the respiratory tract has been demonstrated in various studies (4,5,6), but until now, only limited pieces of information about its effects on other body systems are available. The main goal of our work is to study the influence of acute exposure to SO_2 on the biochemical, hematological, and histological parameters in guinea pigs.

Experimental Part

Two experimental groups (n=8 and 12 respectively) of male guinea pigs of an average weight of 500 g were exposed to SO_2 400ppm for 3hrs, two control groups (n=9 and 10 respectively) were „Sham" exposed to air for 3hrs. The non-invasive polygraphic cardiac recordings of the systolic time intervals were investigated under anaesthesia induced by Ketamin (150 mg/kg i.p.). The general anaesthesia was enhanced by the addition of Urethan (20% solution, 0,65 ml/100 g) and samples of blood and tissues were simultaneously taken for biochemical, hematological and histological analysis.

Samples were taken either 3 or 24 hours after the exposure. Biochemical parameters were determined in plasma/serum by the analyser Hitachi 717 (Boehringer, Mannheim). Hematological parameters were measured by the analyser Coulter T890. Histological pictures of myocardium were obtained from the total transversal cut in the ventricular area and were stained by hematoxylin and Mallory blue trichrom.

Results and Discussion

Acute exposure to SO_2 can produce various changes in the organism. These changes could be reflected by the biochemical, hematological and histological data. The data obtained 3 and 24 hours after the exposure to SO_2 were always compared with the control groups to verify the modifying effects of sulphur dioxide.

Biochemical Parameters

- **Examination performed 3 hours after the exposure**: no significant changes in ion concentrations between the exposed and control group were found, with the exception of a significantly lower potassium level in the exposed group as compared with controls (4,54 mmol/l and 8,3 mmol/l respectively). Other biochemical parameters (enzymes and proteins) did not exhibit any difference.
- **Examination performed 24 hours after the exposure:** lower levels of potassium, calcium, and magnesium (6,71 mmol/l and 4,50 mmol/l, 2,42 mmol/l and 2,15 mmol/l, and 1,36 mmol/l and 1,12 mmol/l respectively) between the exposed and „Sham" exposed group were observed. Other biochemical parameters were not influenced.

Hematological Parameters

Hematological parameters did not tend to indicate any definite changes.

Histological Picture

The histological examination of the myocardium revealed no differences between groups.

The results we obtained show that acute exposure to SO_2 can induce certain changes in the levels of various elements in the body. The blood levels of potassium were significantly decreased 3 and 24 hours after exposure to sulphur dioxide.

The blood levels of calcium, and magnesium were significantly decreased 24 hours after exposure to sulphur dioxide. Therefore it can be concluded that acute exposure of guinea pigs to SO_2 did not induce consistent and significant changes in most of the followed biochemical, hematological and histological parameters. The observed changes in ion concentrations can be due to the stress mechanism.

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THE EFFECT OF ACUTE EXPOSURE TO SULPHUR DIOXIDE (SO_2) ON NON-INVASIVE PARAMETERS OF CARDIAC FUNCTION IN GUINEA PIGS

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Atmospheric pollution is the presence of various components in the atmosphere which may negatively influence the environment and consequently cause health problems. These components can be classified according to various aspects: 1. primary and secondary pollutants, 2. natural and anthropogenic or 3. the often used distinction of „pollutants" is for reducing and oxidizing types. The reducing type is characterized by sulphur dioxide and smoke from incomplete combustion of coal, enhanced by conditions of fog and cool temperatures. The oxidizing type is characterized by hydrocarbons, nitrogen oxides, and photochemical oxidants from the atmospheric reaction products of automobile exhaust. (1, 4)

In our region sulphur dioxide still represents one of the most important pollutants. The main causes are high levels of human activities which include industrial exhalation and burning of fossil fuels. The negative influence of SO_2 on the organism can be manifested during exposure to high concentrations, especially if meteorological inversion (where the upper layers of the atmosphere are warmer than the lower ones, so that mixing and dilution cannot take place) is present.

Many effects of SO_2 on the respiratory system have been described (2, 3, 5, 6), but almost no information about its action on the cardiovascular system is available. The main goal of the present work is to investigate the effects of acute exposure to SO_2 on the cardiovascular system in guinea pigs.

Experimental Part

Two experimental groups (n=8 and 12 respectively) of male guinea pigs of an average weight of 500 g were exposed to SO_2 400ppm for 3hrs, two control groups (n=9 and 10 respectively) were „Sham" exposed to air for 3hrs.

A simple schedule of experiments was adopted. The first measurement of Non-invasive Heart Parameters was done 7 days before the exposure, the second either 3 or 24 hours after the exposure.

Non-invasive Heart Parameters were measured by non-invasive polygraphic cardiac recordings of systolic time intervals under general anaesthesia (Ketamin, 150 mg/kg i.p.). The non-invasive parameters of cardiac function were suggested to be: PEP(pre-ejection period), LVET(left ventricular ejection time), PEP vs LVET ratio, Q2 and RR.

Results and Discussion

Non-invasive parameters of cardiac function (especially the PEP/LVET ratio) were used to evaluate the function of the heart. Each time we compared the SO_2 exposed group of animals with the „Sham" exposed ones and the data obtained before and after the exposure (the first and the second measurements).

- Measurement 3 hours after the exposure: no significant change of non-invasive parameters after SO_2 were present (PEP/LVET ratio 0.2850 before and 0.2874 after the exposure) as well as in the control group (0.2378 and 0.2279 respectively).
- Measurement 24 hours after the exposure: a mild but significant increase in PEP/LVET ratio was found (0.2473 before and 0.2822 after the exposure) and these changes were significantly different from those in the control group (0.2719 and 0.2446 respectively). The increase of PEP and Q2 also occurred in the SO_2 exposed group; a significant difference from the „Sham" exposed animals exists here.
- Comparing data measured 3 and 24 hours after the exposure to SO_2, no significant changes were found.

It is possible to conclude that at the earlier phase (3 hours after the acute exposure to SO_2) sulphur dioxide did not induce significant changes in the cardiac function. A mild, but significant, increase in PEP vs. LVET ratio present in the later phase (24 hours after the exposure) may suggest a possibility of declination in cardiac function.

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PROTECTIVE EFFECTS OF STOBADINE ON BLOOD RETICULOCYTES AND HEART OF CO^{60} IRRADIATED MICE

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Stobadine is a novel drug with antioxidant properties and potential pharmacological use (Štolc et al., 1983, Štolc et al., 1997, Styk et al.,1989, Horáková et al., 1993). The protective action of stobadine against Co^{60} irradiation was studied in ICR mice to contribute to the understanding of its antimutagenic (Chorvatovičová and Bauer, 1994) and cardioprotective effects (Ondrejičková et al., 1991). Stobadine dipalmitate was administered orally at three time intervals, i.e., 2 h or 1 h prior to and immediately after 6.5 Gy Co^{60} exposure. Induction of micronuclei in peripheral blood reticulocytes and the activity of glutathione peroxidase in homogenate of heart was estimated 40 h post irradiation. A significant decrease of micronucleated reticulocytes was observed in the group of mice pretreated with stobadine 2 h ($P<0.05$) or 1 h ($P<0.01$) before irradiation. Co^{60} exposure followed by stobadine administration did not lead to the same protective effect in the micronucleus assay. Exposure of 6.5 Gy Co^{60} caused a significant decrease in the activity of glutathione peroxidase ($P<0.01$). Treatment with stobadine 1 h or 2 h before irradiation resulted in activity enhancement, in comparison with the non-pretreated irradiated group ($P<0.01$). Glutathione peroxidase activity, an indicator of oxidative stress, was significantly decreased after irradiation, but stobadine pretreatment enhanced the activity significantly.

The results of both tests are in concordance and indicate that stobadine is an effective radioprotector. We found that Co^{60} irradiation followed by stobadine administration did not result in the

same radioprotection as did the reversed order of treatment described in this study. Therefore, it is assumed that a radical-scavenging mechanism was involved in the radioprotective effect of stobadine.

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COOKING IN ALUMINUM-COOKWARE AS A SECONDARY SOURCE OF DIETARY ALUMINUM

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There is increasing evidence to indicate that aluminum (Al) can be neurotoxic to several animal species including man [1]. A number of studies have implicated Al as a possible factor in the pathogenesis of some neurological disorders [2]. It is well established that Al is causally implicated in the generally fatal brain disease dialysis encephalopathy, a brain disease seen mostly in patients with renal failure treated with haemodialysis[3]. Al as a possible factor of Alzheimer's disease, degenerative dementia characterized by a deterioration of cognitive function and recent memory loss, is also discussed very often[4].

There is commonly accept that higher amount of Al in diet is a risk factor of some diseases. Al concentrations in food can be incerased by cooking in aluminum-cookware, but too little informations about this problem exist in literature. The present work is a contribution to this questions.

The effect of some natural organic acids on the dissolution of metallic Al was studied with the aim to take whether cooking in Al-cookware can increase content of this metal in diet significantly. Al-foil 5 x 20 cm of average wieght of about 550 Mg was boiled 60 minutes in 300 ml of water solutions of tested acids at concentration of 1 mg/ml. The shortage of Al-foil was estimated by weighing.

The amounts of Al disengagent from Al-foil (total area 200 cm^2) by 60 min boiling with different acids at concentration of 1 mg/ml are summarized in Table I. The most shortage of Al-foil was observed in the presence of the oxalic acid. Further followed lactic, tartaric, ascorbic, citric, and malic acids. The Al-foil was not dissolved in the presence of butyric, acetic, and benzoic acids, as well as in distilled and drinking water. In other experiments was demonstrated, that the shortage of Al-foil is dependent on the time of boiling (linear function of time) and/or acid concentration (nonlinear funcion of concentration). It was also demonstrated that the passivation of Al surface by the film of Al_2O_3 do not guard Al against an acide effect.

All these experiment bring evidences that the amount of Al dissolved from Al-foil by some natural fruit acids might be till more than 30 mg per 100 cm^2 of the surface of dishes. From this results is evident that cooking in Al-cookware can increase the amount of dietary Al up to recomended value. According to contemporary knowledge the recommended amount of Al in diet is no more than 3 mg per day per person[5]. Increasing daily dietary Al

above that value contribute to a consequential health complication of patients with chronic renal failure[6] and it is also risk factor in pathogenesis of Alzheimer's disease[7-9].

Table 1

The amounts of Al disengagent from Al-foil of total area 200 cm ^{2} by 60 min boiling with different organic acids at concentration of 1 mg/ml in total volume of 300 ml.

Acid	Amount of Al ± S.D. (mg)
citric	7.49 ± 0.34
tartaric	15.56 ± 2.59
ascorbic	10.70 ± 1.39
malic	4.99 ± 0.80
oxalic	65.32 ± 13.47
butyric	none
lactic	29.46 ± 5.49
acetic	none
benzoic	none
distilled water	none
drinking water	none

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY DETERMINATION OF DIPOTASSIUM CLORAZEPATE

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Dipotassium clorazepate (Tranxene) is an antianxiety agent (3). Different chromatographic method have been used for the determination of clorazepate in plasma (1, 2, 4). We describe liquid chromatographic assay, which we developed for the experiment performed on rats. Placental transfer of clorazepate and nordiazepam was studied using the rat term placental perfusion system.

Extraction procedure included decarboxylation of clorazepate to nordiazepam in 1M hydrochloric acid. Samples were incubated for 1 h at 37 °C, then 1M sodium hydroxide and borate buffer pH 9 were added. Nordiazepam and internal standard flunitrazepam were extracted into the mixture of diethylether-methylen chloride (2:1). Organic phase was evaporated at 40 °C and the residue was reconstituted in column eluent solution (acetonitrile-methanol-10mM phosphate buffer, 16:9:25, pH 5.7) and 100 µl were injected into the liquid chromatograph (Waters). The separation was performed at a flow-rate of 1.1 ml/min on a reversed-phase Symmetry column (250x4.6 mm I.D., 5 µm particle size). We used the diode array detector to get spectrum in range of 195-600 nm. We used for quantitation the wavelength at 220 nm.

The values of retention time for nordiazepam and flunitrazepam were 13.55 and 9.70 min. The limit of detection of method was 10 ng/ml. The calibration curve was linear for a concentration of 312.5 - 40000 ng/ml. Within-day and day-to-day precision of the method, as measured by the coefficient of variation, ranged from 2.29 to 5.7 % and 6.9 to 8.5 %. The recovery of standard additions (625 ng/ml and 1250 ng/ml) ranged from 96 to 105 %.

The second part of the samples we used for assay without conversion of clorazepate to nordiazepam in hydrochloric acid. We added borate buffer and extracted nordiazepam into the organic phase. This assay is useful for quantitation of nordiazepam as a product of decarboxylation of clorazepate in the stomach or in the plasma (3).

The chromatograms were interference-free from components of rat plasma.

The HPLC procedure proposed here was sensitive and reproducible.

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