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

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

Introduction

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

Arbutin is a glycosylated benzoquinone, isolated from the leaves of bearberry (Arctostaphylos uva ursi L., Ericaceae), wheat, pear skins, majorana, coffee or tea. It was found to possess antiseptic and diuretic properties, the ability to decrease allergic-reaction-induced inflammation and the capacity to potentiate antiinflammatory effects of indomethacine and corticosteroids. Arbutin counteracted reduction of the oxidative burst arising inside neutrophils. RLU – relative luminescence units.

Materials and methods

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

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

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

Table I

<table>
<thead>
<tr>
<th>Arb</th>
<th>Chemiluminescence [millions of RLU*s]</th>
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<tbody>
<tr>
<td></td>
<td>Whole blood</td>
</tr>
<tr>
<td>0</td>
<td>1.53 ± 0.29</td>
</tr>
<tr>
<td>0.0</td>
<td>1.50 ± 0.24</td>
</tr>
<tr>
<td>1</td>
<td>1.14 ± 0.19*</td>
</tr>
<tr>
<td>0.1</td>
<td>1.48 ± 0.23</td>
</tr>
<tr>
<td>1</td>
<td>1.14 ± 0.19*</td>
</tr>
<tr>
<td>0.35</td>
<td>0.35 ± 0.04*</td>
</tr>
<tr>
<td>100</td>
<td>0.11 ± 0.01*</td>
</tr>
</tbody>
</table>

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

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

Besides the inhibition of neutrophil activity, arbutin was found to decrease the concentration of the pro-inflammatory cytokine IL-6. The control plasma level of interleukin-6 was 29.4±2.6 pg ml−1, whereas in the presence of arbutin, the respective IL-6 concentrations were 72.8±12.0 and 35.5±8.8 pg ml−1.

Discussion

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

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

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

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

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12. mezioborová česko-slovenská toxikologická konference
Introduction

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

Subject and methods

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

Cytogenetic analysis: Conventional short-term lymphocyte cultures were made from whole blood in RPMI medium with l-glutamine (Gibco) supplemented with 20 % foetal calf serum (Gibco) and antibiotics. Lymphocytes were stimulated by phytohaemagglutinin (Murex) and incubated at 37 °C and 5 % CO\(_2\).

Chromosome aberrations: The cells were harvested at 48 h; colchicine (Sigma, 0.75 µg ml\(^{-1}\)) was added 2 h before harvest. 100 metaphases per sample were scored for various types of chromosome aberrations\(^7\).

Micronucleus test: Cytochalasin B (Sigma, 6 µg ml\(^{-1}\)) was added 44 h after the start of culture and incubation was harvested at 72 h. Micronuclei were identified according to the criteria of Fenech et al\(^8\).

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

Results

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

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

### Table I

| Chromosome aberrations in lymphocytes of exposed to mineral fibres and controls |
|---------------------------------|-----------------|---------------|-----------|
| **Group**          | **N** | **Number of analyzed cells** | **% of aberrant cells** | **Break/ cells** |
| Rockwool exposed     | 97    | 9700                       | 0.65                  | 0.007          |
| control              | 43    | 4300                       | 0.65                  | 0.007          |
| Glass fibres exposed | 79    | 7900                       | 0.59                  | 0.009          |
| control              | 36    | 3600                       | 0.53                  | 0.007          |
do not experience an overall increase in MN frequency, although when the interaction with occupational exposure was taken into account, heavy smokers were the only group showing a significant increase in genotoxic damage as measured by the micronucleus assay in lymphocytes.

**Conclusion**

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

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1. IARC 81, (2002).
**P18**

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

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

**Introduction and aim of the survey**

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

**Material and methods**

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

**Results**

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

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

**Discussion**

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

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

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**Table I**

Morphine content in blood circulation after one potion application

<table>
<thead>
<tr>
<th>Way of application</th>
<th>Used 6–28 % morphine in natural opium dose and 0.25–0.6 % morphine in poppy heads dose</th>
<th>Used 24.06–33.9 % morphine in natural opium dose and 0.20–0.28 % morphine in poppy heads dose (our samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection</td>
<td>5.1–23.8</td>
<td>20.5–28.3</td>
</tr>
<tr>
<td>Smoking</td>
<td>3.6–33.3</td>
<td>14.3–39.6</td>
</tr>
<tr>
<td>Drinking</td>
<td>35.3–113.0</td>
<td>28.3–52.8</td>
</tr>
</tbody>
</table>
used 20% of them use 24.62 mg to even in 103.86 mg (with morphine amount in our samples). Altogether 20% of them use 24.62 mg to even in 103.86 mg (with morphine amount in our samples). It is likely, that via smoking our subjects have exceeded it even in 28.8 mg (calculated with morphine amount declared in the literature search1,3) or even in 23.8 mg (calculated with morphine amount in our samples). Via “opium tea” drinking users have perhaps exceeded maximum single dose of morphine in their blood either even in 28.8 mg (calculated with morphine amount declared in the literature search1,3) or in 35.1 mg (calculated with morphine amount in our samples). Via „opium tea“ drinking users have perhaps exceeded maximum single dose of morphine in their blood either even in 108.5 mg (calculated with morphine amount declared in the literature search1,3) or even in 23.8 mg (calculated with morphine amount in our samples). According to the literature a drug dependent person with chronic abusing can endure single dose with even 5 g of morphine without a serious acute intoxication6. It is not probable our subjects have achieved this amount.

But also in such way clients of CC have perhaps exceeded maximum single dose of morphine referred-to the Czech Codex 2002. 4.5 mg of morphine, maximum daily one then 15 mg. These ceiling values we can compare with the morphine amount absorbed into users’ blood at them declared quantity of the drug. Again we counted with the poppy heads number confided by our subjects and either with morphine level in natural opium and poppy heads resulted from our samples analysis or with morphine value declared in the literature search. Via single injection application the users have probably exceeded maximum single dose of morphine either in 19.3 mg (calculated with morphine amount declared in the literature search1,3) or even in 23.8 mg (calculated with morphine amount in our samples). It is likely, that via smoking our subjects have exceeded it even in 28.8 mg (calculated with morphine amount declared in the literature search1,3) or even in 35.1 mg (calculated with morphine amount in our samples). Via „opium tea“ drinking users have perhaps exceeded maximum single dose of morphine in their blood either even in 108.5 mg (calculated with morphine amount declared in the literature search1,3) or even in 23.8 mg (calculated with morphine amount in our samples). According to the literature a drug dependent person with chronic abusing can endure single dose with even 5 g of morphine without a serious acute intoxication6. It is not probable our subjects have achieved this amount.

But also in such way clients of CC have perhaps exceeded maximum single dose of morphine referred-to the Czech Codex 2002. A third of them has admitted injection application 1-time or 2-times a day. Whether we have in mind a day number application and an amount of morphine in natural opium 6–28 % (ref.1,3), than we obtained this results: users have perhaps exceeded maximum daily dose of morphine in their blood in 8.8 mg or 32.6 mg depended on usage frequency. Calculating with morphine amount in our samples (24.06–33.9 %) we resulted in 108.5 mg or in 41.6 mg overdosing. A fifth of subjects use to smoke natural opium once to 3-times a day, most often twice a day. Maximum daily dose of morphine has been probably exceeded either in 18.3 mg to even in 85 mg (with morphine amount declared in the literature1,3) or in 24.62 mg to even in 103.86 mg (with morphine amount in our samples of poppy heads). Altogether 20 % of them use to cook the „opium tea“, but they did not told how many times they drank it, it is not possible to find out how many times they could exceed maximum daily dose of morphine. But even one pot of „opium tea“ exceed maximum daily dose either in even 98.0 mg (calculated with morphine amount declared in the literature search1,3) or in even 37.8 mg (with morphine amount in our samples). You can see it in the Table II.

<table>
<thead>
<tr>
<th>Way of application</th>
<th>Used 6-28 % morphine in natural opium dose and 0.25-0.6 % morphine in poppy heads dose1,3</th>
<th>Used 24.06-33.9 % morphine in natural opium dose and 0.20-0.28 % morphine in poppy heads dose (our samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection</td>
<td>Max. Single dose: 5.3× 1.6× or 3.2× Max. daily dose: 6.3× 1.9× or 3.8×</td>
<td>Max. Single dose: 6.3× Max. daily dose: 1.9× or 3.8×</td>
</tr>
<tr>
<td>Smoking</td>
<td>Max. Single dose: 7.4× 2.2× even 6.6× Max. daily dose: 8.8× 2.6× even 7.8×</td>
<td>Max. Single dose: 8.8× Max. daily dose: 2.6× even 7.8×</td>
</tr>
</tbody>
</table>

n.a. – not available

to cook the „opium tea“, but they did not told how many times they drank it, it is not possible to find out how many times they could exceed maximum daily dose of morphine. But even one pot of „opium tea“ exceed maximum daily dose either in even 98.0 mg (calculated with morphine amount declared in the literature search1,3) or in even 37.8 mg (with morphine amount in our samples). You can see it in the Table II.

During one month of the opioid period (June–August) they can apply (according to their frequency of daily usage) 30 even 60 dose by injection/smoking or drink „opium tea“ all day. Opioids addictive potential depends on the dosage, frequency of the usage and the users’ sensibility4,6,7. Natural opium and poppy heads are usually rated as only season drugs, but in the period of their abusing 40 % of subjects do not use another substances, natural opium and poppy heads become the primary drug than. Nevertheless dependence on opioids is interpreted as the most serious connected with tolerance increasing and the strong syndrome abstinence6, they are very dangerous also because of overdosing. Opioid tolerance falls during abstinence fast and dose before being off abusing becomes to be mortal if it is used on the beginning of new opium season9. This kind of overdosing could be an actual danger menacing our users. Detoxification is very rare; substitution can be appreciated as good effect5. None of our subjects has endured reportedly this treatment. Next hazard connected with injection abusing of natural opium is viral hepatitis and HIV infections10. Fourth part of clients admit more frequent needle and paraphernalia sharing during opium season than out of it, possibility of so danger diseases transfer increases markedly.

Conclusions

Morphine quantity in users’ blood circulation after natural opium and poppy heads products applications seems to be considerable. Gathering from it dependence on opioids and tolerance are developing via existing abusing algorithm. Our subjects consider natural opium and poppy heads only as season drugs, but by contrast of it almost half of them admit it as the primary drug during the opium season. This existing way of abusing could be connected...
with so strong dependence that opioids would be the primary drug not only for several months but yearly with all hazards resulted from it.

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PROTECTION EFFECT OF VITAMIN C ON ALCOHOL BINDING TO PHOSPHOLIPID MONOLAYERS

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

Introduction

The mechanism of toxic effect of ethanol chronic uptake has been intensively studied¹⁻³. The simple model of the biological membrane provides well-controlled lipid monolayers at the air – water interface. The Maxwell displacement current technique (MDC) provides novel approach to conformation study of the membrane models. Ethanol is a clear inducer of oxidative stress. Vitamin C (ascorbic acid) is a coenzyme in many oxidative/reduction reactions, it acts as antioxidant, prevents oxidation of unsaturated fat acids. Protective effects of antioxidants have been confirmed by many studies⁴. Vitamin C is an essential compound for humans; it is required for protein synthesis. Ethanol chronic intake influences many systems in the body. The changes in protein functions, that are the basis of membrane channels, enzymes etc., cause loss of cell energy and decrease its communication ability with the environment. Acetaldehyde and free radicals are created by ethanol metabolism on subcellular level, which evokes oxidative stress.

Simultaneous supplementation of ethanol and vitamin C reduces protein and lipid oxidation in the rat kidney, testes, and lungs⁵. These effects were also observed in human glial cells after simultaneous supplementation of ethanol and vitamin C (ref.⁶). Vitamin C prevents membrane dehydration by ethanol.

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

Material and methods

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

Results

Absorption/adsorption of ethanol molecules to phosphocholine (PO₂) group of DPPC molecule occured on DPPC monolayer on the ethanol-water subphase.

After vitamin C supplementation into subphase ethanol-water, it was found that vitamin C molecules influenced interaction between ethanol and DPPC molecules (PO₂ group).

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

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

Surface pressure – area isotherms shows similar behaviour of the DPPC monolayer on alcohol-water mixtures independently of the presence of vitamin C. Binding/adsorption process induces change of electron density distribution across monolayer and thus the molecular dipole moment. We observe small or negligible binding of methanol molecules on oxygen bonds of DPPC.

Discussion

The results of interactions of DPPC monolayer with ethanol and water molecules can change electrical charges on the membrane surface, mechanical properties, permeability, and ion diffusion through the membrane. Patra et al.⁷ suggest that ethanol changes the membrane structure. Ethanol causes conformation changes of structures forming membrane proteins. These structures ensure the performance of their physiological function.

Klemm et Williams⁸ found that ethanol changes the water molecules arrangement around phosphocholine head (PC). Ethanol molecules bind to PC head, water molecules
move and create accumulation around ethanol alkyl group. Chiou et al. observed via FTIR spectroscopy the phenomenon that after ethanol supplementation to the solution of DPPC reversed micelles, part of water molecules bound to DPPC molecules by hydrogen bonds were replaced by ethanol molecules. The authors stated that interaction between alcohols and PC head of membrane lipid caused weakening of the membrane – water interaction and membrane destabilization. Thus ethanol causes membrane dehydration.

Vitamin C can cause two phenomenon: a) vitamin C molecules interact with DPPC molecules or b) vitamin C molecules bind preferentially with ethanol molecules and thus prevent binding of ethanol molecules to DPPC molecules. We believe that vitamin C molecules influence DPPC molecules and that vitamin C molecules adsorb to DPPC monolayer.

From the results concerning the area of DPPC molecule we believe that vitamin C molecules act against decreasing of the size of the shell created by water molecules around PC head of DPPC molecules.

Mechanical properties of the cell membrane play a crucial role in many biological processes. Membrane elasticity is criterion of their mechanical stability. Mechanical properties are largely influenced by ethanol molecules, even though they do not absorb to DPPC monolayer. The elastic modulus measurements revealed constant increasing of the membrane stiffness. Vitamin C does not decrease the DPPC monolayer stiffness distinctly after ethanol molecules uptake. The DPPC monolayer on water is more flexible and elastic against mechanical impact than this monolayer on subphase ethanol-water. The obtained results indicated possible effect of ethanol in vivo on membrane function. Here arises the question if ethanol influences the physiological function of membrane in vivo with concentration close to concentration in plasma or interstitium.

The results show that the protective effect of vitamin C may be other than the antioxidant effect. We believe that we may expect similar protection effect in vitro and in vivo. We used vitamin C in the metabolism-free environment that could have produced free reactive radicals. Thus the antioxidant effect of vitamin C was not demonstrated on relevant level. The question still remains whether ethanol influences the physiological function of a membrane in vivo with concentration close to concentration in plasma or interstitium.

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EFFECT OF CURCUMIN AND QUERCETIN ON OXIDATIVE TISSUE DAMAGE INDUCED BY FERRIC NITRILOTRIACETATE (Fe-NTA)

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Key words: curcumin, quercetin, ferric nitrilotriacetate (Fe-NTA), oxidative damage

Introduction

Ferric nitrilotriacetate (Fe-NTA), iron(III) chelate of nitrilotriacetic acid, is a known prooxidative compound1 and a carcinogen, used as a tool in experimental medicine for kidney carcinogenity studies. It is assumed that the mechanism of its nephrotoxicity is a consequence of iron-stimulated production of free radicals which initiate oxidative stress in the tissue2. Natural antioxidants, e.g. curcumin (Cur) and quercetin (Que) are able to counteract the effect of metal ions which participate in free radical generations. Curcumin (diferuloyl methane), a phenolic compound and a major component of Curcuma longa L., is lately drawing attention for its antioxidant activity and its low toxicity. It is also considered to be a potent cancer chemopreventive agent3. Quercetin (3,5,7,3',4'-penta-hydroxy flavone) is a naturally occurring flavonoid present in fruits and vegetables, known for its antioxidant and free radical scavenging activities4.

The aim of this study was to estimate the effect of curcumin and quercetin on lipid peroxidation, iron content and histopathological changes in kidneys of rats subjected to intraperitoneal ferric nitrilotriacetate administration.

Methods

Male Wistar rats (Anlab Prague) weighing 140–150 g were fed standard diet (Altromin C1000) and drinking water ad libitum. After an adaptation period of 6 days, the animals were assigned to four groups of 9 animals as follows: I. Control; II. Fe-NTA; III. Fe-NTA+Cur; IV. Fe-NTA+Cur, Que. Curcumin was supplemented in the diet (Altromin C1000A, 2500 mg kg⁻¹ of diet) for 8 days; quercetin was administered per os (15 mg kg b.w.⁻¹, dispersed in 0.5 % methylcellulose) once a day for 8 days. On the 6th day of experiment, the freshly prepared Fe-NTA solution (8 mg Fe kg b.w.⁻¹, 1 ml 100 g b.w.⁻¹) was injected intraperitoneally to animals in group II-IV. The experiment was finished 24 h after the last dose of antioxidants, i.e. 72 h after Fe-NTA injection. Both kidneys were quickly excised, one kidney of each animal was immediately frozen and stored at −70 °C until analyzed. The second kidney was fixed in 4 % formaldehyde, tissue specimens then routinely processed, stained with hematoxylin and eosin and used for histopathological examination.

Lipid peroxidation was measured as malondialdehyde (MDA) production formed in the thiobarbituric acid reaction in kidney homogenates5. The level of reduced glutathione (GSH) was estimated in the deproteinized supernatant fraction of kidney homogenate using 5,5'-dithiobis (2-nitrobenzoic acid) and recording absorption at 412 nm (ref6). The iron concentration in the kidney was measured using atomic absorption spectrometry (SpectrAA 220 FS, Varian Australia Ltd.).

The data were expressed as means ± SD. Differences between experimental groups were estimated using unpaired Student’s t-test. Results were considered statistically significant at P<0.05.

Results and discussion

The renal oxidative damage at 72 h after Fe-NTA administration was manifested by a significant increase in lipid peroxidation (fig. 1). Curcumin treatment was found to decrease the level of MDA and quercetin treatment significantly enhanced the protective effect of curcumin (P<0.01).

An increase of renal GSH level was found in Fe-NTA treated animals at 72 h following the injection (fig. 2). Curcumin+quercetin treatment increased renal GSH level compared to Fe-NTA only treated group.

As shown in Table I, a significant increase of iron

![Fig. 1. The effect of curcumin and quercetin treatment on lipid peroxidation in the kidneys of Fe-NTA exposed rats; data represent mean ± SD, n=9; *** P<0.001 FeNTA group vs. Control group; # P<0.05, ### P<0.001 FeNTA+antioxidant group vs. FeNTA group](image-url)
concentration was found at 72 h after a single administration of Fe-NTA. This enhancement was not affected significantly by antioxidants treatment.

Histopathological examination of kidney sections of Fe-NTA treated rats at 72 h after Fe-NTA administration revealed severe tubular epithelial damage with disappearance of distal brush border, without accompanying interstitial changes (fig. 3). The treatment with curcumin alone or in combination with quercetin did not prevent these changes.

Conclusion

The results from this study indicate that the treatment with curcumin and especially the combined treatment with quercetin significantly decreased lipid peroxidation. However, differently from examinations performed by other authors at shorter time period after the Fe-NTA administration (1−12 h)8,9, no evidence of positive effects of antioxidants on Fe-NTA induced morphological changes was found in our experiment at 72h after Fe-NTA injection.

This study was supported by the grant MSM 0021620819.

REFERENCES


Fig. 2. The effect of curcumin and quercetin treatment on reduced glutathione (GSH) level in the kidneys of FeNTA-exposed rats; data represent mean ± SD; n=9; *** P<0.001 FeNTA vs. Control group; # P<0.05 FeNTA+antioxidant group vs. FeNTA group

Table I
Iron concentration in the kidneys of FeNTA and antioxidants treated Wistar rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fe [µg g⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26.7 ± 3.7</td>
</tr>
<tr>
<td>FeNTA</td>
<td>39.5 ± 4.9  ***</td>
</tr>
<tr>
<td>FeNTA + curcumin</td>
<td>36.4 ± 5.9</td>
</tr>
<tr>
<td>FeNTA + curcumin, quercetin</td>
<td>34.7 ± 5.9</td>
</tr>
</tbody>
</table>

Results in µg g⁻¹ of wet tissue weight. Mean ± S.D., n=9, *** P<0.001 vs. Control group

Fig. 3. Light microscopy of renal tissue from Fe-NTA treated rats; hematoxylin-eosin staining; (a) intense zonal necrosis in tubular apparatus, 100×; (b) detail of necrotic changes, 200×
ANTIOXIDANT STATUS IN LUNG OF RATS EXPOSED TO FIBROUS DUST OR CIGARETTE SMOKE

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Key words: lung, antioxidant status, cigarette smoke, fibrous dust

Introduction

The lung is the primary organ at risk of inhaled particles and gases, some of them are contributing factors of various respiratory diseases causation. The evidence of adverse health effects following exposure to asbestos resulted of banning asbestos and replacing it by various types of man-made mineral fibres having suitable technological properties. Cigarette smoke is a complex mixture containing more than 4700 constituents\textsuperscript{1} including polycyclic aromatic hydrocarbons, cadmium, arsenic and other oxidants and toxic substances. Exposure to fibres\textsuperscript{2} or cigarette smoke has been associated with production of reactive oxygen species generated either from the noxes themselves or by activated inflammatory cells. The lung has various protective mechanisms including an antioxidant system designated to metabolize oxidants. However, antioxidant enzyme systems in the lung may be overwhelmed by increased levels of free radicals\textsuperscript{3}. We subjected rats to inhalation of cigarette smoke and to intratracheal instillation of different fibres and evaluated their antioxidant status in lung after finishing the exposures. The effects of different exposures were compared.

Materials and methods

Animals

Male Albino Wistar rats (Velaz, Prague, Czech Republic) were used in all experiments. They were housed under standard laboratory conditions and were given a conventional laboratory diet (TOP-Dovo, Horné Dubové, Slovakia) and tap water \textit{ad libitum}. The study was conducted with the approval of the Animal Committee of the Slovak Medical University and in accordance with the guidelines of the European Convention for the Protection of Vertebrate Animals for Experimental Purposes.

Exposure to fibrous dust

Three different fibrous dusts were used in this experiment: amosite (asbestos), glass fibres and refractory ceramic fibres 3 (man-made mineral fibres). Animals were intratracheally instilled 4 mg of fibres resuspended in 0.2 ml saline. The control group was instilled by the same volume of saline. The exposure lasted 48 hours.

Exposure to cigarette smoke. A whole-body actively ventilated exposure chamber with a cigarette smoke generator and pumps (THRI, Lexington, KY, USA) was used. Smoker animals breathed diluted mainstream tobacco smoke from 8 standard research cigarettes of the 1R1 type whose smoke contained defined components. The animals were exposed daily, 5 days a week. The total length of exposure was 6 months.

Preparing of material and biochemical analysis

After finishing the exposures the animals were exsanguinated in anesthesia and bronchoalveolar lavage was performed. The lavage was centrifuged and the cell free fraction was separated and used for analysis. The lung tissue was homogenized in PBS and the 10 \% homogenate was centrifuged (30 min, 10 000 rpm). The analyses were done in supernatant. All samples were stored in aliquots at \(-80^\circ\text{C}\) till analysis. Superoxide dismutase activity was estimated using Randox kit. Glutathione peroxidase activity was estimated using cumene peroxide as substrate\textsuperscript{4}, total glutathione was determined using the GSH reductase method\textsuperscript{5}, ascorbic acid was estimated spectrophotometrically by 2,4-dinitrophenylhydrazine method\textsuperscript{6} and protein by Lowry et al\textsuperscript{7}.

Statistical analysis. The results were evaluated by Wilcoxon test.

Results

The antioxidant status was evaluated by estimation of two antioxidant enzymes (GSH-Px and SOD) and two non-enzymatic antioxidants (GSH and AA) both in homogenate of lung tissue and in cell free fraction of bronchoalveolar lavage. The results of exposure to 3 different fibrous dusts (one asbestos and two man-made fibres) are summarized and compared with those from control animals in Table I. The results showed greater changes in BALF than in lung tissue homogenate, all parameters after exposure to three studied fibrous dusts were lowered, in case of SOD with statistical significance. Fig. 1. demonstrated the changes evoked by inhalation exposure to cigarette smoke. The changes were more pronounced in non-enzymatic antioxidants with statistical significance in the level of AA in lung tissue and GSH in BALF. The effect of exposure was more reflected in BALF than in lung tissue.
Lung is equipped with antioxidant systems enabling to metabolize oxidants. Intratracheal exposure to three fibrous dusts (which differ in their chemical composition, solubility, size distribution and number of particles per dose) showed similar tendency as inhalation exposure to cigarette smoke: the differences were more pronounced in BALF. BALF contains epithelial lining fluid which is in vital lung on the air-epithelial border and presumed to protect the underlying epithelial cells against oxidative damage. As the response of all tested agents was similar we can speculate that the protective mechanism is universal and focus our next experiments on the role of epithelial type 2 cells which are localized on the epithelial surface and belong from the toxicological point of view to the most important cells.

This work was supported by APVV contract 21-011164 and by Ministry of Health of Slovak Republic contract 2005/29-SZU-07. The authors appreciate the technical assistance of Mária Valentová and Helena Bobeková.

Table I
Antioxidant status in lung tissue homogenate and cell free fraction of bronchoalveolar lavage fluid after intratracheal exposure to 4 mg of fibrous dust for 48 hours

<table>
<thead>
<tr>
<th>Group</th>
<th>GSH-Px [U mg⁻¹prot]</th>
<th>SOD [U mg⁻¹prot]</th>
<th>GSH [nmol mg⁻¹prot]</th>
<th>AA [µmol g⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>0.128 ± 0.005</td>
<td>8.14 ± 0.73</td>
<td>51.58 ± 6.69</td>
<td>0.87 ± 0.03</td>
</tr>
<tr>
<td>amosite</td>
<td>0.120 ± 0.003</td>
<td>7.24 ± 0.38</td>
<td>66.37 ± 9.59</td>
<td>0.95 ± 0.03</td>
</tr>
<tr>
<td>glass fibres</td>
<td>0.118 ± 0.006</td>
<td>6.84 ± 0.29</td>
<td>64.73 ± 4.40</td>
<td>0.84 ± 0.03</td>
</tr>
<tr>
<td>RCF 3</td>
<td>0.120 ± 0.002</td>
<td>7.41 ± 0.50</td>
<td>48.58 ± 7.57</td>
<td>0.95 ± 0.03</td>
</tr>
<tr>
<td>BALF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>7.27 ± 0.52</td>
<td>0.67 ± 0.03</td>
<td>6.82 ± 0.69</td>
<td>0.085 ± 0.010</td>
</tr>
<tr>
<td>amosite</td>
<td>6.51 ± 1.00</td>
<td>0.48 ± 0.01*</td>
<td>6.63 ± 0.62</td>
<td>0.075 ± 0.005</td>
</tr>
<tr>
<td>glass fibres</td>
<td>6.59 ± 1.24</td>
<td>0.44 ± 0.01**</td>
<td>7.15 ± 0.21</td>
<td>0.066 ± 0.006</td>
</tr>
<tr>
<td>RCF 3</td>
<td>5.77 ± 2.00</td>
<td>0.39 ± 0.06*</td>
<td>7.85 ± 2.64</td>
<td>0.061 ± 0.002</td>
</tr>
</tbody>
</table>

Values are given as means ± SEM (n=6), * P<0.05, ** P<0.01
P22
INHIBITORS OF NADPH OXIDASE DECREASE ENDOTOXIN MEDIATED INDUCTION OF INDUCIBLE NITRIC OXIDE EXPRESSION IN MOUSE MACROPHAGES

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Key words: lipopolysaccharide, phagocytes, reactive oxygen species, diphenyleneiodonium chloride, apocynin

Introduction

Endotoxin is an important environmental pollutant significantly contributing to wide range of acute and chronic inflammatory diseases including lung inflammatory disorders1. Examples of endotoxin are lipopolysaccharide (LPS) or lipo-oligo-saccharide found in the outer membrane of various Gram-negative bacteria. Activation of macrophages by endotoxin leads to production of wide range of inflammatory mediators contributing to pathology of inflammatory process1. Among potent inflammatory mediators is nitric oxide (NO) produced by activated macrophages in high quantities due to increased expression of inducible NO synthase (iNOS)2,3. Intracellular signaling pathways activated upon the activation of cells by endotoxin engage mediators sensitive to intracellular redox environment4. Thus, intracellularly produced reactive oxygen and nitrogen species (RONS) produced by NADPH oxidases and NOS are suggested to be important mediators augmenting the response of cells to endotoxin. Through this mechanisms NO could positively promote NOS expression and further increase of NO production.

The aim of this study was to characterize modulation of endotoxin activated macrophage NO production by the inhibition of RONS producing NADPH oxidases and NOS.

Material and methods

Murine macrophages RAW 264.7 were activated by LPS (100 ng/ml) isolated from E. coli (Sigma-Aldrich) for 24 h in the absence and in the presence of NADPH oxidase inhibitor Apocynin and inhibitor of flavoproteins including NADPH oxidases and NOS Diphenyleneiodonium chloride (DPI). Toxicity of these compounds were evaluated based on determination of floating death cells in cell culture media and total amount of protein in cell lysate5. The production of NO was evaluated based on determination of nitrites (metabolites of NO oxidation) in cell culture media by Griess reagent2. Expression of iNOS was determined in cell lysate by Western blot technique using iNOS specific monoclonal antibodies (BD Laboratories)3. Values represent mean ± standard error of the mean. The statistic analysis was performed using Mann-Whitney test and differences at $P<0.05$ and $P<0.01$ were regarded as statistically significant.

Results and discussion

Evaluation of toxicity by both protein determination (Table I) and counting of death floating cells (data not shown) showed slight toxic effects of DPI on RAW 264.7 in two highest tested concentrations, however, without statistical significance. Apocynin did not show any toxic effects on RAW 264.7 (Table I) Interestingly, the LPS

![Fig. 1. Nitrite concentration in culture media](image-url)
The study was supported by a grant No. 524/06/1197 of the Grant Agency of the Czech Republic and research plan AVOZ50040507.

REFERENCES

CONSEQUENCES FOLLOWING INTOXICATION WITH HEPATOTOXIC AND NEPHROTOXIC SUBSTANCES

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Introduction

The aim of our study was to evaluate the severity and consequences of hepatic and kidney damage following intoxication with hepatotoxic and nephrotoxic chemical substances and mushrooms retrospectively in the years between 2000 and 2004 with a focus on their reversibility based on the calls made to the Czech Toxicological Information Centre (TIC).

Methods

A variety of clinical and laboratory parameters were collected: increase of serum alanine aminotransferase level (ALT > 0.75 µkat l⁻¹), serum aspartate aminotransferase level (AST > 0.75 µkat l⁻¹), serum total bilirubin level (> 17.0 µmol l⁻¹), serum creatinine level (> 110 µmol l⁻¹) and decrease of prothrombin time (< 75 %). Follow-up evaluation was indicated at the time of discharge if hepatic or renal functions remained abnormal.

Results

From 2000 till 2004 the TIC received in total 43,044 calls. There were 4,649 (10.80 %) enquiries with suspicion of poisoning with potentially hepatotoxic or nephrotoxic substances. Intoxications with mixtures or drugs were excluded. Only 269 (0.62 %) calls involved intoxication with chemical substances (ethylene glycol, chlorinated hydrocarbons) or with mushroom (Amanita phalloides). Poisoning with Cortinarius orellanus was not recorded. The hepatic or renal damage was recorded in 69 (0.16 %) patients. The laboratory markers had twofold to more than hundredfold increase/decrease.

Ingestion of the toxic substances as a route of poisoning occurred in all but two cases. 66 years old man inhaled carbon tetrachloride vapours from an old fire extinguisher during his work in the household. He also poured his hands with the toxic liquid and drank one litter of beer. He developed renal failure (serum creatinine 948 µmol l⁻¹) with a necessity of repeated haemodialysis during 14 days. Nevertheless his renal function completely improved till 6 months following intoxication. 29 years old woman had been working for 3 months with a mixture of organic solvents containing chloroform and methylene chloride in a small room without ventilation, where she inhaled the vapours of the solvents. She developed jaundice; her AST, ALT and bilirubin levels had tenfold increase above the reference value, but improved until 3 months.

50 adults developed nephrotoxicity following ingestion of ethylene glycol. 74 % of them fully recovered, 20 % did not comply in the follow-up. Hepatic and/or renal damage was recorded in other 17 patients following Amanita phalloides ingestion. 5 patients did not comply in the follow-up. In 33 years old woman renal failure persisted with the necessity of haemodialysis continuing even 4 years after intoxication. Other patients fully recovered.

Of 54 adults, who were followed-up, all but 4 recovered completely, mostly (81 %) till 6 months following intoxication.

Discussion

Our follow-up analysis demonstrated that hepatic or renal function impairment was reversible ad integrum in majority of patients. Recovery of their function could be influenced by the dose ingested and time delay between poisoning and admission to the hospital. All 3 subjects that did not recover after ethylene glycol intoxication had a history of disease that could have impaired the kidney function.

All mentioned hepatotoxic substances injure centrilobular cells of the liver acinus. Our results confirmed that this type of liver damage has usually a good prognosis. On the other hand the harmful effect of some other substances (phosphorus, iron) in the periportal area is mostly fatal because the new hepatocytes are created there.

Prognosis of poisoning with nephrotoxins, which act in the tubular segment of the nephron (ethylene glycol), is more favourable than a damage of the multipotent progenitor cells from the Bowman’s capsule. This establishment explains the irreversible renal failure with lifelong haemodialysis following ingestion of Cortinarius orellanus.

Conclusion

Hepatic or renal function impairment was reversible in almost all patients, usually up to six months following the intoxication. The irreversible damage was observed very rarely, mostly in patients with pre-existing target organ disorders.

This study was supported by Research Project No. MSM 0021620807.

REFERENCES

EFFECTS OF A FLAVONOID STRUCTURE ON CYTOCHROMES P450 INDUCTION

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Key words: cytochrome P450, Western blotting, enzyme assay, flavone, flavanone

Introduction

Flavonoids are a diverse group of naturally occurring phenolic compounds. They are widely distributed in most plants and are an important component of human diet. It has been reported that flavonoids demonstrate a wide variety of biological activities, such as the enzyme-modifying activity, scavenging of reactive intermediates, antioxidant, antibacterial, antimutagenic and antiviral properties. On the other hand, it has been suggested that flavonoids may act as mutagens, prooxidants, and enzyme inhibitors and that they exert cytotoxicity at higher concentrations.

The basic chemical structure consists of two benzene rings (A and C) that are linked by a heterocyclic ring (B) (fig. 1). The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a group differ in the pattern of substitution of the ring A and B. The biochemical and biological properties of flavonoids vary considerably with only minor modifications of the flavonoid structure. The effects of multiple hydroxyl and methoxyl groups substituting the basic flavonoid skeleton have been reported previously.

One of the mechanism by which these compounds may exert their effects is through the interaction with cytochromes P450, monooxygenases metabolizing xenobiotics (e.g. drugs, carcinogens). In the processes of carcinogenesis, flavonoids might increase CYP-mediated carcinogen activation by inducing CYPs or by stimulating their enzymatic activities.

The aim of the present study is to investigate the effects of a series of non-substituted flavonoids (fig. 1) and flavanones (Table I) on the induction and metabolic activities of CYP1A1/2 after p.o. administration. For this purpose, their effects were evaluated by (i) the activities of ethoxyresorufin-O-deethylase EROD (CYP1A1) and methoxyresorufin-O-demethylase MROD (CYP1A2); (ii) the immunochemical identification of the P450 protein.

Material and methods

Flavonoids (α-naphthoflavone, β-naphthoflavone, flavone, naringenin, naringin, hesperetin, hesperidin, flavanone; Sigma Chemical Co., USA) were administered p.o. 60 mg kg⁻¹ body weight to male Wistar rats (140–150 g), dissolved in sunflower oil (1 ml), daily for 5 consecutive days. The control group was treated with 1 ml of the sunflower oil. Microsomal fractions were prepared, immediately after sacrificing the rats, by differential centrifugation according to the method of van der Hoeven and Coon from the whole liver. Protein concentration in microsomes was determined by the method of Smith et al.

The CYP1A1/2 protein amounts present in the microsomes were determined by Western blotting on Immobilon-P membrane using specific chicken anti-CYP1A1 antibody. Ethoxyresorufin-O-deethylase (EROD) and methoxyresorufin-O-demethylase (MROD) activity assays were performed using the method described by Burke and Mayer. Formation of the resorufin product was continuously measured for 10 minutes by monitoring its fluorescence with excitation and emission wavelengths of 530 and 585 nm, respectively. Both these enzyme assays have been performed with PerkinElmer Luminescence Spectrometer LS55.

Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>C3’</th>
<th>C4’</th>
<th>5</th>
<th>7</th>
<th>C2-C3</th>
</tr>
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<tbody>
<tr>
<td>Flavanone</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>single</td>
</tr>
<tr>
<td>Hesperetin</td>
<td>OH</td>
<td>OCH₃</td>
<td>OH</td>
<td>OH</td>
<td>single</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>OH</td>
<td>OCH₃</td>
<td>OH</td>
<td>rutinose</td>
<td>single</td>
</tr>
<tr>
<td>Naringenin</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>single</td>
</tr>
<tr>
<td>Naringin</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>rutinose</td>
<td>single</td>
</tr>
</tbody>
</table>

Fig. 1. Structure of flavones
Results and discussion

The aim of this study was to specify the induction effect of natural and synthetic flavonoids on CYP1A subfamily. Our results show that the induction pattern of the flavonoids varies with their structure. Naturally occurring non-substituted flavonoids, flavone and flavanone; two synthetic flavonoids, β-naphthoflavone and α-naphthoflavone; and citrus flavanones, naringenin, naringin, hesperidin, hesperetin, were selected to study the CYP1A1 and CYP1A2 induction. Hesperidin – hesperetin and naringin – naringenin, were selected as representatives of flavanone glycosides and their aglycones. To mimic the human flavonoid intake, the tested compounds were administered p.o. to rats and CYP1A1/2 content was determined in isolated liver microsomes.

Flavonoid effects on xenobiotic-metabolizing enzyme activities

We investigated the effects of a number of flavonoids on P450-dependent EROD and MROD activities. All four non-substituted flavonoids increased both monooxygenase activities, EROD and MROD (fig. 2). Synthetic flavonoids, β-naphthoflavone and α-naphthoflavone, enhanced the activity of EROD more than the two other natural flavonoids, flavone and flavanone. Flavone increased both activities more than flavanone. It can be explained by the presence of a C2-C3 single bond (ring C) in flavanone and a double bond in flavones (flavone, BNF, ANF). According to our results, the unsubstituted flavonoids are suggested to be able to function as inducers of several CYP (1A and 2B)12,13.

The two glycosides, hesperidin and naringin, affected the activity of CYP1A1 in liver microsomes in contrast to their appropriate aglycones. On the other hand, all four flavanones increased the MROD activity. The different affection can be explained by the presence of glycosides in the molecule.

Immunoblotting studies

Immunoblot analyses were carried out to determine CYP1A1/2 protein expression and subsequently to correlate the observed EROD and MROD activities with content of the corresponding isofrom. The specific primary chicken antibody against CYP1A1 and the secondary antibody conjugated with alkaline fosfatase were used for detection of CYP1A1 and 1A2. As shown in fig. 3, the CYP1A2 protein is known to be several times more abundant in control rat liver microsomes than the CYP1A1 protein. From fig. 3, it is clear that beside the model inducer, β-naphthoflavone, the strongest induction effect on CYP1A1 of all natural flavonoids was determined in flavone-treated rats. Whereas, flavanone did not affect CYP1A1, it markedly induced CYP1A2. The induction of CYP1A2 was observed in all four non-substituted flavonoids. The significant induction effect on CYP1A2 was

![Graph](image1.png)

Fig. 2. Effects of flavonoids on EROD (a) and MROD (b) activities of CYP1A1/2 in rat liver microsomes after p.o. exposure to flavonoids (60 mg kg⁻¹ body weight) for 5 days, ±SD≤10 %

![Graph](image2.png)

Fig. 3. Immunodetection of CYP1A1 and CYP1A2 in liver microsomes from flavonoid-treated rats; electrophoresed microsomal proteins (25 µg) were transferred to Immobilon-P membrane and probed with antibody against CYP1A1 and CYP1A2. Lane 1: flavone; lane 2: flavanone; lane 3: β-naphthoflavone; lane 4: control; lane 5: α-naphthoflavone; lane 6: naringin; lane 7: control; lane 8: hesperidin; lane 9: hesperetin
determined in hesperidin and naringin-treated rats. These results are in accordance with the above mentioned EROD and MROD enzyme assays.

Abbreviations

EROD  ethoxyresorufin-O-deethylase
MROD  methoxyresorufin-O-demethylase
CYP  cytochromes P450
ANF  α-naphthoflavone
BNF  β-naphthoflavone
N  naringenin
NG  naringin
HT  hesperidin
F  flavone
FN  flavanone

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Key words: methotrexate, carnitine, transintestinal transport, in situ intestinal perfusion, rat small intestine

Introduction

Two substances were selected for the definition of the transport processes and their influence in the small intestine in rat. Methotrexate (MTX) is indicated as cytostatic and immunosupressive drug1. Its antiproliferative action temporarily decreases the mucosae cellularity, the villy height and the depth of crypts in small intestine in rat, thus, induces the malabsorption syndrome2,3. L-Carnitine (CRT) is naturally occurring endogenous substance which facilitates the transport of the fatty acids through the inner mitochondrial membrane for the purpose of the β-oxidation4; thus, we also expect its potential influence on the general transport mechanisms.

Material and methods

Drugs

The representative substances of the transport mechanisms: indapamide hemihydrate (IND) as the representative substance of passive diffusion, galanthamine hydrobromide (GAL) and 7-methoxytacrine lactate (MEOTA) as the representatives of the combination of passive diffusion and carrier mechanism.

MTX and L-carnitine hydrochloride (Sigma Aldrich), IND (PRO.MED.CS Praha a.s.), GAL (Sigma Aldrich), MEOTA was synthesised in the Department of Toxicology, Faculty of Military Health University of Defence in Hradec Králové. [3H]-7-methoxytacrine (specific activity 128.5 GBq mmol⁻¹, radiochemical purity >98 %, tritiated in the 1st Faculty of Medicine, Charles University). The drugs was added into the perfusion medium and was detected in perfusate samples. Detected amount of radioactive labelled MEOTA was consecutively re-counted for the amount of radioactive unlabelled MEOTA.

Animals

Male Wistar Han II rats (six animals in each group) weighing 311±69 g. For 24 hours before the study, the animals were not given solid food. The rats were anaesthetised by urethane (intraperitoneal dose of 1.5 g kg⁻¹) during the whole experiment. The experiment was approved by the Ethical committee of the Czech Academy of Sciences.

Application of the intestinal absorption modulating substances

The malabsorption syndrome was induced with intramuscularly application of MTX three days before perfusion in accordance with our previously published method2. In case of CRT application: the first group of animals was orally administrated with CRT using gastric probe (250 mg kg⁻¹) for three consecutive days. The perfusion with model drug (GAL or MEOTA 36 µg ml⁻¹) was carry out on third day, one hour after the CRT application. In the second group of animals the simultaneous perfusion only was performed with model drug and CRT (18.2 mg ml⁻¹ of perfusate). The third group of animals was perfused only with model drug without CRT.

The small intestine in situ perfusion

The perfusion apparatus, applied perfusates and the surgical procedure were used the same as described previously2. For the control of the mucosae physiological state we compared the histology of intestinal barrier among intact animals and animals after the perfusion. After the

![Fig. 1. Histological examination of the mucosae of small intestine](image-url)
overall appreciation of the histological structure of intestine, the heights of villi and crypts and the distance between villi were evaluated using light microscopy. The histological examination of mucosae document that the measured parameters were not significantly altered under the perfusion condition compared to the control animals (fig. 1).

**Bioanalytical procedures**

The HPLC methods were used for the detection of GAL and IND (UV detection, λ = 240 nm, chromatographic column 250×4 mm, Purospher® RP-18 end-capped (5 µm), with pre-column 4×4 mm; mobile phase A: 0.01 M phosphate buffer (pH 3.4) – acetonitrile (63 : 37, v/v), mobile phase B – for the wash of a ballast matters: 0.01 M phosphate buffer (pH 3.4) – acetonitrile (20 : 80, v/v); the flow rate of both mobile phases was 1 ml min⁻¹. The radioactive labelled MEOTA was analyzed using scintillation detection. The samples from portal vein were interfused with 1 ml of a scintillation solution. After 24 hours of stabilization, the measuring of an activity was done in the samples with the use of liquid scintillation spectrometry (BECKMAN LS 5000 ID apparatus). Results were expressed as the mean ± standard deviation. F-test and Student’s t-test were used for statistical evaluations.

**Results**

MTX significantly increased the transport of indapamide on the way of passive diffusion (fig. 2), but the transport of MEOTA was not significantly affected by MTX (fig. 3). CRT significantly increased the absorption of MEOTA and GAL after its previous in vivo premedication. The lower absorption occurred in the case of simultaneous perfusion of these model drugs with CRT (fig. 3 and 4).

**Discussion**

In our previous paper we demonstrated that MTX induced the histological changes in mucosae of the small intestine. These findings are in good correlation with results published by other authors which demonstrated that MTX induced significant mucosa damage within the duodenum, jejunum and ileum of rats and dose-dependent decrease in villus heights. Small intestine damage is believed to result from hypoproliferation and widespread apoptosis of stem cells in intestinal crypts. It is perceptible that MTX induces the malabsorption syndrome, significantly reduces the villus height (thus absorption area) and intestinal wall repair and hence its cellular outfit. It leads to the reduction of the intestinal barrier and thus to the increase the transport of xenobiotics (indapamide) on way of passive diffusion. On the other hand, in our previous experiments we documented that MTX significantly decreases the carrier transport of glucose from lumen of small intestine. The explanation why MTX significantly did not influence the MEOTA and GAL transport may be that both substances are partly transported by passive diffusion and partly by carrier transporting mechanism. The
influence of MTX is finally neutral in result. Due to the fact that the CRT absorption occurs as well as MEOTA and GAL partly via carrier-mediated transport and partly by passive diffusion, it could lead to the over-saturation of carrier system and thereby to the competition upon the absorption of the other compounds. Significantly higher MEOTA and GAL absorption in rats orally premedicated with CRT in vivo for a period of three days suggested that CRT have probable some facilitated effect on the transintestinal transport of MEOTA and GAL and maybe some others substances after its incorporating into the cellular metabolism.

Conclusion

The experiments documented the usability of the rat small intestine perfusion method in the study of transintestinal mechanisms in rat. The MTX application enable to differentiate the simple diffusion and carrier mechanism and thus the way of transintestinal transport of xenobiotics. MTX and CRT are usable in the study of processes of intestinal absorption modulation.

REFERENCES