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Cover page:
Common primrose (*Primula vulgaris*) sprouting among last year's oak leaves. This photo was taken at a bank of the River Drava near Osijek by Ivan Balković. Just like this year's first issue it heralds a new season.
Disclaimer: This photo is intended to evoke the content of this issue of the journal. It is not intended for instructional or scientific purposes.

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Scientific Paper

ALLERGENS FROM *FUSARIUM SOLANI* IDENTIFIED BY IMMUNOBLOTTING IN ASTHMA PATIENTS IN IRAN

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We extracted *Fusarium solani* antigens to evaluate specific anti-*F. solani* IgE in fifty-one patients with asthma (33 men and 18 women) and in 22 non-atopic healthy subjects (15 men and 7 women). *F. solani* strains were cultured in Sabouraud glucose agar and subjected to cell disruption using the freeze-and-thaw method. The obtained cytoplasmic extracts were analysed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Sensitisation to *F. solani* antigens has been evaluated in asthmatic patients using the immunoblotting assay. The SDS-PAGE identified 29 protein bands in the cytoplasmic extracts of *F. solani* isolates, with molecular weights ranging from 24 kDa to 112 kDa. Immunoblotting detected specific anti-*F. solani* IgE antibody in all asthma patients, but not in the control group. The predominant reactive allergens in patients corresponded to the bands with molecular weights of 24 kDa, 58.5 kDa, 64.5 kDa, 69 kDa, 72 kDa, and 97 kDa. Our results suggest that various allergenic components of *F. solani* may produce symptoms of asthma in susceptible individuals and they call for further research.

KEY WORDS: *airborne spores, allergenic sensitisation, fungal allergy, IgE, SDS-PAGE*

Today, more than one fifth of the world population faces IgE-mediated allergic diseases such as asthma, rhino-conjunctivitis, and eczema (1). Asthma is a complex illness resulting from an interplay of several allergenic and non-allergenic factors, and is characterised by airway obstruction and inflammation (2).

Sensitisation to allergens has an important role in the development of asthma (3). Fungal spores and mycelia are well known respiratory allergens. It is estimated that 3 % to 40 % of allergic patients worldwide suffer from fungal allergy (4). By now, the role of fungi in the development of asthma has not been fully investigated. *Alternaria alternata*, *Aspergillus fumigatus*, and *Cladosporium herbarum* have been reported as the most common causes of fungal allergies, and many allergens of these fungi have been described at the molecular level (5, 6). In addition, airborne spores of the *Fusarium* species are also widely dispersed and common in many environments (7, 8). These saprophytic or parasitic fungi often contaminate crops, fruit, and vegetables. They also frequently affect the eyes, skin and nails, and cause systemic infections in immunocompromised hosts (9, 10). Among the *Fusarium* species, *F. solani* plays the most important role in IgE-mediated allergic reaction in patients with nasobronchial allergy (11). O'Neil et al. (12) found that about 24 % of atopic individuals had the skin test and radioallergosorbent test positive to *F. solani* extracts.

The culture filtrate, mycelium, and spore extracts of *F. solani* contain various allergens, either species-specific such as the 65-kDa glycoprotein (13) or shared with related species or genera.

The diagnosis of allergy is usually based on the medical history of the patient, skin prick tests, and *in vitro* tests for allergen-specific IgE in the patient serum. However, most commercial fungal allergen extracts used for *in vivo* and *in vitro* diagnostics are neither pure enough nor standardised. Our study is an attempt to investigate potential allergenic proteins from *F. solani* that could improve screening for asthmatic patients by widening the array of known fungal allergens.

MATERIALS AND METHODS

Chemicals and reagents

All general chemicals and reagents used in the study were purchased from Merck Co. (Darmstadt, Germany), unless specified otherwise.

Strain and cultivation

A total of 12 *F. solani* strains were isolated from the air and foodstuffs from Iran. First the samples were cultured in Sabouraud glucose agar supplemented with 0.5 % chloramphenicol (Sigma Chemical Co., St. Louis, Mo., USA) at 30 °C for 5 days. All *Fusarium* sp. isolates were further subcultured on potato dextrose agar (PDA), Spezieller Nährstoffarmer agar (SNA), and carnation leaf agar (CLA). PDA cultures were incubated at 30 °C for 5 to 7 days, while CLA and SNA cultures were incubated at 25 °C for 2 to 4 weeks.

The cultures were evaluated both by eye and microscopically. For morphological identification of the isolates we followed the criteria of Leslie and Summerell (14). Colony morphology was observed from cultures cultivated on PDA. To assess the morphology of macroconidia, microconidia, conidiogenous cells, and the chlamydospores we used the cultures incubated on SNA and CLA.

Cell fractionation and crude extract preparation

The fungal colonies were cultured in 500 mL of Sabouraud glucose broth and agitated in the shaker at 30 °C for 10 days. At the end of incubation, the fungal mats of each strain containing spores and mycelia were harvested using the filtration technique (Whatmann filter paper; no. 0.45 μm) and washed three times with the PBS buffer (0.15 mol L^{-1}, pH 7.2). A suspension of wet fungal colonies was prepared in a lysing buffer (62.5 mmol L^{-1} Tris, 1 mmol L^{-1} dithiothreitol, 0.2 mg mL^{-1} of phenyl sulfonyl fluoride and 15 % glycerol, pH 6.8). Cells were disrupted by freezing them to -70 °C for 30 min and thawing them over 20-minute intervals, repeating the procedure six times in a row (the so called freeze-and-thaw method). The samples were finally ground by a sterilised mortar for 20 min. Crude antigens were separated from other cell components by centrifugation at 20000g for 20 min, and subsequently centrifuged at 25000g for 30 min. Clear supernatants were obtained and stored at -20 °C until used.

Protein determination

Protein concentration of fungal extracts was measured after reconstituting the extracts in 50 μL and 100 μL of distilled water using the Bradford method (15).

SDS-PAGE analysis

To separate the fungal extracts we used a 17.5 % separation gel with a 5 % stacking gel in a discontinuous buffer system as proposed by Laemmli (16). The extracts were boiled in a reducing sample buffer with 2-β-mercaptoethanol for 5 min. Aliquots of the sample (V=10 μL, which equals to 60 μg of proteins) were loaded on a 0.5 cm gel. Molecular weight standard markers of 14.4 kDa to 93 kDa (Rainbow markers, Amersham International PLC, UK) were also loaded on the gel. The gels were stained with silver nitrate and commassie brilliant blue R-250 (Sigma, St. Louis, USA).

Serum collection

Sera used in this study were pooled from 51 adult patients (33 men and 18 women aged 20 to 60 years) whose asthma had been confirmed by a physician. The majority (62.7 %) were younger than 40 years. They were recruited in the Tehran Allergy Clinic, Iran. The sera were also taken for testing from an age and sex matching control population of 22 healthy subjects (15 men and 7 women) with no personal or family history of atopy. All subjects gave informed consent to participate in the study.

All underwent a detailed clinical examination with special attention to the respiratory system. All subjects also completed a questionnaire with personal data and information related to possible confounding factors such as smoking, drugs used, history of diseases, respiratory and non-respiratory allergic symptoms,

history of oral corticosteroid use and other therapies. All patients had a history of bronchitis, difficulties breathing, and limited lung capacity. None took any kind of medications. Only five patients smoked.

Blood was collected by venipuncture in BD vacutainers (Becton Dickinson, UK) and allowed to clot to facilitate serum collection. Sera collected from each subject were pooled into one sample for each study group, frozen, and stored until use.

Serum IgE against *F. solani* antigens was determined using the immunoblotting assay.

IgE immunoblotting

The isolated *Fusarium* sp. proteins were transferred to nitrocellulose sheets (Schleicher and Shuell, Dassel, Germany) with a transphor apparatus (Hoefer, San Francisco, USA) at 100 V for 2.5 h using a transfer buffer (25 mmol L^{-1} Tris, 192 mmol L^{-1} glycin, 0.03 % SDS, and 20 % methanol, pH 8.3). The nitrocellulose sheets were cut into 0.5 cm strips and incubated overnight at 4 °C with the TBS-Tween 20-BSA buffer (20 mmol L^{-1} Tris, 0.15 mol L^{-1} NaCl, 0.05 % Tween 20, and 1.5 % BSA, pH 7.5). The strips were washed three times with the TBS-Tween 20 buffer at room temperature for 15 min.

Pooled serum was diluted to 1:2 in TBS-Tween 20-BSA and incubated at room temperature for 16 h. After the strips had been washed three times with TBS-Tween 20-BSA, each was added anti-human IgE conjugated with alkaline phosphatase (Sigma, St. Louis, USA) diluted to 1:1000 in TBS-Tween 20-BSA. The strips were then incubated for 5 h and then washed in the alkaline phosphatase buffer (100 mmol L^{-1} Tris-HCl, 100 mmol L^{-1} NaCl, and 5 mmol L^{-1} magnesium chloride) for 10 min. The immunoblot with at least one visible protein band was considered positive (17).

RESULTS

The SDS-PAGE electrophoregram obtained from cytoplasmic extracts of *F. solani* isolates has shown 29 protein bands with molecular weights ranging from 24 kDa to 112 kDa (Table 1 and Figure 1). The gel showed a moderate degree of similarity between the protein banding patterns of various fungal isolates. Overall, the isolates differed in 27 bands whereas two bands were common in all isolates. The most common protein bands were 24 kDa and 32 kDa, followed by 72 kDa and 50 kDa, whereas bands with molecular weights of 108 kDa, 87 kDa and 40 kDa were the least common bands within the *F. solani* isolates.

As isolates 1, 4, 8, and 12 had the highest number of protein bands, they were selected for immunoblotting to detect the presence of IgE against *F. solani* antigens. Immunoblotting of the pooled sera of the asthmatic patients showed a strong reaction to fungal extracts. At the same time, no positive result was observed in controls. IgE responded to 29 different bands in asthmatic patients (Figure 2). The strongest response was to the following six allergens: 24 kDa, 58.5 kDa, 64.5 kDa, 69 kDa, 72 kDa, and 97 kDa.

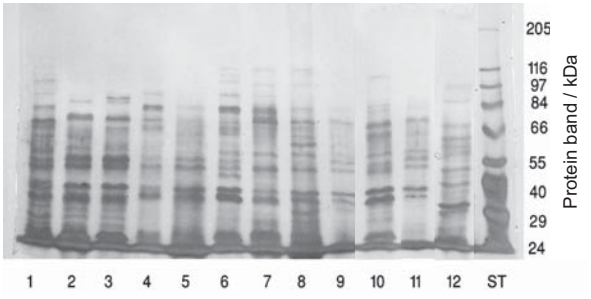

Figure 1 *Protein components of crude extracts of* Fusarium solani *isolated with SDS-PAGE.*

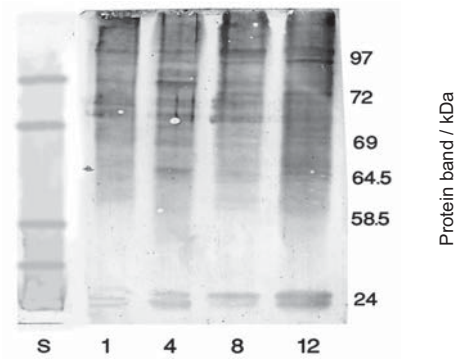

Figure 2 *IgE binding to* Fusarium solani *allergen extracts in the immunoblotting test with pooled serum of 51 patients with asthma.*

DISCUSSION

Members of the genus *Fusarium* are common indoor and outdoor airborne fungi, confirmed to cause bronchial asthma (18). Earlier studies (19, 20) suggest that sensitivity to fungi is common, especially in patients with asthma.

Polyacrylamide gel electrophoresis is frequently used to isolate and identify proteins from different fungal species (21). The same method was successfully employed in the present study. The most important finding is that *F. solani* isolates showed several similar

Table 1 *Bands identified with the SDS-PAGE in each of the 12 isolates of* Fusarium solani.

Isolate No.	Protein bands / kDa	Total number of bands
1	24, 26, 27, 32, 38, 45, 55, 58.5, 64, 69, 72, 81, 94, 100	14
2	24, 26, 28.5, 32, 37, 50, 55, 60, 63, 72, 81	11
3	24, 26, 28.5, 32, 37, 50, 53, 60, 66, 72, 87	11
4	24, 32, 35, 40, 50, 53, 58.5, 66, 72, 81, 94, 100, 112	13
5	24, 32, 35, 50, 53, 64, 72, 81	8
6	24, 32, 35, 61, 64, 66, 72, 81	8
7	24, 28.5, 32, 35, 50, 58.5, 75, 81, 97	9
8	24, 28.5, 32, 35, 50, 58.5, 61, 64, 69, 72, 81	13
9	24, 32, 35, 50, 53, 75	6
10	24, 27, 32, 38, 45, 55, 63, 66, 81, 108	10
11	24, 28, 32, 38, 45, 55, 63, 66	8
12	24, 27, 32, 38, 55, 60, 64, 69, 81, 97	10

protein profiles in cytoplasmic extracts within the range of 24 kDa to 112 kDa. In a previous study by Aghamirian and Zaini (22), somatic proteins UAMH 3317 and UAMH 7419 extracted from *F. solani* produced 16 and 21 protein bands, respectively, with molecular weights of 14 kDa to 100 kDa. In a cytoplasmic extract of *F. solani* isolated from the soil, Bahrain Mandeel et al. (23) identified 18 protein bands ranging from 21 kDa to 100 kDa. In addition, using the SDS-PAGE method, Verma and Gangal (24) demonstrated 18 cytoplasmic protein bands from *F. solani* in India, with the 65-kD protein component as a major allergen. It is difficult to identify a protein in a complex antigen extract on the basis of its molecular weight, and sometimes the same antigen differs in molecular weight between studies. This discrepancy may be related to differences in the calculation of molecular weight, differences in medium composition and incubation temperature or to the presence of several antigens with the same molecular weight (25). Investigation should be continued to further specify the bands.

For clinical diagnosis it is essential to identify the allergenic profiles of the *Fusarium* species, *F. solani* in particular, and see if there are any IgE-cross reactivities between major allergens of this widespread fungus. Immunoblotting is a readily available and simple screening test for IgE binding to major *F. solani* allergens. In this study, we identified six major allergens of *F. solani* with molecular weights of 24 kDa, 58.5 kDa, 64.5 kDa, 69 kDa, 72 kDa, and 97 kDa. In two studies conducted by Verma et al. (11, 26), a 65-kDa allergen of *F. solani* bound IgE from all the sera of asthmatic patients. Verma et al. (27) also demonstrated that peptide named IV-1, a 3.4-kDa fragment of the 65-kDa allergen, was highly allergenic and bound to IgE in the sera of most *F. solani*-positive patients. In another study (28), the same researchers found that a 45-kDa allergen of *F. solani* reacted with patients' sera sensitive to many fungi.

In general, IgE response seems to vary from country to country. Our results disagree with the findings of O'Neil et al. (12) and Hoff et al. (29), who established IgE response to extracts of *Fusarium* species in 24 % and 50 % of atopic patients from the USA and Germany, respectively. The reasons for these differences are not clear, but point to differences in exposure between these countries that may involve climate/geography, *Fusarium* strains, heating, ventilation, and air-conditioning. In addition, the study procedures may have varied, including the allergen extraction methods, patient recruitment criteria, and assays used. Furthermore, immune responses to the allergens may vary between different populations and even races (30), as allergies result from an interplay of various environmental and genetic factors. Nevertheless, this discrepancy between our and the reported studies needs further clarification.

In conclusion, we identified six allergenic bands of *F. solani* (corresponded to 24 kDa, 58.5 kDa, 64.5 kDa, 69 kDa, 72 kDa and 97 kDa proteins), which showed the highest IgE-binding frequency in asthmatic patients. These results suggest that different allergenic components of *F. solani* may produce symptoms of asthma in susceptible individuals and call for further investigation.

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Sažetak

ALERGENI IZ PLIJESNI *FUSARIUM SOLANI* U IRANSKIH BOLESNIKA S ASTMOM UTVRĐENI S POMOĆU IMUNOBLOT-TESTA

Ekstrahirali smo antigene plijesni *Fusarium solani* kako bismo izmjerili imunosni odgovor, tj. razine anti-*F. solani* IgE u 52 bolesnika s astmom (33 muškarca i 18 žena) te u 22 zdrava ispitanika bez atopija (15 muškaraca i sedam žena). Sojevi *F. solani* uzgojeni su na podlozi glukoznoga Sabouraudova agara i podvrgnuti razbijanju stanica s pomoću metode smrzavanja i odmrzavanja. Dobiveni citoplazmatski ekstrakti analizirani su s pomoću elektroforeze u poliakrilamidnom gelu u prisutnosti natrijeva dodecilsulfata (engl. *sodium dodecyl sulphate-polyacrylamide gel electrophoresis*, krat. SDS-PAGE). Senzibilizacija na antigene *F. solani* u bolesnika s astmom utvrđena je s pomoću imunoblot-testa. S pomoću SDS-PAGE-a u citoplazmatskim ekstraktima izolata *F. solani* dokazano je 29 proteinskih vrpca. Molekulske mase razdvojenih proteina kretale su se u rasponu od 24 kDa do 112 kDa. Imunoblot-test otkrio je specifično anti-*F. solani* IgE- protutijelo u svih bolesnika s astmom, ali niti u jednog ispitanika iz kontrolne skupine. Najčešći su alergeni u bolesnika s astmom odgovarali proteinima ovih molekulskih masa: 24 kDa, 58,5 kDa, 64,5 kDa, 69 kDa, 72 kDa i 97 kDa.
Naši nalazi upućuju na to da simptome astme u osjetljivih pojedinaca može izazvati više alergena soja *F. solani* te da ih treba podrobnije istražiti.

KLJUČNE RIJEČI: *alergija na plijesni, IgE, SDS-PAGE, senzibilizacija na alergene, spore u zraku*

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Scientific Paper

EFFECTS OF MYOSMINE ON ANTIOXIDATIVE DEFENCE IN RAT LIVER

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Myosmine [3-(1-pyrrolin-2-yl) pyridine] is an alkaloid structurally similar to nicotine, which is known to induce oxidative stress. In this study we investigated the effects of myosmine on enzymatic and non-enzymatic antioxidative defence in rat liver. Wistar rats received a single *i.p.* injection of 19 mg kg^{-1} of myosmine and an oral dose of 190 mg kg^{-1} by gavage. Nicotine was used as a positive control. Through either route of administration, myosmine altered the hepatic function by decreasing the levels of reduced glutathione, superoxide dismutase, and glutathione peroxidase activities on one hand and by increasing malondialdehyde, catalase, and glutathione reductase activity on the other. Compared to control, both routes caused significant lipid peroxidation in the liver and altered hepatic enzymatic and non-enzymatic antioxidative defences. The pro-oxidant effects of myosmine were comparable with those of nicotine.

KEY WORDS: *lipid peroxidation, oxidative stress, rodents, tobacco alkaloid*

Myosmine [3-(1-pyrrolin-2-yl) pyridine] is the second tobacco alkaloid to nicotine (1). However, myosmine has a much wider distribution over a wide variety of food products such as maize, rice, wheat, cocoa, and milk in a wide range of mass fractions from 0.03 ng g^{-1} in carrots up to 6.26 ng g^{-1} in cream (2). It is therefore not surprising that myosmine has also been detected in human toenail, plasma (up to 5.5 ng mL^{-1}), saliva (up to 4.6 ng mL^{-1}) (3) or breast milk (4). When given orally to Wistar rats, myosmine is metabolised rapidly and completely (5). After nitrosation and/or peroxidation, myosmine bioactivates to 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) and can form pyridyloxobutylated DNA adducts (6) or *N'*-nitrosonornicotine (NNN), which is an oesophageal carcinogen (7). *In vitro* experiments with calf thymus DNA have shown that myosmine is rapidly nitrosated at pH 2 to pH 4 and forms DNA adducts (8). Using the comet assay in human lymphocytes and upper aerodigestive tract epithelial cells, Kleinsasser et al. (9) have demonstrated dose-and time-dependent genotoxic effects of myosmine.

While there are some data about the potential genotoxic and carcinogenic effects of myosmine, little information is available about its toxicity. Organ toxicity of nicotine is known to be related to oxidative stress (10). We assume that because of its structural similarity to nicotine, myosmine might exert similar toxicity by generating reactive oxygen species (ROS).

Zwickenpflug and Tyroller (6) have suggested that because of its imine structure, myosmine seems to be more reactive than other investigated tobacco alkaloids. The authors have shown that when myosmine is mixed with hydrogen peroxide, the latter attacks the carbon nitrogen double bond of the imine

structure, yielding oxaziranes, nitrones, or N-oxides. These metabolic intermediates might be involved in oxidative stress.

The aim of our study was to investigate the effects of myosmine on the enzymatic and non-enzymatic antioxidative defences in rat liver after a single oral and intraperitoneal (*i.p.*) administration.

MATERIAL AND METHODS

Chemicals and enzymes

Myosmine was synthesised at the Institute of Molecular Biology, Bulgarian Academy of Science (BAS), following the method of Brandänge and Lindblom (11). The product was found to be 99.9 % pure; its identity was confirmed by comparing it with commercial myosmine using ultraviolet and infrared spectral analysis, gas chromatography, and high pressure liquid chromatography. All the other reagents used were of analytical grade. Nicotine dihydrogen ditartrate (CAS 65-31-6), 1-chloro-2,4-dinitrobenzene (CDNB), ß-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH), ethylenediaminetetraacetic acid (EDTA), L-glutathione reduced (GSH), L-glutathione oxidised (GSSG), cumene hydroperoxide, thiobarbituric acid (TBA), trichloroacetic acid (TCA), bovine serum albumin (fraction V), glutathione reductase from baker's yeast (*S. cerevisiae*) ammonium sulfate suspension, 100 to 300 units mg^{-1} protein (Biuret) were purchased from Sigma-Aldrich (Taufkirchen, Germany). 2,2'-Dinitro-5,5'-dithiodibenzoic acid (DTNB) was obtained from Merck (Darmstadt, Germany).

Experimental animals

Male Wistar rats weighing (200±10) g were housed under standard laboratory conditions at 20 °C, with 12-h alternating light/dark cycles and free access to food and water. The animals were purchased from the National Breeding Centre, Slivnitza, Bulgaria. All experiments were performed after at least one week of adaptation to this environment. All procedures were approved by the Institutional Animal Care Committee, and the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) (12) were strictly followed throughout the experiment.

Experimental design

Earlier studies have shown that the *i.p.* and oral LD_{50} of myosmine for rats is 190 mg kg^{-1} and 1875 mg kg^{-1}, respectively (13). Before starting the experiment, we ran a pilot study to establish the dose-related effect of myosmine on animal behaviour and liver ALT, AST, and LDH (the results are not shown). A single dose of 1/5, 1/10, or 1/20 of LD_{50} of myosmine was administered *i.p.* and orally to six groups of three rats per group. The dose of 1/20 of LD_{50} did not show any statistically significant behavioural or biochemical changes. The dose of 1/5 of LD_{50} proved very toxic (2 of 3 rats died after *i.p.* injection and 1 of 3 after oral administration) and the dose of 1/10 of LD_{50} was well tolerated and at the same time produced significant changes in the investigated parameters. This is why we used the dose 1/10 of LD_{50} for both routes of administration. For positive control we used nicotine ditartrate in an equitoxic dose of 1/10 of LD_{50} (14).

The rats were then randomised into six groups with six animals in each. Group 1 were control rats treated with physiological saline *i.p.* (1 mL kg^{-1} b. w.). Group 2 were control rats given physiological saline via oral gavage (10 mL kg^{-1} b. w.). Group 3 were rats injected with nicotine dihydrogen ditartrate *i.p.* (1 mg kg^{-1} b. w.). Group 4 were rats administered nicotine dihydrogen ditartrate through oral gavage (6.5 mg kg^{-1} b. w.). Group 5 were rats injected myosmine *i.p.* (19 mg kg^{-1} b. w.), and group 6 were rats administered myosmine through oral gavage (190 mg kg^{-1} b. w.).

Twenty-four hours after the administration of the single dose, the animals were killed by cervical dislocation. Tissue samples were immediately transferred to ice-cold containers, weighed, and homogenised using appropriate buffers (15).

Preparation of liver homogenates for lipid peroxidation (LPO) assessment

Thiobarbituric acid reactive substances (TBARS), expressed as malondialdehyde (MDA) equivalents, were determined as a marker of lipid peroxidation. The method was described by Polizio and Peña (15). Briefly, 1 mL of homogenate was mixed with 1 mL of 25 % trichloroacetic acid (TCA) and 1 mL of 0.67 % thiobarbituric acid (TBA). Samples were then mixed thoroughly, heated in a boiling water bath for 20 min, cooled down, and centrifuged at 4000g for 20 min. The absorbance of supernatant was measured at 535 nm against a blank that contained all the reagents except tissue homogenate. MDA concentration was

calculated using a molar extinction coefficient of $1.56x10^5$ L mol^{-1} cm^{-1} and expressed in nmol g^{-1} of wet tissue.

Preparation of liver homogenates for reduced glutathione (GSH) assessment

GSH was assessed by measuring non-protein sulfhydryls after precipitation of proteins with TCA, using the method described by Bump et al. (16). Briefly, tissues were homogenised in 5 % TCA and then centrifuged at 4000g for 20 min. The reaction mixture contained 0.05 mL of supernatant, 3 mL of 0.05 mol L^{-1} phosphate buffer (pH 8), and 0.02 mL of DTNB reagent. The absorbance was determined at 412 nm and the results expressed as nmol g^{-1} wet tissue.

Preparation of liver homogenates for antioxidant enzyme activity measurement

Rat livers were rinsed in ice-cold physiological saline and minced with scissors. Ten-percent homogenates were prepared in 0.05 mol L^{-1} phosphate buffer (pH 7.4), centrifuged at 7000g for 10 minutes, and the supernatants used for antioxidant enzyme analysis. The protein content of liver homogenates was measured using the method of Lowry (17) with bovine serum albumin as a standard.

Catalase (CAT) activity

CAT activity was assessed as described by Aebi (18). Briefly, 10 µL of homogenate was added to 1990 µL of H_2O_2 solution (containing 6.8 µL of 30 % H_2O_2 + 1983.2 µL 0.05 mol L^{-1} phosphate buffer, at pH 7.4). CAT activity was determined by measuring H_2O_2 decomposition through the decrease in absorbance at 240 nm for 1 min. Enzyme activity was calculated using the molar extinction coefficient of 0.043 L $mmol^{-1}$ cm^{-1} and expressed as µmol L^{-1} min^{-1} mg^{-1} of protein.

Superoxide dismutase (SOD) activity

SOD activity was measured according to Sun and Zigman (19) with slight modifications. The incubation mixture contained 50 mmol L^{-1} carbonate buffer with pH 10.2 at 30 °C. The reaction was started by adding epinephrine base (0.001 mol L^{-1} in 0.02 mol L^{-1} HCl). Epinephrine auto-oxidation was followed spectrophotometrically at 320 nm, using the molar extinction coefficient of 4.02 L $mmol^{-1}$ cm^{-1}. SOD activity is expressed as nanomol of epinephrine that are prevented from auto-oxidation after addition of the sample.

Glutathione peroxidase (GPx) activity

GPx activity was measured by NADPH oxidation, using a coupled reaction system consisting of reduced glutathione, glutathione reductase and cumene hydroperoxide (20). Briefly, 100 µL of enzyme sample was incubated at 30 °C for 5 min with 1.5 mL of 0.05 mol L^{-1} phosphate buffer (pH 7.4), 100 µL of 1 mmol L^{-1} EDTA, 50 µL of 1 mmol L^{-1} GSH, 100 µL of 0.2 mmol L^{-1} NADPH, and 1 U of glutathione reductase. The reaction was initiated by adding 50 µL of cumene hydroperoxide (1 mg mL^{-1}) and the rate of disappearance of NADPH over time was determined by monitoring absorbance at 340 nm using an extinction coefficient of $6.22x10^3$ L mol^{-1} cm^{-1}. The results are expressed in nmol min^{-1} mg^{-1} of protein.

Glutathione reductase activity (GR)

GR activity was measured spectrophotometrically at 340 nm according to Pinto et al. (21). We followed NADPH oxidation using the extinction coefficient of $6.22x10^3$ L mol^{-1} cm^{-1}. The incubation mixture contained 0.05 mol L^{-1} phosphate buffer, pH 7.4, 2.5 mmol L^{-1} GSSG, and 125 µmol L^{-1} NADPH at 30 °C. Enzyme activity is expressed in nmol min^{-1} mg^{-1} of protein.

Glutathione-S-transferase (GST) activity

GST activity was measured using CDNB as the substrate (22). The incubation mixture containing 1.6 mL of 0.05 mol L^{-1} phosphate buffer, 100 µL of 1 mmol L^{-1} GSH, 100 µL of 1 mmol L^{-1} EDTA, and 100 µL of homogenate was incubated at 37 °C for 15 min. After the incubation, 100 µL of 1 mmol L^{-1} CDNB was added and the increase in absorbance with time was recorded at 340 nm. Enzyme activity was measured using the extinction coefficient of $9.6x10^3$ L mol^{-1} cm^{-1} and is expressed as nanomol of CDNB-GSH conjugate per minute per milligram of protein.

Statistical analysis

The statistical analysis was performed using MedCalc (MedCalc Software bvba, Mariakerke, Belgium). The significance of the data was assessed using the non-parametric Mann-Whitney test. Values of $p \leq 0.05$ were considered statistically significant.

RESULTS

The results are expressed as means ±SEM for six rats per group. Figure 1 shows the levels of lipid peroxidation in the liver tissue of control animals and animals treated with myosmine and nicotine. MDA significantly increased in the myosmine and nicotine-treated groups after both *i.p.* injection (+31 % and +21 %, respectively; p≤0.05) and oral administration (+29 % and +33%, respectively; p≤0.05) in respect to control.

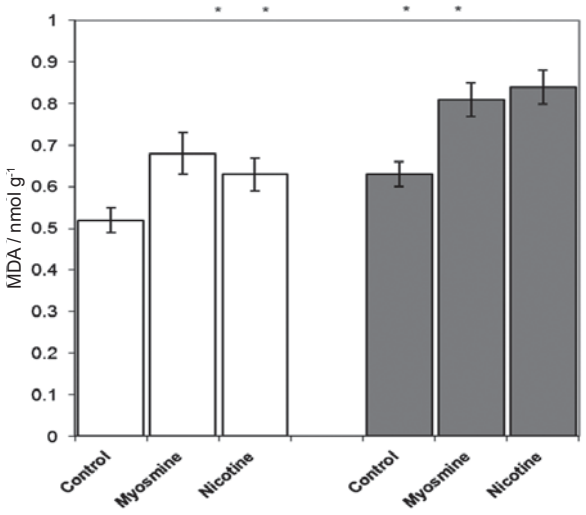

Figure 1 *MDA concentration in rat liver 24 h after* i.p. *injection (□) or oral administration (■) of myosmine or nicotine. Values are presented as the mean ± SEM, n=6. *Significant difference from control values (Mann-Whitney test, p<0.05)*

Myosmine and nicotine treatment depleted GSH -36 % and -14 % after *i.p.* injection and -23 % and -28 % after oral administration, respectively (Figure 2).

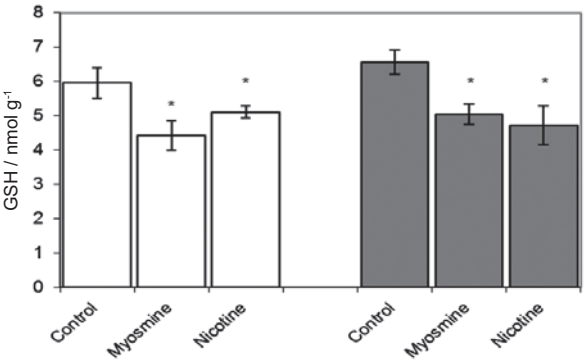

Figure 2 *GSH level in rat liver 24 h after* i.p. *injection (□) or oral administration (■) of myosmine or nicotine. Values are presented as the mean ± SEM, n=6. *Significant difference from control values (Mann-Whitney test, p<0.05)*

The effects on CAT, SOD, and GPx are summarised in Figures 3-5. Significant decreases in the activity of SOD and GPx were noted with both routes of myosmine exposure and were comparable with nicotine. However, myosmine significantly increased CAT activity (24 % after *i.p.* injection and 19 % after oral administration; p≤0.05), while nicotine did the opposite; it decreased CAT by 22 % and 18 % (p≤0.05) after *i.p.* injection and oral administration, respectively.

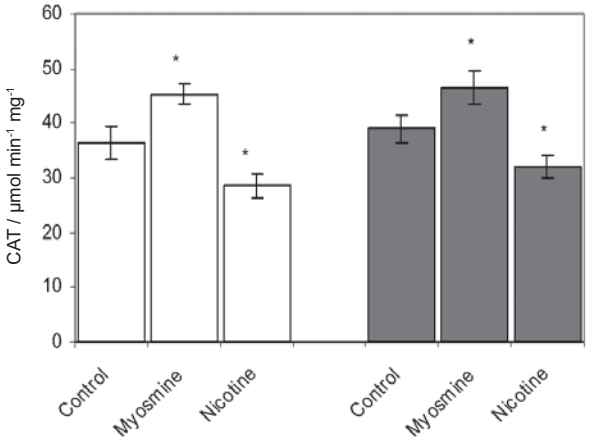

Figure 3 *CAT activity in rat liver 24 h after* i.p. *injection (□) or oral administration (■) of myosmine or nicotine. Values are presented as the mean ± SEM, n=6. *Significant difference from control values (Mann-Whitney test, p<0.05)*

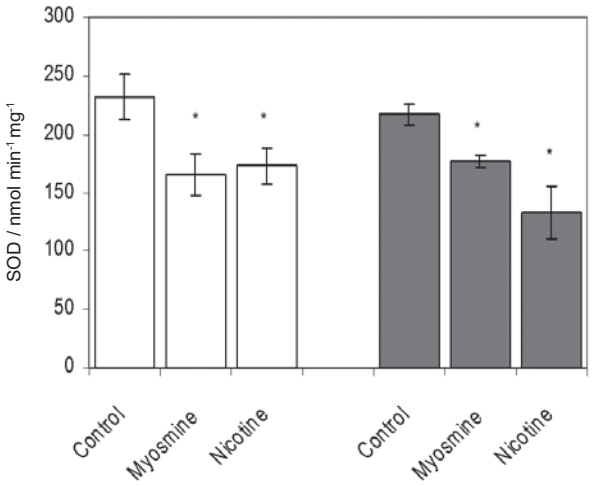

Figure 4 *SOD activity in rat liver 24 h after* i.p. *injection (□) or oral administration (■) of myosmine or nicotine. Values are presented as the mean ± SEM, n=6. *Significant difference from control values (Mann-Whitney test, p<0.05)*

Figures 6 and 7 show the activities of GR and GST after myosmine and nicotine administration. Both significantly increased GR activity through either

route of administration, and to a similar extent, 28 % and 35 %, respectively ($p \leq 0.05$). Neither compound induced significant changes in GST activity after either *i.p.* injection or oral administration.

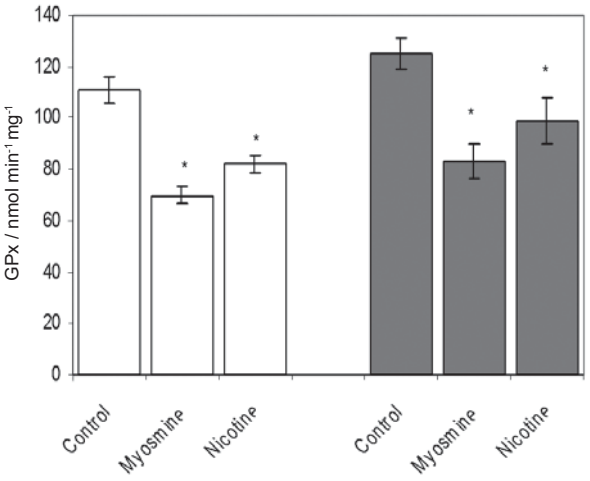

Figure 5 *GPx activity in rat liver 24 h after* i.p. *injection (□) or oral administration (■) of myosmine or nicotine. Values are presented as the mean ± SEM, n=6. *Significant difference from control values (Mann-Whitney test, p<0.05) difference*

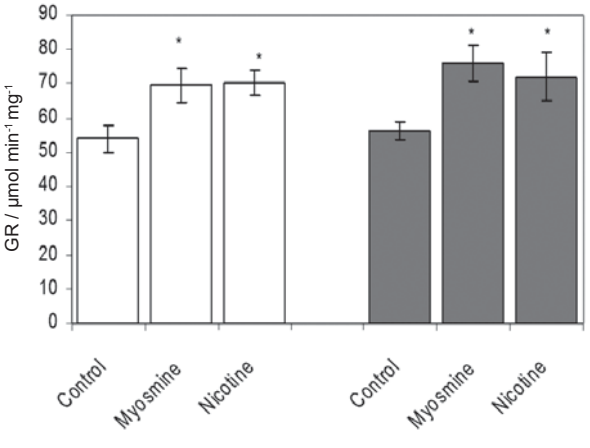

Figure 6 *GR activity in rat liver 24 h after* i.p. *injection (□) or oral administration (■) of myosmine or nicotine. Values are presented as the mean ± SEM, n=6. *Significant difference from control values (Mann-Whitney test, p<0.05)*

DISCUSSION

Oxidative stress is an imbalance between the production and scavenging of ROS and free radicals that can induce lipid peroxidation, DNA fragmentation, and protein oxidation (23). These damages result in the loss of membrane integrity, structural and functional changes in proteins and gene mutations (24). Normally, the affected cells are trying to

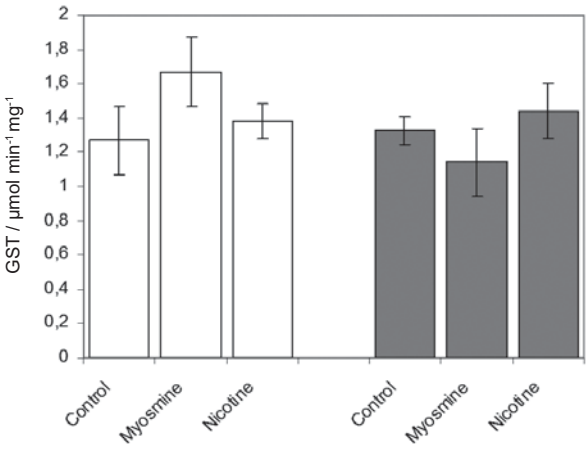

Figure 7 *GST activity in rat liver 24 h after* i.p. *injection (□) or oral administration (■) of myosmine or nicotine. Values are presented as the mean ± SEM, n=6.*

neutralise reactive molecules by deploying their antioxidative defences that include reduced glutathione (GSH), alpha-tocopherol, ascorbic acid, and antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-*S*-transferase (GST).

The formation of reactive oxygen species (ROS) is stimulated by a number of xenobiotics and can result in oxidative stress and cellular injury. As myosmine is structurally similar to nicotine, which is known to induce oxidative stress (24), it is expected to act in a similar way. Its proclivity for oxidation reactions (6) may give rise to lipid peroxidation and cellular damage. In our study, a single dose of nicotine decreased GSH, SOD, CAT and GPx activities and increased MDA level and GR activity in rat liver. These results are in good agreement with the results of Sudheer et al. (26), who reported that nicotine subchronic administration resulted in significant decreases of enzymatic and non-enzymatic antioxidative defences in the liver, lung, and the circulation. Myosmine *i.p.* and oral treatment at equitoxic doses also led to significant increases in MDA and GR and decreased the GSH level and SOD and GPx activities. In contrast to nicotine, myosmine increased CAT activity.

In vivo metabolism of myosmine has been investigated after oral administration in female Wistar rats by Zwickenpflug et al. (5). The authors identified 3-pyridylacetic acid (3-PAA) and 4-oxo-4-(3-pyridyl) butyric acid (keto acid) as the main metabolites and 3-pyridylmethanol, 3-hydroxymyosmine and 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) as minor

metabolites. The relatively high amount of 3-PAA (23 %) suggests that myosmine undergoes a biochemical transformation in the rat stomach (27), leading to free radical formation. The increase in MDA level detected in our study may be related to the increase in free radical formation. De Zwart et al. (28) reported that lipid peroxidation produced several toxic byproducts such as MDA, which can attack cellular targets including DNA, inducing mutagenicity and carcinogenicity. The increased MDA level after myosmine treatment, might also contribute to the mutagenic and genotoxic effects of myosmine (9, 29-31). Glutathione is required to maintain the normal reduced state of cells and to counteract the adverse effects of oxidative stress. It is involved in many cellular processes including the detoxification of endogenous and exogenous compounds and can be monitored as a nonspecific indicator of cellular toxicity. The increased lipid peroxidation in myosmine-treated rats can be regarded as a key event for the observed GSH depletion after *i.p.* and oral administration (see Figure 2). Furthermore, Zwickenpflug and Tyroller (6) suggested that myosmine peroxidation to 3-pyridylmethanol, nornicotyrine, HPB, and keto acid produced highly reactive electrophilic intermediates known as epoxides, and we believe that these electrophiles interact with the large nucleophilic pool of GSH in the liver and deplete it.

In addition to increased lipid peroxidation and decreased GSH level, myosmine significantly decreased the activities of the antioxidant enzymes SOD and GPx (see Figures 3 and 5 respectively) and increased CAT activity (see Figure 4). These enzymes act together and constitute the enzymatic antioxidative defence mechanism against reactive oxygen species (32). The SOD dismutates superoxide radicals ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2) and O_2. Catalase further detoxifies H_2O_2 into H_2O and O_2 (33). Glutathione peroxidase, similar to CAT, detoxifies H_2O_2. Lower SOD and GPx activities in our study could be attributed to several mechanisms. The first is their overconsumption in inactivating the reactive intermediates generated during the metabolism of myosmine. The second is that oxidative stress could inactivate enzyme proteins through these intermediates or could be a consequence of lower *de novo* synthesis of enzyme proteins. The increased hepatic CAT activity may be a compensatory mechanism to get rid of excess peroxides due to low GPx activity. The third is that the activation of myosmine by peroxidative or nitrosative mechanisms could lead to pyridyloxobutylation of proteins, DNA, and RNA (6, 8, 30) and could compromise their functioning.

The significant increase in GR (see Figure 6) could be attributed to increased GSSG production, which we have not measured in this study but which is reasonable to assume (23).

Our study confirms that single *i.p.* and oral administration of myosmine significantly increases lipid peroxidation and compromises the enzymatic and non-enzymatic antioxidative defence systems. Its pro-oxidant effects are comparable with those of nicotine.

Acknowledgements

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Sažetak

DJELOVANJE MIOZMINA NA ANTIOKSIDATIVNE SUSTAVE U JETRI ŠTAKORA

Miozmin (3-(1-pirolin-2-il)piridin) alkaloid je strukturno sličan nikotinu, za koji se zna da potiče oksidativni stres. Istražili smo djelovanje miozmina na enzimske i neenzimske antioksidativne sustave u jetri štakora. Wistar štakori primili su jednokratno pokusni spoj intraperitonealno u dozi od 19 mg kg^{-1}, odnosno na usta u dozi od 190 mg kg^{-1}. Za pozitivnu kontrolu rabili smo nikotin.
Nakon primjene, bez obzira na put, zamijećena je promjena u jetrenoj funkciji u obliku pada razina glutationa, aktivnosti superoksid dismutaze i glutation peroksidaze te rasta razina malondialdehida, aktivnosti katalaze i glutation reduktaze. Ovi nalazi upućuju na to da intraperitonealna i oralna primjena miozmina dovode do značajne lipidne peroksidacije u jetrenome tkivu te promjena u enzimskoj i neenzimskoj zaštiti jetre. Prooksidativno djelovanje miozmina pokazalo se sličnim onomu nikotina.

KLJUČNE RIJEČI: *duhanski alkaloid, glodavci, lipidna peroksidacija, oksidativni stres*

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Scientific Paper

CYTOTOXIC EFFECTS OF IMIDAZOLIUM IONIC LIQUIDS ON FISH AND HUMAN CELL LINES

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Ionic liquids bring a promise of a wide range of "green" applications that could replace conventional volatile solvents. However, before these applications become large-scale, their toxicity needs to be investigated in order to predict the impact on human health and environment. In this study we assessed the cytotoxicity of imidazolium ionic liquids (in the concentrations between 0.1 mmol L^{-1} and 10 mmol L^{-1}) in the ovarian fish cell line CCO and the human tumour cell line HeLa using the MTT cell viability assay. Our results showed that the most cytotoxic ionic liquid was 1-*n*-butyl-3-methylimidazolium bis(trifluoro methylsulphonyl)imide, [BMIM][Tf_2N], followed by 1-*n*-butyl-3-methylimidazolium tetrafluoroborate [BMIM][BF_4], 1-*n*-butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF_6], and 1,3-dimethylimidazolium hexafluorophosphate [MMIM][PF_6]. Generally, the effects were concentration-dependent. They also depended on the type of anion and the *n*-alkyl chain length. The comparison between the fish CCO and human HeLa cell lines suggests that CCO cells provide a good biological system for initial toxicity testing of ionic liquids that could replace *in vivo* bioassays.

KEY WORDS: *CCO cells, HeLa cells, imidazolium ionic liquids, MTT assay*

Ionic liquids are organic salts in liquid state at room temperatures that have recently attracted considerable attention as potential "green" substitutes for conventional volatile solvents, as they do not evaporate and are not flammable (1-2). In addition, ionic liquids are stable thermally, chemically and electrochemically, and can dissolve a wide range of compounds. These desirable properties and almost limitless structural possibilities provide a tremendous potential for application in various fields, such as organic synthesis, biocatalysis, and extraction and separation of biologically important compounds.

However, before their production and use reaches the industrial scale, ionic liquids should be assessed for the impact on human health and environment. One of the useful assessment tools is *in vitro* testing on cell lines, which can elucidate intracellular, molecular, or physiological mechanisms induced by chemical substances (3). Mammalian cell lines are routinely used for basal cytotoxicity studies (4), while continuous fish cell lines are used in the ecotoxicological assessment of chemicals and environmental samples (5). It is suggested that cytotoxicity tests using dedifferentiated cancer cells provide a quick and convenient first information about the toxic potential of chemical substances (6). The effects of ionic liquids on cell viability have already been reported in well-characterised tumour mammalian cell lines such as HeLa (7), CaCo-2 (8), MCF7 (9), and PC12 (10). Investigations of the effects of ionic liquids on the aquatic environment have mostly been based on the evaluation of the growth and survival of different

aquatic organisms (11-12). Even though fish cell lines are becoming the most important *in vitro* tool in aquatic ecotoxicology (13), investigations of ionic liquid cytotoxicity in continuous fish cell lines have not been reported yet.

The aim of our study was to assess the cytotoxicity of imidazolium ionic liquids in the ovarian fish cell line CCO and the human tumour cell line HeLa using the MTT cell viability assay.

MATERIALS AND METHODS

Ionic liquids

The ionic liquids 1-*n*-butyl-3-methylimidazolium tetrafluoroborate [BMIM][BF_4], 1-*n*-butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF_6], and 1-*n*-butyl-3-methylimidazolium bis(trifluoromethylsulphonyl)imide, [BMIM][Tf_2N] used in the experiments were purchased from Acros Organics, USA. 1,3-dimethylimidazolium hexafluorophosphate [MMIM][PF_6] was synthesised by M. Cvjetko in the Laboratory of Cell Culture Technology and Biotransformations (Zagreb, Croatia). The structures of the tested ionic liquids are shown in Table 1.

Materials

Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), and Trypan blue were purchased from Sigma, St. Louis, MO, USA. Heat-inactivated foetal bovine serum (FBS) was purchased from GIBCO, Paisley, Scotland, UK. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich Chemie, Steinheim, Germany. DMSO was purchased from Kemika, Zagreb, Croatia.

Cell lines

The CCO cell line, derived from the ovaries of Channel catfish (*Ictalurus punctatus*, Rafinesque, 1818), was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA; ATCC® number CRL-2772). The HeLa cell line, derived from the human cervical carcinoma, was obtained from the Ruđer Bošković Institute, Zagreb, Croatia. Both cell lines were cultured in 75 mL flasks in DMEM supplemented with 10 % inactivated FBS and maintained at 5 % CO_2. CCO cells were incubated at 30 °C and HeLa cells were incubated at 37 °C.

Cell treatment

Samples of CCO and HeLa cells were taken at the exponential growth phase and counted using the Trypan blue. Cells were then seeded in 24-well plates at a density of $5x10^4$ cells mL^{-1} in 1 mL of media. Stock solutions of the ionic liquids (1 mol L^{-1}) were prepared in the culture medium or DMSO. After 24 h of cell growth, the medium was replaced with fresh medium containing different concentrations of ionic liquids. Both cell types were exposed to ionic liquids in the concentration range from 0.1 mmol L^{-1} to 10 mmol L^{-1}; CCO cells for 72 h and HeLa cells for 48 h. The final DMSO concentration in the medium was 0.1 % for each sample.

Cytotoxicity assay

The cytotoxicity of ionic liquids was measured using the MTT assay as described by Mosmann (14). The absorbance of purple formazan was measured at 570 nm using a spectrophotometer (Helios, Thermo Electro Corporation). The results are given as percentages of the control absorbance.

Statistical analysis

The obtained data are expressed as the mean±SEM of three independent experiments performed in triplicate. We used the one-way analysis of variance (ANOVA) with Dunnett's test and set the probability level of $p<0.05$ as statistically significant. The half maximal effective concentration (EC_{50}), defined as the concentration of ionic liquid that resulted in 50 % growth inhibition, was calculated from the dose-response curves using equations of related trend lines for the MTT assay.

RESULTS

Cytotoxicity of ionic liquids in CCO and HeLa cells

Figure 1a shows the effects of different [BMIM][BF_4] concentrations on CCO and HeLa cells, expressed as percentages of viability. The proliferation of CCO cells was not affected by [BMIM][BF_4] at the concentrations of 0.1 mmol L^{-1} and 0.5 mmol L^{-1}, but in HeLa cells these caused a slight cytotoxic effect.

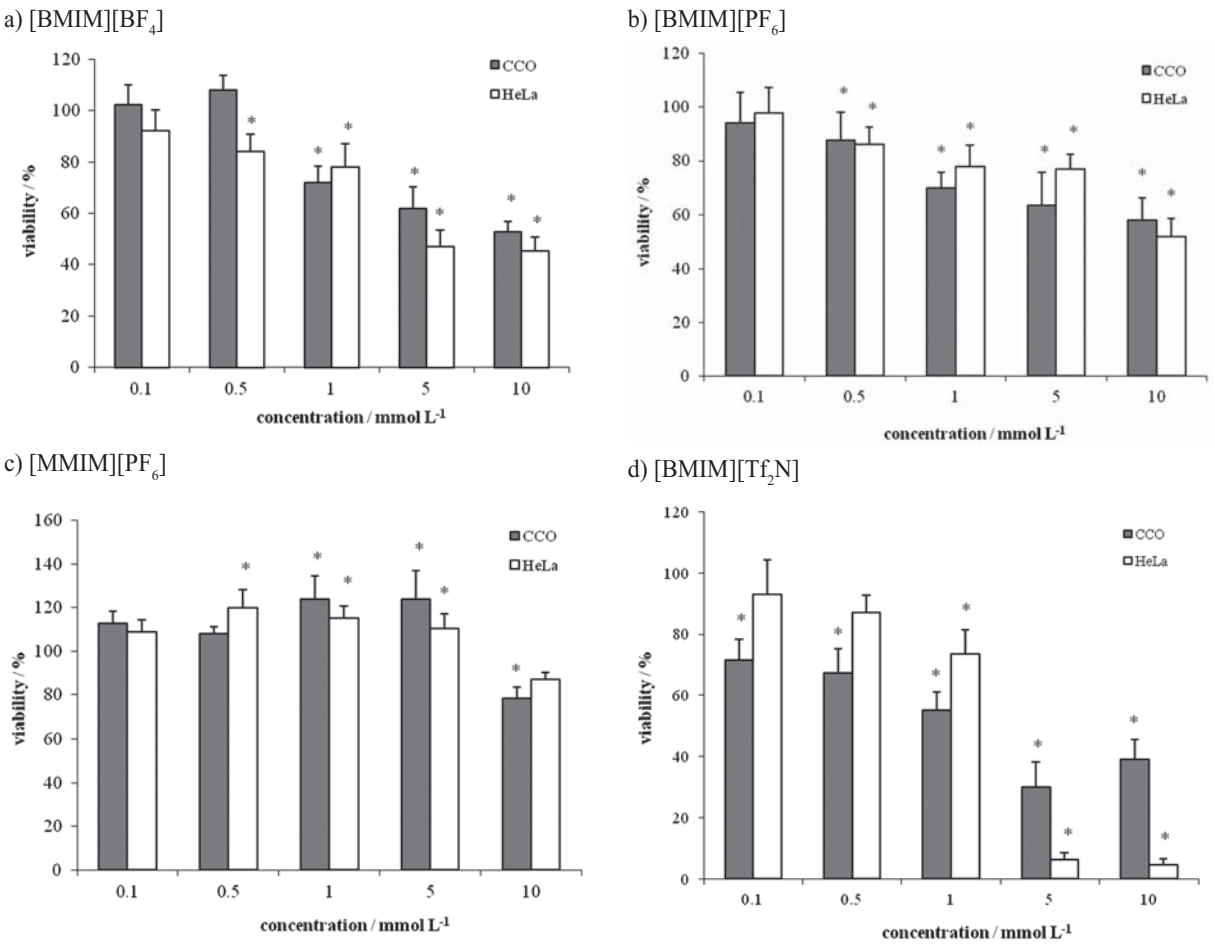


Figure 1 *Effects of [BMIM][BF$_4$], [BMIM][PF$_6$], [MMIM][PF$_6$], and [BMIM][Tf$_2$N] at different concentrations on CCO and HeLa cell viability. Data expressed as a percentage of unexposed control cells±SEM of three replicates for each exposure concentration. * denotes significant difference from control (p<0.05).*

Table 1 *Ionic liquids used in the study*

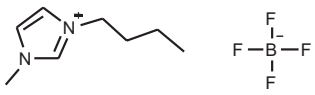
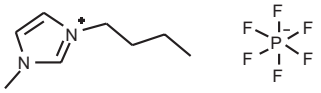
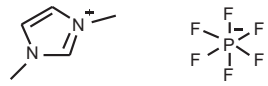
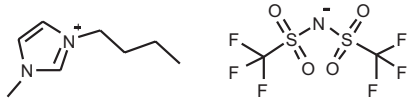
Systematic name	Trade name	Molecular weight	Chemical formula
1-*n*-butyl-3-methylimidazolium tetrafluoroborate	[BMIM][BF$_4$]	226.02	
1-*n*-butyl-3-methylimidazolium hexafluorophosphate	[BMIM][PF$_6$]	284.18	
1,3-dimethylimidazolium hexafluorophosphate	[MMIM][PF$_6$]	242.11	
1-*n*-butyl-3-methylimidazolium bis (trifluoromethylsulphonyl) imide	[BMIM][Tf$_2$N]	419.36	

Table 2 *EC$_{50}$ (mmol L^{-1}) of the selected imidazolium ionic liquids in CCO and HeLa cells*

Ionic liquid	CCO cells	HeLa cells
[BMIM][Tf$_2$N]	3.26±0.18	3.11±0.11
[BMIM][BF$_4$]	5.01±0.32	4.42±0.18
[BMIM][PF$_6$]	10.32±0.28	11.2±0.15
[MMIM][PF$_6$]	-	-

Higher concentrations of [BMIM][BF_4] (1 mmol L^{-1} to 10 mmol L^{-1}) induced a significant cytotoxic effect in both cell lines. Survival rates decreased as [BMIM][BF_4] concentrations increased.

Figure 1b shows viability of CCO and HeLa cells exposed to different [BMIM][PF_6] concentrations (0.1 mmol L^{-1} to 10 mmol L^{-1}). At 0.1 mmol L^{-1}, [BMIM][PF_6] did not cause significant cytotoxic effects in either cell line. Significant cytotoxic effects in both CCO and HeLa cells started at 0.5 mmol L^{-1} and increased in the dose-dependent manner.

Exposure to [MMIM][PF_6] (Figure 1c) led to significant cytotoxic effects only in CCO cells at 10 mmol L^{-1}. At lower concentrations, [MMIM][PF_6] in fact significantly improved cell viability in both cell lines; in HeLa cells at doses up to and including 5 mmol L^{-1} and in CCO cells at 1 mmol L^{-1} and 5 mmol L^{-1}.

Exposure to [BMIM][Tf_2N] produced significant cytotoxic effects in CCO cells at all tested concentrations (Figure 1d). In contrast, significant cytotoxicity in HeLa cells was observed only at higher concentrations (1 mmol L^{-1} to 10 mmol L^{-1}). In addition, at 5 mmol L^{-1} and 10 mmol L^{-1} this effect was much stronger in HeLa cells (95 %) than in CCO cells (65 %).

Comparison of cytotoxicity in CCO and HeLa cells

Table 2 shows the half maximal effective concentrations (EC_{50}) for selected ionic liquids in CCO and HeLa cells, that were calculated from cell viability data. The exception is [MMIM][PF_6] which could not decrease cell viability below 80 % in CCO cells and 85 % in HeLa cells, even at the highest tested concentration. The lowest EC_{50} was calculated for [BMIM][Tf_2N] in both cell lines, followed by [BMIM][BF_4] and [BMIM][PF_6].

DISCUSSION

Even though ionic liquids are originally considered "green solvents", recent studies on their ecotoxicity and degradability has shown that some are not as environmentally friendly as others (15). This calls for a careful and critical assessment that should be able to predict their effects on human health and environment.

Our study compared the cytotoxic effects and EC_{50} of four imidazolium ionic liquids in fish CCO and human tumour HeLa cells. [BMIM][BF_4] had about half the EC_{50} [(5.01±0.32) mmol L^{-1} in CCO cells and (4.42±0.18) mmol L^{-1} in HeLa cells] of [BMIM][PF_6] [(10.32±0.28) mmol L^{-1} for CCO cells and (11.2±0.15) mmol L^{-1} for HeLa cells]. Similar results for HeLa cells have also been reported by Stepnowski et al. (7) and Wang et al. (16).

EC_{50} did not differ much between CCO and HeLa cells, and since there is no available data on the toxicity of ionic liquids in fish cells, this finding may be relevant for the comparison of cytotoxicity data between fish and mammalian cell lines.

We also observed a significant stimulatory effect on CCO and HeLa cell viability by [MMIM][PF_6] at the concentrations of 0.5 mmol L^{-1} to 5 mmol L^{-1}. This phenomenon is known as chemical hormesis, which is characterised by low-dose stimulation and high-dose inhibition (17). A similar hormetic effect of 1-*n*-octyl-methylimidazolium tetrafluoroborate [C_8MIM][BF_4] was observed in IPC-81 leukaemia cells (18) and of 1-*n*-butyl-3-ethylimidazolium tetrafluoroborate [BEIM][BF_4] in HeLa cells (7).

It seems that *n*-alkyl chain length correlates with toxicity, as [BMIM][PF_6] was more toxic in both cell lines than [MMIM][PF_6], which is consistent with the results reported by Ranke et al. (18).

It also seems that cytotoxicity may be related to the anion; the lowest EC_{50} in both cell lines was found for [Tf_2N]. Kumar et al. (9) showed that the [Tf_2N] anion was more toxic in MCF7 cells than the bromide anion, and this toxicity could be associated with hydrolytic cleavage that resulted in the formation of free fluoride ions (19).

HeLa cells showed higher sensitivity when exposed to 5 mmol L^{-1} and 10 mmol L^{-1} of [BMIM][Tf_2N] than the CCO cells. This might point to HeLa-specific mechanisms of [BMIM][Tf_2N] action. Similarly specific mechanisms of action have been observed for other cell lines and imidazolium liquids (18).

Castaño et al. (20) compared the sensitivity of *in vitro* basal cytotoxicity tests (MTT assay) in fish and mammalian cell lines. These cells showed similar sensitivity when exposed to a range of chemicals. Although our results with CCO and HeLa cells are in agreement with this study, future *in vitro* cytotoxicity studies should include more cell lines to provide a more comprehensive information about the effects of ionic liquids in specific mammalian and fish cell lines.

In conclusion, our results obtained with CCO and HeLa cell lines show that the toxicity of the selected ionic liquids depends on the dose, anion type, and *n*-alkyl chain length. This has been the first study to assess the cytotoxicity of ionic liquids in fish CCO cells and it suggests that fish cell lines could be a good biological system for initial toxicity testing of ionic liquids that could replace *in vivo* fish bioassays. However, future studies on other fish cell lines should answer if differences between fish species and tissues/organs could affect toxicity data and their interpretation.

Acknowledgements

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Sažetak

CITOTOKSIČNI UČINCI IONSKIH TEKUĆINA NA STANIČNIM LINIJAMA RIBA I LJUDI

S obzirom na širok raspon primjena ionskih tekućina, prethodno je potrebno ispitati njihovu toksičnost i mogući utjecaj na zdravlje ljudi i okoliš. U ovom radu ispitana je citotoksičnost odabranih imidazolijevih ionskih tekućina na stanicama ovarija riba CCO i ljudskoj tumorskoj staničnoj liniji HeLa primjenom MTT-metode. Izlaganje stanica različitim koncentracijama ionskih tekućina [(0,1 do 10) mmol L^{-1}] rezultiralo je uglavnom citotoksičnim učincima ovisnima o koncentracijama ionske tekućine. Na temelju dobivenih vrijednosti EC_{50} najtoksičnija ionska tekućina je 1-*n*-butil-3-metilimidazolijev bis(trifluorome tilsulfonil)imid [BMIM][Tf2N], zatim 1-*n*-butil-3-metilimidazolijev tetrafluoroborat [BMIM][BF4],1-*n*-butil-3-metilimidazolijev heksafluorofosfat [BMIM][PF6] i 1,3-dimetilimidazolijev heksafluorofosfat [MMIM][PF6]. Općenito, toksičnost je bila ovisna o koncentraciji, tipu aniona i duljini *n*-alkilnog lanca. Nakon usporedbe rezultata toksičnosti na CCO i HeLa-stanicama smatramo da CCO-stanice mogu biti dobar biološki sustav za početna ispitivanja toksičnosti ionskih tekućina u cilju zamjene *in vivo* testova na ribama.

KLJUČNE RIJEČI: *CCO-stanice, HeLa-stanice, imidazolijeve ionske tekućine, MTT-metoda*

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Scientific Paper

BOAT PRESSURE WASHING WASTEWATER TREATMENT WITH CALCIUM OXIDE AND/OR FERRIC CHLORIDE

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The aim of this study was to investigate the efficiency of (1) chemical precipitation by calcium oxide, (2) coagulation/flocculation by ferric chloride (FC), and (3) the combination these two methods in reducing the toxicity of wastewater generated by boat pressure washing. All three methods gave satisfactory results in the removal of colour, turbidity, Cr, Fe, Cu, Zn, and Pb. The concentrations of heavy metals were lowered below national limits with 1 g of CaO, 2.54 mg of Fe^{3+} in the form of $FeCl_3 \times 6H_2O$, and the combination of 0.25 g of CaO and 5.08 mg of Fe^{3+} per 50 mL of wastewater. Both CaO (1.50 g per 50 mL of wastewater) and FC proved efficient, but their combination yielded a significantly better performance: 99.41 %, 100.00 %, 97.87 %, 99.09 %, 99.90 %, 99.46 % and 98.33 % for colour, turbidity, Cr, Fe, Cu, Zn, and Pb respectively. For colour, Cr, Cu, Zn, and Pb removal efficiencies increased in the following order: FC<CaO<CaO+FC, while this order for turbidity and Fe was as follows: CaO<FC<CaO+FC. As expected, all three methods increased the concentration of total dissolved solids in the final effluent. Our results suggest that the combined treatment of marina wastewaters with calcium oxide followed by ferric chloride is efficient, cost-effective, and user-friendly.

KEY WORDS: *antifouling paints, CaO, chromium, colour, copper, lead, heavy metals, turbidity, zinc*

The major source of heavy metals in the sediments around shipyards and marinas are the antifouling paints (1) that have been extensively used for boat protection for over the last 100 years (2). Antifouling paints contain strong agents that kill algae and other organisms and prevent them from attaching to the hull (2). Efficient protection usually requires that the hull be treated once a year (1). Old paint is removed by pressure washing that generates 100 L to 150 L of wastewater per boat. This amount of wastewater contains about four kilograms of paint distributed over particles of different size (3). The major biocide present in all paints is copper (Cu). Other metals like zinc (Zn), chromium (Cr), titanium (Ti), and lead (Pb) can also be present, but their concentration varies with the brand and the type of paint (1). A number of studies (reviewed in ref. 1) have determined their genotoxic effects on marine environment, including extinction of local species.

There are over a hundred marinas along the Croatian Adriatic coast and most still release boat pressure washing wastewater directly into the sea.

Our group has been testing several physico-chemical methods of wastewater treatment with red mud as coagulant, first in the laboratory (4, 5) and then full-scale (1). The average removal efficiency of the

full-scale treatment was 99.6 % for Pb and Cu and 99.9 % for Zn. In a pilot-scale trial, Walker et al. (2) managed to remove over 98 % of chemical oxygen demand (COD) from shipyard wastewaters using dolomite and dolomitic sorbents. Ottosen et al. (3) efficiently lowered Cu, Zn, and Sn levels in dockyard wastewaters to below regulatory limits using the coagulation/flocculation method with ferric chloride.

Coagulation/flocculation with alum and ferric sulphate was also efficient (99.8 %) in removing tributyltin from shipyard wastewaters in a laboratory and a full-scale system (6). In another study (7), organotin species was successfully removed from shipyard wastewaters by electrochemical oxidation using niobium coated with boron-doped diamond and titanium coated with iridium dioxide anodes (7). Vreysen et al. (8) combined adsorption with a bentonite-type adsorbent and coagulation/flocculation with activated carbon in powder and removed up to 98.7 % of Cu and 99.4 % of Zn from shipyard wastewaters.

The aim of this study was to develop and test a cost-effective and user-friendly laboratory-scale method combining precipitation with calcium oxide (CaO) followed by neutralisation and coagulation/flocculation with ferric chloride (FC) for the removal of inorganic/organic contaminants from wastewaters generated in marinas by boat pressure washing

MATERIALS AND METHODS

Wastewater sampling and storage

Wastewater generated from boat pressure washing was collected in the Marina Kaštela, Kaštel Gomilica, Croatia. The effluent was collected in a channel with fine grate at the end. To obtain representative samples, eight boats with different paint coatings (different colour and brand) were washed. One hundred litres of wastewater was sampled. Before purification, wastewater was homogenised as described in our previous research (9).

Purification experiments

All purification experiments were conducted at 22 °C. The treatment with CaO was performed as follows; aliquots of wastewater (50 mL) were mixed with 0.25 g, 0.50 g, 1.00 g, or 1.50 g of CaO (Lička tvornica vapna, Ličko Lešće, Croatia) (12). After adding 1.50 g of calcium oxide, we lowered the pH to 9 by adding 4 mol L^{-1} of hydrochloric acid (Kemika, Zagreb, Croatia). The suspension was mixed for 10 minutes on a magnetic stirrer (30 MAG 12, Labline Stock Centre, Mumbai, India; 200 rpm) and left to settle for 30 minutes. After floc sedimentation, clear water was decanted and analysed.

Treatment with ferric chloride (FC), $FeCl_3 \times 6H_2O$ (Kemika, Zagreb, Croatia), was performed as follows; aliquots of wastewater (50 mL) were mixed with the 1.27 mg, 2.54 mg, 3.81 mg, or 5.08 mg of Fe^{3+} added in the form of ferric chloride. Due to the drop in pH caused by Fe^{3+} hydrolysis, we had to adjust it to 8 by adding ammonium hydroxide (Kemika, Zagreb, Croatia). The suspension was mixed on a magnetic stirrer for 15 minutes and left to settle for 30 minutes.

For the combined treatment, we mixed wastewater aliquots (50 mL) with 0.25 g, 0.50 g, 1.00 g, or 1.50 g of CaO on a magnetic stirrer without pH adjustment. After 10 minutes, we added 5.08 mg of Fe^{3+} into each beaker and mixed on a magnetic stirrer for another 15 minutes, and let it settle for 30 minutes.

All experiments were done in triplicate. In all cases, the relative standard deviation (RSD) was less than 10 %.

Sample preparation and analysis

Untreated and treated wastewater was prepared for the analysis as follows: 5 mL of untreated effluent and 100 mL of purified effluent were adjusted to pH 3 by adding hydrochloric acid (Kemika) or ammonium hydroxide (Kemika) preconcentrated (9) with ammonium-pyrolidinedithiocarbamate (APDC) (Merck, Schuchardt, Germany). Wastewater was filtered through Millipore micro filters and analysed using a MINIPAL4 X-ray spectrometer (PANalytical, Almelo, Nederland) (10-12). Colour and turbidity were determined using a HACH DR890 colorimeter (Hach Company, Loveland, Colorado, USA) (9, 12), while the pH and total dissolved solids (TDS) were determined using a PHT-027 water quality multi-parameter monitor (Kelilong Electron, Fuan Fujian, China) (9, 12).

Statistical analysis

For statistical analysis we used the STATISTICA 7.0 software package. The level of significance in all tests was set to $P<0.05$. Differences between treatment methods were tested using the analysis of variance and the Student-Newman-Keuls test.

RESULTS AND DISCUSSION

Chemical precipitation with CaO

Table 1 shows the results of the analysis of boat pressure washing wastewater. The baseline colour and turbidity and the respective concentrations of Fe, Cu, and Zn were 1.98, 16.23, and 5.58 times higher than the limit values for wastewaters to be discharged into a natural recipient.

Adding 0.25 g of CaO into 50 mL of wastewater resulted in 98.71 %, 98.31 %, 62.84 %, 81.60 %, 90.60 %, 95.42 %, and 59.36 % removal of colour, turbidity, Cr, Fe, Cu, Zn, and Pb, respectively (Figure 1). In spite of good removal efficiencies obtained for all seven parameters, the concentration of Cu in the final effluent was still 1.5 times higher than the maximum allowed level. The concentrations of all metals were lowered below the limit after adding 1 g of CaO. A further increase to 1.50 g of CaO improved the final removal efficiency for colour and turbidity to 99.07 % and 99.54 %, respectively. Removal efficiency of heavy metals increased linearly with the amount of CaO. It increased the most for Fe, followed by Cu and Zn. The highest removal efficiencies for Cr, Fe, Cu, Zn, and Pb were 86.97 %, 96.77 %, 97.81 %, 98.76 %, and 84.10 %, respectively.

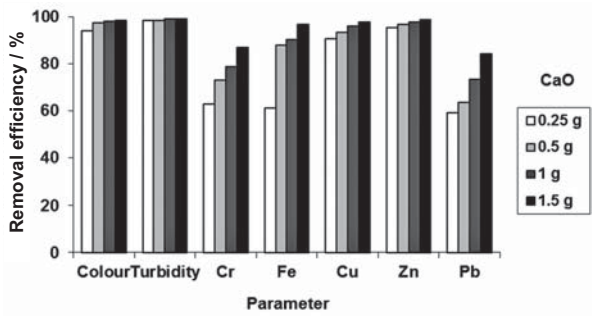

Figure 1 *CaO treatment removal efficiency. CaO amount expressed per 50 mL of wastewater.*

Coagulation/flocculation with ferric chloride

With ferric chloride (Figure 2) we obtained better removal efficiencies for colour and turbidity than for heavy metals. Acceptable levels of heavy metals were obtained after adding 2.54 mg of Fe^{3+}. Removal efficiency increased linearly with further increases in the amount of coagulant for all seven parameters, and the final was as follows: 98.76 % for colour, 99.85 % for turbidity, 78.99 % for Cr, 97.35 % for Fe, 96.77 % for Cu, 98.53 % for Zn, and 78.99 % for Pb. All measured parameters in the treated effluent below the maximum allowed level for wastewater suitable for discharge into the environment (Table 1).

Table 1 *The values of the measured parameters, at baseline (before treatment) and after the treatment of 50 mL of wastewater with 1.50 g calcium oxide, 5.08 mg Fe^{3+} in the form of $FeCl_3 \times 6H_2O$ and their combination against maximum allowed levels that can be released into a natural recipient (13).*

Measured parameter	Untreated effluent	Treated effluent			Maximum allowed levels (13)
		1.50 g CaO per 50 mL of wastewater	5.08 mg Fe^{3+} per 50 mL of wastewater	(1.50 g CaO+5.08 mg Fe^{3+}) per 50 mL of wastewater	
Colour / PtCo	8370	78	104	49	colourless on visual inspection
Turbidity / NTU	1300	6	2	0	clear on visual inspection
Cr / mg L^{-1}	0.890	0.116	0.187	0.019	0.5
Fe / mg L^{-1}	3.968	0.128	0.105	0.036	2
Cu / mg L^{-1}	8.118	0.178	0.262	0.008	0.5
Zn / mg L^{-1}	11.162	0.138	0.164	0.060	2
Pb / mg L^{-1}	0.790	0.124	0.187	0.013	0.5
TDS / mg L^{-1}	4850	8340	6540	7500	-
pH	7.84	8.25	7.93	7.43	6.5-9
Sludge volume / mL	-	6.4	12.8	15	-

PtCo-platinum cobalt units; NTU - nephelometric turbidity units; TDS-total dissolved solids

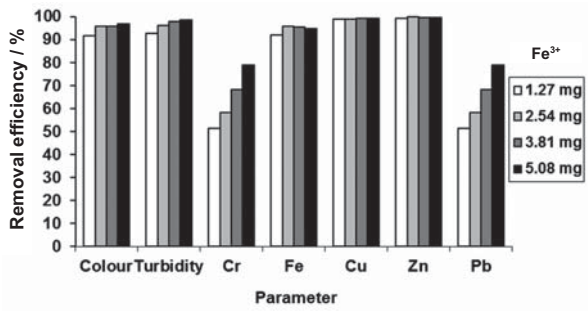

Figure 2 *$FeCl_3 \times 6H_2O$ treatment removal efficiency. Fe^{3+} amount expressed per 50 mL of wastewater.*

Combined treatment with CaO and ferric chloride

In combination with 5.08 mg of Fe^{3+} CaO lowered the concentrations of all heavy metals below the discharge limit as early as 0.25 g per 50 mL (Figure 3). Any further increase in the amount of CaO was

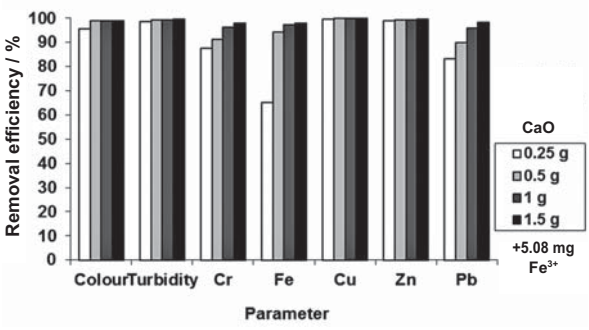

Figure 3 *Combined treatment removal efficiency. CaO and Fe^{3+} added in the form of $FeCl_3 \times 6H_2O$.*

followed by a significant linear increase in the removal efficiency for Fe, Cr, and Pb, while the removal efficiency for the other four parameters increased slightly. The final removal efficiencies with the highest doses of CaO (1.50 g per 50 mL) were 99.41 %, 100.00 %, 97.87 %, 99.09 %, 99.90 %, 99.46 %, and 98.33 % for colour, turbidity, Cr, Fe, Cu, Zn, and Pb, respectively.

Figure 4 compares the best removal efficiencies of the three treatment methods. The analysis of variance showed a statistically significant difference ($P<0.05$) in mean removal efficiencies between the treatment methods for all parameters. Significant differences were confirmed by the Student-Newman-Keuls test. The most efficient method in removing all seven parameters was the combined treatment. For colour, Cr, Cu, Zn, and Pb, the removal efficiencies increased in the following order: FC<CaO<CaO+FC, while for turbidity and Fe this order was as follows: CaO<FC<CaO+FC. Better performance of CaO than

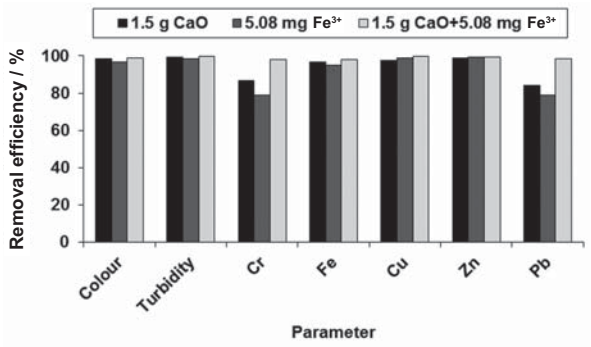

Figure 4 *Comparison of the best removal efficiencies between the three treatment methods (treatment of 50 mL of wastewater with 1.50 g CaO, 5.08 mg Fe^{3+} in the form of $FeCl_3 \times 6H_2O$ and their combination).*

FC in the removal of Cu and Pb is probably related to the heat released by hydration of CaO that destroyed Cu and Pb organic complexes (10). Consequently, elements liberated from the organic ligands into the solution could be removed easily by hydroxide precipitation and coagulation/flocculation. The destruction of organic matter during the hydration of CaO could also explain better removal performance of colour by CaO compared to FC (12).

Taking into account the cost of CaO and FC and their consumption per m^3 of treated water, the approximate cost of the combined treatment is 10.3 € per m^3. From what we learn, this is at the lower half of the price range for this kind of wastewater treatment, but we have no data to support it.

With the combined treatment Cr, Fe, Cu, Zn, and Pb levels were 26, 56, 63, 33, and 38 times lower than the maximum allowed level set by the Croatian regulations (13), respectively.

Our earlier toxicological study (14) showed that the treated effluent with similar Cr, Fe, Cu, Zn, and Pb levels did not cause any significant toxic effect on HEp2 and HeLa human cell lines or on human white blood cells. Similarly, treated wastewater in our earlier studies produced no significant toxic effects on either bacterial cell lines (TA98 and TA100) or HEp2 and HeLa human cell lines (15) or on duckweed (16). Gajski et al. (17) found that the leachate of solidified sewage sludge containing 0.110 mg L^{-1} of Cr, 0.240 mg L^{-1} of Fe, 0.066 mg L^{-1} of Cu, 0.080 mg L^{-1} of Zn, and 0.060 mg L^{-1} of Pb did not cause significant toxic effects on human blood lymphocytes determined by the DNA diffusion assay, micronucleus test, and the comet assay. Medley and Clements (18) found no significant toxic effect of Zn concentrations lower than 0.200 mg L^{-1} on diatom communities. Zinc at values

similar to ours also did not have a toxic effect on a benthic macroinvertebrate community studied by Clements and Kiffney (19).

In addition, our concentrations in the treated wastewater are significantly lower than the LC_{50} values for Cu, Zn, Pb, and Cr obtained by Calabrese et al. (20). All this suggests that a discharge of effluents treated by a combination of CaO and FC in the final concentrations described in our study will not have a toxic effect on local marine life.

Sludge treatment

The sludge formed during the purification must be dewatered by filtration on filter presses, stored in a pool, and handed over to the authorised waste collection service for further disposal.

CONCLUSION

Combined treatment yielded the best performance in the removal of organic (colour, turbidity) constituents and heavy metals from boat pressure washing wastewater. However, CaO treatment alone yielded the highest TDS increase and generated the lowest volume of sludge. The remaining concentrations of heavy metals in the effluent following the combined treatment are lower than or comparable with previously published data (14-17), and we do not expect any toxic effects on the environment and humans. All in all, the combined treatment of marine wastewater with CaO and FC has turned out to be efficient, cost-effective, and user-friendly.

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Sažetak

OBRADA OTPADNIH VODA OD PRANJA BRODOVA KOMBINACIJOM FIZIČKO-KEMIJSKIH METODA

Radi smanjenja toksičnosti otpadnih voda koje nastaju pranjem brodova premazanih bojama protiv obraštaja primijenjene su tri metode obrade: (1) kemijsko taloženje s pomoću kalcijeva oksida, koagulacija/flokulacija s pomoću željezova klorida (FC) i (3) kombinacija ovih dviju metoda. Sve tri metode dale su zadovoljavajuće rezultate u uklanjanju boje, mutnoće, kroma, željeza, bakra, cinka i olova. Koncentracije teških metala niže od graničnih vrijednosti postignute su nakon tretmana s 1 g CaO ili 2,54 mg Fe^{3+} dodanog u obliku $FeCl_3 \times 6H_2O$ ili kombinacijom od 0,25 g CaO i 5,08 mg Fe^{3+} na 50 mL otpadne vode. Optimalne vrijednosti uklanjanja boje, mutnoće, Cr, Fe, Cu, Zn odnosno Pb s pomoću CaO (1,50 g na 50 mL) bile su 99,07 %, 99,54 %, 86,97 %, 96,77 %, 97,81 %, 98,76 % odnosno 84,10 %, dok su u slučaju željezova klorida te vrijednosti iznosile 98,76 %, 99,85 %, 78,99 %, 97,35 %, 96,77 %, 98,53 % odnosno 78,99 %. Značajno viši stupanj uklanjanja postignut je kombinacijom navedenih dvaju pristupa čime je postignuta maksimalna učinkovitost uklanjanja i to 99,41 % boje, 100,00 % mutnoće, 97,87 % kroma, 99,09 % željeza, 99,90 % bakra, 99,46 % cinka i 98,33 % olova. Za boju, krom, bakar, cink i olovo učinkovitost uklanjanja raste ovim redoslijedom: FC <CaO <CaO + FC dok za mutnoću i željezo raste u ovom nizu: CaO <FC <CaO + FC. Sukladno očekivanju, sve tri metode povećavaju koncentraciju ukupne otopljene tvari u konačnom ispustu.

Naši rezultati pokazuju da je primijenjeni način pročišćavanja otpadnih voda iz marina kombinacijom kalcijeva oksida i željezova klorida učinkovit s obzirom na stupanj uklanjanja, s povoljnim odnosom stupnja pročišćavanja i cijene te jednostavan za primjenu.

KLJUČNE RIJEČI: *boja, boje protiv obraštaja, CaO, mutnoća, teški metali, željezo(III) klorid*

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Short Communication

THE EFFECTS OF TAURINE ON PERMETHRIN-INDUCED CYTOGENETIC AND OXIDATIVE DAMAGE IN CULTURED HUMAN LYMPHOCYTES

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Permethrin (PM) is a common pyrethroid pesticide used to control pests in agriculture, forestry, horticulture, health care, homes, and textile industry. It is confirmed as a strong mutagen in animals and humans. Taurine (TA) is an amino acid found in mammalian tissues that protects the cell against DNA damage. In this study, we investigated whether supplementation of human lymphocyte cultures with TA (in the concentrations of 25 μg mL^{-1}, 50 μg mL^{-1} and 100 μg mL^{-1}) provided any protection against PM toxicity applied in the concentration of 200 μg mL^{-1}. Genotoxicity was assessed using the micronucleus (MN) and sister chromatid exchanges (SCE) tests. In addition, we measured the total antioxidant capacity (TAC) and total oxidative stress (TOS) levels in the plasma to determine oxidative effects. PM increased SCE and MN levels and altered TAC and TOS levels. TA alone did not affect SCE and MN levels compared to controls, regardless of the concentration applied. In addition, it increased TAC levels without changing TOS levels. Moreover, it significantly buffered the negative cytogenetic and oxidative effects induced by PM in a clear dose-dependent manner. In conclusion, this study is the first to evidence the beneficial effects of TA against PM-induced DNA and oxidative damages *in vitro*.

KEY WORDS: *antioxidant, micronucleus, pesticide, sister chromatid exchanges*

Because of widespread use it is important to keep (re)evaluating the genotoxic properties of pesticides (1, 2). A number of studies (3-6) has suggested that pesticides such as DDT, cypermethrin, and lambda-cyhalothrin produce oxidative stress by generating free radicals and by inducing tissue lipid peroxidation (LPO) in mammals and other organisms. Permethrin (PM), the most popular insecticide among the synthetic pyrethroids, has been used worldwide to control a wide range of insects in agriculture, forestry, public health, and homes (**7**). Mammalian and non-mammalian bioassays and toxicology studies have confirmed its carcinogenic potential (8, 9). PM exposure leads to DNA damage in humans and experimental animals (1, 10, 11). It induces oxidative damage to purine bases in the heart cells (12) and produces single- and double-strand breaks in striatum cells (13). In addition, PM can cause a genotoxic response through oxidative stress (14).

Much of the current research is focused on finding substances capable of countering the genotoxicity of man-made or natural mutagens. These include various vitamins, propolis, sulphhydryl compounds, and plant products (15-19). Taurine (TA) (2-aminoethanesulfonic acid) is found in high concentrations in various mammalian tissues (20). Sung et al. (21) reported that

TA suppressed oxidant-induced tissue injuries by stabilising the biomembrane and by scavenging free radicals (21). This has been supported by other studies that associate its protective antioxidant properties with direct scavenging of free radicals and indirect preserving of membrane permeability (22, 23). TA has therefore become a promising option to treat chemically induced illnesses (14, 24-27). Recent investigations have shown that TA protects cells from the toxic effects of cadmium (Cd), paracetamol, and arsenic (As) that are mediated by free-radical attack (28-31).

This is why we assumed that TA could provide protection against PM-induced genotoxic and oxidative damages. To test this assumption, we treated human lymphocytes with PM and with TA and determined the frequencies of sister chromatid exchanges (SCEs) and micronuclei (MNs), as both assays are widely used in genotoxicity testing and biomonitoring (32, 33). In addition, we wanted to see how treatment with PM and TA affected the total antioxidant capacity (TAC) and total oxidative stress (TOS) levels in the plasma.

MATERIAL AND METHODS

Blood sampling

Blood was sampled by venipuncture from three healthy non-smoking women aged 22 to 25 years. Blood sampling has been approved by the institutional ethics committee and has been performed according to the Declaration of Helsinki.

Experimental design

Taurine (TA; $C_2H_7NO_3S$; CAS No. 107-35-7; Sigma-Aldrich® Chem. Co. St. Louis, MO, USA) was dissolved in distilled water and tested in the concentrations of 25 μg mL^{-1}, 50 μg mL^{-1}, and 100 μg mL^{-1}. Permethrin (PM; $C_{21}H_{20}Cl_2O_3$, CAS No. 52645-53-1; Riedel-de Haen®, Seelze-Hannover, Germany) was dissolved in a mixture of 95 % ethanol and distilled water and tested in the concentration of 200 μg mL^{-1}. These concentrations are based on earlier reports; specifically by Undeger and Basaran (1), who have evidenced the mutagenic effects of PM at 200 μg mL^{-1} while Chesney (34) evidenced the protective effects of TA in the concentrations of 25 μg mL^{-1}, 50 μg mL^{-1}, and 100 μg mL^{-1}. The compounds were added to the lymphocyte cultures just before incubation for cytogenetic analysis. Cultures were incubated for 72 h at 37 °C according to standard protocols for SCE and MN tests. The blood used for determining TAC and TOS levels was incubated with the tested compounds for 2 h at 37 °C. Negative control samples were treated with distilled water only. Positive control samples were treated with ascorbic acid ($C_6H_8O_6$, CAS No. 50-81-7, Sigma®; 10 μmol L^{-1}) in the TAC analysis and with hydrogen peroxide (H_2O_2, CAS No. 8007-30-5, Sigma®; 25 μmol L^{-1}) in the TOS analysis.

SCE assay

We slightly modified the protocol proposed by Evans and O'Riordan (35). Peripheral blood lymphocyte cultures supplemented with 5-bromo-2'-deoxyuridine (Sigma®) were incubated at 37 °C in complete darkness for 72 h. Microscope slides were prepared in triplicate using the standard procedure, air-dried, and stained according to fluorescence plus Giemsa (FPG) method (36). Scorings of SCE were performed under a light microscope on coded slides. To determine the number of SCEs per cell at least, 25 well-spread, second-division metaphases were analysed as described in previous studies (37-39). To ensure the same scoring criteria (40), all microscopy was performed by a single well-trained observer.

MN assay

We followed the protocol proposed by Fenech and Morley (41). Cytochalasin B (Sigma®) was added on hour 44 of cultivation. We used duplicate cultures for each concentration. After incubation, the lymphocytes were fixed and placed directly on slides using a cytospin. Air-dried slides were stained with May Grünwald-Giemsa, coded, and analysed under a light microscope applying the criteria reported by Fenech (42). We examined at least 2000 binucleated lymphocytes per concentration for the presence of one, two, or more micronuclei. Here too the slides were analysed in triplicate.

TAC and TOS analysis

Plasma samples, obtained from the lymphocyte cultures 2 h after incubation with PM and TA, were analysed using commercial kits (Rel Assay Diagnostics®, Gaziantep, Turkey) for automated Trolox-equivalent total antioxidant capacity (TAC) assay and the total oxidant status (TOS) assay (43-46).

The major advantage of the TAC assay is that it measures the antioxidant capacity of all antioxidants in a biological sample and not just of a single compound (43). In this test, antioxidants in the sample reduce dark blue-green coloured 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate (ABTS) radical to its colourless form. The change in absorbance at 660 nm corresponds to the total antioxidant level in a sample. The assay is calibrated with a stable antioxidant standard solution of vitamin E analog, (Trolox-equivalent) (44).

The TOS assay used here is based on the oxidation of the ferrous ion-chelator complex to ferric ion (Fe^{3+}), which is mediated by oxidants contained in the tested sample. The reaction is further enhanced by other molecules from the reaction medium. The reaction of Fe^{3+} with chromogen in an acidic medium produces a coloured complex. Its intensity corresponds to the total amount of oxidants in the sample and can be measured spectrophotometrically. The TOS assay is calibrated with hydrogen peroxide and the results are expressed in terms of micromolar hydrogen peroxide equivalent per litre (45).

Statistics

Statistical analysis was performed using the SPSS software (version 12.0, SPSS, Chicago, IL, USA). The results are expressed as mean ± standard deviation (S.D.). Groups were compared by one-way analysis of variance followed by Duncan's multiple range test. The level of significance was set at $P<0.05$.

RESULTS

Table 1 shows the effects of TA and PM on oxidant status in lymphocyte cultures that were determined by TAC and TOS analysis. Total antioxidant capacity decreased with the addition of PM. In the positive control (10 μmol L^{-1} of ascorbic acid) it was twice as high as in the negative control. Similarly, TA caused a significant and dose-dependent increase in TAC compared to negative control. In addition, TA showed dose-dependent inhibitory effects on the oxidative damage caused in lymphocytes by PM. On the other hand, the positive control showed about a threefold increase in TOS compared to the negative control. PM also increased the TOS level, but TA did not.

Figures 1 and 2 show the anti-genotoxic effects of lymphocyte treatment with TA alone and in combination with PM. Permethrin increased SCE frequency compared to the negative control, while TA alone (in the concentrations of 25 μg mL^{-1}, 50 μg mL^{-1} and 100 μg mL^{-1}) did not change it. When given with PM, it significantly reduced SCE frequency compared to lymphocytes treated with PM alone (Figure 1).

Similar effects were observed with MN frequency. It significantly ($p<0.05$) increased in lymphocytes treated with PM alone and was unaffected by TA at any of the tested concentrations. In the cells treated with PM and TA together, TA decreased MN frequency compared to the negative control (Figure 2).

DISCUSSION

Pyrethroid pesticides have recently been reported to cause apoptosis (47), disrupt the endocrine system (48), change nitrogen metabolism (49), increase mitochondrial membrane permeability (50), and alter constitutive nitric oxide release (51). Higher SCE and MN frequencies and higher TOS plasma levels in our

Table 1 *Total antioxidant capacity (TAC) and total oxidative stress (TOS) levels in cultured human lymphocytes simultaneously exposed to permethrin (PM) and taurine (TA).*
Different superscripts denote significant differences within each column at the p<0.05 level. Values are expressed as means ± standard deviations of three experiments.

Treatment	TAC Trolox Equiv. / mmol L^{-1}	TOS H_2O_2 Equiv. / μmol L^{-1}
Negative control	6.5±0.7^{d}	11.5±3.2^{a}
Positive control	13.71±0.94^{g}	39.25±4.63^{f}
PM	4.4±0.7^{a}	17.2±4.5^{e}
TA1	7.6±0.8^{e}	11.0±3.9^{a}
TA2	8.1±0.9^{ef}	11.6±4.7^{a}
TA3	8.8±1.2^{f}	11.6±4.4^{a}
PM + TA1	4.8±0.7^{ab}	15.4±5.1^{d}
PM + TA2	5.4±0.8^{b}	14.2±5.3^{c}
PM + TA3	5.9±0.9^{c}	12.1±4.4^{b}

PM: 200 μg mL^{-1}; TA1: 25 μg mL^{-1}; TA2: 50 μg mL^{-1}; TA3: 100 μg mL^{-1}

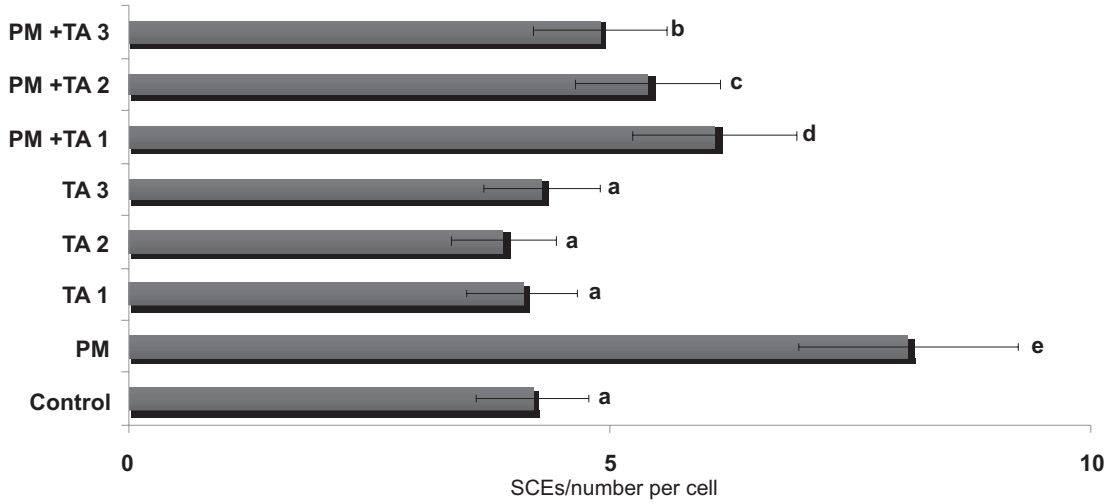

Figure 1 *SCE frequency in cultured human lymphocytes simultaneously exposed to permethrin (PM) and taurine (TA). PM: 200 μg mL^{-1} permethrin; TA1: 25 μg mL^{-1} of taurine, TA2: 50 μg mL^{-1} of taurine, TA3: 100 μg mL^{-1} of taurine; means of three measurements marked by different letters differ significantly (P<0.05)*

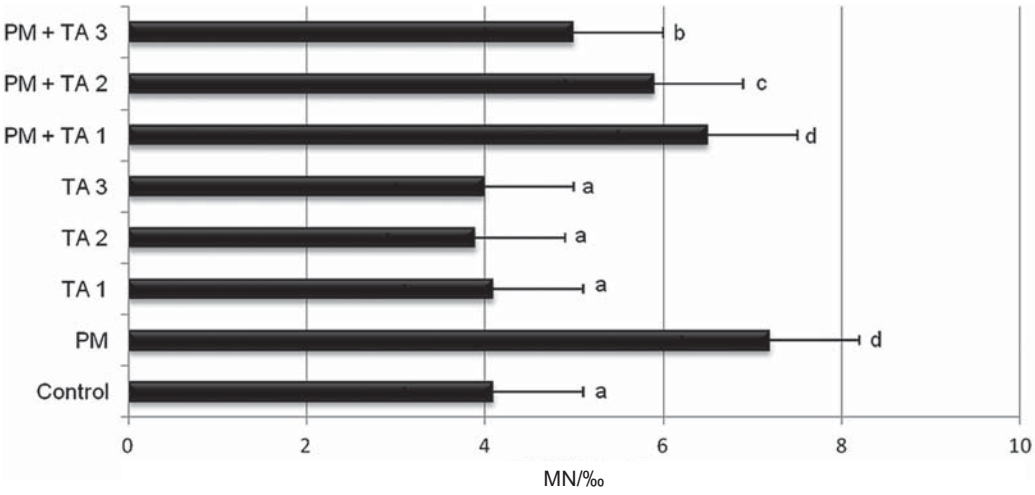

Figure 2 *MN frequency (‰) in cultured human lymphocytes simultaneously exposed to permethrin (PM) and taurine (TA). For abbreviations see Figure 1.*

study confirm the genotoxic and oxidative action of PM in human lymphocytes. Similarly, earlier studies (1, 52, 53) reported that PM induced MN formation and chromosomal aberrations in human peripheral blood lymphocytes and caused nuclear DNA damage in rats (13). In an experiment with rat adrenal pheochromocytoma (PC12) cells (54), it increased the production of reactive oxygen species (ROS) and malondialdehyde (MDA), which is known as a marker of lipid peroxidation (LPO). At the same time, the authors observed lower antioxidative activity of superoxide dismutase, catalase, and glutathione).

Free radicals damage the cell by, for example, irreversibly damaging lipids in cell membranes and disturbing the structure of proteins. Free radical derivatives damage both the purine-pyrimidine bases and the deoxyribose skeleton in the DNA. The most harmful oxidative lesion of the DNA is 8-hydroxyguanine (8-OH-G), which is involved in mutagenesis, carcinogenesis, and aging (55-60).

The increase in SCE and MN frequency observed in our study could be attributed to the pro-oxidative effects of PM. This is supported by decreased TAC and increased TOS levels in PM-treated cells. Recent studies (12, 13) also associate oxidative stress induced by PM with DNA damage. Moreover, it is known that toxic oxygen metabolites produced by normal human white blood cells cause the formation

of SCEs and MNs in cultured mammalian cells (61-63). The production of toxic oxygen metabolites in lymphocytes could be set off by a variety of chemicals, including PM.

The results of our study also suggest that TA plays an important protective role. Two hypotheses could explain the modulating effect of TA against PM-induced genotoxicity. The first is that TA acts as an antioxidant in a dose-dependent manner (24). Through antioxidative pathways it seems to modify both target and receptor cell homeostasis (64-66). Plasma and extracellular fluid TA concentrations typically range from 10 μmol L^{-1} to 100 μmol L^{-1} (67). Although this amino acid is not incorporated into proteins, TA intracellular concentrations can reach up to 80 mmol L^{-1}, depending on the tissue type (68). Adding TA does not make it toxic in cells where it is already present in high concentration. Moreover, supplementation with TA (150 mg kg^{-1} or ~1.87 g L^{-1}) in a recent study (20) prevented oxidative stress induced by doxorubicin. However, concentrations this high may lead to saturation. Another evidence in favour of the antioxidative action of TA is that the activities of SOD, CAT, and glutathione peroxidase (GSH-Px) normalise in TA-supplemented cultures, and this normalisation reduces LPO levels (69). The second hypothesis is that TA has a cytoprotective role. It is based on the study by Eppler and Dawson (70), who suggest that TA stabilises membrane receptor proteins, reduces protein carbonyl formation, and inhibits oxidative damage to DNA. Both hypotheses have been supported by Cozzi et al. (71), who found that TA protected the DNA by scavenging ROS. Similarly, Du et al. (72) found that TA supplementation had protective effects in high-fat diet-induced obese rats due to its antioxidant nature. In addition, TA showed anti-mutagenic properties in the *Salmonella* Ames tester strain assay and against aluminium sulphate (73, 74).

CONCLUSION

We have demonstrated for the first time that TA protects human lymphocytes against PM-induced toxicity. TA significantly attenuated DNA damage related to the overproduction of ROS in PM-treated lymphocytes. Supplementation with TA may eventually be useful in individuals who are exposed to synthetic pyrethroids, but the mechanisms of its beneficial effects remain to be elucidated by future studies.

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Sažetak

DJELOVANJE TAURINA PROTIV CITOGENETIČKOG I OKSIDATIVNOG OŠTEĆENJA U KULTURAMA LJUDSKIH LIMFOCITA UZROKOVANIH PERMETRINOM

Permetrin je piretroidni pesticid koji se često rabi za suzbijanje nametnika u poljoprivredi, šumarstvu, povrtlarstvu, zdravstvenoj zaštiti, domovima i tekstilnoj industriji. Poznat je kao snažan mutagen u životinja i ljudi. Taurin je aminokiselina koja se nalazi u tkivu sisavaca i štiti stanicu od oštećenja DNA. Svrha je ovog istraživanja bila saznati hoće li taurin (u koncentracijama od 25 μg mL^{-1}, 50 μg mL^{-1} te 100 μg mL^{-1}) zaštititi ljudske limfocite od toksičnoga djelovanja permetrina koji je dodan kulturama u koncentraciji od 200 μg mL^{-1}. Genotoksično djelovanje ocijenili smo s pomoću mikronukleus (MN)-testa i testa izmjena sestrinskih kromatida (engl. *sister chromatid exchanges*, krat. SCE). Da utvrdimo oksidativno djelovanje, izmjerili smo ukupni antioksidativni kapacitet (engl. *total antioxidant capacity*, krat. TAC) i ukupni oksidativni stres (engl. *total oxidative stress*, krat. TOS) u plazmi. Permetrin je povećao učestalost SCE i MN te promijenio TAC i TOS. Sam taurin, bez obzira na koncentraciju, nije utjecao na učestalost SCE i MN u odnosu prema kontroli. Usto je podigao TAC, a da pritom nije utjecao na TOS. Štoviše, značajno je ublažio štetno citogenetičko i oksidativno djelovanje permetrina, a učinkovitost mu je bila izravno povezana s primijenjenom koncentracijom. Ovo je prvo *in vitro* istraživanje koje je pokazalo povoljno djelovanje taurina protiv oksidacijskoga djelovanja permetrina i oštećenja DNA koje on uzrokuje.

KLJUČNE RIJEČI: *antioksidans, izmjene sestrinskih kromatida, mikronukleus-test, pesticidi, TAC, TOS*

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Professional paper

POLLEN COUNTS IN SLAVONSKI BROD, CROATIA DURING THE POLLINATION PERIOD 2008 TO 2010

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Hay fever or pollinosis is the allergic reaction of the human body to allergic pollen grains and is a seasonal phenomenon. Pollen concentrations depend on the climate, geographic features, and vegetation. Trees, grass, and weed pollinosis is frequent in Croatia, common ragweed (*Ambrosia artemisifolia* L.) pollinosis in particular. Continuous monitoring of pollen air concentrations can provide timely information to the general public and can help sensitised patients and their physicians to prevent or alleviate allergic reactions. This is the task of health ecology services such as our Public Health Institute of the Brod-Posavina County. This article reports pollen concentrations in Slavonski Brod measured in March 2008 to November 2008, March 2009 to October 2009, and April 2010 to October 2010 and discusses the increasing exposure to ragweed and ways to control it.

KEY WORDS: *aerobiology, allergy, Europe, grass pollinosis, ragweed, tree pollinosis, weed pollinosis*

Europe faces an increasing incidence of pollen allergy, particularly in the urban areas (1). Hay fever or pollinosis is the allergic reaction of the human body to allergenic pollen grains and is a seasonal phenomenon (2). From spring to autumn, pollen is in the air and its concentrations depend on the climate, geographic features, and vegetation (3-6). Pollinosis in Croatia is between the typical Mediterranean and central European, with grass (central European) and cypress pollen (Mediterranean) as the most frequent causes of pollinosis (1). Over the past few years, however, Croatia has seen a strong increase in the pollination of common ragweed (*Ambrosia artemisiifolia* L.), the source of the most potent pollen allergen in the country (7).

Information about the levels of pollen grains in the air could be very useful for sensitised patients and physicians, as it can improve prevention and therapy of seasonal allergic symptoms (9-11).

The aim of this study was to establish air levels of tree, grass, and weed pollens in Slavonski Brod during pollination in 2008 to 2010.

METHODS

This study reports pollen concentrations in Slavonski Brod measured in March 2008 to November 2008, March 2009 to October 2009, and April 2010 to October 2010. For pollen sampling we used a seven-day Hirst-type volumetric pollen and spore trap (Lanzoni s.r.l., Italia) (12). The sampler was placed on the roof of the general hospital "Dr Josip Benčević" in Slavonski Brod. It uses a vacuum-pump to pass 10 L

of air through the orifice (2 mm x 14 mm) always directed into the wind. Inside is a drum with a tape coated with silicon oil to catch pollen grains. This adhesive tape moves at the speed of 2 mm h^{-1}. Twice a week (on Mondays and Thursdays), we removed this tape, cut it to 48 mm strips that correspond to 24-hour pollen sampling. The strips were placed on glass slides and embedded in a medium prepared by dissolving 70 g polyvinil alcohol (Gelvatol, Burkard Scientific, UK) with 4 g phenol (*p. a.*, Kemika, Croatia), in 200 mL of distilled water. After the overnight rest, 100 mL of glycerol (GRAM-MOL d.o.o., Croatia) was added and warmed up in a water bath until the solution turned liquid and clear. Then we added four drops of alcohol solution of basic fuchsin (Kemika, Croatia) per 100 mL of the medium. Two hours later, samples were examined under a light microscope (Olympus Corporation, Japan) at 400x magnification to determine pollen type against laboratory standards and bibliographical sources (13-16) and count per day. Pollen count is the number of pollen grains per cubic meter of air. We compared the pollen count with a standard pollen rating scale (PRS, Table 1) to determine whether it was low, moderate, high, or very high (17, 18).

The start of the pollen season was defined as the first date on which at least one pollen grain per cubic meter of air was recorded for at least five consecutive days (19).

RESULTS

We identified 25 pollen taxa with moderate and highly allergenic grains, including: *Abies* (fir), *Aesculus* (chestnut), *Alnus* (alder), *Ambrosia* (ragweed), *Artemisia* (mugwort), *Betula* (birch), *Carpinus* (hornbeam), *Castanea* (*chestnut*), *Chenopodium* (goosefoot), *Corylus* (hazel), *Fagus* (beech), *Fraxinus* (ash), *Juglans* (nut), *Picea* (spruce), *Pinus* (pine), *Plantago* (plantain), *Platanus* (plane), *Poaceae* (grass), *Populus* (poplar), *Quercus* (oak), *Salix* (willow), *Taxus/Juniperus* (cypress/juniper), *Tilia* (lime), *Ulmus* (elm), *and Urtica* (pellitory).

The highest pollen count of all pollen grain types (trees, grasses, weeds) was recorded in 2008 (Figure 1). Weed pollen dominated in every pollination period with the exception of 2009, when tree pollen prevailed (Figure 2).

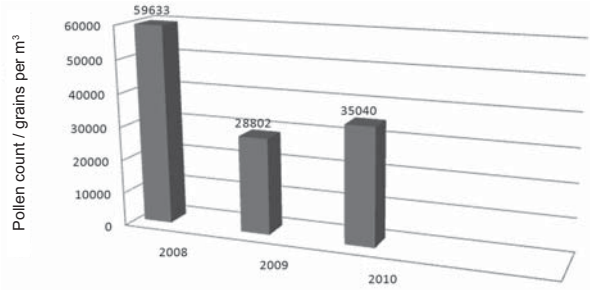

Figure 1 *Total pollen counts of all types of plants in the air of Slavonski Brod during the pollination period 2008 to 2010*

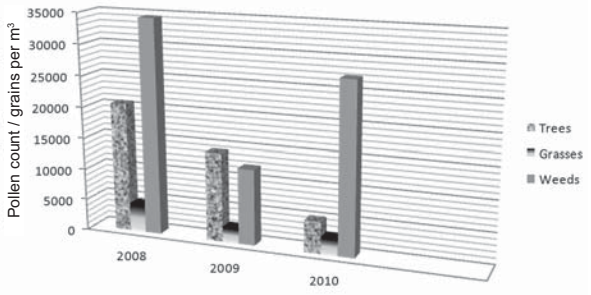

Figure 2 *Total pollen counts for trees, grasses, and weeds in Slavonski Brod during the pollination period 2008 to 2010*

Figure 3 shows the pollination season for all pollens and all three years. In 2008, tree pollination started in the beginning of March. By the beginning of the summer, it declined and was replaced by much lower grass pollination. This explains why the curve has dropped. In August, weed pollen took over with

Table 1 *Pollen rating scale categories* (*18*)

Pollen rating scale (PRS)	Pollen density / grains m^{-3}			Allergy sufferers
	trees	grasses	weeds	
low	1 to 15	1 to 5	1 to 10	extremely sensitive people will have symptoms
moderate	16 to 90	6 to 20	11 to 50	many sensitive people will have symptoms
high	91 to 1500	21 to 200	51 to 500	most sensitive people will have symptoms
very high	>1500	>200	>500	almost all sensitive people will have symptoms

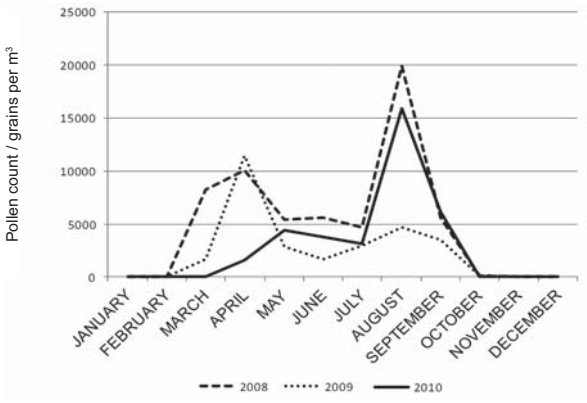

Figure 3 *Pollination period of all plant types (trees, grasses, weeds) in Slavonski Brod from 2008 to 2010*

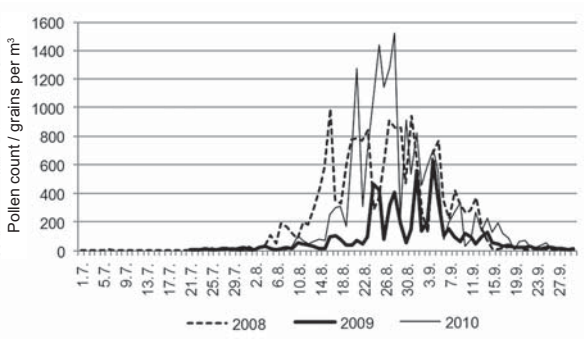

Figure 4 *Ragweed pollen counts in Slavonski Brod during the pollination period 2008 to 2010*

a high ragweed pollen count and this is why the curve has risen again. The curve for 2009 looks slightly different, with a higher peak in the spring than in the late summer and autumn, because tree pollen count was higher than weed pollen count. The highest peak in 2010 was reached in August and September, because ragweed pollen count was particularly high that season, which is even clearer in Figure 4 that shows the pollination of ragweed for all three years.

Figure 5 shows the daily variations in ragweed pollen counts on peak days for every year. The peak concentrations were recorded in the mornings.

Table 2 shows quantitative trends of the main pollen types (period of occurrence, duration, concentration on a peak day, and peak day).

Table 2 *Levels of eight dominating plant taxa dominated in Slavonski Brod during the pollination period 2008 to 2010*

Taxa	Period of occurrence	Duration / days	Concentration in a peak day / grains m^{-3}	Peak day
	2008: 5 March to 1 May	57	134	29 March
Taxus	2009: 17 March 17 to 29 May	73	1456	3 April
	2010: 23 April to 30 May	37	51	1 May
	2008: 9 March to 25 May	77	1836	29 March
Betula	2009: 28 March to 15 May	48	276	5 April
	2010: 23 April to 27 May	34	47	1 May
	2008: 13 March to 1 June	80	105	11 April
Acer	2009: 26 March to 17 May	52	227	12 April
	2010: 23 April to 29 May	36	41	1 May
	2008: 10 March to 4 June.	86	548	11 April
Platanus	2009: 9 April to 3 May	24	194	17 April
	2010: 23 April to 30 May	37	66	6 May
	2008: 11 March to 11 June	92	135	27 May
Quercus	2009: 2 April to 30 May	58	137	17 April
	2010: 23 April to 1 June	39	115	4 May
	2008: 1 April to 13 September	165	249	30 May
Poaceae	2009: 13 April to 13 September	153	165	19 May
	2010: 26 April to 11 September	138	156	26 May
	2008: 14 March to 10 September	190	410	14 August
Urtica	2009: 8 April to 15 September	160	159	16 July
	2010: 29 April to 29 September	153	185	15 August
	2008: 22 June to 26 October	126	994	16 August
Ambrosia	2009: 13 July to 15 October	94	625	4 September
	2010: 26 July to 15 October	81	1520	28 August

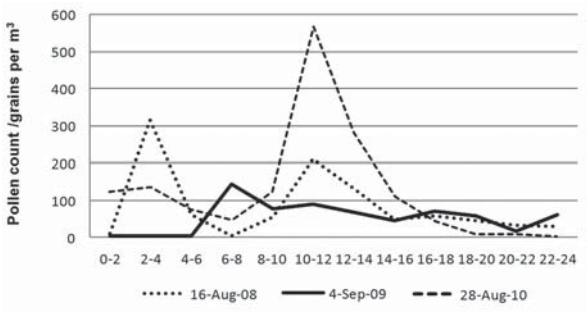

Figure 5 *Daily variation of ragweed pollen counts in Slavonski Brod on peak days from 2008 to 2010*

DISCUSSION

Tree pollen types dominated by the taxa *Quercus, Betula, Platanus, Acer*, and *Taxus* in the spring coincide with earlier findings in other two continental towns of Vinkovci and Bjelovar (10, 20). Grass pollen took over in May and June, as tree pollen counts subsided. Grass pollen counts dominated until July and August to be taken over by weeds, mostly ragweed, followed by the taxa *Artemisia, Plantago*, and *Chenopodium*.

The highest ragweed concentration in our study of 1520 pollen grains per cubic meter was recorded on 28 August 2010. For comparison, the highest concentration recorded in continental Zagreb in 2003 was 883 pollen grains per cubic meter (18). In Zadar, a Mediterranean-type town of Croatia, the highest ragweed pollen count in 2008 was 435 grains per cubic meter (21).

Even when ragweed pollination was not in the season (July/August), its pollen count dominated over other weed and in the peak days exceeded 600 to 1500 grains per cubic meter, that is, the threshold (see pollen rating scale in Table 1) associated with symptoms of hay fever or asthma that develop in sensitised patients. This is not surprising, as every single plant of ragweed produces enormous amounts of pollen (18).

On peak days, ragweed pollen counts were the highest in the morning. This pattern is similar to patterns reported in the continental towns of Zagreb, Samobor, and Ivanić Grad (18) and may reflect variations in meteorological parameters, as observed by Recio et al. (9).

CONCLUSIONS

Considering that the prevalence of allergic diseases is on the rise, and that it is largely related to sensitisation to pollen, there are several ways to reduce the risk of exposure for the general public. One of the measures is stern control of *Ambrosia artemisiifolia* L. as the most potent allergen source, decreed by the Croatian Ministry of Agriculture, Forestry and Water Management in 2006 (25). This control includes pulling young plants out at the end of May and the beginning of June when they reach 20 cm in height. Alternatively, during the vegetation period, the plants should be mowed before the pollination season starts (26). Another measure is making information on pollen levels public and regular; this is the task of health ecology services such as our Public Health Institute of the Brod-Posavina County. Clearly, this information should contain advice to physicians and sensitised patients about how to keep exposure at bay and adjust their daily activities and treatment, where necessary (27).

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Sažetak

KRETANJE PELUDNIH ALERGENA NA PODRUČJU SLAVONSKOG BRODA ZA VRIJEME POLINACIJSKIH SEZONA 2008.-2010.

Svrha je ovog rada prikazati kretanje peludnih alergena na području grada Slavonskog Broda, s obzirom na to da je pelud jedan od najčešćih prirodnih alergena koji može uzrokovati velike zdravstvene teškoće. Služba za zdravstvenu ekologiju Zavoda za javno zdravstvo Brodsko-posavske županije od kolovoza 2007. godine provodi monitoring koncentracije peludnih zrnaca u zraku na području Slavonskog Broda. Metodologija uzorkovanja peluda standardizirana je volumetrijska metoda. Dnevna koncentracija svih vrsta peluda izražava se kao broj peludnih zrnaca u kubnom metru zraka. Utvrđeno je 25 biljnih vrsta koje se dijele prema podrijetlu na drveće, trave i korove. Najviše peludnih zrnaca (svih vrsta zajedno) zabilježeno je 2008. godine. Prevladavao je pelud korova, s naglaskom na ambroziju. U proljeće se u zraku pojavljuje samo pelud drveća, početkom ljeta pelud trava i korova, dok tokom samog ljeta i jeseni dominira pelud korova. S obzirom na to da se Slavonski Brod nalazi u kontinentalnom dijelu Hrvatske, moglo bi se očekivati da će se tijekom godine u zraku pojaviti pelud svih biljnih vrsta. Kontinuirano praćenje koncentracije peluda u zraku omogućuje pravodobno obavješćivanje javnosti i uvelike može pomoći i liječnicima i pacijentima u preveniranju alergijskih reakcija.

KLJUČNE RIJEČI: *aerobiologija, alergija, ambrozija, biljke, Europa*

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Professional Paper

THE USE OF MERCURY-BASED MEDICAL DEVICES ACROSS CROATIAN HEALTHCARE FACILITIES

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In 2009, we conducted a survey to assess the use of mercury-based thermometers and sphygmomanometers and their disposal in Croatian healthcare facilities. The questionnaire addressing the use of mercury-based medical devices, waste management, preferences between mercury-based and electronic devices, and the knowledge on mercury toxicity was filled by ward nurses affiliated with 40 (71.4 %) out of 56 contacted healthcare facilities. Only one of these facilities had given up the use of mercury-containing medical devices at the time. As many as 84.6 % of the nurses believed that broken devices did not increase the risk of mercury exposure, even though 90 % claimed they were aware of mercury toxicity. In fact, 69.4 % of the nurses preferred mercury-containing devices on account of their precision and reliability and because they received little training in the use of electronic devices.

Breaking of thermometers and sphygmomanometers is common in healthcare facilities. The number of broken thermometers and sphygmomanometers was estimated to 278 and five per month, respectively. Only 18 (46.2 %) of the surveyed healthcare facilities claimed to have had a proper disposal procedure for mercury from broken devices. Nurses, who most often handle these devices and collect mercury spills, are primarily exposed to mercury vapours via inhalation. Croatia has adopted the EU Directive 76/769/EEC intended to reduce mercury exposure in the living and working environment. Our survey suggests that all healthcare professionals need training in proper management of broken mercury-based medical devices, nurses in particular. To reduce the risk of exposure, all Croatian healthcare facilities should implement guidelines for staff protection and programmes to gradually replace mercury-based with electronic devices.

KEY WORDS: *nurses, occupational exposure, sphygmomanometers, thermometers*

Occupational exposure to toxic substances may have adverse health effects on healthcare professionals. They are exposed to a wide variety of mechanical, chemical, physical, and biological risks. These risks arise not only from the direct contact with the diseased, but also from unfavourable occupational settings and behavioural patterns at work (1). The first step in lowering these risks is to recognise them (2-5).

Elemental mercury (Hg^0) is one of the major toxic hazards directly associated with health care. The Agency for Toxic Substances and Disease Registry (ATSDR) has ranked it the third most toxic substance (6). In the past, various forms of mercury were used to treat syphilis and worm infestations, and mercuric chloride solution was one of the first antiseptics (7). Industrial development triggered a more extensive use of mercury and mercury-based compounds, which were used for both diagnostic and therapeutic purposes. For example, Hg^0 was used in oesophageal dilators and Cantor/Miller Abbott tubes. Today, mercury can still be found in amalgam fillings, reagents, lab chemicals, batteries, certain types of

bulbs, fluorescent tubes, and a number of other products (7, 8). Due to its high density, air stability, and uniform heat-induced spreading, it has often been used for metric equipment, including thermometers, sphygmomanometers, and barometers (9).

At room temperature, Hg^0 is a liquid that evaporates, unless sealed in a container such as a mercury-based medical device. Inhalation is the primary route of occupational exposure to Hg^0 and about 80 % is absorbed (8, 10). Mercury spills can penetrate flooring cracks and be hard to clean up. Even in small amounts such as from a broken thermometer, these spills can contaminate indoor air above the recommended levels and present an occupational risk, unless cleaned and properly removed (11).

Mercury-based medical devices such as thermometers and sphygmomanometers break often in healthcare facilities (12, 13). Among healthcare professionals, people who are most often exposed to Hg^0 vapours are the nurses who handle these instruments on a daily basis for a number of years and are responsible for collecting spills (1, 14). A US study conducted by the National Institute for Occupational Safety and Health (NIOSH) in 1980 to 1983 estimated that occupational exposure to Hg^0 occurred in as many as 67,551 professionals (21,153 of whom were women). Most were affiliated with healthcare facilities and chemical plants either as nurses or chemistry technicians (6).

Hg^0 has no other than toxic effects on the human body (10, 15, 16). In recognition of direct mercury-related adverse human health effects, programmes to reduce mercury use and encourage proper management and disposal of mercury-containing wastes have been implemented throughout the world. Both the United States and some EU countries have long abandoned a number of mercury-containing products and compounds. Whenever possible, mercury-based medical devices are being replaced with electronic and infrared equipment (17, 18).

The aim of this survey was to see how nurses in Croatia saw the use of mercury-based medical devices, the cleanup and disposal of Hg^0 spills, and mercury exposure in their healthcare facilities.

SUBJECTS AND METHODS

The survey was performed in 2009 and included 519 nurses affiliated with 40 (71.4 %) of the 56 healthcare facilities we contacted and which were listed by the Croatian Ministry of Health and Social Welfare. These facilities included three clinical hospital centres, three clinical hospitals, two clinics, 17 general hospitals, 13 special hospitals, and two health resorts and medical rehabilitation centres. The questionnaire collected data on the use of mercury-based thermometers and sphygmomanometers, the

Table 1 *Views on harmful mercury-related health effects and preferences in choosing medical devices, reported by the interviewed nurses*

Answers	Number of participants	Percentage of answers
No additional exposure at workplace	439	84.6
Additional exposure at workplace	80	15.4
Total number of participants	**519**	**100**
Hg is completely harmless	52	10.0
Hg is slightly harmful	40	7.7
Hg is profoundly harmful	151	29.1
Hg is extremely harmful	276	53.2
Total number of participants	**519**	**100**
Favour mercury-based thermometers	360	69.4
Dislike mercury-based thermometers	159	30.6
Total number of participants	**519**	**100**
Favour mercury-based sphygmomanometers	340	65.5
Dislike mercury-based sphygmomanometers	179	34.5
Total number of participants	**519**	**100**

number of broken mercury-based medical devices per month, and the disposal and management of broken devices. Ward nurses were asked to share their views about the health impact of mercury spills and their preference between mercury-based and electronic medical equipment. They were also asked whether they considered their occupational mercury exposure to be greater than the exposure of the rest of medical staff.

The nurses were all informed about the purpose of the study and its protocol and participated on a voluntary and anonymous basis. The study was approved by the Ethics Committee of each participating healthcare facility.

RESULTS

Mercury-based thermometers and sphygmomanometers were used in 39 of the 40 surveyed healthcare facilities. In these 39 facilities, electronic medical equipment was also used, but only in certain wards. Mercury-based devices had been fully abandoned and replaced with electronic devices in only one of the surveyed healthcare facilities.

Knowledge on Hg toxicity and common patterns of mercury-based device use

Table 1 shows answers on mercury toxicity and personal attitudes and preferences. Even though most were aware of mercury toxicity, they still preferred mercury-based over electronic equipment, as they found it more precise and reliable, if slower to read.

Table 2 shows the estimates about the number of broken thermometers a month. This figure ranged between 2 and 278. Yearly, this figure ranged between 4 and 3336.

The monthly number of damaged/broken sphygmomanometers ranged from 0 to 5, and the annual figure ranged from 1 to 94.

Disposal and management of mercury-based medical devices

Broken mercury-based medical devices and mercury spills were properly disposed of into separate containers and managed as hazardous chemical waste in only 18 (46,2 %) out of 39 respondent healthcare facilities. Only one healthcare facility fully abandoned the use of mercury devices. At the remaining 21 healthcare facilities, 81 of 259 wards (31.3 %) properly handled the spills, while the remaining 178 (68.7 %) disposed of Hg^0 inappropriately. Inappropriate disposal methods included placing broken devices in containers for infectious waste (65.17 %), in containers originally meant for broken glass storage (18.54 %), in communal waste (15.17 %), pouring the spills down the drain (0.56 %), and storing broken devices together with cytostatic waste (0.56 %) (Figure 1).

DISCUSSION

To increase the awareness of the hazards of mercury in healthcare facilities and the healthcare sector on the whole, the World Health Organization (WHO) has recommended regular monitoring and assessment of mercury use in these settings (18). The US Environmental Protection Agency (EPA) has recommended replacing mercury-containing medical devices with mercury-free alternatives.

The EU member states have recommended reducing the use of mercury-based medical equipment to reduce the adverse health effects of occupational mercury exposures. This survey has shown that mercury-based medical devices are used in virtually all Croatian healthcare facilities. By comparison, 46 % of Irish healthcare facilities have completely abandoned these devices and switched to mercury-free alternatives (19).

Table 2 *Estimated number of monthly broken thermometers in healthcare facilities divided in 5 groups*

Number of broken thermometers per month	Healthcare facilities	
	Number	Percentage
0 to 50	20	51.3
51 to 100	7	17.9
101 to 150	4	10.3
151 to 200	5	12.8
251 to 300	3	7.7

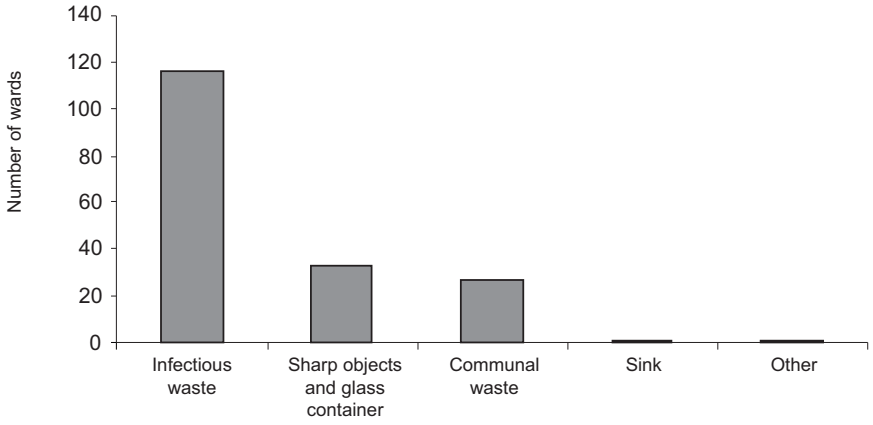

Figure 1 *Ways of improper disposal of broken mercury thermometers among wards (N=178) recorded in 21 hospitals*

Many physicians and nurses believe that mercury sets "the gold standard" against which all alternative thermometers and sphygmomanometers should be compared. In our survey, 69.4 % of the nurses preferred mercury-based medical devices. One reason for this preference might be that electronic devices currently in use are not fully adjusted to best serve hospital needs and are sometimes imprecise and unfit for repetitive measurements. The other reason is that the staff has not received proper training in electronic equipment maintenance and use, and that technical support leaves much to be desired.

However, Croatian healthcare facilities have started to switch to alternative devices such as infrared tympanic thermometers, which are far more precise and can quickly repeat measurements. It is reasonable to assume that, once they are satisfied with performance, nurses will more readily accept mercury-free devices.

A large number of studies have concluded that mercury-free measuring devices produce the same degree of accuracy as mercury devices, provided they are properly maintained and calibrated (17, 18).

The average amount of mercury to be found in thermometers spans from 0.5 g to 1.5 g; wall-mounted and transportable sphygmomanometers contain 110 g to 200 g of mercury, while hospital lab thermometers contain 3 g to 4 g (20). According to some studies (8), the average number of thermometers broken in hospitals with 300 to 500 beds is 70 a month or 840 a year. This means that these hospitals release up to 3 kilograms of mercury into the environment a year and pose not only occupational, but also environmental threat. The number of broken thermometers in Croatian hospitals with about 350 beds is estimated to between 160 and 180, or as many as 2,000 a year (21). By comparison, the Irish healthcare services witnessed no Hg spills at all in 2007 (19).

To get the broader picture of the issue, there are 31,578 nurses in Croatia alone. They play a crucial role in proper containment and management of mercury spills and mercury-containing waste (1). Yet, in our survey nurses of only 18 Croatian healthcare facilities reported proper disposal of broken mercury-based medical devices. Clearly, the rest requires urgent training minimise potential mercury exposure and improve the safety not only of the staff, but also of patients of all ages. In Croatia, only the occupational limits for mercury body burden have been determined, but not for the general population. Most of the regulatory standards are related to safety at work. For inorganic mercury the occupational limit is 0.05 mg m^{-3}. The biological limit for blood mercury is 30 mg L^{-1} and for urine mercury 50 mg g^{-1} creatinine (22).

In Croatia, medical waste management is subject to the Waste Management Act (23), the Regulation on Waste Types (24), and the Guidelines for the Management of Waste Generated by Healthcare Services (25). Given the overall lack of knowledge on proper mercury disposal, it is likely that many mercury spills are not reported. Insufficient knowledge on managing, collecting, and waste disposal procedures to be followed after a mercury spill from broken mercury-based devices may result in the contamination of the working environment and substantial exposure to toxic mercuric vapours (1). Given that 84.6 % of the surveyed nurses do not associate spills with their exposure to mercury, hazardous waste management

should urgently be introduced to healthcare professional curricula. Our findings are supported by the Irish study reporting that over 50 % of hazardous waste is inappropriately disposed of (19). Each ward should receive not only a proper training and procedures, but also sealable overpack containers for storing broken devices and spills. The collected waste should promptly be removed by a company duly licensed for hazardous waste management, contracted by the healthcare facility.

The latest Croatian regulations, adopted in April 2010, bring a new inventory of hazardous chemicals that takes mercury-based medical devices off the free market (26) in line with the EU Directive 76/769/EEC (28). However, this will not stop their use in Croatia. They will be used as long as they function properly. The results of our survey suggest the need for monitoring and measuring mercury pollution across Croatian healthcare facilities. All healthcare professionals, nurses in particular, should receive training in mercury-related toxicity, potential sources of exposure, and proper management of mercury-containing waste. All healthcare professionals should also receive training in the benefits of using mercury-free alternatives. This can be orchestrated on the national level, following the current guidelines for the protection of healthcare professionals.

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Sažetak

UPOTREBA ŽIVINIH MJERNIH UREĐAJA U ZDRAVSTVENIM USTANOVAMA U HRVATSKOJ

Živa je štetni čimbenik izravno povezan s provođenjem zdravstvene zaštite. Tijekom 2009. provedeno je istraživanje u zdravstvenim ustanovama RH, s ciljem procjene uporabe živinih mjernih instrumenata, toplomjera i tlakomjera te načina odlaganja razbijenih uređaja. Upitnik o uporabi živinih uređaja, zbrinjavanju otpada, sklonostima uporabi živinih, odnosno elektroničkih mjernih uređaja te pitanja o poznavanju toksičnosti žive, ispunile su odjelne medicinske sestre iz 40 (71,4 %) od 56 zdravstvenih ustanova. Samo u jednoj ustanovi živini se mjerni uređaji uopće ne rabe. Čak 84,6 % ispitanica smatra da nisu dodatno izložene živi iz razbijenih uređaja, iako je 90 % svjesno toksičnosti Hg. Zbog njihove preciznosti, pouzdanosti i nedostatka edukacije o uporabi i održavanju elektroničkih uređaja prednost uporabi živinih uređaja daje 69,4 % medicinskih sestara. Razbijanje toplomjera i tlakomjera čest je incident u zdravstvenim ustanovama. Procijenjeni broj mjesečno razbijenih toplomjera bio je do 278, a razbijenih tlakomjera do 5. U samo 18 (46,2 %) ustanova pravilno se odlagala živa iz razbijenih uređaja. Medicinske sestre koje najčešće rukuju uređajima i prikupljaju živu najizloženije su živinim parama putem inhalacije. U Hrvatskoj su doneseni pravni akti s namjerom smanjenja prisutnosti žive u životnom i radnom okolišu. Time je stupila na snagu EU direktiva 76/769/EEZ-a o smanjenju proizvodnje i prometa uređaja koji ju sadržavaju. Rezultati upućuju na potrebu edukacije svih zdravstvenih radnika, posebno medicinskih sestara, o zbrinjavanju razbijenih živinih mjernih uređaja. Radi smanjenja potencijalne izloženosti i osiguranja boljih zdravstvenih uvjeta na radnome mjestu sve hrvatske zdravstvene ustanove trebaju provoditi smjernice za zaštitu radnika i programe za smanjenje uporabe žive uporabom zamjenskih toplomjera i tlakomjera dostupnih na tržištu.

KLJUČNE RIJEČI: *medicinske sestre, tlakomjeri, toplomjeri, profesionalna izloženost, zbrinjavanje otpada, živa*

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Review

THE ROLE OF INTERFERON-GAMMA RELEASE ASSAY IN TUBERCULOSIS CONTROL

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Tuberculosis is still one of the major global public health threats. Countries with low incidence must focus on exhausting the reservoir of future cases by preventing reactivation. Therefore, it is important to identify and effectively treat those individuals who have latent tuberculosis infection and who may develop active disease. The tuberculin skin test has been the standard for detection of immune response against *M. tuberculosis* since the beginning of the 20th century. The new millennium has brought advancement in the diagnosis of latent tuberculosis infection. The name of the new blood test is interferon-gamma release assay (IGRA). Croatia is a middle-incidence country with a long decreasing trend and developed tuberculosis control. To reach low incidence and finally eliminate tuberculosis, its tuberculosis programme needs a more aggressive approach that would include intensive contact investigation and treatment of persons with latent tuberculosis infection. This article discusses the current uses of IGRA and its role in tuberculosis control.

KEY WORDS: *Croatia, enzyme-linked immunospot assay, latent tuberculosis infection, QuantiFERON-TB Gold In-Tube, tuberculin skin test*

Tuberculosis (TB) control is the most effective if TB is detected and treated early. National tuberculosis programmes (NTP) in high-incidence countries without sufficient resources are therefore focused on early detection and treatment. In turn, tuberculosis elimination strategies in low-incidence countries focus on exhausting the reservoir of future cases by preventing reactivation. They seek to identify and effectively treat individuals who have latent tuberculosis infection (LTBI) and who may develop active disease in their lifetime (1).

Croatia is a middle-incidence country with a long decreasing trend and advanced tuberculosis control (2). To reach low incidence and finally eliminate tuberculosis, its NTP needs a more aggressive approach that would include intensive contact investigation to identify, treat, and follow up individuals with LTBI according to the national guidelines (3).

There is no possibility to be sure that a person thought to have latent tuberculosis infection actually carries viable *M. tuberculosis* (4). What can be identified is a cell-mediated memory against mycobacterial antigens. For over 100 years this immune response against *M. tuberculosis* has been screened for using the tuberculin skin test (TST). It involves intradermal injection of purified protein derivative (PPD), which is a mixture of more than 200

antigens that lead to a delayed hypersensitivity reaction, visible as a local skin induration. The antigen spectrum of PPD is shared by the *M. tuberculosis* complex, Bacillus Calmette-Guerin (BCG)-subtype of *M. bovis*, and nontuberculous mycobacteria. As BCG vaccination has been a part of the Croatian national immunisation programme since 1948, TST has shown limited performance in diagnosing LTBI, as it gets confounded by prior BCG vaccination. With a new test called interferon-gamma release assay (IGRA), the new millennium has brought advance in identifying immunological response against antigens of *Mycobacterium tuberculosis* to clinical practice and tuberculosis control (5). A number of studies, many of them referred to in this article, have found IGRA a promising assay, but have also raised many questions to be answered. This is not surprising, as the nature of tuberculosis and immune response are still not fully understood.

Tests based on immunological memory, be they TST or IGRA, share an important limitation; they do not directly detect the presence of *M. tuberculosis*, but identify immune response to recent or remote infection with *M. tuberculosis* (4). However, IGRA overcomes the main limitation of TST, which is the lack of species specificity. It measures interferon-gamma (IFN-gamma), a cytokine released by sensitised T-cells in response to mycobacterial antigens present in the *M. tuberculosis* complex and absent from the BCG-strain and from the most nontuberculous mycobacteria except *M. kansasii*, *M. marinum* and *M. szulgai.*

Two types of IGRAs are currently available: the ELISpot test (*enzyme linked immunospot assay*), that directly counts the number of IFN-gamma secreting T-cells (commercially available as the T-SPOT.*TB*, Oxford Immunotec Ltd, Abingdon, UK) and the ELISA test (*enzyme linked immunosorbent assay*), that measures the concentration of secreted IFN-gamma. Several generations of the ELISA test have been developed by Cellestis Ltd, Carnegie, Australia.

The first was with PPD as the antigen, which is not longer commercially available; the second was QuantiFERON®-TB Gold (QFT-G), with early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10); and finally QuantiFERON®-TB Gold In-Tube (QFT-G-IT), with ESAT-6, CFP-10, and TB7.7.

This latest generation has currently been used in Croatia by a limited number of hospitals and public health institutes. It is more sensitive than the earlier one, probably because of the additional TB7.7 antigen (83 % *vs.* 93 %, p=0.006, respectively) (6).

QFT-G-IT TESTING

The testing procedure is as follows; three millilitres of collected blood is distributed in three tubes (1 mL each): the nil tube (heparin-only negative control tube), the TB antigen tube, and the mitogen tube (phytohaemagglutinin positive-control tube). Immediately after filling, the tubes have to be shaken ten (10) times to coat the entire tube wall with blood that will solve the antigens on the wall.

Within 16 h of blood collection, the tubes need to be incubated for 16 h to 24 h and ELISA test performed. The results are software-reported automatically as positive if the reaction to TB antigens is $\geq$0.35 IU mL^{-1} after subtracting the nil control (7). This points to a likely *M. tuberculosis* infection. For now, the cut-off of $\geq$0.35 IU mL^{-1} IFN-gamma has been taken as an optimal combination of sensitivity and specificity. The positive result does not distinguish between recent and remote infection or between active TB and LTBI. In this respect, it is no better than TST. The reason why that QFT-G-IT does not say much about the activation of the immune response in tuberculosis is that it uses peripheral blood that contains much less memory T-cells than the site of infection in active disease (8). Therefore, interpretation should not rely on the positive result alone, but should include risk factors to compensate for suboptimal sensitivity and the possibility of cross-reaction with three nontuberculous mycobacteria.

A negative result suggests that *M. tuberculosis* infection is not likely. However, if TB is suspected, negative result alone can not rule out active disease (8). IGRA conversion usually takes four to seven weeks following exposure to TB, but conversions can also take 14 to 22 weeks (9).

With an indeterminate result, LTBI can neither be excluded nor confirmed. If blood cells have not responded to a positive control there may have been an error in performing the test or the subject's immune system may be suppressed. By including an internal positive control (mitogen tube), it is possible to distinguish between indeterminate results and those that are truly QFT-negative. In contrast, a negative TST does not differentiate between immune suppression or false test performance and truly negative result (7). Indeterminate results are usually associated with

young (<5 years) and old (>80 years) age and immunosuppression, such as that owed to HIV infection or immunosuppressant therapy (10, 11). New guidelines recommend to retest the person with indeterminate result. If the result remains indeterminate, this may point to T-cell anergy (12).

THE MOST IMPORTANT IGRA FEATURES

Many studies have reported on IGRA's performance. They differ in regard to TB incidence, prevalence of infection, patient age, status of the immune system, prevalence of HIV-coinfection, antigens used (older or newer generation), and tuberculin units used during TST. This article has focused on newer studies published in prestigious scientific journals, performed in settings similar to ours.

Sensitivity

The sensitivity and specificity of IGRAs or TST for LTBI cannot be reliably estimated because there is no gold method for the diagnosis of LTBI. This is why two surrogates for infection are used to estimate sensitivity. One is active TB. In a recent meta-analysis (13), pooled sensitivity of QFT-G-IT, T-SPOT.*TB*, and TST was 80 %, 81 %, and 65 %, respectively. Similar meta-analysis are consistent; the sensitivity of both IGRAs are usually higher than that of TST (6, 11, 14-16).

Even though not all individuals exposed to TB will become infected, the second approach to estimate sensitivity is based on the assumption that some of them will, depending on the frequency, length, and proximity of contact with infectious TB patient (17). Exposure to a TB patient is often used as the infection surrogate to estimate test performance in an epidemic or contact investigation. Even though more studies have estimated T-SPOT.TB sensitivity than that of QFT, they all show that both tests better correlate with TB exposure than TST and do not depend of BCG vaccination (11, 16, 18, 19). To conclude, even though either IGRA is more sensitive than TST, their sensitivities are suboptimal and we still need to find a more sensitive method.

Specificity

In areas with low TB incidence the prevalence of LTBI is expected to be very low among unexposed individuals. Therefore, where LTBI has not been established, test specificity is assessed in healthy subjects without known exposure. A recent meta-analysis (20) reported that QFT specificity ranged between 92 % to 100 % and pooled specificity was 98 %. Pai et al. (14) reported that pooled specificity of QFT tests was higher among non BCG-vaccinated individuals (99 %) than among vaccinated (96 %), while pooled specificity of T-SPOT.*TB* was 93 %. TST specificity was high in populations that had not received BCG (97 %), but usually low among BCG-vaccinated individuals (59 %) (16). Overall, IGRAs have a higher specificity than TST, particularly in BCG-vaccinated population.

PREDICTIVE VALUE OF IGRA IN THE DEVELOPMENT OF ACTIVE DISEASE

Preventive treatment of IGRA-positive contacts in comparison with IGRA-negative contacts will be reasonable only if their risk of progression to tuberculosis is higher. At the moment, no correlation has been established between IFN-gamma responses and the stage or degree of infection, the level of immune responsiveness, or the probability of progression to active disease. However, some findings suggest that QFT may be able to predict progression to active disease better than TST (21). In recent contacts with infectious cases, IGRA-positive tests better correlate with *M. tuberculosis* exposure than TST-positive findings. During a follow-up of an average of 3.7 years after exposure to TB, 12.9 % of QFT-positive contacts who refused chemoprevention developed TB in comparison with 3.1 % of TST positives (>5 mm of induration) (21). Several studies suggest that IGRAs are superior to TST in predicting active TB in BCG-vaccinated recent contacts (8, 11). However, if active TB is suspected, other diagnostic parameters are necessary to confirm TB (eg. bacteriological confirmation).

Higuchi et al. (22) showed that after 3.5 years of follow-up, none of BCG-vaccinated contacts who were both QFT-negative and TST-positive had developed active disease. The negative predictive value for *M. tuberculosis* infection is >95 % for both TST and IGRA negative results (23, 24). This suggests that LTBI and active TB can be excluded with a high degree of certainty in immunocompetent individuals, if both tests are performed and both have negative results (25). In immunocompromised individuals, however, this prediction is very limited (8, 23, 24).

AGREEMENT BETWEEN IGRA AND TST

Current research has shown that the level of agreement between the IGRAs and TST is lower if the tested population received BCG. This is not surprising because TST is confounded by prior BCG vaccination. Agreement between T-SPOT.*TB* and TST is moderate to high, with kappa values ranging from 0.51 to 0.72, and between QFT test and TST is more variable, with kappa values ranging from 0.19 to 0.87 (11).

However, most studies have focused on the agreement in positive vs. negative findings and have failed to view results as continuous variables. TST induration can be viewed not only as a positive or negative outcome, but also as a range of diameters. The same goes for INF-gamma results; instead dividing them into positive and negative along the cut-off value of 0.35 IU mL^{-1} , they can be compared directly. Pai et al. (26) have shown that changes in cut-offs of both tests can change the level of their agreement.

REPRODUCIBILITY OF IGRA

Reproducibility of IGRAs is particularly interesting in terms of within-person variability of T-cell responses in serial testings performed in some countries (27, 28). IGRAs are highly dynamic tests, and T-cell responses, especially weakly positive, tend to vary. Conversions, reversions, and non-specific variations have been reported for both IGRA and TST serial testing (18, 29-31). The clinical significance and prognosis of IGRA conversion and reversion are not clear, and it is difficult to determine an optimal cut-off to distinguish true infection from biological variability (27, 28, 30).

Reproducibility may be evaluated through dichotomous (positive vs. negative) results or through continuous IFN-gamma values. Reproducibility is usually analysed in two ways: a same sample is tested by two separate QFT-G-IT assays (so called test-retest variability) and the same person is tested repeatedly over a short period of time (short-term, within-person variability). When QFT-G-IT results are interpreted using dichotomous results, then test-retest and within-person reproducibility are very high, as most of the results are well below or well above the cut-off of 0.35 IU mL^{-1} IFN-gamma. Discordance is mostly present in subjects who had IFN-gamma values around the cut-off point (28, 29).

Using continuous IFN-gamma values to interpret QFT-G-IT results, reproducibility was moderate to high (28) or high (32). A similar study also showed that weakly positive IGRA results were subject to variation over time more than negative results which generally remained negative. Variation in strongly positive IFN-gamma values will probably not have any clinical significance, but weakly positive IFN-gamma values may complicate interpretation of repeated results (30).

With dichotomous test results, conversion (which may lead to LTBI treatment) is simply defined as a change from negative to positive. Findings from reproducibility studies suggest that QFT conversion has to meet two conditions: there should be a change from a negative to a positive result and the baseline IFN-gamma value should increase at least 30 % (28). The US Centers for Disease Control and Prevention recommend that both the standard qualitative test interpretation and the quantitative assay measurement should be reported together with the criteria used for test interpretation (27).

IGRA IN CHILDREN

In terms of LTBI and active TB, children run a high risk of developing active TB after being infected, and TB diagnosis is often difficult due to the paucibacillary nature of disease. Weak cellular immune response is usually the reason why IGRA results are more often indeterminate in children than in adults (33). Some even prefer TST in diagnosing LTBI in smaller children over IGRA (27, 34, 35). This preference stems from the fact that TST obtains a stronger immunological response, as it stimulates central memory cells over a longer incubation time, while QFT responses result from effector cells in peripheral blood. In addition, the Th1 system, that produces interferon-gamma, is immature in children while the immune response of the Th2 system (interleukin 4, IFN-gamma, tumour necrosis factor alpha, interleukin 10, interleukin 12, and granulocyte colony-stimulating factor) is active and is more likely detected by TST (19, 36). On the other hand, several studies suggest that at higher levels of exposure to TB, the proportion of children with positive results in both IGRAs is higher, independent of the BCG status (11, 19, 36).

In a recent meta-analysis (37), pooled sensitivity of TST, QFT, and T-SPOT TB in children was 80 %, 83 %, and 84 %, while pooled specificity was 85 %, 91 %, and 94 %, respectively. All tests had lower sensitivity in young or HIV-infected children.

An Australian study (19) reported a high level of agreement between IGRAs (93 %), but often low agreement between IGRAs and TST (75 %). The size of TST induration and IFN-gamma levels were significantly associated with TB contact history, while age influenced only the TST results.

One study (38) directly compared T-SPOT.*TB*, QFT-G-IT, and TST performance in immunocompromised children in a setting with low TB incidence. T-SPOT.*TB* and QFT-G-IT yielded a high percentage of indeterminate findings (13.5 % and 20 %, respectively) and agreed in 62 % of the cases. Excluding the indeterminate results, the IGRAs agreement with TST was low and their performance was not associated with age, sex, white blood cell count, or treatment duration.

Indeterminate results reported for immunocompetent children in Italy, tested mostly during contact investigation, were more frequent with ELISA-based, QFT-G and QFT-G-IT tests than with T-SPOT.*TB*. (12.6 % *vs*. 2.3 % and 16.4 % *vs*. 1.5 %, respectively). The extent of exposure to *M. tuberculosis* (household *vs*. casual contacts or recent immigrants) was not associated with the rate of indeterminate results for any test (33).

In general, studies evaluating IGRA performance in children are few, and many questions remain unanswered. This is probably the reason why many national guidelines recommend caution in the use of IGRA in children (UK, Netherlands, Switzerland, Canada, France, and Japan) (20).

To conclude, sensitivity may be higher in children with a high risk of infection especially in children below five years of age and in immunocompromised children when IGRA testing is done in addition to the TST (4, 39). Whichever test turns positive, it points to true infection. Low-risk children may benefit from IGRA as a confirmation of positive TST in terms of increased specificity and reduced risk of a false diagnosis of LTBI (4).

IGRA IN IMMUNOCOMPROMISED INDIVIDUALS

The risk of progression to active disease is higher in infected immunosuppressed patients that in general population. Therefore, this is the target group for the screening and treatment of LTBI. Different diseases and conditions have been found to have impaired cell-mediated immunity such as HIV-infection, transplantation-related immunosuppressive therapy, anti TNF-alpha or corticosteroid treatment, malignancy, and chronic renal failure/haemodialysis. Only a limited number of studies focused on the accuracy of IGRAs in LTBI diagnosis in these groups of patients.

HIV-infection is the most important risk factor for TB in infected patients. In HIV-positive patients, the sensitivity of QFT is generally lower and the percentage of indeterminate results higher than in HIV-negative individuals. Test sensitivity rises as CD4 cell count drops. A recent meta-analysis (20) showed that IGRA's pooled sensitivity in HIV-infected TB patients was much higher than that of TST (70 % *vs.* 45 %, respectively). Another study (40) showed that T-SPOT.*TB* test was positive in 44.2 % immunosuppressed, HIV-negative, non-BCG vaccinated persons, who had been nosocomial contacts of smear-positive TB patient, while TST was positive in 17.4 % (agreement 67.8 %) In addition, T-SPOT.*TB* test produced only 4.3 % indeterminate results, regardless of the level of immunosuppression.

In a BCG-vaccinated population studied by Kim et al. (41), immunosuppression was significantly associated with the lower percentage of positive TST results (10.3 % in immunocompromised *vs*. 27.7 in immunocompetent patients), but not with positive QFT-G-IT results (21.4 % *vs*. 25.5 %, respectively). However, its association was significant with indeterminate QFT-G-IT results (21.4 % in immunocompromised *vs.* 9.6 % in immunocompetent patients). Indeterminate QFT-G-IT results were associated with laboratory findings such as anaemia, lymphocytopenia, hypoproteinemia, and hypoalbuminemia. The agreement between QFT-G-IT and TST was lower in the immunocompromised than in the immunocompetent patients.

Generally, only a few studies have addressed the performance of IGRAs in these patients. Expert opinion is that immunocompromised patients could benefit from the simultaneous use of IGRAs and TST. This approach is based on the need to increase test sensitivity. Indeterminate findings do not exclude TB infection. In fact, an indeterminate result in the absence of mitogen response may suggest anergy rather than absence of infection (12).

IGRA RESPONSES DURING THERAPY

In a study by Dyrhol-Riise et al. (42), 85 % of patients receiving preventive treatment with isoniazid and rifampicin for three months were still positive to QFT-G-IT three and 15 months after treatment. Moreover, IFN-gamma levels were comparable at baseline and the three and 15 months after preventive treatment. The authors concluded that the test was not useful for monitoring preventive therapy. Similarly, Pai et al. (43) showed that 73 % of patients were positive to QFT-G-IT at baseline, 81 % after two months of treatment, and 79 % at the end of a standard six-month treatment.

Changes in IFN-gamma were also inconclusive**.** Both studies suggest that IGRAs are not reliable markers to monitor the effect of therapy.

IGRA IN CONTACT INVESTIGATION

The study of QFT-G-IT performance by Dyrhol-Riise et al. (43) conducted in a low-incidence country showed that almost 70 % of the participants with positive TST results were QFT-G-IT-negative. The highest percentage of QFT-G-IT-positive results was found among household contacts (59.5%) and among those who had TST induration ≥15 mm (47.5%), which is usually taken as a true positive finding. Positive QFT-G-IT results were associated with immigrants born in non-western countries with high or intermediate prevalence, then with the time of TB exposure, and with previous TB disease.

A large German comparative study (44) of TST, QFG-G-IT, and T-SPOT.*TB* performance in persons exposed to pulmonary tuberculosis showed that cumulative exposure time, positive smear and/or coughing of the source, age, and foreign origin of the contact were good predictors of positive QFG-G-IT and T-SPOT.*TB* findings. The two IGRAs agreed in 93.9 % of cases (κ=0.85). Assuming that positivity to both IGRAs was true infection, the sensitivity of TST was 72 % and 39.7 % at respective cut-offs of >10 mm and >15 mm. The authors concluded that either QFG-G-IT or T-SPOT.*TB* would reduce the number of LTBI suspects by approximately 70 % in comparison with TST. In addition, the authors suggested that testing should be limited to contacts of smear-positive sources with >8 hours of cumulative exposure and contacts of smear-negative sources with >40 hours of cumulative exposure. However, the testing should also include contacts closely exposed to smear-positive sources, regardless of the cumulative time of exposure, as this may also lead to LTBI. The authors also pointed out that active TB could develop in IGRA-positive but TST-negative untreated patients. In other words, regardless of the cut-off point, TST may fail to detect infected contact. By raising the cut-off from 5 mm to 10 mm in BCG-vaccinated contacts, 25 % of the tests turned false negative and the authors concluded that IGRAs were more accurate indicators of LTBI than TST. In line with that observation, new German guidelines (35) recommend that only IGRA is used to test adult contacts who were at least partially BCG-vaccinated, while TST is preferred for children younger than five years.

COST-EFFECTIVENESS ANALYSIS OF IGRA IN PRACTICE

A British study (45) compared the cost-effectiveness of screening for TB with IGRAs and TST alone and in combination. Examining the cost alone, the TST/IGRA dual strategies cost less than IGRA alone strategies (about 160,000 £ *vs*. 200,000 £ per 1000 contacts, respectively). However, IGRA alone strategies are more specific for those who are really infected, reduce the number of people who need to be treated, and are more effective in preventing post-exposure TB. When the cost of these strategies is compared with effectiveness, the TST/IGRA dual strategies are the most cost-effective (approximately 37,000 £ per one case of prevented TB). TST alone is the least cost-effective (approximately 47,500 £ per one case of prevented TB). However, dual strategies are less effective in preventing active disease than screening with IGRA alone (45).

A similar study from Germany (46) also found the TST/QFT-G-IT strategy the most cost-effective in reducing the disease burden.

IGRA IN NATIONAL TUBERCULOSIS PROGRAMMES

Implementation of IGRAs in national tuberculosis programmes varies in terms of testing strategies and testing inclusion criteria. The US Centers for Disease Control and Prevention have suggested replacing TST with IGRAs in testing homeless persons, drug users,

and persons who have received BCG as a vaccine or cancer therapy. Either a TST or an IGRA may be used without preference to test contacts of TB patients or for periodic screening of persons who might have occupational exposure to *M. tuberculosis* (27). Either the TST or an IGRA are recommended in Japan (except in children below five years of age), Denmark (for child contacts), Australia (for refugees) and France. In Switzerland and Denmark IGRA has replaced TST in patients receiving anti-TNF-alpha therapy. Canada, UK, Italy, Switzerland (for contacts), the Netherlands (for contacts and immigrants), Korea, Norway (for contacts), and Croatia recommend using IGRAs to confirm positive TST results (3, 34, 47).

BOOSTING EFFECT OF TST ON IGRA

Many studies have pointed out that TST can boost IGRA result because both tests contain the same *M. tuberculosis*-specific antigens (48-53). This is relevant if TST and IGRA are used one after the other, as in two-step testings. This boosting effect has been observed on day three post TST and later (47, 54), and is more pronounced in IGRA-positive individuals. However, as many as two to twelve percent of otherwise IGRA-negative individuals experience a boost to IGRA-positive results after TST, which renders interpretation of two-step screening difficult (47). Current data can not answer when the boosting effect of TST on IGRA results stops, but the conventional wisdom is that it wanes after three months. Van Zyl-Smit et al. (47) conclude that IGRA testing performed before or within 72 hours of TST will not be subject to the boosting effect. In other words, the optimal time to collect blood for IGRA is at the time of reading TST.

IGRA IN TESTING HEALTHCARE WORKERS

The increased risk of TB in healthcare workers is well known (55, 56). In the US, serial screening for LTBI is part of the national guidelines and some hospital infection control programmes (57). In Croatia, healthcare worker screening has been performed only as part of contact tracing activities, while regular serial screening does not make part of the national tuberculosis programme.

Ever since the IGRAs have become part of TB screening, there is no general agreement about their role for this purpose. Regardless of many advantages over TST, the use of IGRAs for serial testing is limited, as there are no optimal cut-offs to distinguish new infections from nonspecific variations, and it is difficult to interpret conversion and reversion (29, 58-62).

Cut-off recommendations range between 0.2 IU mL^{-1} and 0.7 IU mL^{-1} of IFN-gamma, and retesting of borderline positive results is recommended before LTBI treatment (31, 63). However, many agree that repeated results in serial testing should be interpreted with caution, especially in countries with low TB incidence (31, 62).

CONCLUSIONS AND POLICY IMPLICATIONS

Immune-based tests such as TST and IGRAs share one important limitation. They do not directly detect *M. tuberculosis.* Instead, they detect remote or recent sensitisation to *M. tuberculosis*. A positive IGRA result may not necessarily indicate active tuberculosis, and a negative IGRA result does not exclude active disease. This also applies to TST. However, the negative predictive value of both tests (TST+IGRA combined) is high. Their positive predictive value depends on the prevalence of *M. tuberculosis* in a given population. Therefore, the gold standard for diagnosing active tuberculosis continues to be bacteriologically confirmed *M. tuberculosis*, but IGRA has a great potential in helping to identify LTBI.

The sensitivity of IGRAs may vary, but in general it is as high as or higher than TST sensitivity. All IGRAs have excellent specificity, and are certainly superior to TST, particularly in BCG-vaccinated patients. Furthermore, the agreement between TST and IGRAs is the lowest in these patients. Generally, the agreement between T-SPOT.*TB* and TST is higher than between QFT and TST, but these findings call for further investigation.

Although reproducibility of IGRAs is generally high, conversions and reversions may affect interpretation and procedures are still unclear in this respect.

IGRA sensitivity in children is suboptimal. To maximise sensitivity in children with a high risk of infection, especially in children below five years of

age and in immunocompromised children, IGRA testing is recommended in addition to the TST. Low-risk children may benefit from IGRAs as a confirmation of the positive TST in terms of increased specificity and reduced risk of a false diagnosis of LTBI. Children tend to have more IGRA-indeterminate results, which makes interpretation difficult.

Expert opinion is that immunocompromised patients could benefit from simultaneous use of IGRAs and TST. Indeterminate findings do not exclude TB infection. As a compromised immune system is too weak to respond to antigen stimulation, indeterminate results may actually point to LTBI, although these results should not be interpreted as positive.

In contact tracing studies, IGRA showed a good correlation with the infectiousness and the intensity of exposure to the source. IGRAs should not be used in monitoring TB or LTBI therapy or as a test of cure. Dual screening strategies (IGRA following positive TST) are more cost-effective than TST alone. It is generally accepted that TST may boost IGRA results, especially if IGRA is performed more than three days after TST. Therefore, the optimal time to collect blood for IGRA is at the time of TST reading.

IGRAs are an important advancement in the search for better diagnostic tests for LTBI. However, their performance still leaves much to be desired.

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Sažetak

ULOGA TESTOVA OTPUŠTANJA INTERFERONA GAMA U NADZORU NAD TUBERKULOZOM

Tuberkuloza je i danas jedan od vodećih javnozdravstvenih problema. Zemlje s niskom incidencijom fokusiraju se na iscrpljivanje rezervoara budućih slučajeva sprječavanjem reaktivacije bolesti. To se odnosi na traženje i učinkovito liječenje inficiranih osoba, primarno onih koje su u riziku od obolijevanja nakon infekcije. Tuberkulinski test je od početka 20. stoljeća bio standard u otkrivanju imunosnog odgovora na kontakt s *Mycobacterium tuberculosis*. Novo tisućljeće donijelo je određeni napredak u obliku novih testova za dijagnozu latentne tuberkulozne infekcije, krvne testove otpuštanja interferona gama. Hrvatska je zemlja srednje incidencije tuberkuloze s dugogodišnjim silaznim trendom i razvijenim protutuberkuloznim aktivnostima. U težnji prema niskoj incidenciji i u konačnici eliminaciji tuberkuloze potrebne su opsežnije aktivnosti unutar državnog programa nadzora nad tuberkulozom, uključujući intenzivnu obradu kontakata i probir na postojanje latentne tuberkulozne infekcije. Ovaj rad razmatra trenutačnu uporabu IGRE (engl. *interferon - gamma release assay*) i njezinu ulogu u nadzoru nad tuberkulozom.

KLJUČNE RIJEČI: *Hrvatska, ELISpot, latentna tuberkulozna infekcija, QuantiFERON-TB Gold In-Tube, tuberkulinski kožni test*

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Review

A SYSTEMATIC REVIEW OF ALUMINIUM PHOSPHIDE POISONING

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Every year, about 300,000 people die because of pesticide poisoning worldwide. The most common pesticide agents are organophosphates and phosphides, aluminium phosphide (AlP) in particular. AlP is known as a suicide poison that can easily be bought and has no effective antidote. Its toxicity results from the release of phosphine gas as the tablet gets into contact with moisture. Phosphine gas primarily affects the heart, lungs, gastrointestinal tract, and kidneys. Poisoning signs and symptoms include nausea, vomiting, restlessness, abdominal pain, palpitation, refractory shock, cardiac arrhythmias, pulmonary oedema, dyspnoea, cyanosis, and sensory alterations. Diagnosis is based on clinical suspicion, positive silver nitrate paper test to phosphine, and gastric aspirate and viscera biochemistry. Treatment includes early gastric lavage with potassium permanganate or a combination with coconut oil and sodium bicarbonate, administration of charcoal, and palliative care. Specific therapy includes intravenous magnesium sulphate and oral coconut oil. Moreover, acidosis can be treated with early intravenous administration of sodium bicarbonate, cardiogenic shock with fluid, vasopresor, and refractory cardiogenic shock with intra-aortic baloon pump or digoxin. Trimetazidine may also have a useful role in the treatment, because it can stop ventricular ectopic beats and bigeminy and preserve oxidative metabolism. This article reviews the epidemiological, toxicological, and clinical/pathological aspects of AlP poisoning and its management.

KEY WORDS: *human poisoning, mechanism of toxicity, phosphine, phosphides, pesticides*

Aluminium phosphide (AlP) is a well known, highly effective outdoor and indoor insecticide and rodenticide. It is readily available in Asian markets such as India. Although its use has been banned in Iran, it is still used to protect rice (hence the local name "rice tablet") and stored grains from rodents and other household pests.

Moisture in the air mixes with phosphide grains and sets off phosphine (hydrogen phosphide, phosphorus trihydride, PH_3), which is the active form of the pesticide. Tablets, pellets, or compressed discs contain phosphide and other substances such as ammonium carbonate. If it comes into contact with an acid, phosphine is released even more vigorously. Two kinds of acute poisoning have been reported: indirect inhalation of phosphine released during approved use or direct ingestion of metal phosphides (1, 2).

In an autopsy study of unnatural deaths in Northwest India (1), AlP was found to be the most common suicidal poison, causing 68.4 % of total deaths due to poisoning between 1992 and 2002. This epidemic of suicidal AlP poisoning has also been confirmed by Gupta and Ahlawat (2). Between 1977 and 1987, barbiturates (33.3 %), organophosphates (23.8 %), and copper sulphate (14.3 %) were the most common causes of death by poisoning and between 1987 and

1997 they were replaced by organophosphates (45 %) and AlP (26.5 %). Since 1992, AlP has taken over the lead (80 %). The incidence of suicidal deaths increased from 10.9 % in 1987 to 1992 to 15.7 % between 1997 and 2002, with a peak incidence of 18.2% in 1992 to 1997, when AlP became available on the free market. Of all fatal poisonings, suicidal and accidental between 1987 and 1997, AlP accounted for 26.5 %. The rate of AlP poisonings in Iran is also high; of 471 cases reported between 2000 and 2007, 146 (31 %) were fatal (3), which is even more serious considering that AlP has been banned for marketing in Iran. In the European countries such as UK, however, AlP is available in the form of tablets, but supply is restricted under the 1998 Pesticides Act to qualified users (4). In the European countries suicides by AlP ingestion are rare and have been reported in Denmark (5), Germany (6), France (7), and the UK (4).

AlP as a solid fumigant may be synthesised as dark gray or dark yellow crystals and can take the form of tablets, pellets, granules, or dust. It is marketed as dark grey 3 g tablets consisting of AlP (56 %) and carbamate (44 %), under the brand names such as Celphos, Alphos, Quickphos, Phosfume, Phostoxin, Talunex, Degesch, Synfume, Chemfume, Phostek, and Delicia (8). Phosphine gas has a foul odour resembling decaying fish or garlic because of substituted phosphines and diphosphines.

Ready availability of this fumigant insecticide in Asian countries makes it an important public health concern, especially because no specific treatment or antidote is available. The survival rate is low, but updating knowledge of health professionals and general public may help reduce the risk of poisoning (9).

RESEARCH

We looked up the terms aluminium, aluminium phosphide, and phosphine in bibliographical databases such as the TUMS digital library, Pubmed, Scopus, and Google Scholar. This review includes relevant articles published between 1990 and 2011.

Epidemiology

Every year, about 300,000 deaths due to pesticide poisoning are reported worldwide (10). Most reports of acute pesticide poisoning are based on hospital admission records and reflect only a fraction of the real incidence. Most reports of AlP poisoning refer to the young adult population from rural Asian areas (4). In Asia, about 25 million agricultural workers report an episode of poisoning every year (11). In a study conducted in Tehran, Shadnia et al. (3) found that of 77,958 poisonings, 471 people were poisoned with AlP , of whom 146 (31 %) died. This makes AlP a major concern in the Iranian population (3, 12).

Of 188 cases of phosphine poisoning reported in Germany between 1983 and 2003, 28 % were intentional, mostly by ingestion, which ended in two fatalities, whereas 65 % were accidental, mostly by inhalation, due to inappropriate self-protection from the released gas, which resulted in only transient gastrointestinal (GI) and respiratory symptoms (13). In the UK, the majority of 93 AlP poisonings reported to the National Poisons Information Service between 1997 and 2003 were accidental and concerned limited exposure to phosphine gas in agricultural locations (4). Only one fatal outcome was reported in this case series.

Mechanism of action

The exact mechanism of action of AlP is still unknown. However some initial studies on different animals showed that phosphine mainly binds cytochrome oxidase and changes the valences of the haem component of haemoglobin (14). It also induces oxidative stress and boosts extra-mitochondrial release of free oxygen radicals (15) that results in lipid peroxidation and protein denaturation of the cell membrane (16, 17) in various organs. Abdollahi et al. (18) believe that oxidative stress is one of the main mechanisms of action of AlP toxicity that is somehow similar to that of organophosphate (OP) compounds. Furthermore, AlP reduces glutathione, which is one of the main antioxidant defences. In fact, AlP and OP alike cause a toxic stress that is accompanied by changes in glucose metabolism (19, 20). Al-Azzawi et al. (21) showed that *in vitro* exposure to phosphine leads to reduction of human serum cholinesterase activity, depending on the duration and phosphine concentration. On the other hand, some studies found no change in erythrocyte cholinesterase activity in accidental phosphine inhalation cases (22).

Figure 1 shows a plausible mechanism of AlP toxicity.

Toxicokinetics

Judging by the rapid systemic toxicity, phosphine is quickly absorbed after oral ingestion. Phosphine is

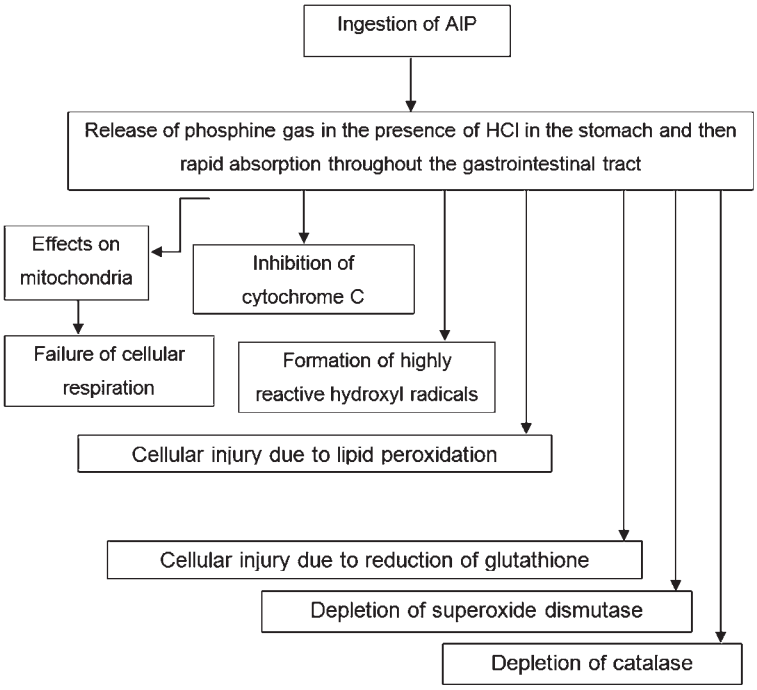

Figure 1 *The mechanisms of AlP toxicity.*

released as soon as AlP or other phosphide salts get in contact with hydrochloric acid of the stomach (23).

In vitro studies (24-27) suggest that phosphides are absorbed as microscopic particles of unhydrolysed salt that permanently interact with free haemoglobin and haemoglobin in intact erythrocytes (rat and human) to produce a haemichrome (a methaemoglobin derivative resulting from distorted protein conformation). In addition, Potter et al. (26) report that Heinz bodies (denatured haemoglobin aggregates) are formed when phosphide concentration *in vitro* exceeds 1.25 μg mL^{-1}.

Reports of *in vivo* phosphide poisoning showed intravascular complications such as haemolysis and methaemoglobinaemia, which support the involvement of erythrocytes in the biotransformation of phosphine in humans (24). Phosphine is excreted in the urine as hypophosphite and is also exhaled in the unchanged form (8).

Organ toxicity

The lethal dose of AlP is around 0.5 g. Those who survived had either taken a very small amount, the tablet expired or phosphine gas evaporated because the tablet had been exposed to air. AlP poisoning affects most of the organs. Early symptoms include nausea, vomiting, retrosternal and epigastric pain, dyspnoea, anxiety, agitation, and garlic breath (28-30). The early signs of fatal toxicity (90 % to 100 %) are shock and peripheral circulatory failure (31). Histopathological changes such as central venous congestion, degeneration of hepatocytes, and mononuclear infiltration are usually seen in the liver of poisoned patients. Furthermore, Mehrpour et al. (23) report alveolar thickening and dilated capillaries in the lung, degeneration of Nissl granule in the brain cytoplasm, degenerated eccentric nucleus in the cortex, and congestion within glomerulus and intraparenchymal part of the kidney.

Gastrointestinal toxicity

The early gastrointestinal symptoms of AlP ingestion include haematemesis, vomiting and epigastric pain. Endoscopy reveals corrosive lesions of the oesophagus and stomach, severe gastric erosions, duodenal erosions, and oesophageal strictures or fistula. Dysphagia is a common late complication (32-36). In our previous study, we described a family (a 35-year-old woman with her 18-year-old daughter and a 6-year-old son) who were accidentally poisoned with phosphine gas. They all

had intensive abdominal pain, hyperglycaemia, hypotension, and severe thirst, and the boy died because of cardiopulmonary arrest before admission to the hospital (37).

A case of benign oesophagobronchial fistula secondary to AlP poisoning in a 17-year-old boy was reported recently. The patient presented with acute dysphagia and severe cough following every swallow of either a liquid or solid five to six days after AlP tablet ingestion (38).

In another report (39), dysphagia was observed in 38.87 % of survivors after a mean interval of 38.6 days from the day of AlP poisoning. Oesophageal strictures were seen in 32.2 % of the survivors. Two patients reported a tracheo-oesophageal fistula 20 cm to 22 cm from the incisors (39).

Hepatic toxicity

Jaundice as a clinical and laboratory indicator of liver damage is sometimes observed, but reports are controversial (31). Jaundice can even be a manifestation of another disturbances such as intravascular haemolysis (29). A more common finding is transient elevation of serum aspartate and alanine aminotransferase (40-43). The main histopathological findings in the liver at autopsy of fatal phosphine poisoning include cytoplasmic vacuolisation of hepatocytes and sinusoidal congestion. Nuclear fragmentation and sinusoidal clusters of polymorphonuclear leukocytes in the liver were also reported (44).

Respiratory toxicity

Tachypnoea, dyspnoea, crepitations and rhonchi are common signs of respiratory toxicity. Respiratory distress syndrome and other types of pulmonary oedema are common in adults, accompanied by protein-rich or haemorrhagic pleural effusions (2, 31, 45-47).

Cardiac toxicity

Post-mortem reports of cardiac toxicity include heart failure (6), profound and refractory hypotension (42), heart congestion, subendocardial infarction or pericarditis, separation of myocardial fibres by oedema, fragmentation of the fibres, non-specific vacuolation of myocytes, focal necrosis, and neutrophil and eosinophil infiltration (41, 48, 49). Other signs and symptoms include, increased left ventricle, hypokinesia of the left ventricle and septum, akinesia, lower ejection fraction, severe hypotension, raised systemic venous pressure, normal pulmonary artery wedge pressure, inadequate systemic vasoconstriction, and electrocardiographic (ECG) abnormalities (2, 50-52) such as dysrhythmia, ST-T wave changes and conduction defects. Sinus tachycardia dominates in the first three to six hours of poisoning, followed by ST-T changes and conduction disturbances between hour 6 and twelve, and then by arrhythmias (49). Siwach et al. (53) found ventricular tachycardia in 40 %, ventricular fibrillation in 23.3 %, supraventricular tachycardia in 46.7 %, and atrial flutter/fibrillation in 20 % of AlP-poisoned patients.

Electrolyte and metabolic abnormalities

Hypokalaemia (primary or secondary to vomiting), metabolic acidosis or mixed metabolic acidosis, respiratory alkalosis, and acute renal failure have often been reported (24). Hyperkalaemia, hypo- and hypernatraemia have also been observed in AlP poisoning, and these changes were associated with a higher mortality rate (54).

In experimental models, AlP may alter glucose homeostasis; it significantly decreases plasma glucose in AlP-treated rats (55) and horses (56). Hypoglycaemia has also been reported in other studies (40, 57-59). Some studies have reported a hyperglycaemic effect of AlP (9, 37, 60) and some suggested that hyperglycaemia may be an important prognostic factor in AlP poisoning (61, 62), although not specific for AlP alone (20). In our earlier study, blood glucose was significantly higher in people who died of AlP poisoning than in those who survived (61). Controversial findings of blood sugar may reflect a wide variety of changes in magnesium, calcium, phosphate, citrate, and cortisol levels (60). Some studies reported increased blood magnesium in AlP poisoning (31, 52, 63) and other reported hypomagnesaemia (64-67). Moreover, some studies (68, 69) found no change in blood magnesium. These differences between reports may also point to differences between analytical methods used.

Mehrpour et al. (23) have also reported that arrested oxidative phosphorylation and poor tissue perfusion may lead to lactic acidosis.

Other effects

Hepatitis, pancreatitis, acute adrenocortical insufficiency, acute tubular necrosis, and disseminated intravascular coagulation are less common findings

in AlP poisoning (24). Refractory shock may result in cerebral anoxia that usually presents itself as drowsiness, delirium, and coma. Another uncommon sign is methaemoglobinaemia (27, 70).

There are interesting reports about spontaneous ignition and burns that occurred after oral poisoning with AlP (71-73). Phosphine gas is inflammable, depending on temperature and pressure. The absolute limit of flammability for phosphine in air is 1.8 % (17.9 mg mm^{-3}). When this limit is exceeded, phosphine can explode. Diphosphine, which is a product of the reaction between AlP and acid or moisture, burns spontaneously, reacting instantly with oxygen in the air, and this is one of the causes of explosions occurring during fumigation of storage grains (73). To prevent self-ignition AlP is mixed in the tablets with aluminium carbonate in the ratio 56:44, respectively (72).

Sequelae in survivors?

Only a few articles have described long-term disabilities due to AlP poisoning. Brautbar and Howard (74) described two patients, one with a long-term peripheral neuropathy, weakness and loss of sensation in the left-side extremities, severe headaches, fatigue, and dizziness and the other with the obstructive airway disease and headache (74). Kurzbauer and Kiesler (75) described a case of AlP poisoning with neurological abnormalities, such as Romberg, Rossolimo reflex on the left side, and bilateral Babinski. The neurological symptoms continued for 1.5 years post AlP exposure. Jain et al. (39) reported dysphagia, oesophageal strictures, and tracheo-esophageal fistula in survivors a month after AlP exposure.

Diagnosis of poisoning

Although the diagnosis of AlP poisoning is often based on clinical suspicion or reports, a range of chemical and analytical tests are there to confirm it. One simple and sensitive spot test for the detection of phosphine gas in gastric fluid or breath is the silver nitrate test (76). Another test that can be performed on the gastric content is the ammonium molybdate test, which is both qualitative and quantitative (77). Gas chromatography is the most specific and sensitive method for detecting the presence of phosphine in blood/air and can detect even minute amounts of phosphine in the air (78).

Other salts of phosphide such as zinc phosphide (ZnP) are also available in some countries that have the same mechanism of toxicity as AlP, but lower human mortality (79). Analytical tests help us to distinguish between AlP and ZnP poisoning and make a more accurate prognosis of the outcome. Hydrochloric acid is added to the sample (gastric contents or tissue), the mixture is heated, ammonium chloride and ammonium hydroxide added to the filtrate, and if the resulting gelatinous precipitate is white, it indicates the presence of Al (4).

Management of poisoning

Without a specific antidote, the management of AlP poisoning is very limited, mostly supportive. It is important do diagnose it as early as possible (see above). Before starting the treatment, medical staff should protect themselves with a full-face mask and rubber gloves. In cases of inhalation poisoning, the patient should be transferred to a well-ventilated space or fresh air. Contaminated clothes should be removed, and skin and eyes washed with tap water immediately. Gastric lavage with potassium permanganate, activated charcoal+sorbitol solution, and coconut oil can be performed in the first emergency step (80). Potassium permanganate (1:10 000 solution) oxidises phosphine gas in the stomach to phosphate, and reduces the amount of lethal phosphine gas (9, 80). Although charcoal is generally used for reducing phosphine absorption in the gastrointestinal system, no study has proved its efficacy in humans.

Bajwa et al. (81) recommended extensive gastric lavage with a mixture of sodium bicarbonate solution and coconut oil. The acidic environment of the stomach stimulates the conversion of AlP to phosphine gas, and lavage with sodium bicarbonate can be helpful.

Cardiac monitoring should include blood pressure and ECG to prevent arrhythmias and maintain tissue perfusion and oxygenation. One of the most common results of AlP toxicity is myocardial injury and haemodynamic instability (82).

Another necessary step in the management of AlP poisoning is early resuscitation with fluid and vasoactive agents to control central venous pressure (CVP) or pulmonary artery wedge pressure (PAWP). Norepinephrine or phenylephrine, dopamine, and dobutamine can be used to treat hypotension and refractory shock, while anti-arrhythmic agents, DC cardioversion, and temporary pacemaker should address the arrhythmia. Intra-aortic balloon pump

(IABP) is a good way to mechanically support the heart, especially in toxic myocarditis with refractory shock (84). Trimetazidine has also proven itself effective recently in stopping ventricular ectopic beats and preserving oxidative metabolism (67). In addition, digoxin can be used to stabilise the left ventricular heart failure (82).

Early diagnosis of organ damage is another important aspect in the management, as AlP poisoning affects virtually all organs in the body. Acute lung injury may need endotracheal intubation and mechanical ventilation. Cyanosis not responding to oxygen therapy may be a sign of methaemoglobinaemia that requires therapy with intravenous methylene blue (1 % solution) in the dose of 2 mg kg^{-1} of body weight over five minutes. For metabolic acidosis, intravenous sodium bicarbonate should be considered, whereas severe acidosis, volume overload or renal failure may require haemodialysis. However, haemodialysis is probably not very effective in removing phosphine (8). These new therapeutic approaches have been summarised in Table 1.

Magnesium supplementation

It is difficult to decide whether to give supplemental magnesium or not. Treatment with magnesium sulphate has been reported to reduce mortality by up to 50 % (2, 49, 65, 66). Magnesium stabilises the cell membrane and acts as an anti-oxidant. A study published in 1994 (69) compared treatments of AlP-poisoned patients and concluded that the survival rate of those who received supplemental magnesium was not significantly better than of those who did not (42 % *vs.* 40 %, respectively). In contrast, in another case control study (65), magnesium improved survival of patients who ingested high doses of AlP. Moreover, a recent study (84) on a rat model showed that $^{25}Mg^{2+}$-carrying nanoparticle (^{25}MgPMC16) significantly increased blood pressure and heart rate of rats poisoned with AlP. This study also demonstrated that ^{25}MgPMC16 increased intracardiac magnesium levels, reduced lipid peroxidation, and improved mitochondrial function.

Hyperinsulinaemia-euglycaemia and hyperventilation oxygenation

Some authors propose that a combination of hyperinsulinaemia-euglycaemia and hyperventilation oxygenation is worthy of more extensive evaluation as a therapy for AlP poisoning (85).

N-acetylcysteine

Different studies in rats (86, 87) and humans (66) have revealed that *N*-acetylcysteine can help as it replenishes cellular glutathione and magnesium, in addition to its antioxidant properties. In rats exposed to AlP, *N*-acetylcysteine increased survival time and reduced myocardial oxidative injury (4).

Coconut and almond oil

There are reports of the positive clinical effects of coconut oil against AlP poisoning in humans (9, 86). Its mechanism of action is unclear, but it may form a protective layer around the gastric mucosa and prevent the absorption of phosphine gas. In addition, coconut oil may dilute HCl in the stomach and reduce the breakdown of phosphide.

Saidi and Shoajaie (88) reported that intragastric lavage with sweet almond oil considerably reduced the mortality of rats poisoned with AlP. It also significantly lowered plasma cholinesterase levels. The authors suggested that sweet almond oil should be given orally immediately after AlP ingestion, but this has yet to be confirmed in humans.

Hyperbaric oxygenation

Saidi et al. (89) found that hyperbaric oxygenation improved the survival time in rats poisoned with AlP. However, its efficacy in humans has not been investigated.

Digoxin

It has been hypothesised that treatment with digoxin (rapid digitalisation) would increase myocardial contractility and blood pressure (90), countering thus the direct effects of AlP on cardiac myocytes which lead to refractory cardiogenic shock. Mehrpour et al. (82) have recently reported about successful treatment of an 18-year-old girl who had ingested a 3 g AlP tablet. On hospital admission her blood pressure was undetectable and she had a severe left ventricular systolic dysfunction. She received dopamine (10 μg kg^{-1} min^{-1}) and digoxin (0.5 mg) every six hours during the first day and continued with 0.25 mg per day on the following days. The ECG parameters became normal on day three, and she was discharged on day 10 fully recovered.

Hydroxyethyl starch

Another option for the management of AlP poisoning is hydroxyethyl starch (91). Leakage of

Table 1 *New treatment strategies for AlP poisoning*

Study	Type of study	New Treatment	Other therapeutic measures	Effects	Conclusion
Mehrpour et al., 2011 (82)	Case Report	***Digoxin*** 0.5 mg initially followed by 0.5 mg at 6 h intervals	Gastric decontamination with $KMnO_4$, activated charcoal and sodium bicarbonate; administration of *i.v.* Mg sulphate and Ca gluconate	Resolved cardiogenic shock due to left ventricle failure	Administration of digoxin as an adjustment therapy can improve the out come
Saidi et al., 2011 (89)	Experimental (Rats)	***Hyperbaric oxygen***	-	Increasing survival time	Administration of hyperbaric oxygen may be also effective in humans
Baeeri et al., 2011 (84)	Experimental (Rats)	**$^{25}Mg^{2+}$-carrying nanoparticle**	Na bicarbonate (4 mmol kg^{-1}, *i.v.*)	Increased blood pressure and heart rate; increase in antioxidant power, Mg level in the plasma and the heart; reduction in lipid peroxidation and ADP/ATP ratio	^{25}MgPMC16 at 0.025 LD_{50} + Na bicarbonate was the most effective combination
Saidi and Shojaie., 2011 (88)	Experimental (Rats)	Intragastric irrigation with ***sweet almond oil***	-	Protective role for plasma cholinesterase inhibition in AlP poisoning, decreased mortality rate	Significant reduction of mortality
Soltaninejad et al., 2011 (95)	Case Report	***Vitamin C*** (1 g at 6 h intervals, *i.v.*) + ***methylene blue*** (1 mg kg^{-1} of 1 % solution)	Supportive care, Na bicarbonate, norepinephrine, Mg sulfate, Ca gluconate	Twelve hours after treatment with vitamin C, the methaemoglobin concentration decreased from 46 % to 33 %. High doses of methylene blue, the methaemoglobin concentration decreased to 23 %	Administration of vitamin C followed by methylene blue may have a role in successful treatment of methaemoglobinaemia and haemolysis following phosphine poisoning
Bajwa et al., 2010 (81)	Case Series (33 patients)	***Extensive gastric lavage*** with aliquots of 50 mL of ***coconut oil*** and 50 mL of ***sodium bicarbonate solution*** with simultaneous aspiration	Strict monitoring + Supportive treatment	Survival rate 42 %	Recommendation to intensivists and physicians to use this particular regimen of gastric decontamination

Table 1 *Continuation*

Study	Type of study	New Treatment	Other therapeutic measures	Effects	Conclusion
Siddaiah et al., 2009 (83)	Case Report	***IABP****	Supportive care, inotropes and mechanical ventilation	IABP was used for cardiovascular support until the effects of AlP resolved	IABP used for treatment of cardiogenic shock due to AlP poisoning can improve the outcome
Azad et al., 2011 (96)	Experimental (Rats)	***NAC**** (6.25 mg kg^{-1} min^{-1}, *i. v.* for 30 min)		Significantly increased survival time, stabilization of blood pressure and heart rate, decreased MDA*** level and increased GSH Px**** levels	NAC increased the survival time by reducing myocardial oxidative injury
Azad et al., 2011 (96)	Experimental (Rats)	***L-NAME***** (1 mg kg^{-1} min^{-1}, *i. v.* for 60 min)		Significant rise in blood pressure but precipitated ECG abnormalities. Pre- and post-treatment of L-NAME with AlP neither improved the survival time nor the biochemical parameters despite significant rise in blood pressure	L-NAME showed no protective effects in rats exposed to AlP
Mittra et al., 2001 (97)	Experimental (Rats)	***Atropine*** (1 mg kg^{-1}, intra peritoneal) + ***pralidoxime*** (5 mg kg^{-1}, intra peritoneal) administered five minutes after AlP exposure	-	Increased survival time. Plasma cholinesterase levels were inhibited in rats poisoned with AlP as compared to controls	Atropine and pralidoxime can increase survival time
Dueñas et al., 1999 (67)	Case Report	Oral dose of 20 mg trimetazidine twice daily	Mg sulphate intravenously at 3 g over 30 min	Resolved dysrhythmia due to AlP poisoning after 48 h [ventricular premature complexes (>600 h^{-1}) with periods of bigeminy]	Ventricular dysrhythmias were treated solely with oral trimetazidine tresulting in rapid disappearance of all electrocardiographic abnormalities

***IABP:** *Intra-aortic Balloon Pump*
****NAC**: *N-Acetylcysteine*
*****MDA**: *Malonyldialdehyde*
******GSH Px**: *Glutathione peroxidase*
*******L-NAME**: *N-omega-nitro-L-arginine methyl ester*

fluids from intravascular to extravascular space that leads to a strong refractory hypotension is one of the post-mortem findings in AlP poisoning. Hydroxyethyl starch remains in the intravascular space as a colloid rather than as a crystalloid and thus reduces the extravascular leak of albumin and fluids (91). However, no experiment has yet investigated the use of starch in the management of AlP poisoning.

Prognosis of AlP poisoning

The mortality in adults who have ingested 500 mg of AlP or over is between 30 % and 100 %. The higher the blood phosphine, the higher the mortality. Patients having blood phosphine levels equal to or less than 1.067±0.16 mg survived, and this dose seems to be the lethal threshold of phosphine toxicity (92). Survival may increase if a very small amount of AlP is ingested or the tablet has expired or was exposed to air (12). Vomiting and early supportive care also increase the survival rate (8).

Poor prognosis is indicated by hyperglycaemia, high simplified acute physiology score (SAPS II), hypotension, acidosis, leukocytosis, hyperuraemia, ECG abnormalities, high acute physiology and chronic health evaluation score (APACHE II), low Glasgow coma scale, acute renal failure, low prothrombin rate, hyperleukocytosis, methaemoglobinaemia, use of vasoactive drugs, lack of vomiting after ingestion, and use of mechanical ventilation (4, 61, 70, 93, 94).

To conclude, acute AlP poisoning is a worldwide problem. The understanding of its mechanisms of toxicity and clinical effects has improved in the recent years. An antidote proper for phosphine poisoning is still unavailable. A number of possibilities for treatment have been tried out or experimented with, but they all need further validation (see Table 1). Meanwhile, preventive measures might help to control the risk of poisoning in humans such as limited access to phosphide compounds, regulations to ban its use as a pesticide, and keeping health professionals abreast with the latest knowledge about early management of phosphide poisoning.

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Sažetak

SUSTAVNI PREGLED OTROVANJA ALUMINIJEVIM FOSFIDOM

Svake godine u svijetu oko 300.000 ljudi umre od trovanja pesticidima. Najčešći pesticidni spojevi su organofosfati i fosfidi, ponajviše aluminijev fosfid. On je poznat kao otrov samoubojica, jer ga je lako nabaviti, a nema djelotvornoga protuotrova. Toksičnost duguje otpuštanju fosfinskoga plina u trenutku kada tableta dođe u dodir s vlažnim okružjem. Fosfinski plin ponajviše djeluje na srce, pluća, probavni sustav i bubrege. Znakovi i simptomi trovanja obuhvaćaju mučninu, povraćanje, uznemirenost, bol u trbuhu, lupanje srca, refraktorni šok, srčane aritmije, plućni edem, dispneju, cijanozu i osjetilne promjene. Dijagnoza se zasniva na kliničkom opažanju, pozitivnom nalazu testa na fosfin sa srebrnim nitratom, aspiratu iz želuca te biokemiji crijeva. Otkrije li se rano, liječenje obuhvaća ispiranje želuca kalijevim permanganatom, odnosno kombinacijom kokosova ulja i natrijeva bikarbonata, aktivni ugljen te palijativnu skrb. Usmjereno liječenje obuvhaća intravensku primjenu magnezijeva sulfata i kokosova ulja. Korisnim se može pokazati liječenje acidoze ranom intravenskom primjenom natrijeva bikarbonata i liječenje srčanoga šoka odgovarajućom tekućinom i vazopresorom te refraktornog šoka, intraaortalnim balonom odnosno digoksinom.
Korisnim se može pokazati i trimetazidin, budući da sprječava ventrikularne ekstrasistole i bigeminiju te čuva oksidativni metabolizam. Ovaj članak donosi pregled različitih vidova trovanja aluminijevim fosfidom, uključujući njegovu epidemiologiju, toksičnost, kliničke znakove i simptome te medicinsku obradu.

KLJUČNE RIJEČI: *fosfin, pesticidi, toksičnost, trovanje*

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Review

DIPEPTIDIL-PEPTIDAZA IV (DPP IV/CD26) I UPALNE BOLESTI CRIJEVA

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Upalne bolesti crijeva (Crohnova bolest, ulcerozni kolitis, nedeterminirani kolitis) skupina su kroničnih autoimunosnih upalnih bolesti obilježenih ponavljanim upalama različitih dijelova gastrointestinalnog trakta koje su važan javnozdravstveni problem današnjice. Unatoč brojnim temeljnim i kliničkim istraživanjima etiologija ovih bolesti, kao i sama patogeneza upale ostaju nedovoljno razjašnjene. Dosadašnja su istraživanja potvrdila uzročno-posljedičnu vezu između medijatora upalnog odgovora i molekula uključenih u regulaciju njihove biološke aktivnosti, osobito proteaza. Cilj ovoga preglednog rada jest sažeti prikaz dosadašnjih saznanja o različitim aspektima upalnih bolesti crijeva, s posebnim naglaskom na potencijalnu ulogu i uključenost dipeptidil-peptidaze IV, odnosno molekule CD26 (DPP IV/CD26) u mehanizme nastanka upalnih procesa u probavnom sustavu. Dan je i pregled životinjskih modela kolitisa koji su znatno pridonijeli razumijevanju i terapiji ovih bolesti, s osobitim naglaskom na mišji model ulceroznog kolitisa (DSS-kolitis) te Crohnove bolesti (TNBS-kolitis).

KLJUČNE RIJEČI: *Crohnova bolest, DSS-kolitis, molekula CD26, TNBS-kolitis, ulcerozni kolitis, životinjski modeli upalnih bolesti crijeva*

Upalne bolesti crijeva (UBC) skupina su kroničnih autoimunosnih upalnih bolesti nedovoljno poznate etiologije, obilježene ponavljanim upalama različitih dijelova gastrointestinalnog trakta i nepredvidim tijekom (1). Bolest se može pojaviti u bilo kojoj dobnoj skupini i u oba spola, a zbog neprestanog porasta incidencije i povezanosti s brojnim izvancrijevnim manifestacijama u velikoj mjeri smanjuje kvalitetu života oboljelih i velik je javnozdravstveni problem današnjice (2, 3).

U ovisnosti o zahvaćenom odsječku probavnog sustava te proširenosti i trajanju upale, u kliničkoj se praksi razlikuju dva patomorfološka oblika UBC-a: Crohnova bolest (CB) i ulcerozni kolitis (UK). Glavne razlike između ovih bolesti jesu njihova lokalizacija, priroda upalnih promjena, imunopatološke osnove te patogeneza (4). Najstarija saznanja o CB-u i UK opisuju ih kao jedinstvenu bolest koja zahvaća crijeva, a kao zasebni entiteti jasno se opisuju zadnjih stotinjak godina. Međutim iako su u kliničkoj praksi CB i UK prepoznati kao dvije zasebne bolesti s različitim kliničkim, anatomskim i histološkim karakteristikama, zbog nemogućnosti jasne klasifikacije u jedan od oblika UBC-a, 10 % do 15 % oboljelih svrstava se u kategoriju "nedeterminirani kolitis" (5), jer je unatoč velikom napretku medicinske znanosti još i danas u mnogim slučajevima teško povući granicu i postaviti točnu dijagnozu, kao i uzrok nastanka bolesti (6).

Dosadašnja su istraživanja potvrdila uzročno-posljedičnu vezu između medijatora upalnog odgovora te molekula uključenih u regulaciju njihove biološke

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aktivnosti, osobito proteaza. Postoje dokazi o potencijalno vrlo važnoj ulozi dipeptidil-peptidaze IV odnosno molekule CD26 (DPP IV/CD26) u mehanizmima nastanka UBC-a te povezanosti ove molekule s jačinom i aktivnošću bolesti (7-9). Cilj je ovoga preglednog rada sažeti prikaz dosadašnjih saznanja o različitim aspektima UBC-a, s posebnim naglaskom na potencijalnu ulogu i uključenost multifunkcionalne molekule DPP IV/CD26 u mehanizme nastanka upalnih procesa u probavnom sustavu. Dan je i pregled životinjskih modela UBC-a koji su znatno pridonijeli razumijevanju i terapiji ovih bolesti s osobitim naglaskom na mišji model UK izazvan natrijevim dekstran-sulfatom te mišji model CB-a uzrokovan trinitrobenzensulfonskom kiselinom.

UPALNE BOLESTI CRIJEVA

Epidemiologija

Epidemiološka istraživanja upućuju na visoku incidenciju CB-a i UK u industrijaliziranim zemljama poput skandinavskih zemalja, Velike Britanije, sjeverozapadne Europe, Sjeverne Amerike i Australije te nisku incidenciju u sjevernoj i srednjoj Africi, Aziji i Južnoj Americi (10). Međutim različita stopa incidencije može označavati i različitu gensku pozadinu stanovnika različitih područja Zemlje. Unatoč tomu, okolišni čimbenici igraju vrlo važnu ulogu, a u prilog tomu govore podaci o rastućoj incidenciji bolesti kod imigranata iz zemalja niske incidencije u zemlje visoke incidencije (11).

UBC pokazuje bimodalnu dobnu distribuciju. Najčešće se dijagnosticira u dobi između petnaeste i četrdesete godine života, ali se pojavljuje još jedna manje izražena učestalost pojave bolesti između pedesete i osamdesete godine života. UBC se javlja češće u adolescenata nego u mlađe djece; početak je bolesti prije desete godine života rijedak, u svega oko 2 % slučajeva, a u 30 % slučajeva pojavljuje se kod mladih ljudi u dobi između deset i devetnaest godina. Nadalje, bolest podjednako zahvaća i muškarce i žene, ali je UK nešto učestaliji kod muškaraca, dok je CB nešto češći kod žena. Populacijska istraživanja pokazala su da je UBC učestaliji u pripadnika bijele rase nego u crnoj i orijentalnoj rasi. Zapažena je viša incidencija kod Židova nego kod ostalih populacija koje žive u istoj regiji (12).

Incidencija i prevalencija upalnih bolesti crijeva u svijetu te u Republici Hrvatskoj zadnjih desetljeća značajno rastu, na što upućuju novija epidemiološka istraživanja (13). Incidencija upalnih bolesti crijeva u svijetu kreće se od 1,6 do čak 24,5 na 100000 stanovnika za UK te od 0,9 do 9,2 na 100000 stanovnika za CB (14). U Hrvatskoj incidencija UK iznosi 4,3 na 100000 stanovnika, dok incidencija CB-a iznosi 7,0 na 100000 stanovnika (15).

Etiologija i čimbenici rizika

Unatoč brojnim temeljnim i kliničkim medicinskim istraživanjima etiologija UBC-a, kao i čimbenici koji dovode do nastanka pojedinoga patomorfološkog oblika bolesti, do danas nisu sasvim poznati. Postoji nekoliko čimbenika rizika za koje je dokazana uzročno-posljedična povezanost s UBC-om, a dosadašnje spoznaje upućuju na to da genski čimbenici i uvjeti okoliša poput prehrane, mikroorganizama i načina života mogu biti značajno uključeni u njezin razvoj (16). Kako je zasigurno riječ o multifaktorskoj bolesti, ne može se sa sigurnošću utvrditi koji je od čimbenika rizika presudan u nastanku i razvoju upale u gastrointestinalnom sustavu. Međutim općenito je prihvaćena pretpostavka da bolest nastaje kao neadekvatan, odnosno pretjeran imunosni odgovor na određene bakterijske antigene ili antigene iz hrane kod genetski predisponiranih pojedinaca pod utjecajem čimbenika okoliša (11).

Među najvažnijim čimbenicima rizika od nastanka UBC-a ističu se genetska predispozicija, imunoregulacijske nepravilnosti, čimbenici ranog djetinjstva, izloženost infekcijama u dječjoj i odrasloj dobi, prehrana, životne navike te tzv. zapadnjački način života (1, 16). Bolest je jasno povezana s urbanim načinom života, razvijenijim zemljama i teorijom tzv. "pretjerane higijene", a češća je u regijama sjevernih zemljopisnih širina (17).

Genetska predispozicija

Važna karika u nastanku upalnih procesa u probavnom sustavu jest genetska predispozicija (18), a vjeruje se da su CB i UK heterogene poligenske bolesti koje dijele neka područja genske podložnosti (19). Najvjerojatnije je fenotip UBC-a određen s nekoliko faktora, uključujući i interakciju među alelima, kao i utjecaj ostalih gena i čimbenika okoliša. Posljedično tomu, prisutnost jednoga mutiranoga gena ne znači da će sa sigurnošću doći do razvoja UBC-a, niti predodređuje pojedinca kod kojeg će doći do razvoja bolesti (1, 20).

Utvrđeno je nekoliko gena za koje je dokazano postojanje značajne povezanosti s nastankom UBC-a. Osobito se ističu geni na kromosomu broj 1, 3, 4, 5, 6, 7, 10, 12, 14, 16, 19 te kromosom X (21, 22). Jedan od gena najjasnije povezanih s patogenezom UBC-a je IBD-1, koji je područje podložnosti na pericentromerskoj regiji kromosoma 16 (23). Detaljnijim analizama utvrđena je oligomerizacijska domena 2 koja veže nukleotid (engl. *nucleotide-binding oligomerization domain 2*, NOD2), odnosno gen koji kodira sekvencu odgovarajućeg NOD2-proteina. NOD2, poznat i kao CARD15 (engl. *caspase activation and recruitment domain* 15, CARD15). To je polimorfan gen čiji je produkt uključen u regulaciju imunosnih zbivanja te je prvi otkriveni gen s utvrđenom jasnom uzročno-posljedičnom vezom s UBC-om (24). Do danas je otkriveno više od 60 mutacija ovoga gena među kojima su 3 neposredno povezana s razvojem CB-a (25). Procjenjuje se da je defekt NOD2-gena prisutan u 17 % do 27 % oboljelih od CB-a (24). Međutim nije sasvim jasan mehanizam koji povezuje mutacije ovoga gena i UBC (1).

Istraživanja vezana uz CB i UK pokazala su veći stupanj obolijevanja u monozigotnih blizanaca nego u dizigotnih. Međutim pokazano je da je stopa pojavnosti bolesti u blizanaca veća za CB, što je dovelo do zaključka o snažnijem utjecaju genskih komponenata (19). Saznanja dobivena istraživanjima provedenima na blizancima upozorila su na činjenicu da UBC ne slijedi karakteristično Mendelovo pravilo nasljeđivanja, već su kompleksnoga poligenskog podrijetla (23).

Kod UBC-a izražena je obiteljska tendencija razvoju bolesti, a pojavnost UBC-a unutar iste obitelji iznosi 20 do 30 %. Postoji povećana prevalencija UBC-a među rođacima prvog i drugog koljena. U obiteljima gdje postoji visoka stopa pojavnosti UBC-a, 75 % bolesnika obolijeva od istog oblika bolesti. Osim toga, uočena je veća pojavnost ostalih genski uvjetovanih bolesti kod oboljelih od UBC-a.

Poremećaji imunoregulacijskih mehanizama

UBC je karakteriziran imunoregulacijskim nepravilnostima mukoze, najvjerojatnije genetski predodređenima, za koje se pretpostavlja povezanost s bakterijskim antigenima prisutnima u crijevu (26). Fiziološka povezanost između komenzalnih bakterija u crijevu i organizma domaćina simbiotska je (20), a u zdravom epitelu crijeva postoji tolerancija na prisutnost potencijalno proupalnih komponenata luminalnih bakterija. Postoji nekoliko teorija koje djelomično daju mogući odgovor na problematiku poremećaja imunoregulacije u crijevu: disfunkcija imunosnog odgovora organizma prema uobičajenim sadržajima lumena crijeva, infekcija specifičnim patogenom i/ili poremećaj u propusnosti mukozne barijere, odnosno povećan protok luminalnih antigena u unutarnje slojeve stijenki crijeva.

Neadekvatan imunosni odgovor organizma prema uobičajenim sadržajima, odnosno antigenima u lumenu crijeva čini se najvjerojatnijim lokalnim patološkim mehanizmom koji dovodi do nastanka ranih događaja povezanih s razvojem upalnih promjena u UBC-u. U zdravom organizmu izloženost komenzalnim bakterijama utišava izražaj proupalnih gena i sprječava aktivaciju imunosnog odgovora usmjerenog na velik broj prisutnih bakterijskih antigena, kao i antigena podrijetlom iz hrane (27). Kod oboljelih od UBC-a ne postoji ova tolerancija imunosnog sustava na antigene prisutne u crijevu, već izloženost luminalnoj mikroflori dovodi do pretjerane aktivacije imunosnog sustava organizma, osobito imunosnih stanica prisutnih u mukozi crijeva, što dovodi do kroničnoga, destruktivnog upalnog procesa (28).

Infekcija specifičnim patogenom koja dovodi do nastanka UBC-a zasad nije sa sigurnošću utvrđena, no postoje indikacije da određene patogene bakterije, poput *Mycobacterium paratuberculosis, Listeria monocytogenes i Helicobacter hepaticus* (28) mogu biti uključene u patogenezu UBC-a. Nekoliko objavljenih znanstvenih članaka povezuje CB i preboljele ospice u ranoj životnoj dobi (21). Veća incidencija CB-a u zimskom periodu također upućuje na povezanost bolesti s određenim patogenim mikroorganizmima (29). Nadalje, pokazano je da laboratorijske životinje čuvane u specifičnim sterilnim uvjetima ne mogu oboljeti od UBC-a, dok isti sojevi laboratorijskih životinja u uobičajenim uvjetima, dakle uz prisutnost komenzalnih bakterija, obolijevaju od UBC-a induciranjem modela bolesti (30). Čak i infekcijom laboratorijskih životinja čuvanih u specifičnim sterilnim uvjetima jednim ili s nekoliko bakterijskih sojeva, nakon indukcije bolesti ubrzo dolazi do razvoja upalnih promjena u crijevu (31). Nadalje, pokazano je postojanje različitih imunoregulacijskih odgovora na jedan te isti bakterijski soj. Stoga se može zaključiti da su u oboljelih od UBC-a različiti bakterijski sojevi uključeni u nastanak upalnih promjena kod različitih pojedinaca (1, 31, 32).

Brojna epidemiološka istraživanja definirala su UBC kao „bolest prekomjerne čistoće". Za razliku od ostalih poligenskih, imunosno posredovanih bolesti poput astme, multiple skleroze i reumatoidnog artritisa, UBC pokazuje obrnuto proporcionalnu povezanost sa stupnjem čistoće. Naime, niži stupanj čistoće, osobito u ranijoj životnoj dobi, čini se protektivnim u etiopatogenezi, s obzirom na to da djeca koja odrastaju u višim socioekonomskim standardima imaju veću predispoziciju za razvoj UBC-a (33). Ova se pojavnost tumači činjenicom da poboljšana ili pak pretjerana higijena utječe na mikrobiološku floru općenito, a time i na luminalne bakterije, što jednostavno dovodi do smanjene izloženosti određenim kritičnim bakterijskim sojevima te razvoja netolerancije prema njima (32, 34).

Nedostatna funkcija crijevnog epitela u oboljelih od UBC-a dokazano je povezana s povećanom permeabilnošću epitela crijeva za različite antigene, što dovodi do stimulacije imunosnog sustava mukoze (28). Kod ljudi površina crijeva iznosi od 150 do 200 četvornih metara, što je golema površina koja je svakodnevno izložena različitim antigenima, uključujući i približno 10^{14} mikroorganizama koje nalazimo u lumenu crijeva (35).

Zdrav epitel crijeva s čvrstim poveznicama među stanicama tvori učinkovitu barijeru protiv prodiranja luminalnih mikroorganizama i različitih antigena u dublje slojeve crijeva. Međutim ako zbog promjene u propusnosti crijevnog epitela bakterijski produkti i/ili antigeni prodru u unutrašnjost crijeva, dolazi do direktnog kontakta s imunosnim stanicama i aktivira se imunosni odgovor (20). Potom dolazi do lučenja proupalnih citokina, što će ponovno stimulirati novačenje dodatnih stanica u slojevima crijeva. Navedeni mehanizam obuhvaća citokine koji sudjeluju u oslabljivanju čvrstih veza između endotelnih stanica, što naposljetku olakšava nakupljanje neutrofila iz periferne cirkulacije u mukozu crijeva (27).

Istraživanja koja uključuju životinjske modele UBC-a upozorila su na tendenciju razvoja snažnih upalnih procesa upravo u područjima gdje postoje poremećaji propusnosti sluznice crijeva (23). Bakterije iz lumena crijeva zatim dodatno pojačavaju defekt poremećaja propusnosti crijeva, što naposljetku stvara začarani krug koji rezultira ponavljanim upalnim procesima gastrointestinalnog trakta (29).

OSTALI ČIMBENICI RIZIKA

Zapadnjački način života

Područja s najvećom prevalencijom oboljelih od UBC-a jesu razvijene, industrijalizirane zemlje (3). Isto tako, zabilježen je veći broj oboljelih u gradskim nego u ruralnim sredinama, a postoji i varijacija incidencije i prevalencije na relaciji sjever-jug. Zanimljivost je pak porast incidencije u zemljama južnih regija, poput Azije. Izraziti porast broja novooboljelih u dvadesetom stoljeću govori u prilog teoriji utjecaja okolišnih čimbenika na pojavnost bolesti (36). Vjeruje se da su ove pojave zapravo posljedica prihvaćanja zapadnjačkog načina života, poput promjena u prehrambenim navikama, pušenja, izloženosti sunčevim zrakama, onečišćenja okoliša te industrijalizacije (10).

Također, jedan od aspekata zapadnjačkog načina života te čimbenika koji može pridonijeti pojavnosti UBC-a jest i zanimanje pojedinca (1). Veći postotak obolijevanja i smrtnosti od UBC-a zapažen je kod zaposlenika koji obavljaju stresne poslove i imaju zanimanja koja iziskuju veću psihičku napetost, osobito ona sjedilačkog tipa. S druge strane, nešto manja stopa pobolijevanja od UBC-a zabilježena je kod zaposlenika koji se bave poljoprivrednim zanimanjima i obavljaju poslove koji su obilježeni boravkom na otvorenom prostoru (37, 38).

Utjecaj prehrane

Teorija o postojanju određenih antigena iz hrane, ali i bakterijskih, koji mogu potaknuti kaskadu imunosnih reakcija koje naposljetku dovode do upalnih promjena u gastrointestinalnom traktu općeprihvaćena je, međutim ne postoje točna saznanja o kojim je antigenima riječ (39). Istraživanja vezana uz prehranu i etiopatogenezu UBC-a općenito su nedorečena, a često i nekoherentna. Međutim kvaliteta prehrane nedvojbeno je čimbenik koji utječe na razvoj i tijek UBC-a (40). Prehrana temeljena na namirnicama s visokim sadržajem zasićenih masnih kiselina i rafiniranih ugljikohidrata, prema objavljenim istraživanjima može povećati rizik od obolijevanja od UBC-a čak tri do četiri puta (41). S druge strane, namirnice s visokim sadržajem višestrukonezasićenih masnih kiselina, osobito omega-3, poput eikozapentaenske i dokozaheksaenske kiseline, mogu djelovati preventivno, ali i djelomično kurativno kod oboljelih od UBC-a pa i drugih kroničnih bolesti (42).

Pušenje

Među svim čimbenicima okoliša potencijalno uključenim u etiopatogenezu UBC-a najčvršći dokazi postoje za utjecaj pušenja (43). Povezanost pušenja duhanskih proizvoda i patogeneze UBC-a kompleksna je, no brojna su istraživanja potvrdila istu činjenicu: pušenje je protektivan faktor kod oboljelih od UK, dok je s druge strane čimbenik rizika i pridonosi nastajanju i egzacerbaciji CB-a (44). Ovi su utjecaji, čini se, ovisni o količini duhanskog dima koja se aktivno ili pasivno unosi u organizam, a rezultati su koherentni u različitim zemljopisnim područjima (45). Paradoksalno, osobe koje su prestale pušiti imaju 1,7 puta veći rizik od razvoja UK od osoba koje nikada nisu pušile duhanske proizvode. Isto tako, bivši pušači češće bivaju hospitalizirani za razliku od aktivnih pušača, a vjerojatnost da će biti podvrgnuti kolektomiji dvostruko je veća nego kod oboljelih koji nikada nisu bili aktivni pušači (46).

Dodatni čimbenici rizika potencijalno povezani s patogenezom UBC-a jesu činjenica je li osoba dojena u novorođenačkoj dobi, cijepljenja i bolesti u ranom djetinjstvu, uzimanje farmaceutskih pripravaka (ponajviše antibiotika, oralnih kontraceptiva i nesteroidnih protuupalnih lijekova), prethodna apendektomija, infekcije crijevnim parazitima, stres te ostali čimbenici vezani uz okoliš i način života pojedinca (16). Međutim rezultati istraživanja na ovom području proturječni su, često i kontroverzni. Iako postoji povezanost čimbenika rizika s patogenezom UBC-a, još nije definirana njihova točna uzročno-posljedična povezanost (47).

Patogeneza upalnih bolesti crijeva

CB i UK najvjerojatnije nastaju kao posljedica genetske predispozicije koja uzrokuje abnormalni imunosni odgovor na luminalne mikroorganizme s pomoću raznih čimbenika okoliša. Tip imunosnog odgovora odredit će ekspresiju bolesti. Najvjerojatnije promijenjena sluznica crijeva kod UK te abnormalna epitelna permeabilnost kod obje bolesti olakšavaju pristup produktima metabolizma te bakterijskim produktima u podsluznicu. Ovdje ih prerađuju stanice koje prezentiraju antigen (APC), što dovodi do ekspresije antigena na staničnoj površini limfocita T i njihove aktivacije. Kod zdravih će osoba posljedični rezultat biti imunosna tolerancija, odnosno kontrolirana upala. U slučaju UBC-a, zbog poremećaja u imunoregulaciji, doći će do nekontroliranog i prolongiranog upalnog odgovora. Pretpostavlja se da je ključni trenutak poremećene imunoregulacije manjak supresorskih limfocita T. Iako nema direktnog dokaza o defektnoj T-staničnoj regulacijskoj ulozi ni u CB-u ni u UK, dokazano je da je prirođena imunost nedostatna u CB-u (30).

Nadalje, povećan izražaj nuklearnih transkripcijskih faktora uvjetuje prekomjerno lokalno nakupljanje posrednika upale, kao što su citokini, leukotrijeni, tromboksani, čimbenici rasta, prostaglandini, neuropeptidi, dušikovi oksidi, aktivatori trombocita te proteaze. Oštećenje sluznice pak olakšava prodiranje luminalnog sadržaja u unutrašnje slojeve, što pojačava upalni odgovor.

Različitu imunosnu pozadinu dvaju oblika UBC-a jasno potvrđuju patohistološke promjene poput granuloma u CB-u i neutrofilne infiltracije s destrukcijom epitela u UK. Za CB karakterističan je tip Th1 imunosnog odgovora koji uključuje lučenje IFN-γ, TNF-α, IL-2, IL-8 i IL-12. Th1-stanični odgovor novijim je istraživanjima proširen utjecajem medijatora Th17-imunosnog odgovora, koji je pod regulacijom IL-17 (31). Lučenje IL-17 stimulirano je lučenjem IL-6, TGF-β te IL-23 od strane prirođenih imunosnih stanica i APC-a, osobito dendritičkih stanica. Bakterijska kolonizacija stimulira ekspresiju IL-23-gena u dendritičkim stanicama ileuma (32), a dokazane su povišene koncentracije IL-23 i IL-17 u CB-u. Koncentracija IL-27, citokina povezanog s IL-12, također je povišena u oboljelih od CB-a. Nadalje, lučenje IL-21 inducirano je s IL-12 te je također povišeno u CB-u. Poput IL-12, i IL-21 stimulira T-bet, intracelularni transkripcijski faktor koji je ključan u Th1-staničnoj diferencijaciji i aktivaciji (33).

S druge strane, za UK je karakterističan atipičan Th 2-tip imunosnog odgovora, koji daje uglavnom humoralni imunosni odgovor, a posredovan je lučenjem citokina poput IL-4, IL-5, IL-6, IL-10, IL-13 (10). Stoga je logična povezanost UK s autoimunosnim bolestima poput Hashimotova tiroiditisa i sistemskog eritematoznog lupusa. Povećana humoralna imunost kod UK obilježena je i visokim koncentracijama imunoglobulina te povećanom proizvodnjom autoantitijela (npr. p-ANCA, engl. *perinuclear neutrophil cytoplasmic antibody*).

Aktivacija NFκB stimulira izražaj mnogobrojnih molekula relevantnih za patogenezu UBC-a. Najvažniji medijatori upalnih procesa među njima jesu IL-1β, TNF, IL-6, IL-8 i ostali kemokini, ICAM1 i druge adhezijske molekule te kostimulacijske molekule, uključujući CD40, CD80, CD86 i inducibilni T-stanični kostimulator (ICOS). Ekspresija svih ovih

proupalnih medijatora povećana je u UBC-u (34). S druge strane, koncentracije citokina koji induciraju Th1 i Th17-imunosne odgovore povišene su u CB-u, ali ne i u UK. Dok je razina IFN-γ u mukozi oboljelih od CB-a povišena, kod oboljelih od UK nije značajnije izmijenjena (35). Koncentracije citokina povezanih s generalnom upalom, poput IL-1, IL-6 i TNF-α, povišene su u oba oblika UBC-a.

Istraživanja na molekularnoj razini značajno su pridonijela novim saznanjima o mehanizmima nastanka UBC-a. Dosadašnja su istraživanja, uključujući i ona koja obuhvaćaju bolesnike te istraživanja provedena na životinjskim modelima upalnih bolesti crijeva, pokazala da proteaze imaju važnu, ali još nedovoljno jasnu ulogu u patogenezi UBC-a (48, 49). Pokazano je da peptidaze stanične površine imaju presudnu ulogu u razgradnji medijatora uključenih u održavanje integriteta sluznice, kao i u nastanku upalnih promjena (50, 51). Među njima nalazi se i dipeptidil-peptidaza IV (DPP IV), odnosno molekula CD26 (DPP IV/CD26) (52), član porodice prolin-selektivnih dipeptidil-peptidaza.

PORODICA PROTEINA SLIČNIH DIPEPTIDIL-PEPTIDAZI IV

Prolin-selektivne dipeptidil-peptidaze skupina su serinskih proteaza koje sudjeluju u regulaciji različitih biokemijskih procesa putem hidrolize N-terminalnih dipeptida s polipeptidnih i proteinskih lanaca koji sadržavaju aminokiselinu prolin na pretposljednjem mjestu (53). Porodicu DPP IV proteina čine DPP IV/CD26, FAP (protein aktivacije fibroblasta), DPP8 i DPP9 te DPL1 i DPL2, koji nemaju enzimsku ulogu (tablica 1). Nazivaju se još i „homolozi aktivnosti i strukture dipeptidil-peptidaze IV“ (engl. *dipeptidyl peptidase IV activity and structure homologues*), zbog slične enzimske aktivnosti i/ili strukturne podudarnosti te visoke homologije na razini DNA i aminokiselina (54). Preklapanje specifičnosti prema supstratima te određenih komponenata strukture upućuje na njihovu važnost u smislu kooperacije, interakcije s ostalim biološki važnim molekulama, kao i evolucijske očuvanosti (55).

Protein aktivacije fibroblasta alfa (FAP-α, sepraza) dijeli oko 50 % sličnosti u sekvenciji s DPP IV i sadržava gotovo identičan broj (760) aminokiselina. Gen koji kodira ovaj protein nalazi se u neposrednoj blizini gena za DPP IV (2q24.3). Poput DPP IV, enzimska aktivnost FAP-α ovisna je o dimerizaciji (56). Osim što ima aktivnost sličnu DPP IV FAP-α ima endopeptidaznu sposobnost cijepanja denaturiranog kolagena tipa I i III, zbog čega mu se pripisuje mogućnost regulacije izvanstaničnog matriksa tumorskog mikrookoliša (57). Međutim za razliku od DPP IV, FAP-α izražen je na relativno ograničenom broju tkiva, i to onima koja ulaze u procese remodeliranja i regeneracije, poput određenih stanica jetre i embrionalnih mezenhimalnih stanica. Izražen je na više od 90 % stanica humanih epitelnih karcinoma, poput karcinoma pankreasa, dojki, pluća i kolona (55, 58). Miševi s deficitom FAP-α normalnog su fenotipa što se tiče tjelesne težine, težine organa, histoloških karakteristika glavnih organa, kao i hematoloških osobina (59).

DPP8 i DPP9 ubikvitarno su izraženi enzimi s dipeptidil-peptidaznom aktivnošću sličnom DPP IV. Dijele 26 % aminokiselinske homolognosti s DPP IV i FAP-α, a međusobno su 61 % homologni (60). DPP8 i DPP9 solubilni su citoplazmatski proteini za razliku od DPP IV/CD26 i FAP-α. Izražaj glasničke RNA DPP8 i DPP9 pokazuje široku distribuciju u humanim tkivima s najvišom koncentracijom u testisima i posteljici za DPP8 te u skeletnim mišićima, srcu, jetri i leukocitima periferne krvi za DPP9. Izražaj DPP8 i DPP9 u miša pronađen je u kolonu, mozgu, koži i timusu (61).

DPL1 i DPL2 članovi su porodice DPP IV bez enzimskih svojstava zbog nedostatka triptofana i nukleofilnoga serinskog ostatka potrebnih za katalitičko djelovanje. Izraženi su u mozgu i organima endokrinog sustava. DPL1 se prvotno nazivao DPPX i DPP6. Dosadašnja saznanja o njihovim funkcijama nedostatna su, no vjeruje se da je DPL1 važan u organogenezi. Poznato je da nedostatak DPL1-gena u miša uzrokuje letalan ishod u homozigotnih embrija, dok u heterozigota uzrokuje defekt pigmentacije (62).

DIPEPTIDIL-PEPTIDAZA IV/MOLEKULA CD26 (DPP IV/CD26)

Obilježje membranski vezanih peptidaza jest posjedovanje specifičnog profila izražaja u pojedinim tkivima, vrstama stanica ili pak staničnim dijelovima, što odražava funkciju svake pojedine stanice. Kod nekih je membranski vezanih proteaza pronađen i topljivi analog koji se može locirati intracelularno i ekstracelularno, uključujući i biološke tekućine poput

Tablica 1 *Karakteristike članova porodice humanih proteina sličnih DPP IV (prilagođeno prema ref. 63)*

Svojstvo	DPP IV	FAP	DPP8	DPP9
Hidroliza X-Gly-Pro	✓	Slabo	✓	✓
Hidroliza X-Ala-Pro	✓	✓	✓	✓
Hidroliza X-Arg-Pro	✓	✓	Slabo	Slabo
Razgradnja kemokina	✓	NP	NP	NP
Želatinazna aktivnost (kolagen tipa I)	×	✓	×	×
Dimerni oblik	✓	✓	×	×
Vezanje adenozin-deaminaze	✓	×	×	×
Izražaj mRNA u tkivima odraslih osoba	Ubikvitarno	Ubikvitarno	Ubikvitarno	Ubikvitarno
Izražaj proteina u tkivima odraslih osoba	Ubikvitarno	Serum, gušterača	NP	NP
Izražaj proteina na aktiviranim fibroblastima	✓	✓	NP	NP
Izražaj na fetalnim mezenhimalnim stanicama	✓	✓	NP	NP
Izražaj na aktiviranim Itovim stanicama jetre	×	✓	NP	NP
Izražaj na limfocitima	✓	×	✓	✓

✓*, DA;* ×*, NE; NP, nije poznato*

seruma, urina, sline, sjemene i amnionske tekućine (54).

Tipičan predstavnik membranski vezanih proteaza je DPP IV/CD26 (EC 3.4.14.5). Nalazimo je izraženu na površini brojnih stanica tkiva različitih organa, ali i u cirkulaciji, na sistemskoj i lokalnoj razini. Specifičnost ove molekule jest mogućnost cijepanja dipeptida s N-terminalnog dijela lanca biološki važnih supstrata koji na pretposljednjem mjestu u primarnoj strukturi odnosno sekvenciji aminokiselina sadržavaju prolin ili alanin (64).

Dugi niz godina vjerovalo se da je glavna uloga DPP IV/CD26 razgradnja konačnih metabolita u procesu probave. Pripisivala joj se jedinstvena uloga u metabolizmu polipeptida s visokim sadržajem prolina. Brojnim daljnjim istraživanjima utvrđena je njezina važna uloga i u imunosnom odgovoru organizma (65). Nakon dobivenih saznanja o biološkim učincima važnih bioaktivnih molekula, supstrata DPP IV/CD26 te mogućnosti njihove aktivacije odnosno inaktivacije, što dovodi do modulacije neuroimunobiokemijskog odgovora organizma, istraživanja vezana uz ovu molekulu eksponencijalno su rasla (66).

Prvi opis DPP IV/CD26 zabilježen je 1966. u znanstvenoistraživačkom radu Hopsu-Havua i Glennera koji su u homogenatu jetre štakora otkrili enzim sa svojstvom otpuštanja naftilamina iz Gly-Pro-2-naftilamida. Zbog navedenog su svojstva ovaj enzim nazvali glicil-prolin naftilamidaza (67). Shrader i Stacy otkrili su 1977. funkciju vezanja neodređenog proteina s adenozin-deaminazom (ADA) te ga nazvali protein koji veže adenozin deaminazu (engl. *adenosin deaminase binding or complexing protein*, ADAbp, ADAcp). Tek je 1993. utvrđeno da je riječ o kompleksu DPP IV-ADA (68).

Godine 1977. prvi je put dokazana aktivnost DPP IV na limfocitima periferne krvi (69). Jedanaest godina kasnije otkriveno je da je DPP IV identična leukocitnom površinskom antigenu CD26, nakon čega je nekoliko monoklonskih protutijela koja prepoznaju DPP IV grupirano pod nazivom "CD26". Godinu dana kasnije utvrđeno je da se DPP IV sastoji od dvije jednake podjedinice, od kojih svaka sadržava svoje aktivno središte (70).

Ova je molekula godine 1985. karakterizirana kao receptor za kolagen i fibronektin (71). S obzirom na to da je slijed aminokiselina Gly-Pro učestao u kolagenu, pretpostavilo se da ima važnu metaboličku ulogu u biokemijskim procesima u kojima je uključen kolagen. Međutim kako je ustanovljeno da DPP IV/CD26 nema sposobnost cijepanja Pro-Pro i Pro-Hyp veza koje ponajviše slijede Gly-Pro sekvence u kolagenu, točna fiziološka uloga ovog enzima dugo je ostajala nepoznata (72).

Daljnjim istraživanjima ove molekule cjelokupna cDNA humane CD26 prvi je put objavljena 1992. godine (73). Godine 2001. pokazana je sposobnost direktnog vezanja CD26 na citoplazmatsku domenu tirozinske fosfataze, odnosno molekule CD45 (74). Današnja

saznanja opisuju molekulu CD26 kao transmembranski glikoprotein s funkcijom serinske proteaze te ujedno i jedinstven hematopoetski diferencijacijski antigen. DPP IV/CD26 jedini je član porodice prolil-oligopeptidaza čiji je izražaj pronađen na leukocitima (75). Daljnjim je istraživanjima dokazano da dijeli mnoga zajednička strukturna i regulacijska svojstva s molekulom CD10 (neutralnom endopeptidazom), molekulom CD13 (aminopeptidazom N) te molekulom BP-1/6C3 (aminopeptidazom A) (76).

Istraživanja provedena u posljednja tri desetljeća pokazala su široki spektar područja djelovanja i funkcionalnih osobitosti DPP IV/CD26 u biokemijskim, imunološkim i neuroendokrinim međudjelovanjima. U mnogim slučajevima, učinci ne moraju nužno biti vezani samo uz katalitičko djelovanje ove molekule, već uz brojne, dosad još nedostatno poznate, mogućnosti djelovanja na području stanične adhezije, međustanične komunikacije te prijenosa signala u stanicu (77).

Lokalizacija DPP IV/CD26

Molekula CD26 konstitutivno je izražena na brojnim stanicama različitih tkiva i organa poput gastrointestinalnog, neurološkog, reproduktivnog i ostalih organskih sustava. Imunohistokemijskim analizama, DPP IV/CD26 dokazana je gotovo u svim endotelnim stanicama kapilara različitih organa i tkiva (77). Humana DPP IV/CD26 relativno je ubikvitarna molekula, osobito izražena na zrelim timocitima, aktiviranim limfocitima T, B, NK-stanicama i makrofagima. Molekula CD26 izražena je na *in vivo* i *in vitro* aktiviranim CD4+ i CD8+ humanim limfocitima T (78). Otprilike 56 % CD4+ i 35 % CD8+ stanica limfocita periferne krvi, 74-81 % CD4+ te 12-19 % CD8+ limfocita T aktiviranih s pomoću fitohemaglutinina izražava CD26 (79). Izražena je i na mirujućim limfocitima T u znatno manjoj mjeri, ali njezin izražaj raste 5 do 10 puta nakon stimulacije antigenima poput anti-CD3 i interleukina 2 (IL-2) (62, 64). Najveća razina izražaja molekule CD26 pronađena je na imunosnim stanicama koje izražavaju aktivacijske markere poput CD25, CD71, CD45RO te CD29 (80).

Izražaj molekule CD26 na limfocitima B vrlo je nizak, no također raste nakon stimulacije mitogenima ili proteinima Staphylococcusa aureusa (81). Poput limfocita T i B, i NK-stanice izražavaju CD26 u niskoj razini, ali se izražaj može povećati za oko 30 % stimulacijom s IL-2. Utvrđeno je da 10 % CD16+ svježe izoliranih NK-stanica izražava molekulu CD26 (82). Izražaj molekule CD26 utvrđen je i na klonovima stanica s NK-aktivnošću te subpopulacijama NK-stanica pacijenata nakon transplantacije koštane srži (83, 84). Podaci dobiveni uporabom inhibitora DPP IV/CD26 upućuju na činjenicu da je molekula CD26 uključena u regulaciju proliferacije NK-stanica, ali ne i u njihovu citotoksičnu ulogu (85).

Molekularna obilježja DPP IV/CD26

Gen koji kodira humanu DPP IV/CD26 nalazi se na dužem kraku drugog kromosoma (2q24.3). Dužina mu iznosi približno 70 kb, a sadržava točno 26 egzona, čija se veličina prostire od 45 b do 1,4 kb (86). Karakteristično je da se na 5' kraju ne nalaze ni TATA kutija ni CAAT kutija, no područje veličine oko 300 parova baza koje iznimno obiluje citozinom i gvaninom (oko 72 %) sadržava potencijalna vezna mjesta za nekoliko transkripcijskih faktora, poput NFκB, AP2 i Sp1 (86, 87).

Molekula DPP IV/CD26 humanog podrijetla klasificira se kao integralni transmembranski protein tipa II. Relativna molekulska masa dimera iznosi oko 240000, a sastavljena je od dva jednaka monomera. Sadržava ukupno 766 aminokiselina (u štakora 767), što je ustanovljeno sekvencioniranjem cDNA (88). Od ukupnog broja aminokiselina, 22 (VLLGLLGAAALVTIITVPVVLL) otpadaju na transmembransku hidrofobnu domenu, na koju se nastavlja kratak intracelularni, hidrofilni ostatak sastavljen od svega šest aminokiselina (MKTPWK) na N-terminalnom kraju proteinskog lanca. Preostalih 738 aminokiselinskih ostataka sadržava devet potencijalnih glikozilacijskih mjesta.

Strukturna obilježja DPP IV/CD26

Transmembranski glikoprotein DPP IV/CD26 u organizmu se nalazi ponajviše u obliku dimera sastavljenog od dvije jedinice monomera od kojih svaka sadržava dvije podjedinice: alfa-betahidrolaznu domenu, koja obuhvaća aminokiselinske ostatke od 501. do 766. te betapropelernu domenu koja se prostire od 59. do 497. aminokiselinskog ostatka molekule DPP IV/CD26. DPP IV/CD26 posjeduje devet potencijalnih mjesta za glikozilaciju koja se nalaze najvećim dijelom na betapropelernoj domeni, u blizini područja dimerizacije (89). Slika 1, prilagođeno prema (90), prikazuje shematsku strukturu dimerizirane transmembranske DPP IV/CD26.

Kristalna struktura DPP IV/CD26 pomogla je u rasvjetljavanju funkcionalne uloge dimerizacije DPP

IV/CD26. Naime, pokazano je da dimerizacija nije nužna za oblikovanje aktivnog središta. S druge strane, dimerizacija i tetramerizacija utječu na ostale komponente, uključujući supstrate proteolize te vezanje adenozin-deaminaze, a najvjerojatnije i mogućnost međustanične komunikacije. Osim toga, dimerizacija DPP IV/CD26 pojačava afinitet receptora prema ligandu, što može biti od iznimne važnosti u procesima prijenosa signala u stanicu (89).

Osim dimerizacije, detaljnijim istraživanjima otkrivena je i sposobnost oligomerizacije membranski vezane i solubilne DPP IV/CD26. Zbog geometrijske specifičnosti tetramerizacija na površini stanice zahtijeva membranski vezani i solubilni oblik DPP IV/CD26, lociran na površini dviju različitih stanica. DPP IV/CD26 može na ovaj način sudjelovati u međustaničnoj komunikaciji, i to na način da modulira kontakt između dviju stanica u procesu međusobne komunikacije putem tetramerizacije dvaju DPP IV/CD26 dimera prisutnih na njihovoj površini. Važnu ulogu u ovom procesu može imati i solubilni oblik ove molekule, s obzirom na to da može interferirati u vezanju membranski vezanih oblika.

Solubilna DPP IV/CD26

Osim u membranski vezanom obliku na površini različitih stanica, DPP IV/CD26 prisutna je u solubilnom (topljivom) obliku u cirkulaciji (u serumu), slini, urinu, sinovijalnoj tekućini, sjemenoj i amnionskoj tekućini te ostalim biološkim tekućinama. Podrijetlo solubilne DPP IV/CD26 nije sa sigurnošću utvrđeno, ali pretpostavlja se da potječe s površine stanica koje su u doticaju s krvlju. Vjeruje se da postoji proteolitički mehanizam koji pridonosi otpuštanju membranski vezane DPP IV/CD26 u cirkulaciju, no zasad nije poznat (55).

Solubilna DPP IV/CD26 pleiotropna je molekula koja zadržava identična proteolitička svojstva poput membranski vezane. Razlikuju se u nedostatku 22-iju aminokiselina transmembranskog dijela te 6 aminokiselina unutarstaničnog dijela cjelovite DPP IV/CD26 (63).

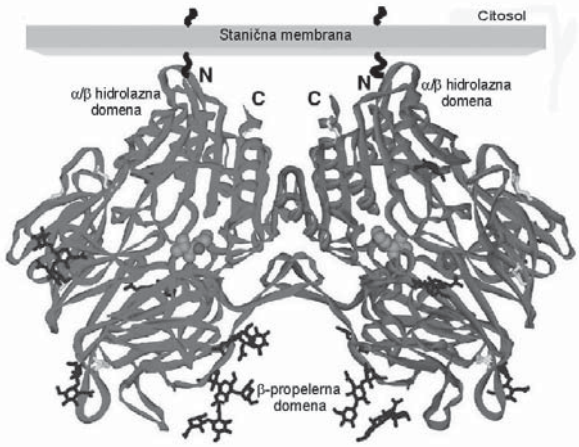
Aktivno središte DPP IV/CD26

Aktivno središte DPP IV/CD26 čini katalitička trijada serin (aminokiselinski ostatak 630), asparaginska kiselina (aminokiselinski ostatak 708) te histidin (aminokiselinski ostatak 740). Osim toga, tirozinski ostatak hidrolazne domene nuždan je za katalitičku aktivnost ovog enzima, a kinetička istraživanja pokazala su i da sudjeluje u stabilizaciji intermedijarnih oblika supstrata. Nadalje, dvije glutaminske kiseline, Glu205 i Glu206 sudjeluju u stvaranju međumolekularnih elektrostatskih veza s N-terminalnim dijelom supstrata, što ostavlja mjesta samo za dvije aminokiseline prije nego što peptid dospije do aktivnog mjesta. Ovaj mehanizam objašnjava i dipeptidilnu katalitičku aktivnost DPP IV/CD26 (91).

Betapropelerna domena prekriva aktivno središte te stoga djelomično ograničava pristup supstratima, što omogućuje i određenu regulaciju biološke aktivnosti ovog enzima. Postoje dva glavna puta do aktivnog središta molekule DPP IV/CD26: kroz prolaz β-propelerne domene te kroz bočni prolaz širine 14 Å do 15 Å (90). Udaljenost od površine DPP IV/CD26 do aktivnog središta iznosi 20 Å od bočnog prolaza te 37 Å od β-propelerne domene. Nakon razgradnje supstrata, nastali produkti napuštaju aktivno mjesto, a da pritom put ulaska i izlaska ne moraju biti jednaki (89).

Specifičnost prema supstratima

Mnoge biološki aktivne molekule poput citokina, kemokina, hormona, neuropeptida i faktora rasta sadržavaju evolucijski očuvani prolin na pretposljednjem mjestu polipeptidnog lanca (92). Ovakva struktura omogućuje im relativno visoku mogućnost regulacije njihova biološkog djelovanja te postojanost u zaštiti razgradnje od strane enzima koji ne posjeduju prolin-specifičnu peptidaznu aktivnost (93). Međutim upravo ih ovakva struktura čini pogodnim supstratima za katalitičko djelovanje DPP IV/CD26 (72).



Slika 1 *Struktura transmembranske DPP IV/CD26 u obliku dimera, shematski prikaz (prilagođen prema ref. 90)*

Pokazano je da su peptidi manje molekulske mase vrlo dobri supstrati DPP IV/CD26. Porastom broja aminokiselina u primarnoj strukturi polipeptidnog lanca smanjuje se i mogućnost dopiranja do aktivnog središta, a time i mogućnost njihove katalitičke razgradnje od strane DPP IV/CD26 (51). Tablica 2. prikazuje popis i karakteristike značajnih regulacijskih peptida, supstrata DPP IV/CD26 u sisavaca.

Funkcije DPP IV/CD26

Uvriježena su četiri područja djelovanja DPP IV/CD26 u kojima, prema dosadašnjim saznanjima, ova molekula ostvaruje svoju biološku ulogu:

1) Proteoliza – u svojstvu serinske proteaze, DPP IV/CD26 sudjeluje u hidrolitičkoj razgradnji pojedinih biološki aktivnih peptida, a samim time u njihovoj aktivaciji i inaktivaciji. Na ovaj način može utjecati na modulaciju neuroimunobiokemijskog odgovora organizma u fiziološkim i patološkim procesima (64).
2) Uloga u međustaničnoj komunikaciji, komunikaciji između stanice i izvanstaničnog matriksa te međudjelovanja stanične površine i virusnih komponenata. DPP IV/CD26 opisuje se i kao protein koji veže kolagen i fibronektin (95), zatim kao koreceptor za HIV-1 u međudjelovanju s adenozin-deaminazom (96) te važan čimbenik metastaziranja tumora (97).
3) Prijenos signala u stanicu – pokazano je da DPP IV/CD26 ima ulogu koreceptora u kaskadnim reakcijama prijenosa specifičnih signala kroz membranu (65).
4) Kompleksni neuroimunobiokemijski mehanizmi, zasad još nedostatno poznati – rezultati objavljenih istraživanja upućuju na uključenost DPP IV/CD26 u mehanizme stanične proliferacije i diferencijacije (98), programirane stanične smrti odnosno apoptoze te složenim mehanizmima aktivacije limfocita T *in vitro* i *in vivo*, osobito u autoimunosnim procesima (98, 99). Utvrđena je uključenost DPP IV/CD26 u neuroimunoendokrine procese, što otvara put novim istraživanjima i terapijskim mogućnostima (100).

Zbog svoje kompleksnosti u djelovanju te izrazite multifunkcionalnosti DPP IV/CD26 i porodica DPP IV proteina vrlo su važno područje u brojnim istraživanjima i temeljnim i kliničko-farmaceutskim. Molekula DPP IV/CD26 prva je membranski vezana peptidaza za koju je utvrđena uloga u procesu prijenosa signala u stanicu. Kako ovaj transmembranski glikoprotein sadržava kratku citoplazmatsku domenu, iako bez uobičajenih signalnih motiva (65, 77), vjeruje se da su upravo taj kratki transmembranski dio i unutarstanični dio uključeni u kaskadne reakcije prijenosa signala u stanicu. Različite grupe istraživača opisale su uključenost DPP IV/CD26 u regulaciju diferencijacije i rasta limfocita T, kao i njihove aktivacije (82, 101, 102).

Važna komponenta prijenosa signala u stanicu putem molekule CD26 jest i agregacija s adenozin-deaminazom. Adenozin-deaminaza (ADA; EC 3.5.4.4) enzim je uključen u metabolizam purina. Katalizira hidrolitičku deaminaciju adenozina ili 2'-deoksiadenozina u inozin ili 2'-deoksiinozin i amonijak. Kongenitalni defekti ADE uzrokuju tešku kombiniranu imunodeficijenciju, karakteriziranu nedostatkom funkcionalnih limfocita T i B (103).

S obzirom na kratku citosolnu domenu molekule CD26, potrebna mu je molekula s kojom se može povezati u cilju efikasne provodnje signala (104). Pokazano je da upravo interakcija DPP IV/CD26 i ADE rezultira kostimulacijskim signalima kod limfocita T. Asocijacija CD26-ADA na površini limfocita T može imati kostimulacijsku ulogu prilikom aktivacije T-staničnog CD3 antigenskog receptora (103). Rezultati provedenih istraživanja pokazali su da prilikom povećane koncentracije ADE dolazi do povećanog lučenja interferona γ (IFN-γ), čimbenika tumorske nekroze α (TNF-α) te interleukina 6 (IL-6), dok nema značajnijeg učinka na interleukine 2 i 10 (IL-2 i IL-10) (104).

Osim u slučaju povezivanja s ADOM Ishii i sur. (74) pokazali su da se signalizacija putem molekule CD26 djelomično odvija i putem asocijacije s tirozinskom fosfatazom odnosno molekulom CD45. Poznato je da je molekula CD45 involvirana u aktivaciju limfocita T te je stoga kompleks CD26-CD45 karika u nizu signalnih kaskada uključenih u imunosni odgovor.

Klinička važnost i terapijska primjena

Mogućnost specifične katalitičke razgradnje ciljnih biološki aktivnih supstrata, kao i uključenost u imunomodulacijske procese čine DPP IV/CD26 molekulom od velikog značenja u kliničkom smislu, kao i potencijalnim, a ponegdje već i potvrđenim kandidatom za terapijsku primjenu (105-107).

Dosadašnja su istraživanja upozorila na izmijenjene vrijednosti aktivnosti i izražaja DPP IV/CD26 u različitim bolestima, poput upalnih bolesti crijeva, reumatoidnih bolesti, neuroloških bolesti, karcinoma, metaboličkih bolesti i mnogih drugih, kao što je sažeto

Tablica 2 *Popis i karakteristike značajnih regulacijskih peptida, supstrata DPP IV/CD26 u sisavaca (prilagođeno prema ref. 72 i 94)*

Supstrat DPP IV/CD26	N- -terminalni kraj lanca	Broj amino-kiselina	Uspješnost katalize[a]	Biološki učinak	Literaturni izvor
Xaa-Pro-peptidi					
Neuropeptid Y, NPY	YP-S…	36	+++	Inaktivacija za Y1-receptor	(147, 148)
Peptid YY, PYY	YP-I…	36	+++	Inaktivacija za Y1-receptor	(148)
Enterostatin	VP-DP-R	5	++	Inaktivacija	(149)
Beta-kazomorfin	YP-F…	7	+++	Inaktivacija	(150)
Interleukin 6 (humani)	VP-PG-E...	184	++	Inaktivacija	(94, 151)
Interleukin 6 (mišji)	FP-TS-Q...	187	++	Inaktivacija	(94, 151)
Interleukin 10 (humani)	SP-G...	160	++	Inaktivacija	(94, 151)
Propeptid tripsinogena	FP-T…	8	+	Nije sasvim poznat, aktivacija?	(150)
Bradikinin	RP-P…	9	Nije sasvim poznata	Nije sasvim poznat	(152)
Supstancija P	RP-KP-Q…	11	++	Nije sasvim poznat	(152, 153)
Peptid koji oslobađa gastrin	VP-LP-A…	27	+	Nije sasvim poznat	(150)
Endomorfin 2	YP-FF-NH_2	4	++	Inaktivacija	(154)
Tyr-melanostatin	YP-LG-NH_2	4	++	Inaktivacija	(150)
Aprotinin	RP-D…	58	+	Nije sasvim poznat	(150)
RANTES[b]	SP-Y…	68	+	Inaktivacija za CCR-1 i CCR-3-receptore	(155, 156)
Granulocitni kemotaktični protein 2, GCP-2	GP-V…	73	+	Nije sasvim poznat	(156)
SDF-1-α[c]	KP-V…	68	+	Inaktivacija za CXCR4-receptor	(155, 157)
SDF-1-β[c]	KP-V…	72	+	Inaktivacija za CXCR4-receptor	(157)
Kemokin deriviran s makrofaga, MDC	GP-YG-A…	69	+	Inaktivacija za CCR4-receptor	(158)
Monocitni kemotaktični protein 1, MCP-1	QP-DA-…	76	Nije sasvim poznata	Nije sasvim poznat	(156)
Monocitni kemotaktični protein 2, MCP-2	QP-DS…	76	+	Inaktivacija	(155)
Monocitni kemotaktični protein 3, MCP-3	QP-VG…	73	Nije sasvim poznata	Nije sasvim poznat	(156)
Eotaksin	GP-A…	74	+	Inaktivacija za CCR3-receptor	(92, 155, 159)
Interferon γ-inducibilni protein 10, IP-10	VP-L…	77	+	Nije sasvim poznat	(94)
Čimbenik rasta 1 sličan inzulinu	GP-E…	70	Nije sasvim poznata	Nije sasvim poznat	(72)

Tablica 2 *Nastavak*

Supstrat DPP IV/CD26	N- -terminalni kraj lanca	Broj amino- kiselina	Uspješnost katalize[a]	Biološki učinak	Literaturni izvor
Prokolipaza	VP-DP-R…	101	+	Nije sasvim poznat, aktivacija?	(150)
Interleukin 2	AP-T-…	133	Nije sasvim poznata	Nije sasvim poznat	(94, 150)
Interleukin 1-β	AP-V…	153	Nije sasvim poznata	Nije sasvim poznat	(93)
α_1-mikroglobulin	GP-VP-T	168	Nije sasvim poznata	Nije sasvim poznat	(150)
Prolaktin	LP-I…	198	+	Nije sasvim poznat	(94, 150)
Tripsinogen	FP-T	231	+	Nije sasvim poznat, aktivacija?	(150)
Korionski gonadotropin	AP-D… (α-lanac)	243	+	Nije sasvim poznat	(150)
Xaa-Ala-peptidi					
Peptid histidin-metionin	HA-E…	27	++	Inaktivacija	(160)
Čimbenik oslobađanja hormona rasta (1-29)	YA-D…	29	++	Inaktivacija	(161)
Čimbenik oslobađanja hormona rasta (1-44)	YA-D…	44	++	Inaktivacija	(160)
Peptid 1 sličan glukagonu, GLP-1	HA-E…	30	++	Inaktivacija	(160)
Peptid 2 sličan glukagonu, GLP-2	HA-E…	34	++	Inaktivacija	(122)
Gastrični inhibicijski peptid, GIP	YA-E…	42	++	Inaktivacija	(160)
Xaa-Ser-peptidi					
Vazoaktivni intestinalni peptid, VIP	HS-DA-VF...	28	++	Inaktivacija za VPAC1-receptor	(147)
Interleukin 10 (mišji)	MS-RG-Q...	160	++	Inaktivacija	(94, 151)

[a] *zaključak prema podacima dostupnima u literaturi: +, dobra; ++, vrlo dobra; +++, izvrsna uspješnost katalize*
[b] *RANTES, od engl.* regulated on activation normal T-cell expressed and secreted
[c] *SDF, od engl.* stromal cell-derived factor

u tablici 3. (107). Međutim uloga ove molekule u fiziološkim i patološkim međudjelovanjima do danas nije potpuno razjašnjena.

Inhibitori DPP IV/CD26

Većina inhibitora DPP IV/CD26 može se podijeliti u tri glavne skupine: I) reverzibilni analozi (npr. pirolidin, tiazolidin), II) kovalentno vezani analozi, III) reverzibilni nepeptidni heterociklični inhibitori. Istraživanja na ovom području osobito su intenzivna i dosada su dala već vrlo važne rezultate te naposljetku rezultirala kliničkom primjenom inhibitora DPP IV/CD26. Potencijal terapijske primjene inhibitora DPP IV/CD26, kao i njegova klinička važnost zaista su veliki, a rasvjetljavanje uloge ove molekule u različitim fiziološkim i patološkim procesima presudno i u tom smislu (108).

Područje koje je zasigurno najistraženije u smislu terapijske primjene modulacije aktivnosti DPP IV/CD26 jest dijabetes tipa 2 (109). Naime, određeni inhibitori aktivnosti DPP IV/CD26 poput sitagliptina, saksaglikpita i vildagliptina već su u kliničkoj primjeni u terapiji dijabetesa u smislu sprječavanja razgradnje supstrata, ponajviše inkretina, koji sudjeluju u održavanju koncentracije glukoze u krvi stalnom i pomažu u glikemijskoj kontroli (110, 111). Prvotna ideja o inhibiciji aktivnosti DPP IV/CD26 u cilju terapije dijabetesa tipa 2 nastala je iz saznanja o

Tablica 3 *Aktivnost serumske DPP IV/CD26 u određenim fiziološkim, odnosno patološkim stanjima*

Fiziološko / patološko stanje	aktivnost serumske DPP IV/CD26
Regeneracija jetre (u štakora)	Povišena
Disfunkcija jetre (humana)	Povišena
Karcinom pankreasa i žučnih vodova (humani)	Povišen
Hepatom (humani, u štakora)	Povišena
Encefalitis (mišji)	Povišena
Odbacivanje transplantata (u štakora)	Povišena
Osteoporoza (humana)	Povišena
Upalna bolest crijeva (humana)	Snižena
Depresija (humana)	Snižena
Reumatoidni artritis (humani, u miša)	Snižena
Sistemski lupus erythematosus (humani)	Snižena
Dijabetes mellitus (humani)	Snižena/povišena
Hipertenzija (humana)	Snižena
Trudnoća (humana)	Snižena
Neonatalna vs. odrasla dob (humana)	Snižena
Karcinom usne šupljine (humani)	Snižena
Imunosupresija (u štakora)	Snižena
Mijeloidna leukemija (humana)	Bez promjene
AIDS	Bez promjene
Fibromialgija	Bez promjene

mogućnosti katalitičke razgradnje inkretinskog hormona, peptida 1 sličnog glukagonu (GLP-1). GLP-1 je gastrointestinalni inkretinski hormon koji se u cirkulaciju otpušta postprandijalno i potiče lučenje inzulina stimulirano glukozom. Osim toga, ima niz drugih djelovanja u metaboličkom smislu, od odgađanja pražnjenja želuca do inhibicije lučenja glukagona, a sveukupnost njegova djelovanja pridonosi osjećaju sitosti. Nadalje, GLP-1 utječe na β-stanice gušterače potičući njihovu neogenezu i inhibirajući njihovu apoptozu te potiče biosintezu inzulina, što ga čini izvrsnim kandidatom u terapiji dijabetesa, a to je kliničkim istraživanjima i potvrđeno (112). Osim GLP-1, važan inkretinski hormon je i inzulinotropni peptid ovisan o glukozi (engl. *glucose-dependent insulinotropic peptide*, GIP) te niz ostalih hormona koji sudjeluju u održavanju homeostaze glukoze. Vjeruje se da sinergistično djelovanje GIP-a i GLP-1 uzrokuje čak 60 % do 70 % postprandijalnog lučenja inzulina (113). Međutim biološki poluživot ovih inkretina vrlo je kratak (svega 1 do 2 minute za GLP-1 te 7 minuta za GIP), jer podliježu katalitičkoj razgradnji zbog enzimskog djelovanja DPP IV/CD26, što dovodi do njihove inaktivacije (112). Stoga je niz istraživanja doveo do razvoja inhibitora DPP IV/CD26, u cilju poboljšanja metaboličke kontrole i očuvanja homeostaze glukoze u oboljelih od dijabetesa tipa 2 (111).

Dosadašnje zapažene nuspojave inhibitora DPP IV/CD26 relativno su dobroćudne, a ponajviše se ističe mogućnost nastajanja hipoglikemije i posljedičnog hipoglikemijskog šoka. Najteža moguća nuspojava terapije inhibitorima DPP IV/CD26 uključuje alergijske reakcije koje mogu biti i kobne. Uporaba inhibitora DPP IV/CD26 međutim nije indicirana u oboljelih od dijabetesa tipa 1 (111).

DPP IV/CD26 I UPALNE BOLESTI CRIJEVA

Dosadašnja istraživanja upućuju na promijenjene vrijednosti aktivnosti i izražaja DPP IV/CD26 kod oboljelih od UBC-a (8, 9, 114). Pokazano je da stupanj sniženja serumske aktivnosti DPP IV/CD26 korelira s jačinom i aktivnošću bolesti i kod CB-a i kod UK te se stoga potencijalno može rabiti u kliničke svrhe kao neinvazivan i ekonomski prihvatljiv marker ovih bolesti. Međutim unatoč različitoj imunosnoj pozadini pojedinog entiteta upalnih bolesti crijeva, serumska aktivnost DPP IV/CD26 nije se pokazala dobrim markerom za dijagnostičko razlikovanje CB-a i UK (9). No unatoč brojnim istraživanjima uloga ove molekule u patogenezi UBC-a, kao i drugim kroničnim upalnim bolestima, ostaje nerazjašnjena (115).

Temeljem dosadašnjih saznanja predložena su dva mehanizma putem kojih bi DPP IV/CD26-molekula

mogla biti uključena u patogenezu UBC-a, a uključuju ulogu DPP IV/CD26 u svojstvu serinske proteaze te kao kostimulacijske, odnosno signalne molekule. Kao serinska proteaza DPP IV/CD26 utječe na biološku aktivnost mnogih molekula koje osim što imaju imunomodulacijsku ulogu, pokazano je da sudjeluju i u patogenezi UBC-a, uključujući RANTES, SDF-1α i TNF-α, IL-6 i IL-10 (116). Modulacijom citokinskog odnosno kemokinskog odgovora pretpostavlja se da DPP IV/CD26 tijekom razvoja ili cijeljenja kolitisa utječe na aktivnost pojedinih imunosnih stanica i upalnih medijatora (7). Nadalje, budući da je oštećenje sluznice jedna od glavnih posljedica tijekom razvoja humanog oblika UBC-a, posljednjih godina istražuju se medijatori koji utječu na proces oporavka sluznice. Dosadašnja saznanja upućuju na to da supstrat DPP IV/CD26, peptid 2 sličan glukagonu (GLP-2), ima vrlo važnu ulogu (117). GLP-2 član je porodice peptida sličnih glukagonu, a sastoji se od 33 aminokiseline. Putem enteroendokrinih stanica izlučuje se u lumen crijeva, a upravo je stoga najvažnije područje djelovanja GLP-21-33 probavni sustav, od želuca do debelog crijeva. Pokazano je da sudjeluje u regulaciji mnogih procesa u gastrointestinalnom traktu, uključujući apsorpciju nutrijenata, permeabilnost epitela, staničnu proliferaciju te apoptozu (118, 119). Istraživanja provedena na eksperimentalnim modelima oštećenja intestinalne sluznice u miša i štakora pokazala su da egzogena primjena GLP-21-33 pospješuje rast epitela mukoze i u tankom i u debelom crijevu (120). S druge strane, smanjuje smrtnost stanica epitela, ublažuje oštećenja sluznice, utječe na izražaj citokina, smanjuje bakterijsku septikemiju u upali te ublažuje jačinu oštećenja sluznice crijeva u eksperimentalnome mišjem modelu kolitisa (121). Stoga je citoprotektivni učinak GLP-21-33 temelj potencijalne uporabe ovog peptida u svrhu liječenja oštećenog ili upaljenog mukoznog epitela crijeva kod ljudi. Međutim glavna je zapreka brza inaktivacija GLP-21-33 od strane DPP IV/CD26 kao serinske proteaze u inaktivan oblik GLP-23-33 (122). Inaktivacija GLP-2 ujedno je najveći problem povezan s primjenom GLP-2 u terapijske svrhe te se stoga otvara mogućnost primjene DPP IV/CD26 inhibitora (117).

S obzirom na to da je DPP IV/CD26 transmembranski glikoprotein koji ima kratak citoplazmatski rep, pretpostavlja se da imunomodulacijski učinak ostvaruje ne samo enzimskom aktivnošću nego i kao kostimulacijska i signalna molekula (64). Pretpostavlja se da kostimulatornu funkciju ostvaruje putem prethodno spomenutog vezanja ADE ili direktnom interakcijom s citoplazmatskom domenom CD45 (74, 123). DPP IV/CD26 inducira monomerizaciju molekule CD45 i time aktivira prijenos signala preko T-staničnog receptora (64). Aktivacijom molekule CD45 dolazi do fosforilacije na T-staničnom receptoru pridruženih tirozinskih kinaza, p56Lck, Zap-70, c-Cbl, Erk1/2 koje mijenjaju fosforilacijsko stanje ostalih signalnih molekula. Rezultat su prijenos signala, aktivacija T-limfocita i lučenje IL-2 (74). Istraživanja su pokazala da molekula DPP IV/CD26 nije stabilan površinski marker te da aktivacija limfocita T inducira povećan izražaj DPP IV/CD26 na njihovoj površini u početku proliferacije te dostiže svoj maksimum nakon tri dana. Nakon toga izražaj ponovno opada i stanice prestaju proliferirati jedanaestog dana kultivacije. Povećanje izražaja DPP IV/CD26 nakon stimulacije odnosno tijekom aktivacije i proliferacije limfocita izraženije je na stanicama Th1 nego na Th 2-stanicama imunosnog odgovora.

POKUSNI MODELI UPALNIH BOLESTI CRIJEVA

Različitost i brojnost čimbenika za koje se pretpostavlja da imaju potencijalnu ulogu u etiologiji UBC-a uključujući gensku heterogenost humane populacije te kompleksnost gen-gen i gen-okolišnih interakcija, značajno otežavaju razumijevanje etiopatogenetskih mehanizama koji vode do razvoja UBC-a. Istraživanja etiopatogeneze upalnih bolesti na životinjskim modelima sežu unatrag gotovo pola stoljeća i potrebno je istaknuti da su upravo ova istraživanja pružila prvi ozbiljan pokušaj rješavanja imunosne osnove upalnih bolesti. Tijekom posljednjih deset godina prepoznata je važnost životinjskih modela bolesti, zabilježen je ubrzani razvoj pokusnih modela te je do danas poznato više od 60 modela UBC-a. Zbog sličnosti s humanim oblicima UBC-a pokusni modeli kolitisa omogućavaju proučavanje ranih događaja vezanih uz međudjelovanje različitih upalnih medijatora, utvrđivanje imunosnih mehanizama, kao i identifikaciju pojedinih gena (tablica 4). Upalni proces crijeva u pokusnim modelima može se razviti: 1) spontano; 2) potaknuto: nakon primjene kemijskog agensa (npr. intrarektalna aplikacija octene kiseline); 3) prijenosom imunosnih stanica iz jednog soja pokusnih životinja u drugi i 4)

genetičkom manipulacijom (selektivne insercije ili delecije određenih gena).

Kao rezultat genetičke manipulacije u određenom broju eksperimentalnih životinja UBC se razvija spontano, a da bismo ih razlikovali od sojeva miševa kod kojih spontane mutacije dovode do razvoja kolitisa, uveden je termin „inducirani mutanti". Upravo ova kategorija danas uključuje najveći broj eksperimentalnih modela, a dobivena saznanja uvelike su pridonijela razumijevanju mehanizama koji vode razvoju kolitisa. Nadalje, genetičkom manipulacijom potvrđena je pretpostavka da će samo mutacije gena koje čine ključne karike u patogenezi dovesti do spontanog razvoja bolesti. Među navedenim modelima ističu se miševi s deficitom IL-2, IL-10 i TGF-β1. Sadlack i sur. su 1993. godine prvi pokazali da otprilike 50 % miševa kojima se inaktivira gen za IL-2 ugine između četvrtog i devetog tjedna života, dok se u ostalih između šestog i petnaestog tjedna razvije kronični oblik kolitisa koji zahvaća kolon, od rektuma do cekuma, a patohistološke promjene gotovo su identične s promjenama prisutnim u humanom obliku kolitisa (124). Miševi s deficitom IL-10 obole od kolitisa, ali za razliku od miševa s deficitom IL-2 upalni proces zahvaća osim kolona i duodenum te proksimlni dio jejunuma (125). U oba modela pokazana je aktivacija CD4+ Th1-stanica, uz smanjenje broja T-regulatornih stanica te se pretpostavlja da ove promjene pokreću mehanizme koji vode razvoju bolesti.

Budući da je etiologija UBC-a kompleksna, a mehanizmi razvoja kolitisa u pojedinome modelu različiti, izbor pokusnog modela važan je korak u praćenju i interpretaciji mehanizama čija se uloga ispituje. Široki spektar manifestacija crijevne upale u poznatim životinjskim modelima govori u prilog velikoj različitosti UBC-a u ljudi. Idealni eksperimentalni model UBC-a trebao bi što bolje imitirati humani oblik ulceroznog kolitisa ili Crohnove bolesti, biti jednostavan u izvođenju te imati visoku reproducibilnost (126). Dosadašnja saznanja upućuju na činjenicu da nema dobrog ili lošeg modela UBC-a, da nema modela na kojem se mogu proučavati sve komponente razvoja UBC-a te stoga izbor prikladnog modela za određeno istraživanje ovisi o segmentu bolesti koji se želi istražiti. Upoznavanje životinjskih modela i kompleksa upalnih medijatora služi kao putokaz za razvoj novih hipoteza i terapijskih strategija.

MODEL CROHNOVE BOLESTI U MIŠA

Model kolitisa u miša induciranog trinitrobenzensulfonskom kiselinom (TNBS-kolitis, model Crohnove bolesti u miša, engl. *Crohn-like colitis in mice*) kemijski je induciran pokusni model upalne bolesti crijeva pri čemu dolazi do oštećenja epitelnih stanica, ulceracija i transmuralne upale koja

Tablica 4 *Odabrani pokusni modeli kolitisa*

Pokusni model kolitisa	Lokalizacija	Upala	Klinička sličnost
Spontani			
C3H/HeJ/Bir miševi	cekum, kolon	akutna, kronična	CB, UK
SAMP1/Yit miševi	ileum	akutna, kronična	CB
Kemijski inducirani			
Octena kiselina	kolon, rektum	akutna	UK
TNBS/etanol	kolon	akutna, kronična	CB
DSS	kolon	akutna, kronična	UK
Ciklosporin A	kolon	akutna, kronična	CB, UK
Oksazolon	kolon	akutna, kronična	UK
Prijenos imunosnih stanica			
prijenos $CD45Rb^{high}$	kolon > ileum	kronični > akutni	CB
Transgenični miševi			
IL-2 - deficijentni miševi	kolon	akutna, kronična	UK
IL-10 - deficijentni miševi	tanko crijevo, kolon	kronična	UK
αβ TCR - deficijentni miševi	kolon	akutna, kronična	UK

CB, Crohnova bolest; UK, ulcerozni kolitis; TNBS, trinitrobenzensulfonska kiselina; DSS, natrijev dekstran sulfat; IL, interleukin; TCR, T - stanični receptor; TGF-β, transformirajući faktor rasta β.

je uglavnom lokalizirana u distalnom dijelu kolona. Rektalna primjena otopine 2,4,6-trinitrobenzensulfonske kiseline (TNBS, $C_6H_3N_3O_9S$, Mr=293.17) u etanolu uzrokuje primarno Th1-oblik imunosnog odgovora, koji je specifičan za CB u ljudi (7, 127). Zbog velike sličnosti kliničkih, histoloških i imunosnih karakteristika kod ljudi te reproducibilnosti u životinja, pogodan je pokusni model za proučavanje ranih događaja vezanih uz nastanak, razvoj i cijeljenje CB-a (127, 128).

Mehanizam djelovanja

Mehanizam toksičnog djelovanja etanolne otopine TNBS-a na crijevnu mukozu nije u cijelosti razjašnjen. Međutim poznato je da etanol u ovoj formulaciji pri rektalnoj primjeni djeluje prije svega kao agens koji razara površinu mukoze crijeva te omogućava prodiranje TNBS-a u dublje slojeve stijenki crijeva. TNBS tada lokalno djeluje kao hapten, vežući se na endogene proteine mukoze kolona, što dovodi do nastanka proteina modificiranih TNBS-om u intersticiju crijeva te na taj način potiče lokalni imunosni odgovor putem aktivacije limfocita T i makrofaga (127, 129). Pritom dolazi i do razgradnje TNBS-a te nastanka veće količine slobodnih kisikovih radikala, što potiče kaskadu citotoksičnih reakcija, dodatno pridonoseći razvoju upalnih procesa koji naposljetku često završavaju staničnom smrću (129). Nadalje, pokazano je da interakcija -NO_2 skupine iz TNBS-a sa specifičnim flavoproteinima, uz prisutnost molekularnog kisika, dovodi do nastanka superoksidnog aniona te vodikova peroksida. Njihovom reakcijom s određenim faktorima, poput iona željeza, dolazi do stvaranja snažnih slobodnih radikala koji mogu izravno oštetiti makromolekule od vitalne važnosti za stanicu, poput DNA, proteina i lipida (130).

Uspostava TNBS-modela kolitisa

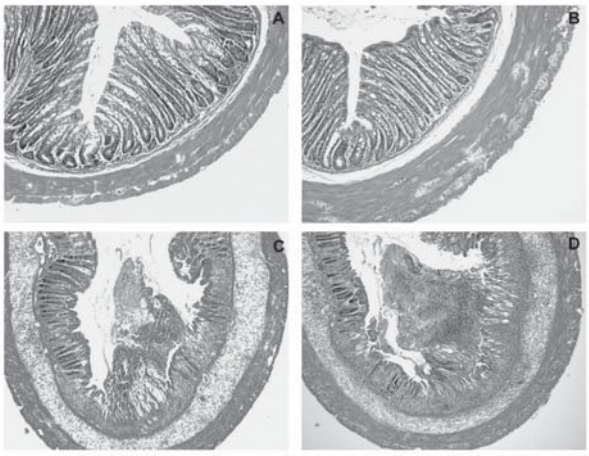
U svojim smo istraživanjima uspostavili TNBS-kolitis u miša s deficitom CD26 (CD26-/-) i divljem tipu miša (C57BL/6), primjenom klizme koja sadržava 5 %-tni TNBS otopljen u 50 %-tnom etanolu u jednakim omjerima. Ovu smo koncentraciju odredili prethodnim pilot-pokusima kao optimalnu, u smislu najvećeg pobolijevanja pokusnih životinja uz najmanju stopu smrtnosti. Klizma je uvedena pokusnoj životinji 4 cm proksimalno od analnog otvora s pomoću polipropilenskog katetera, u općoj anesteziji. Pokusne životinje iz kontrolnih skupina bile su podvrgnute identičnoj proceduri, ali im je rektalno uvedena otopina etanola odnosno fiziološka otopina. Prilikom izvođenja pokusa poštivana su sva načela i propisi rada s laboratorijskim životinjama. Etička komisija Medicinskog fakulteta Sveučilišta u Rijeci prethodno je odobrila sve planirane postupke s laboratorijskim životinjama.

Kolitis se kod pokusnih životinja obaju sojeva manifestirao lošim kliničkim stanjem, gubitkom tjelesne mase, promjenom u konzistenciji stolice u kojoj se mogla vidjeti krv te eventualno prisutnost rektalnog krvarenja. Simptomi su bili najintenzivniji drugog dana od aplikacije otopine TNBS-a u etanolu. Makroskopske promjene također su bile najizraženije drugog dana, a manifestirale su se kao skraćenje i zadebljanje kolona te lokalizirana, žarišna upala završnog dijela debelog crijeva.

Patohistološke karakteristike

Patohistološka analiza potvrdila je postojanje transmuralne upale vrlo slične onoj u humanoj Crohnovoj bolesti. Histomorfometrijska analiza preparata debelog crijeva potvrdila je smanjenje broja kripta, njihovo proširenje te smanjenje dubine u skupinama životinja s induciranim kolitisom. Drugi dan od aplikacije etanolne otopine TNBS-a akutna je faza kolitisa i karakterizirana je skraćenjem i zadebljanjem kolona te lokaliziranim, žarišnim upalnim promjenama ponajviše u završnom dijelu debelog crijeva. Upalne promjene diskontinuiranog su karaktera, s područjima zdravog tkiva između područja zahvaćenih upalom. Navedene promjene zapažene su u oba soja pokusnih životinja kojima je induciran kolitis. Životinje u kontrolnim skupinama tretirane fiziološkom otopinom nisu pokazivale upalne promjene stijenke debelog crijeva. Kod kontrolne skupine životinja tretiranih otopinom etanola u nekim se preparatima drugog dana pokusa moglo uočiti razvijanje upalnog procesa, ali je uvijek bila zahvaćena samo sluznica uz infiltrate granulocita u lamini propriji i pojavu edema, no bez oštećenja površnog epitela, dok kod životinja iz kontrolne skupine tretiranih fiziološkom otopinom nisu zamijećene patohistološke promjene (slika 2A i B).

Pregledom preparata kolona CD26-/- i C57BL/6-miševa u skupinama koje su bile tretirane otopinom TNBS-a u etanolu utvrđene su različite patohistološke promjene. Upalne promjene bile su najintenzivnije drugog dana od primjene otopine TNBS-a u etanolu te je taj dan karakteriziran kao akutna upala, što je u skladu s opažanjima u literaturi (90, 127, 131). Intraluminalna aplikacija etanolne otopine TNBS-a



Slika 2 *Patohistološke promjene kolona u CD26-deficijentnom (A, C) i C57BL6 (B, D) soju miša. A, B: zdrava sluznica kolona dva dana nakon primjene fiziološke otopine. C, D: upalne promjene dva dana nakon primjene etanolne otopine TNBS-a (7). Parafinski preparati pripremljeni su prema standardnom protokolu i obojeni hematoksilin-eozinom (7). Povećanje 10x (A, B), 4x (C, D)*

kod životinja obaju sojeva izazvala je nastajanje istog tipa promjena u stijenci kolona (slika 2C i D). Može se uočiti dijelom nekrotična sluznica, obilno prožeta neutrofilnim granulocitima, kao i raspadnutim leukocitima, uz žarišna krvarenja i nastanak nekrotičnog detritusa. Promjene ne zahvaćaju sluznicu cijelog lumena crijeva, već na nekim mjestima postoji održani epitel uz normalni izgled kripta. U području podsluznice postoji izražen edem uz granulocitnu infiltraciju tkiva. Mogu se uočiti brojni eritrociti te nakupljanje fibrina. Infiltrati upalnih stanica nalaze se i u području obaju slojeva mišićnice.

Na preparatima kolona obaju sojeva životinja u kasnijim danima pokusa vidljivo je vrlo brzo cijeljenje sluznice uz postepenu obnovu kripta i površnog epitela, smanjenje i nestanak upalnog infiltrata te gubitak edema. Stanice koje nalazimo tijekom cijeljenja u lamini propriji mononuklearnog su tipa. Čest su nalaz nakupine limfatičnog tkiva i u podsluznici i u lamini propriji.

MODEL ULCEROZNOG KOLITISA U MIŠA

Model kolitisa induciran primjenom natrijeva dekstran sulfata (DSS) *per os* relativno je dugo poznati model koji se zbog niza prednosti često rabi za proučavanje patogeneze UK. Prednosti ovog modela očituju se u jednostavnosti induciranja kolitisa i pouzdanosti s obzirom na vrijeme pojavljivanja, u intenzitetu kliničkih simptoma i patohistoloških promjena te njihove sličnosti s promjenama u humanom obliku UK. Kombiniranjem različitih koncentracija i dužine primjene DSS-otopine može se izazvati akutni ili kronični oblik kolitisa, a dugotrajna primjena niskih doza dovodi do razvoja malignih bolesti kao što su adenom, adenokarcinom ili papilom. Slijed događaja u razvoju karcinoma identičan je sa slijedom humanog oblika karcinoma kolona, a isto kao i kod ljudi, na pokusnome modelu pokazan je pozitivan odgovor na standardnu terapiju UK kao što su metronidazol (132), 5-aminosalicilati (133), ciklosporin (134) i anti-TNF-terapija (135).

Akutni kolitis induciran DSS-om prvi je put uspostavljen 1985. godine na štakoru (136), a nakon toga je prilagođen i primijenjen i u miša (137), dok je model kroničnog kolitisa prvi put uspostavljen 1992. godine u hrčka (138). Zbog niza prednosti danas je DSS-model jedan od najčešćih eksperimentalnih modela koji se rabe za izazivanje kolitisa u miša, hrčka, štakora ili gvinejskih praščića. Pokazano je da DSS-kolitis može biti izazvan i u miševima s deficitom Rag-2 te se pretpostavlja da DSS dovodi do oštećenja integriteta sluznice crijeva i gubitka njezine osnovne funkcije, a imunosna reakcija sekundarna je pojava. U akutnoj fazi DSS-kolitisa dolazi do aktivacije Th1-imunosnog odgovora, ali kasnije, u kroničnoj fazi upale, pojavljuje se kombinirani Th1/Th 2-odgovor. U oba slučaja, kao odgovor na oštećenje, dolazi do lučenja velikih količina TNF-α i IL-6 (139).

Dekstran je složeni polimer glukoze koji sintetiziraju bakterije, najčešće Leuconostoc spp i Streptococcus spp iz saharoze (140). Građen je od osnovnoga, glavnog lanca i pobočnih grana, a molekulska mu se masa kreće od 5000 pa sve do 1400000 Da. DSS je derivat dekstrana koji nastaje kao rezultat esterifikacije s klorsulfonskom kiselinom, a sumpor čini otprilike 17 % molekule, što odgovara omjeru dviju sulfatnih skupina na jednu glukoznu jedinicu molekule dekstrana te time raspon molekulske mase dekstrana utječe na konačnu molekulsku masu DSS-a. Kitajima i sur. (141) pokazali su varijacije u intenzitetu i lokalizaciji kolitisa nakon primjene 5 %-tne otopine DSS-a različitih molekulskih masa (5000, 40000 i 500000 Da) tijekom sedam dana. Utvrđeno je da je intenzitet kolitisa bio najveći nakon primjene DSS-a molekulske mase 40000 Da, a da se kolitis nije mogao izazvati nakon primjene DSS-a molekulske mase 500000 Da. Nadalje, ispitivane su karakteristike kolitisa izazvane 4 %-tnom otopinom DSS-a

molekulske mase 9000 - 20000 Da u odnosu prema 36000 - 50000 Da u Sprague-Dawley štakora te su također utvrdili razlike u intenzitetu upalnog procesa. Temeljem navedenoga zaključeno je da je molekulska masa DSS-a važan čimbenik koji utječe na razvoj, lokalizaciju, proširenost i intenzitet upalnog procesa (133).

Budući da je pokazano da različiti mišji sojevi imaju različitu osjetljivost na DSS, odnosno razvijanje bolesti, pretpostavlja se da su genetski i imunosni čimbenici u razvoju kolitisa izrazito kompleksni te ih treba istražiti, a DSS-model, zbog niza prednosti, jedan je od životinjskih modela koji pruža dobru mogućnost za takva istraživanja.

Mehanizam djelovanja

Precizan mehanizam kojim DSS dovodi do razvoja kolitisa nije potpuno razjašnjen. Temeljem dosadašnjih saznanja predloženo je nekoliko mehanizama prema kojima DSS primarno uzrokuje promjene na razini sluznice crijeva, a imunosna je reakcija sekundarni proces (137). Prvi predloženi mehanizam sastoji se u pretpostavci da DSS utječe na propusnost sluznice, a u prilog tomu govori i činjenica da je prva promjena koja nastaje kao rezultat primjene DSS-a gubitak bazalnih kripta te njihovo razdvajanje od muskularis mukoze. Kitajima i sur. (141) pokazali su na mišjem modelu DSS-kolitisa da je povećana mukozna propusnost prvi događaj nakon kojega slijedi nakupljanje upalnih stanica, odnosno razvoj upalnog infiltrata. Ova su saznanja Venkatraman i sur. (142) potvrdili i na štakorskome modelu kolitisa. Poritz i sur. (143) otišli su korak dalje i pokazali 50 %-tno sniženje ekspresije proteina čvrstih veza (engl. *tight junction protein 1*), već nakon jednodnevne primjene DSS-a, a razvojem kolitisa, odnosno petog dana eksperimentalnog perioda, primjenom Western blot tehnike, ovaj se protein više nije mogao detektirati, što dodatno upućuje na ulogu povećane propusnosti sluznice u patogenezi DSS-kolitisa.

Drugi predloženi mehanizam djelovanja DSS-a sastoji se u njegovoj direktnoj toksičnosti za sluznicu crijeva, ovisnoj o koncentraciji, što vodi k promjeni razine integrin α4 i M290 podjedinica na epitelnim stanicama remeteći njihovu interakciju s γδ-intraepitelnim limfocitima (144), a za koje se pretpostavlja da imaju zaštitnu ulogu u borbi protiv različitih čimbenika koji mogu oštetiti sluznicu crijeva, uključujući i DSS (144, 145).

Navedena istraživanja potvrđuju pretpostavku da su razaranje sluznice kao fizičke zapreke i posljedično povećanje propusnosti nužni koraci koji prethode infiltraciji upalnih stanica u dublje slojeve sluznice crijeva te da primarno oštećenje nastaje na razini epitelnih stanica nakon čega slijedi aktivacija imunosnog odgovora i razvoja kolitisa posredovanog DSS-om (146). Uloga bakterijske flore u razvoju DSS-kolitisa također je nejasna budući da je pokazano da primjena metronidazola i ciprofloksacina ublažava simptome akutnog, ali ne i kroničnog DSS-kolitisa (132).

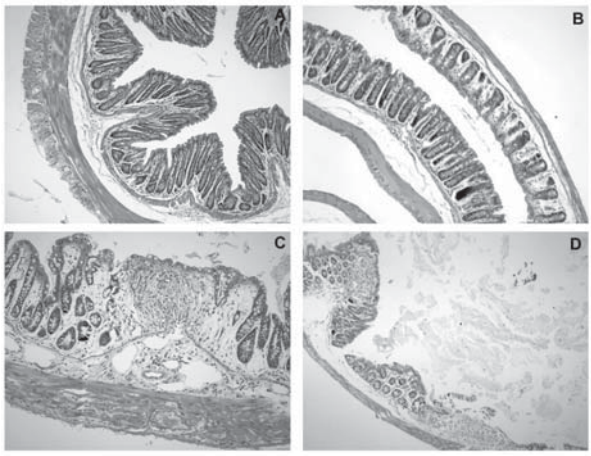
Uspostava DSS-modela kolitisa

U našim istraživanjima model induciranog kolitisa izazvan je u divljem tipu miša (C57BL/6) i u CD26-/- miševima, natrijevim dekstran-sulfatom (DSS; MW 50 kDa; MP Biomedicals, SAD), otopljenim u vodi za piće u koncentraciji od 3 % (131). Koncentracija otopine DSS-a odabrana je na osnovi prethodnih studija (141) te potvrđena pilot-pokusom. Otopinu DSS-a miševi su pili *ad libitum* sedam dana, a svježa se otopina pripremala svakoga drugog dana. Kontrolnu skupinu činili su miševi (C57BL/6 i CD26-/-) koji su tijekom sedam dana konzumirali pitku vodu te su žrtvovani u istim vremenskim razmacima kao i miševi kod kojih je izazvan kolitis.

Uspostava, razvoj i cijeljenje kolitisa u miševa tretiranih DSS-om u odnosu prema kontrolnoj skupini pratili su se na temelju više različitih parametara kao indirektnih markera upale koji uključuju: dnevno mjerenje tjelesne mase, pregled konzistencije stolice i pojave rektalnog krvarenja. Nakon žrtvovanja izolirano je debelo crijevo te je izmjerena njegova dužina i masa, a dobiveni podaci bili su parametri kojima su se pratile lokalne promjene, odnosno promjene nastale zbog razvoja kolitisa odnosno upale.

Patohistološke karakteristike

Patohistološkom analizom sluznice debelog crijeva utvrđeno je da primjena 3 %-tne otopine DSS-a tijekom sedam dana dovodi do razvoja epitelnog oštećenja, ulceracija, promjene broja kripta te upale u obje ispitivane skupine miševa (slika 3C i D), koje nisu vidljive u kontrolnim skupinama (slika 3A i B). Kao i kod humanog oblika kolitisa upalni je proces ograničen na kolon, s vidljivim područjima krvarenja i ulceracijama. Upala uglavnom zahvaća mukozu i u pojedinim se dijelovima širi u dublje slojeve, submukozu i laminu muskularis mukoze. Površinske ulceracije, edem, distorzija kripta i apscesi udruženi



Slika 3 *Patohistološke promjene kolona u CD26-deficijentnom (A, C) i C57BL6 (B, D) soju miša. A, B: zdrava sluznica kolona. C, D: upalne promjene sedam dana nakon primjene otopine DSS-a. Parafinski preparati pripremljeni su prema standardnom protokolu i obojeni hematoksilin-eozinom (7). Povećanje 4x (A), 20x (B, C, D)*

s infiltracijom mukoze i submukoze upalnim stanicama glavne su karakteristike prisutnog upalnog procesa. U ranim fazama razvoja kolitisa, odnosno trećeg dana nakon početka davanja otopine DSS-a počinju se javljati prve promjene u lamini propriji sluznice debelog crijeva. Pojavljuju se upalne stanice, uz edem što dovodi do smanjenja broja kripta na milimetar sluznice crijeva. Tijekom prvog tjedna upala zahvaća mukozu i submukozu, a često je uključena i tunica muscularis. Sedmi dan pokusa uočava se akutni upalni odgovor i površne erozije sluznice kolona s eksfolijacijom epitela te pojavom edema, hemoragije i infiltracije polimorfonuklearnim leukocitima u području lamine proprije. Žlijezde su plitke i široko otvorene. Napredovanjem bolesti javljaju se područja potpuno bez kripta s jakim upalnim infiltratom. Na tim je mjestima uočen nedostatak površnih epitelnih stanica. Upala je okarakterizirana transmuralnom infiltracijom s fokalnom nekrozom, infiltracijom neutrofilima, opsežnom fibrozom i zadebljanjem stijenke. Jako izraženi limfni čvorići i difuzni infiltrati limfocita nalaze se u lamini propriji, ali i u podsluznici uz oštećenje lamine muskularis mukoze.

Patohistološke promjene smanjuju se za vrijeme cijeljenja kolitisa: tijekom trećeg tjedna histološke promjene u kolonu pokazuju povlačenje akutnog upalnog odgovora i prisutnost određene količine kroničnog upalnog infiltrata u mukozi i submukozi. Javlja se i fibrozno cijeljenje oštećenja uz postupno povećanje broja kripta na kontrolnu veličinu. U kroničnom upalnom infiltratu nalaze se limfociti, plazma-stanice, a u nekim slučajevima nalazimo i male granulome u lamini propriji.

ZAKLJUČAK

UBC su važan javnozdravstveni problem današnjice, a unatoč brojnim pretkliničkim i kliničkim istraživanjima etiopatogeneza ovih bolesti ostaje nedovoljno poznata, time i terapija nedovoljno učinkovita. Dosadašnja su istraživanja upozorila na potencijalno važnu ulogu DPP IV/CD26 i porodice proteina sličnih DPP IV u mehanizmima nastanka upale u probavnom sustavu, kao i mogućnost manipulacije aktivnosti ovih molekula u terapijske svrhe. Radi dobivanja novih saznanja na ovom području, ključnu ulogu imaju životinjski modeli UBC-a, koji nam omogućuju istraživanja specifičnih karakteristika pojedinih aspekata ovih kroničnih bolesti. Daljnja istraživanja na ovom području trebala bi dovesti do razjašnjavanja mehanizama nastanka UBC-a, a time i do unaprjeđenja terapijskih mogućnosti, što bi u konačnici dovelo do znatnog poboljšanja kvalitete života oboljelih.

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Summary

DIPEPTIDYL PEPTIDASE IV IN INFLAMMATORY BOWEL DISEASES (DPP IV/CD26)

Inflammatory bowel diseases (Crohn's disease, ulcerative colitis, undetermined colitis) are a group of chronic autoimmune inflammatory diseases distinguished by recurrent inflammation of various parts of the gastrointestinal (GI) system and presenting a significant public health problem. Despite large basic and clinical research, the aetiology of these diseases and the pathogenesis of inflammation itself remain elusive. Previous studies have confirmed a causal relationship between mediators of inflammatory response and molecules involved in the regulation of their biological activity, especially proteases. The aim of this review is to summarise earlier findings on different aspects of inflammatory bowel diseases, paying particular attention to the involvement of dipeptidyl peptidase IV (CD26 molecule, DPP IV/CD26) in the etiopathogenesis of inflammatory processes in the GI tract. Animal studies of colitis have significantly contributed to the understanding and treatment of these diseases, investigations of ulcerative colitis (DSS-colitis) and Crohn's disease (TNBS-colitis) on the murine model in particular.

KEY WORDS: *animal models of colitis, CD26 molecule, Crohn's disease, DSS-colitis, TNBS-colitis, ulcerative colitis*

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Technical Paper

IZVJEŠĆE CENTRA ZA KONTROLU OTROVANJA ZA RAZDOBLJE OD 1. SIJEČNJA DO 31. PROSINCA 2011. / REPORT OF THE POISON CONTROL CENTRE FOR THE PERIOD 1 JANUARY - 31 DECEMBER 2011

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Ovo izvješće Centra za kontrolu otrovanja Instituta za medicinska istraživanja i medicinu rada nastavak je izvješća objavljenih u prethodnim brojevima ovog časopisa. U njemu se navode osnovni statistički podaci o broju poziva primljenih u Centru za kontrolu otrovanja tijekom dvanaestomjesečnog razdoblja od siječnja do prosinca 2011. godine, uključujući podatke o tražiteljima informacija, kao i podatke o bolesnicima i osobinama otrovanja.

Tijekom navedenog razdoblja u Centru je zabilježeno 1560 poziva kojima su se tražile informacije o 1595 slučajeva. U većini slučajeva (89 % od ukupnog broja poziva) tražitelji informacija bili su zdravstveni radnici (liječnici i medicinske sestre), a samo u 11 % slučajeva službene osobe drugih profila (npr. policija) ili privatne osobe.

Prosječna dob bolesnika bila je 14 godina (medijan 3,5 godina), u rasponu od novorođenačke dobi do 91 godinu, a najzastupljenije dobne skupine bile su dojenčad i predškolska djeca (od rođenja do uključivo pete godine života; 46 % od ukupnog broja bolesnika u kojih je dob bila poznata) i odrasle osobe (40 %). Kao i prethodnih godina, u svim dobnim skupinama osim u adolescenata bio je nešto više zastupljen muški spol (57 % muških osoba *vs*. 43 % ženskih osoba). U adolescenata bilo je više slučajeva otrovanja/izloženosti u djevojaka nego u mladića (74 % *vs*. 26 %).

Ukupni broj slučajeva bio je veći tijekom proljeća i ljeta (u prosjeku 140 slučajeva na mjesec) u odnosu prema hladnom dijelu godine, tj. siječanj, veljaču, studeni i prosinac (u prosjeku 108 slučajeva na

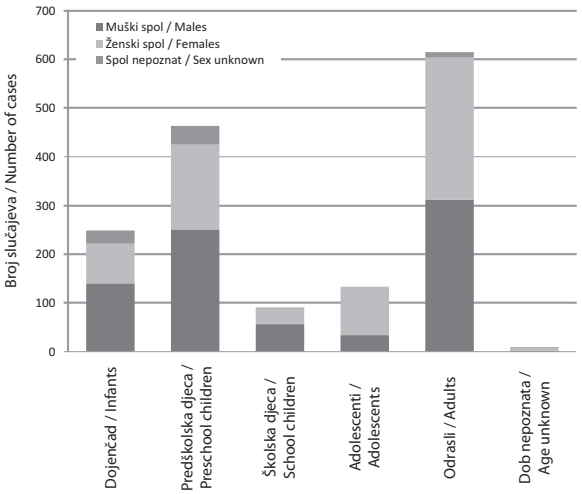
This report of the Poison Control Centre at the Institute for Medical Research and Occupational Health continues previous reports published in this journal. It brings basic annual statistics that include the number of calls received by the Poison Control Centre from January to December 2011, as well as the information on callers, patient and poisoning profiles.

In 2011, the Poison Control Centre received 1560 phone calls reporting 1595 cases. In most cases (89 % of total number of calls), the callers/information users were health care professionals (medical doctors and nurses) and only 11 % were persons of other profiles (police and general public).

The average age of patients was 14 years (median 3.5 years), ranging from newborns to 91 years, and the most prevalent age groups were infants and preschool children (from birth to 5 years; 46 % of the total number of patients with known age) and adults (40 %). As noted in previous years, men were slightly more common patients than women (57 % *vs.* 43 %) in all age groups, except for adolescents. In adolescents, more women than men were reported for poisoning (74 % *vs*. 26 %).

More reports were received in the spring and summer (average of 140 cases per month) than in January, February, November, and December (average of 108 case per month). Their distribution according to cause/exposure was even throughout the year, except for pesticides and inedible plants.

The most prevalent substances were drugs (41 % of all cases) and household chemicals (32 % of all



Distribucija slučajeva otrovanja u ljudi prema dobi i spolu /Age andsexdistributionof human exposurecases
Dobne skupine definirane su ovako: dojenčad – do uključivo23 mjeseca života; predškolska djeca – od 2 do uključivo 5godina; školska djeca – od 6 do uključivo 12 godina;adolescenti – od 13 do uključivo 17 godina; odrasli – navršenih18 godina i stariji / Age groups are defined as: infants–till23 months; preschoolchildren– 2 to 5 years; schoolchildren– 6 to 12 years; adolescents– 13 to 17 years; adults– 18 and more years

mjesec). Distribucija slučajeva prema uzrocima nije se znatnije razlikovala tijekom godine, izuzevši izloženost pesticidima i nejestivim biljkama.

Najzastupljenije tvari koje su uzrokovale otrovanja bile su lijekovi (41 % od ukupnog broja slučajeva s poznatim uzrokom otrovanja) i kućne kemikalije (32 % od ukupnog broja slučajeva s poznatim uzrokom otrovanja). Od lijekova najzastupljeniji su bili psihoaktivni lijekovi (43 % od ukupnog broja slučajeva otrovanja lijekovima) uključujući neuroleptike, benzodiazepine, antidepresive i hipnotike, zatim analgetici i nesteroidni protuupalni lijekovi (18 %) i lijekovi za kardiovaskularne bolesti (11 %).

Izloženost pesticidima bila je najviša od travnja do lipnja (u prosjeku 19 slučajeva na mjesec) s najvećim brojem slučajeva u svibnju (21 slučaj). Tijekom ostalog razdoblja u godini prosječni broj slučajeva izloženosti pesticidima bio je znatno niži, u prosjeku 6 slučajeva na mjesec. Od ukupno 109 osoba izloženih pesticidima najveći broj imao je blage simptome (54 %) ili je bio asimptomatski (40 %). Samo u 5 % bolesnika (5 slučajeva) zabilježeni su teži simptomi, ali bez smrtnih ishoda. Pesticidi koji su uzrokovali teže kliničke slike bili su antikoagulantni rodenticid, organofosforni i piretroidni insekticidi, herbicid iz skupine derivata fenoksioctene kiseline i jedan neidentificirani herbicid.

Uzroci otrovanja ili izloženosti u ljudi / Causes of human poisoning or exposure

Tvar / Substance	Broj slučajeva / Number of cases (% od ukupno / % of total)
Lijekovi / Drugs	631 (40.5)
Kućne kemikalije / Household chemicals[a]	495 (31.8)
Pesticidi / Pesticides[b]	109 (6.9)
Industrijske kemikalije / Industrial chemicals	106 (6.8)
Biljke / Plants[c]	52 (3.3)
Sredstva ovisnosti / Drugs of abuse	14 (0.9)
Životinje / Animals	10 (0.6)
Hrana / Food[e]	7 (0.4)
Gljive / Mushrooms	6 (0.4)
Ugljikov monoksid / Carbon monoxide	2 (0.1)
Alkohol / Alcohol	1 (0.06)
Kombinacije / Combinations[d]	47 (3.0)
Ostalo / Miscellaneous	59 (3.8)
Nepoznato / Unknown	20 (1.3)
UKUPNO / TOTAL	1559 (100.0)

Ukupni broj slučajeva (1559) ne uključuje 34 poziva u kojima su se tražile informacije iz edukativnih razloga / The total number of cases (1559) does not include 34 phone calls asking information for educational reasons
[a] Uključujući sredstva opće uporabe koja sadržavaju pesticide / Including pesticide-containing chemicals sold to general public
[b] Ne uključuje sredstva opće uporabe koja sadržavaju pesticide / Does not include pesticide-containing chemicals sold to general public
[c] Bez gljiva / Mushrooms excluded
[d] Istodobna izloženost ili otrovanje dvjema ili s više skupina tvari (najčešće lijekovima i alkoholu) / Concomitant exposure to or poisoning with two or more substance categories (mostly drugs and alcohol)

cases). Most commonly reported drugs were psychoactive drugs (43 % of all drug poisonings), including neuroleptics, benzodiazepines, antidepressants and hypnotics, followed by analgesics and non-steroidal anti-inflammatory drugs (18 %) and cardiac drugs (11 %).

Exposure to pesticides was higher from April to June (average of 19 cases per month), with a peak in May (21 cases) and much lower over the rest of the year (average of 6 cases per month). Most of the 109 patients exposed to pesticides had only mild symptoms (54 %) or were asymptomatic (40 %). Only 5 patients developed serious symptoms, but with no lethal outcomes. Pesticides causing serious clinical

Broj slučajeva prema uzrocima otrovanja ili izloženosti u pojedinim dobnim skupinama / Number of cases according to causes of poisoning or exposure in different age groups

Tvar / Substance	Broj slučajeva / Number of cases (% od ukupno / % of total)		
	Dojenčad i predškolska djeca / Infants and preschool children	Školska djeca i adolescenti / School children and adolescents	Odrasli / Adults
Lijekovi / Drugs	250 (35.1)	138 (61.9)	242 (39.4)
Kućne kemikalije / Household chemicals	340 (47.8)[a]	28 (12.6)[b]	121 (19.7)[c]
Pesticidi / Pesticides	31 (4.4)	3 (1.3)	75 (12.2)
Industrijske kemikalije / Industrial chemicals	23 (3.2)	8 (3.6)	74 (12.1)
Biljke / Plants	40 (5.6)	5 (2.2)	7 (1.1)
Sredstva ovisnosti / Drugs of abuse	-	10 (4.5)	4 (0.7)
Životinje / Animals	-	4 (1.8)	5 (0.8)
Hrana / Food[c]	5 (0.7)	1 (0.4)	1 (0.2)
Gljive / Mushrooms	2 (0.3)	2 (0.9)	2 (0.3)
Ugljikov monoksid / Carbon monoxide	-	1 (0.4)	1 (0.2)
Alkohol / Alcohol	-	-	1 (0.2)
Kombinacije / Combinations	-	9 (4.0)	38 (6.2)
Ostalo / Miscellaneous[d]	19 (2.7)	10 (4.5)	29 (4.7)
Nepoznato / Unknown	2 (0.3)	4 (1.8)	14 (2.3)
UKUPNO / TOTAL	712 (100.0)	223 (100.0)	614 (100.0)

Dobne skupine definirane su na slici. Ukupni broj slučajeva (1549) ne uključuje 34 poziva u kojima su se tražile informacije iz edukativnih razloga, te 10 slučajeva u kojima je dob bila nepoznata / Age groups are defined on Figure. The total number of cases (1549) does not include 34 phone calls asking information for educational reasons and 10 cases in which age was unknown

[a] najzastupljenija su bila sredstva za pranje i čišćenje, higijensko-kozmetički proizvodi, igračke i školski pribor, silikagel, baterije, i živa iz toplomjera / the most prevalent were detergents and cleaning agents, cosmetics, toys and school accessories, silica gel, batteries, and thermometer mercury

[b] najzastupljenija su bila sredstva za pranje i čišćenje, živa iz toplomjera, higijensko-kozmetički proizvodi, igračke i školski pribor, i silikagel / the most prevalent were detergents and cleaning agents, thermometer mercury, cosmetics, toys and school accessories, and silica gel

[c] najzastupljenija su bila sredstva za pranje i čišćenje, kiseline i lužine, higijensko-kozmetički proizvodi, živa iz toplomjera, i insekticidi i repelenti u kućanstvu / the most prevalent were detergents and cleaning agents, corrosives, cosmetics, thermometer mercury, and household pesticides and repellents

[d] uključujući suzavac, sredstva za gašenje požara, požarne plinove, gnojiva, i petarde / including tear gas, fire-extinguishing substances, combustion gases, fertilisers, and firecrackers

Nije primijećen uobičajeni trend povećanja broja slučajeva ingestije nejestivih gljiva tijekom kasnog ljeta i jeseni, možda zbog oskudnijih oborina tijekom tog razdoblja u 2011. godini. Ingestija nejestivih biljaka, među ostalima adama, božićne zvijezde, oleandera, mahonije, hortenzije, bazge i tise te perkutana izloženost agavi, bila je najčešća u srpnju i kolovozu (ukupno 18 zabilježenih slučajeva).

Daleko najčešći put unosa bio je ingestijom (81 % od ukupnog broja slučajeva u kojima je put izloženosti bio poznat), a zatim udisanjem (8 %).

Od ukupnog broja slučajeva u kojih je klinička slika bila poznata (1496 slučajeva) 48 % bilo je asimptomatsko, 42 % imalo je samo blage simptome (primjerice iritaciju probavnog trakta, glavobolju ili iritaciju kože i dišnog sustava), a 6 % slučajeva imalo

symptoms were an anticoagulant rodenticide, organophosphate and pyrethroid insecticides, a herbicide containing phenoxyacetic acid derivative and one unidentified herbicide.

This year we did not observe the usual increase in inedible mushroom ingestion over the late summer and autumn, possibly due to scarce rainfall. Ingestions of inedible plants such as giant upright elephant ear, poinsettia, oleander, mahonia, hortensia, elder and yew, as well as percutanous exposure to agave, were more frequent in July and August (18 reported cases in total).

Among cases with known route of exposure, ingestion was by far the most prevalent (81 %), followed by inhalation (8 %).

Put izloženosti u registriranim slučajevima otrovanja ili izloženosti u ljudi / Route of exposure in registered cases of human poisoning or exposure

Put izloženosti / Route of exposure	**Broj slučajeva / Number of cases (% od ukupno / % of total)**
Ingestijom / Ingestion	1268 (81.3)
Inhalacijom / Inhalation	127 (8.1)
Kožom / Dermal	37 (2.4)
Konjunktivom oka / Conjunctival	13 (0.8)
Ugriz ili ubod / Bite or sting	13 (0.8)
Parenteralno / Parenteral	8 (0.5)
Rektalno / Rectal	5 (0.3)
Nazalno / Nasal	4 (0.3)
Gingivalno / Gingival	2 (0.1)
Strano tijelo / Foreign body	1 (0.1)
Majčino mlijeko / Mother's milk	1 (0.1)
Više putova / Combination	60 (3.8)
Nepoznato / Unknown	20 (1.3)
UKUPNO / TOTAL	1559 (100.0)

Ukupni broj slučajeva (1559) ne uključuje 34 poziva u kojima su se tražile informacije iz edukativnih razloga / The total number of cases (1559) does not include 34 phone calls asking information for educational reasons

je teške simptome (teži poremećaji središnjega živčanog sustava, korozivna oštećenja gastrointestinalnog trakta, teški dišni simptomi). Zabilježena su dva smrtna slučaja u odraslih osoba, jedan zbog suicida ingestijom antifriza te drugi zbog suicida ingestijom sredstva za zaštitu bilja koje sadržava bentazon i dikambu. Za 63 slučaja (4 %) nisu bili dostupni podaci o kliničkim simptomima. Najčešći uzroci težih oblika otrovanja bili su lijekovi (u 43 od 89 slučajeva s težom kliničkom slikom), i to ponajprije psihoaktivni, a zatim kućne kemikalije (11 slučajeva), pesticidi (5 slučajeva), industrijske kemikalije (5 slučajeva) te kombinacije dviju ili više skupina tvari, najčešće lijekova i alkohola (8 slučajeva).

Omjer između suicidalnih i akcidentalnih otrovanja u ukupnom broju slučajeva bio je veći od 1:3. U skupini adolescenata taj omjer bio je obrnut, s oko 3 i pol puta više suicidalnih u odnosu prema akcidentalnim otrovanjima. U odraslih zabilježen je vrlo sličan omjer akcidentalnih i suicidalnih otrovanja (230 *vs*. 218 slučajeva). Šezdeset i pet posto slučajeva otrovanja kod adolescentica bili su pokušaji suicida.

Profesionalna izloženost otrovnim tvarima zabilježena je u 48 slučajeva, većinom muškog spola (43 muškarca i 5 žena). Za razliku od protekle tri godine tijekom kojih je na prvome mjestu bila izloženost radnika plinovima i dimovima koji se oslobađaju tijekom radnog procesa, tijekom 2011. godine kao najučestalija zabilježena je izloženost kiselinama i lužinama, a na drugome mjestu bila je

From the total number of cases with known clinical symptoms (1496), 48 % were asymptomatic, 42 % had only mild symptoms (such as gastrointestinal irritation, headache, or irritation of the skin and respiratory system), and 6 % had severe symptoms (such as serious disturbances of the central nervous system, corrosive injuries of the gastrointestinal tract, or severe respiratory symptoms). Two fatal outcomes were recorded in adults, one due to suicidal ingestion of antifreeze and the other due to suicidal ingestion of a plant protection product containing bentazone and dicamba. In 63 cases (4 %) data on clinical symptoms were not available. The most prevalent causative agents responsible for severe clinical presentations in non-occupational exposures were drugs (43 out of 89 cases with severe clinical presentation), mostly psychoactive, then household chemicals (11 cases), pesticides (5 cases), industrial chemicals (5 cases), and combinations of substances, mostly drugs and alcohol (8 cases).

The ratio between suicidal and accidental poisonings for all cases was greater than 1:3. In the adolescent group it was reverse, with about 3.5 times more suicidal than accidental poisonings. A very similar number of accidental and suicidal poisonings was recorded in adults (230 and 218 cases, respectively). Attempted suicide accounted for 65 % of all adolescent poisonings in women.

Occupational exposure to toxic substances was reported in 48 cases (43 men and 5 women). Unlike

Okolnosti otrovanja ili razlog traženja informacije / Circumstances of exposure cases or type of information request

Okolnosti otrovanja/razlog traženja informacije Circumstances of exposure/ type of information request	**Broj slučajeva / Number of cases (% od ukupno / % of total)**
Slučajno / Accidental	1045 (65.6)
Suicidalno / Suicidal	326 (20.5)
Profesionalno / Occupational	48 (3.0)
Abuzus / Substance abuse	36 (2.3)
Edukativni razlog poziva / Educational reason	34 (2.1)
Iatrogeno / Iatrogenic	19 (1.2)
Psihijatrijski bolesnik / Psychiatric patient	18 (1.1)
Napad /Assault	9 (0.6)
Nuspojava lijeka / Drug side effect	8 (0.5)
Nepoznato / Unknown	50 (3.1)
UKUPNO / TOTAL	1593 (100.0)

Ukupni broj slučajeva ne uključuje 2 poziva u kojima su se tražile informacije o izloženosti u životinja / Does not include 2 phone calls asking information regarding exposure in animals

Klinička slika otrovanja po dobnim skupinama / Medical outcome by patient age

Simptomi / Symptoms	**Dojenčad / Infants**	**Predškolska djeca / Preschool children**	**Školska djeca i adolescenti / School children and adolescents**	**Odrasli / Adults**	**Nepoznata dob / Age unknown**	**UKUPNO / TOTAL (% od ukupno / % of total)**
Nema / Asymptomatic	181	346	71	148	3	749 (48.0)
Blagi / Mild	57	99	125	369	6	656 (42.1)
Teški / Severe	2	4	17	66	-	89 (5.7)
Smrt / Death	-	-	-	2	-	2 (0.1)
Nepoznato / Unknown	9	10	10	29	1	63 (4.0)
UKUPNO / TOTAL	249	463	223	614	10	1559 (100.0)

Ukupni broj slučajeva (1559) ne uključuje 34 poziva u kojima su se tražile informacije iz edukativnih razloga / The total number of cases (1559) does not include 34 phone calls asking information for educational reasons

izloženost plinovima i dimovima. Profesionalno otrovanje bilo je praćeno težom kliničkom slikom u pet slučajeva, i to zbog izloženosti lužnatom dezinficijensu za automatske muzilice, amonijaku, dimovima pri zavarivanju, nitrolaku te vodikovu peroksidu. Nije bilo profesionalnih otrovanja sa smrtnim ishodom.

Samo dva poziva odnosila su se na izloženost otrovnoj tvari u životinja. Tražene su informacije u vezi s izloženosti veterinarskom insekticidu koji sadržava imidakloprid i permetrin u jedne mačke te insekticidu za komunalnu higijenu na bazi imidakloprida u jednog psa.

the previous three years in 2011, occupational exposure to fumes and gases came second to exposure to acids and alkali. Severe occupational poisoning was reported in five cases due to exposure to alkaline disinfectant for automated milking machine, ammonia, welding fumes, nitrocellulose lacquer, and hydrogen peroxide. No occupational poisoning was fatal.

Only two calls were related to exposure in animals, i.e. to veterinary insecticide containing imidacloprid and permethrin in one cat and to a public health insecticide containing imidacloprid in one dog.

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IN MEMORIAM

KATJA WILHELM (1928.-2011.)

Dr. sc. Katja Wilhelm (rođ. Kuiš) rođena je u Splitu 19. ožujka 1928., a umrla u Zagrebu 2. srpnja 2011. Radila je u Laboratoriju za toksikologiju Instituta za medicinska istraživanja i medicinu rada u Zagrebu od 1958. godine. Apsolvirala je na zagrebačkom Poljoprivredno-šumarskom fakultetu 1950. godine i diplomirala biologiju na Prirodoslovno-matematičkom fakultetu Sveučilišta u Zagrebu godine 1955. Doktor medicinskih znanosti iz područja farmakologije postaje 1965. obranivši doktorski rad „Biološka svojstva nekih novih oksima – reaktivatora kolinesteraze“. Bila je voditeljica Laboratorija za toksikologiju od 1969. do umirovljenja 1982. godine. Usavršavala se u Guildfordu (Velika Britanija) 1970. i 1971. godine. Bila je republički koordinator projekta “Pesticidi” u okviru SEV-a, istraživač na projektima koje je financirala Komisija za medicinsko-naučna istraživanja (KOMNIS) čije je sjedište bilo u Vojnomedicinskoj akademiji u Beogradu i član Znanstvenog vijeća Sveučilišta u Zagrebu.

Nakon umirovljenja bavila se humanitarnim radom. Bila je predsjednica humanitarne organizacije “Djeca prva” u kojoj je sudjelovala u provođenju psihosocijalnih programa za obitelji izbjeglih i prognanih. Bila je i prva predsjednica zagrebačke podružnice “Hrvatska udruga leukemija i limfomi”. Sudjelovala je u svim programima te udruge i u osnivanju podružnica po cijeloj Hrvatskoj. Uvela je posjećivanje hematoloških bolesnika koji se tijekom Božića zateknu u bolnicama. Prigodom desete obljetnice osnivanja „Hrvatska udruga leukemija i limfomi“ dodijelila joj je plaketu kao zaslužnoj članici Udruge koja je uvijek svojom vedrinom i toplom riječi znala razveseliti i ohrabriti bolesnike.

Maja Peraica

IN MEMORIAM
Arh Hig Rada Toksikol 2012;63:A3-A5

BOŽO METZGER (1913.-2012.)

Našega dragog prvog počasnog člana Hrvatskoga društva za zaštitu od zračenja (HDZZ) Bože Metzgera nema više među nama. Kada bi se, u najkraćem, pokušali predstaviti uloga i značenje pokojnika, bilo bi to ovako: prof. dr. Metzger bavio se uglavnom načelima i mjerama zaštite od zračenja, kao i mjerama dozimetrije i primjenom nuklearne energije (sve u sklopu medicinske fizike), a gotovo čitavo vrijeme primijenjenom elektronikom u području radiotehnike. Napisao je 39 članaka, 48 članaka iz primijenjene elektronike, kao i 5 knjiga i 11 udžbenika i skripata. Popis pohvala i nagrada je povelik (21 naslov) od čega treba posebno izdvojiti Plaketu prvomu počasnom članu HDZZ-a, Povelju o počasnom članstvu u Hrvatskome fizikalnom društvu, Spomen-diplomu Medicinskog fakulteta Sveučilišta u Zagrebu, Plaketu Veterinarskog fakulteta Sveučilišta u Zagrebu. Ipak, naš B. Metzger zaslužuje da se o njemu ne piše samo u najkraćim crtama.

Božo Metzger rođen je u Karlovcu, 4. ožujka 1913. Nakon što je započeo školovanje u Karlovcu, zadnja tri razreda pučke škole završio je u Mariboru, gdje je završio i prva tri razreda realne gimnazije. Nakon toga je polazio gimnaziju u Vinkovcima, četvrti i peti razred, dok je šesti, sedmi i osmi razred Državne I. muške realne gimnazije polazio u Zagrebu gdje je i maturirao šk. god. 1930/31. Godine 1931. upisao je studij fizike na Filozofskom fakultetu u Zagrebu. Završio je studij na znanstvenoj grupi IV (Fizikalna grupa) polaganjem diplomskog ispita 1936. godine. Doktorsku je disertaciju pod nazivom Nov način uklanjanja fadinga i smetnja za automatsko primanje radiotelegrafskih i vremenskih signala na kratkom valu primio Savjet Filozofskog fakulteta u Zagrebu na svojoj IV. sjednici 7. lipnja 1939. Odluka o prihvaćanju donijeta je nakon pismenog referata prof. dr. Stanka Hondla i prof. dr. Stjepana Škreba. Nakon toga pristupilo se dvosatnom usmenom ispitu koji je bio u dva dijela. Prvi dio bio je u svezi s tezom, a ispitivati su mogli svi članovi odbora za prijavljenog kandidata (dekan i 3 člana odbora iz predmeta bliskih tezi). Drugi dio ispita sastojao se od ispita iz povijesti filozofije. Božo Metzger obranio je disertaciju 17. veljače 1940. godine objavivši je iste godine u časopisu Rad Jugoslavenske akademije znanosti i umjetnosti.

B. Metzger počeo je raditi kao asistent dnevničar u Fizikalnom zavodu tadašnjega Filozofskog fakulteta (od 1936. g.) kod sveučilišnog profesora fizike S. Hondla. Metzger je, na nagovor prof. Hondla, god. 1941. predao prijavu na natječaj za docenturu za Tehničku fiziku na Tehničkom fakultetu, a njegov kolega Lopašić na Veterinarskome. No, valja istaći da su nakon što su posumnjali da neće uspjeti kod docenture oba poželjela ostati asistenti kod Hondla. No on je odgovorio da to oni neće doživjeti i izrekao već poznatu rečenicu: „Mijenjajmo dame.“ Tako je Metzger dospio na Veterinarski fakultet (1942. g.), a Lopašić kasnije (1950. g.) na Tehnički fakultet. Nakon što je postao docent Veterinarskog fakulteta Metzger je na Medicinskom fakultetu (od 1945. g.) predavao honorarno fiziku za medicinare, i to sve do 1979. godine. Kao konzilijarni fizičar u Kliničkoj bolnici “Dr. Mladen Stojanović” – danas “Sestre milosrdnice” (od 1958., čim je ondje bila postavljena tzv. “kobaltova bomba”) B. Metzger vodi dozimetriju i zaštitu od radijacije. Nakon što je otišao s Veterinarskog fakulteta 1963. preuzeo je dužnost voditelja Odsjeka za primjenu radioaktivnih izotopa u Zavodu za radiologiju i nuklearnu medicinu bolnice “Dr. Mladen Stojanović” (gdje je radio od 1963. do 1979., kada je umirovljen).

Metzger je živio u vrijeme kada su se autoriteti (moralni i intelektualni), kao i stečena baština poštovali. Filozofski ideal traženje istine u njegovo vrijeme bio je još itekako cijenjen budući da je fizika bila u okrilju filozofije jer se studirala u sklopu Mudroslovnog (pa Filozofskog) fakulteta. Prema svjedočenjima studenata prof. Metzger bio je vrlo precizan u izlaganju gradiva i uvijek usredotočen na bitno. U razgovoru s njime mogli su njegovi suradnici i sugovornici uočiti duhovitost stavova i komentara o stručnim pitanjima iz oblasti kojom se bavio. Bio je i humanist o čemu govori činjenica da je prije više od 70 godina zagovarao i promicao zaštitu zdravlja u svezi s obrazovanjem uz isticanje prevladavanja razlika između teorije i prakse u nastavnim programima stručnih škola. Tu je bio itekako suvremen jer ta problematika ni dandanas nije riješena.

Ja sam imao čast i zadovoljstvo više od pedeset puta osobno se sastati s njim. Mogao sam se uvjeriti da je B. Metzger težio uvijek sve većim visinama znanja, širio je kulturne horizonte, držao do stečenih autoriteta (tuđih i svog) i neprestano tražio istinu iskreno voleći svoj posao. U toj zauzetosti i ljubavi činio je čak i ono što nije morao. Učinio je to kada je upisao medicinu kako bi kod prof. Perovića slušao anatomiju da bi u potpunosti bio upoznat s pogubnošću ionizirajućeg zračenja koje je padalo na nezaštićeno ljudsko tijelo. Isto tako je strogo upozoravao liječnike na potrebu nošenja dozimetara pa makar je zbog tog poštenog upozorenja dobivao prijekore od nadređenih.

Osim posla volio je i život. Znao je da se vrednote trebaju stjecati, a pravednost ispunjavati. Stoga je svojim dugim životom stvorio naslijeđe koje tek svojim djelovanjem trebamo očuvati. Hvala mu na tome i neka i dalje vrednote i pravednost koje nam je podario žive u svima nama koji smo ga poznavali. Ako i oni mlađi koji dolaze iza nas budu mislili i djelovali tako, bit će Metzgerovo naslijeđe dalekosežno očuvano.

Umro je u Zagrebu, u Domu za starije i nemoćne osobe – FIDELITAS dana 7. siječnja 2012., a pokopan je tri dana kasnije na zagrebačkom groblju Mirogoj.

Više životopisnih detalja o B. Metzgeru može se naći u članku: Dragutin Mayer i Božo Metzger – velikani znanosti o zračenju i zaštiti od zračenja u Hrvatskoj, autorâ B. Hanžeka i Z. Franića, Arhiv za higijenu rada i toksikologiju, vol. 61, str. 479-498, Zagreb 2010. Ako znatiželjnik želi dobiti potpuniju informaciju o B. Metzgeru, preporučujem monografiju naslova: Mjesta dodira: fizika i medicina, podnaslova: Kroz život i djelo B. Metzgera (urednici akad. M. Pećina i prof. dr. S. Fatović- Ferenčić), u ediciji: Rasprave i građa za povijest znanosti, knj. 11, Zagreb 2011.

Branko Hanžek

ANNOUNCEMENTS

HGD2012 – TREĆI KONGRES HRVATSKIH GENETIČARA UZ MEĐUNARODNO SUDJELOVANJE

Grad Krk, Hrvatska, 13.-16. svibnja 2012.

Kongres koji će se održati od 13. do 16. svibnja 2012. godine u gradu Krku organizira Hrvatsko genetičko društvo. Organizacijski odbor čine: Verica Garaj-Vrhovac (predsjednica), Ana Bielen, Goran Gajski, Marko Gerić, Mladen Ivanković (tajnik), Grozdana Kuleš i Tomislav Vladušić, dok su članovi Znanstvenog odbora: Jasna Franekić (predsjednica), Andreja Ambirović-Ristov, Višnja Besendorfer, Hrvoje Fulgosi, Verica Garaj-Vrhovac, Stipan Jonjić, Davorin Kajba, Maja Osmak, Miroslav Radman, Ivica Rubelj, Ivan-Krešimir Svetec, Hrvoje Šarčević, Đurđica Ugarković, Siniša Volarević, Dušica Vujaklija i Ksenija Zahradka. U raznolikom znanstvenom programu sudjelovat će priznati domaći i strani znanstvenici koji se bave temeljnim molekularnim znanostima o životu i primjenom tih znanosti u biotehnologiji, farmaciji, medicini i zaštiti prirode. Znanstvene teme Kongresa su: Primijenjena genetika, Bioraznolikost/adaptacija, Genetika raka, Molekularna genetika, Genetika biljaka, Interakcije genoma i okoliša/ekologija, Populacijska genetika, Mutacije/DNA popravak/rekombinacija i Epigenetika. Program Kongresa obuhvaća predavanja pozvanih predavača, znanstvenika i mladih istraživača, prezentaciju posterskih priopćenja, promotivne izložbe te razna društvena događanja.

Kongres će se održati u hotelu „Valamar Koralj Romantic Hotel***“ u Ulici Vlade Tomačića bb, Krk; http://www.valamar.com/hr/koralj-hotel-krk. Za sve sudionike Kongresa osiguran je smještaj po promotivnim cijenama.

Visina kotizacije iznosi 1 500 kn, dok je za studente kotizacija 750 kn, a za osobe u pratnji 640 kn. Sažeci radova bit će objavljeni u Knjizi sažetaka uz mogućnost objave u jednom od predloženih časopisa.

Za daljnje informacije molimo vas da provjerite web-stranicu Društva: http://www.genetika.hr/ ili kontaktirajte: Vericu Garaj-Vrhovac (vgaraj@imi.hr ili na broj telefona +385 1 4682 631) ili Jasnu Franekić (jfran@pbf.hr ili na broj telefona +385 1 4863 013.).

Verica Garaj Vrhovac

4TH CROATIAN CONGRESS OF TOXICOLOGY WITH INTERNATIONAL PARTICIPATION

Primošten, Hrvatska, 2.-5. listopada 2012.

Hrvatsko toksikološko društvo organizira 4. hrvatski toksikološki kongres s međunarodnim sudjelovanjem pod pokroviteljstvom Instituta za medicinska istraživanja i medicinu rada i Hrvatskoga zavoda za toksikologiju i antidoping.

Predviđena su plenarna predavanja koja će održati pozvani predavači, usmena priopćenja i posterske prezentacije. Službeni jezik Kongresa je engleski.

Sažeci se mogu prijavljivati u okviru ovih tema: Klinička toksikologija, Radiotoksikologija, Toksini prirodnog podrijetla, Reproduktivna toksikologija, Toksikologija hrane, Imunohematotoksikologija, Industrijski otrovi, Toksikologija metala i polumetala, Pesticidi, Organoklorirani, organofosforni i triazinski spojevi, Genetička toksikologija, Ekotoksikologija, Forenzička toksikologija, Nanotoksikologija, Toksikokinetika, Mutageni i antimutageni i Biomonitoring.

Sažeci se mogu poslati elektroničkom poštom do 1. lipnja 2012. godine na ove e-mail adrese: htd@htd.hr ili crotox@htd.hr.

Registracija po sniženoj cijeni moguća je do 15. lipnja 2012. godine.

Više informacija o skupu nalazi se na web-adresi: http://www.htd.hr/kongres2012.html

Marin Mladinić

4TH CROATIAN CONGRESS OF TOXICOLOGY WITH INTERNATIONAL PARTICIPATION

Primošten, Croatia, 2-5 October 2012

The Croatian Toxicological Society will hold the 4th Croatian Congress of Toxicology with international participation under the auspices of the Institute for Medical Research and Occupational Health and the Croatian Institute for Toxicology and Antidoping.

The scientific programme will consist of plenary lectures, oral presentations, and posters in the following topics: Clinical toxicology, Radiotoxicology, Anthropogenic toxins, Reproductive toxicology, Food toxicology, Immuno-hematotoxicology, Industrial poisons, Toxicology of metals and metalloids, Pesticides, Organochlorines, organophosphorus and triazine compounds, Genetic toxicology, Ecotoxicology, Forensic toxicology, Nanotoxicology, Toxicokinetics, Mutagens and antimutagens, and Biomonitoring. The official conference language is English.

Abstracts must be sent by e-mail to *htd@htd.hr* or *crotox@htd.hr* before June 2012. Registration at a reduced price is available until 15 June 2012.

For further information please visit: *http://www.htd.hr/kongres2012.html*

Marin Mladinić

HDIR-2 "OD BAZIČNIH ISTRAŽIVANJA DO KLINIKE": DRUGA KONFERENCIJA HRVATSKOG DRUŠTVA ZA ISTRAŽIVANJE RAKA S MEĐUNARODNIM SUDJELOVANJEM

Zagreb, 8.-9. studenoga 2012.

S iznimnim zadovoljstvom najavljujemo Drugu konferenciju Hrvatskog društva za istraživanje raka (HDIR), člana Europskog udruženja za istraživanje raka (EACR, European Association for Cancer Research). HDIR broji gotovo 100 članova i po drugi put organizira međunarodnu konferenciju iz područja translacijske i personalizirane medicine. Prva konferencija HDIR-1 "Od bazičnih istraživanja do klinike" održana je 2010. u Institutu "Ruđer Bošković" te je odaziv bio neočekivano velik, 175 sudionika s više od 50 postera iz zemlje i svijeta. Ovogodišnja konferencija planira se kao dvodnevni događaj u Hotelu International u Zagrebu te se predviđaju 4 glavne sekcije: "Prijenos signala u tumorima", "Imunologija tumora", "Rezistencija tumora" te "Genetika i epigenetika tumora". Predavači su renomirani hrvatski i svjetski znanstvenici: Oscar Burrone (Italija), Tanja Čufer (Slovenija), Jozo Delić (Francuska), Ivan Đikić (Njemačka), Zdenko Herceg (Francuska), Zorica Juranić (Srbija), Varda Rotter (Izrael), Damir Vrbanec (Hrvatska).

Uz predavanja predviđena je i posterska sekcija gdje će mlađi sudionici moći prikazati svoja dostignuća te u produktivnome znanstvenom okruženju kritički raspraviti o svojim rezultatima. Cilj je HDIR-2 konferencije promocija istraživanja tumora u Hrvatskoj i poticanje komunikacije između bazičnih istraživanja i kliničkog liječenja u istraživanju tumora. Naglasak ove konferencije bit će na međunarodnoj komunikaciji i suradnji. Ova konferencija pruža mogućnost sudionicima da uspostave suradnju međusobno, ali i s kolegama iz drugih nacionalnih društava za istraživanje raka unutar EACR-a.

Više informacija o konferenciji možete pronaći na web-stranicama Hrvatskog društva za istraživanje raka (http://www.hdir.hr) te na web-stranicama konferencije (http://www.hdir-2.hdir.hr).

Sonja Levanat

HDIR-2 "FROM BENCH TO CLINIC": THE SECOND MEETING OF THE CROATIAN ASSOCIATION FOR CANCER RESEARCH WITH INTERNATIONAL PARTICIPATION

Zagreb, 8-9 November 2012

It is our great pleasure to announce the Second Meeting of the Croatian Association for Cancer Research (HDIR), a constituent society of the European Association for Cancer Research (EACR). HDIR has almost 100 members, and this is the second time it organises an international meeting in translational and personalised medicine. The first meeting, HDIR-1 "From Bench to Clinic" was held in 2010 at the Ruđer Bošković Institute. The interest far exceeded our expectations; 175 people from Croatia and abroad participated with more than 50 posters.

This year's conference is planned as a two-day event in Hotel International in Zagreb. It will consist of four major sections: Cancer Signalling, Immunity and Cancer, Cancer Resistance and Cancer Genetics & Epigenetics. The invited speakers are respected scientists and clinicians from Croatia and abroad: Oscar Burrone (Italy), Tanja Čufer (Slovenia), Jozo Delić (France), Ivan Đikić (Germany), Zdenko Herceg

(France), Zorica Juranić (Serbia), Varda Rotter (Israel), and Damir Vrbanec (Croatia).

There will also be a poster session for younger participants to present their work and discuss it in a productive scientific environment. The aim of HDIR-2 conference is to advance tumour research in Croatia and promote communication between basic research and clinical practice. The emphasis will be on international communication and cooperation. This conference will bring participants to collaborate more closely.

More information about the conference is available at the HDIR webpage (http://www.hdir.hr) and the conference webpage (http://www.hdir-2.hdir.hr).

Sonja Levanat

REPORT

5. HRVATSKI KONGRES MEDICINE RADA S MEĐUNARODNIM SUDJELOVANJEM "ZDRAVLJE, RAD I ZAJEDNICA"
Hvar, Hrvatska, 28. rujna - 2. listopada 2011.

Pod visokim pokroviteljstvima predsjednika Ive Josipovića i Vlade Republike Hrvatske od 28. rujna do 2. listopada 2011. godine u Hvaru je održan 5. hrvatski kongres medicine rada s međunarodnim sudjelovanjem pod naslovom Zdravlje, rad i zajednica. Na Kongresu je sudjelovalo oko 200 sudionika iz osam zemalja: Hrvatske, Austrije, Italije, Nizozemske, Njemačke, Rumunjske, Rusije i Srbije. Specijalisti medicine rada i kliničkih struka te stručnjaci u području zaštite na radu izmijenili su u 81 radu niz spoznaja važnih ne samo za znanstvenu i stručnu zajednicu već i za širu javnost. Teme devet pozvanih predavača, 42-ju usmenih izlaganja, 30 postera, dviju radionica i okruglog stola raspoređene su u 9 cjelina s vrlo dinamičnim raspravama i konstruktivnim zaključcima od kojih iznosimo najvažnije.

Ovim Kongresom stručnjaci iz RH uključili su se u rad EU-kampanje pod nazivom "Zdrava koža@rad" i rad Europske inicijative za prevenciju profesionalnih kožnih bolesti pod pokroviteljstvom Europske akademije za dermatovenerologiju. Istaknuta je važnost pridržavanja mjera osobne zaštite na radu, ali i nepridržavanja ovih mjera kao svjetskog problema, posebno na radnim mjestima s velikim udjelom nekvalificiranih radnika te na radnim mjestima s uvjetima koji pogoduju razvoju sindroma prenaprezanja koji se pojavljuju sve češće kao profesionalne bolesti. Upozoreno je na potrebu unapređenja zaštite zdravlja zaposlenih u poljoprivredi i članova poljoprivrednih domaćinstava. Primjeri dobre prakse koji su zaživjeli u području zaštite zdravlja radnika trebaju biti podloga za uvođenje sustavnijeg nadzora nad zdravljem i sportskom sposobnošću sportaša, poglavito sad kad je specijalizacija medicine rada i sporta zajednička i kad već postoji znatan broj specijalista s odgovarajućom specijalizacijom u praksi. Upozoreno je na važnost osiguranja pravilne prehrane radnika te na sve veći problem osteoporoze kod muškaraca. Zaključeno je da će se poduprijeti Hrvatsko reumatološko društvo koje predlaže da se denzitometrija uvede kao standardna pretraga za muškarce. Predstavljena je Međunarodna klasifikacija funkcioniranja Svjetske zdravstvene organizacije koja daje okvir za procjenu zdravlja i nesposobnosti na individualnoj i populacijskoj razini. Mobing i stres te njihova zastupljenost i utjecaj na radnu sposobnost zdravstvenih radnika izdvojili su se ponovo upozoravajući na važnost zaštite mentalnog zdravlja te suradnje specijalista medicine rada i psihijatrije. Zaključeno je da je nužno ukloniti neusklađenosti nalaza psihijatara i specijalista medicine rada i sporta u procjeni radne sposobnosti koje su česte u praksi. Ozljede na radu u djelatnosti zdravstvene zaštite nedovoljno se nadziru te su nužne mjere podizanja svijesti zaposlenih u zdravstvu. U svrhu očuvanja zdravlja i radne sposobnosti medicinskih sestara i tehničara potrebno je unaprijediti organizaciju rada, edukaciju, uvesti programe za smanjenje stresa i prilagoditi radne zahtjeve osobnim mogućnostima. Potrebno je pratiti broj, kretanje i strukturu hrvatskih radnika migranata, ali i imigranata u Republici Hrvatskoj. Zaključeno je da se brojni specijalisti medicine rada susreću s njima u svakodnevnoj praksi, no ne postoji sustavna evidencija na državnoj razini. Predloženo je da bi evidenciju za imigrante mogla voditi epidemiološka služba Zavoda za javno zdravstvo, kao i evidenciju stanja procijepljenosti, cijepljenja i obolijevanja od zaraznih

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Arh Hig Rada Toksikol 2012;63:A9-A10

bolesti. Istaknuta je kompleksnost vještačenja u području sigurnosti na radu i zaštiti zdravlja radnika te potreba za multidisciplinarnim vještačenjem.

Na Okruglom stolu "Osiguranje zdravstvene zaštite radnika u Republici Hrvatskoj" istaknuto je da otkada je sustav za zaštitu zdravlja na radu ugrađen u Hrvatski zavod za zdravstveno osiguranje (HZZO), zakonskim i organizacijskim promjenama sustav "naizgled" funkcionira, ali nažalost ne samo da nema poboljšanja, nego u pojedinim dijelovima stagnira i nazaduje:

- nova organizacija osiguranja zdravstvene zaštite na radu od 2011. g. ne daje poticaj za unapređenje zaštite na radu poslodavcima i radnicima, a ni specijalistima medicine rada (npr. obilazak radnog mjesta, edukacija radnika i sl.);
- u zdravstvenoj zaštiti na radu zanemaruje se aspekt mentalnog zdravlja;
- dostupni statistički podaci osiguratelja nedostatni su za procjenu stanja ozljeda i profesionalnih bolesti i zaštite na radu;
- potrebno je sagledati mogućnosti da specijalisti medicine rada u sustavu osiguranja budu konzilijarni specijalisti;
- potrebno je sustavno riješiti problem upućivanja liječnika na specijalizaciju iz medicine rada i sporta;
- koncesiju u medicini rada treba završiti po zakonskim odredbama do kraja godine, a nije razvidno na koga se odnosi koncesija i pod kojim uvjetima;
- veliki praktični problem je organizacija pregleda za zanimanja koja zahtijevaju "konzilijarni" pristup, tj. sudjelovanje više specijalista različitih specijalnosti.

Zaključak je da je potrebno sagledati nove mogućnosti organizacije osiguranja zdravstvene zaštite na radu. Hrvatsko društvo za medicinu rada (HDMR) treba donijeti prijedloge za poboljšanje sustava i djelovati na njihovoj realizaciji. U tu svrhu najavljen je sastanak predstavnika HDMR-a s rukovoditeljem Službe za zaštitu zdravlja na radu HZZO-a Krešimirom Hubakom. Nacionalno vijeće za zaštitu zdravlja na radu pri tome i dalje treba biti neizostavni partner HDMR-a za unapređenje sustava za zaštitu zdravlja radnika na radu.

Sažeci svih radova prezentiranih na Kongresu dostupni su na internetskoj stranici http://www.5hkmr.hr/

Jadranka Mustajbegović, Azra Huršidić Radulović i Jelena Macan

UPUTE AUTORIMA*

Profil časopisa

Arhiv za higijenu rada i toksikologiju – Archives of Industrial Hygiene and Toxicology (krat. *Arh Hig Rada Toksikol*) znanstveni je časopis koji objavljuje recenzirane rukopise iz područja biomedicinskih znanosti, posebno medicine rada, toksikologije i zdravstvene ekologije. Rukopisi se objavljuju uz pretpostavku da nisu predani za objavljivanje ili već objavljeni u nekome drugom tiskanome ili elektroničkome mediju.

Časopis objavljuje znanstvenoistraživačke radove, kratka priopćenja (kraći oblik znanstvenoga rada), stručne i pregledne radove te opažanja i pisma uredništvu. Znanstvenoistraživački radovi podnose se na engleskome, a ostali radovi mogu biti na engleskome ili hrvatskome. Najave i izvještaji sa znanstvenih i stručnih sastanaka te prikazi knjiga također se objavljuju. Ti prilozi mogu biti pisani na hrvatskome, engleskome ili slovenskome.

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Rukopisi su u cijelosti dostupni na internetu na stranicama online izdavača Versita (*http://www.versita.com/science/medicine/aiht*) te Portala znanstvenih časopisa Republike Hrvatske – HRČAK (*http://hrcak.srce.hr/aiht*).

Priprema rukopisa

Opsežne upute za pripremu rukopisa tzv. *Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Writing and Editing for Biomedical Publication issued by the International Committee of Medical Journal Editors* dostupne su na <http://www.icmje.org>.

Znanstvenoistraživački radovi u pravilu se sastoje od ovih dijelova: uvod, metode, rezultati, rasprava i zaključak.

U radovima koji se odnose na istraživanja u ljudi autori su dužni navesti podatke o odgovarajućem etičkom povjerenstvu koje je odobrilo istraživanje te da su provedena ispitivanja u skladu s etičkim načelima Helsinške deklaracije. U istraživanjima na životinjama treba navesti da su poštivana odgovarajuća načela Zakona o dobrobiti životinja koja se odnose na pokusne životinje.

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INSTRUCTIONS TO AUTHORS*

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The journal publishes scientific papers, short communications (a shorter form of a scientific paper), professional papers, reviews, case reports, observations, technical papers and letters to the editor. Scientific papers should be written in English, and manuscripts in other categories may be in either Croatian or English. Announcements, book reviews and meeting reports are also accepted, and may be written in Croatian, English or Slovene.

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Full-text articles are available online at Versita (*http://www.versita.com/science/medicine/aiht*) and on the Portal of Scientific Journals of Croatia – HRČAK at *http://hrcak.srce.hr/aiht*.

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The text of the manuscript should be typed with margins wide enough for annotations, and pages should be numbered. The paragraph line space should be 1.5. Units should be quoted according to the International System of Units (SI), and abbreviated terms should be written in full when first mentioned.

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* Revised in March 2012

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Journals / časopisi

- *personal author(s) / osoba kao autor*

  Ashani Y, Segev O, Balan A. The effect of fluoride on the scavenging of organophosphates by human butyrylcholinesterase in buffer solutions and human plasma. Toxicol Appl Pharmacol 2004;194:90-9.

- *organization as author / organizacija kao autor*

  The Cardiac Society of Australia and New Zealand. Clinical exercise stress testing. Safety and performance guidelines. Med J Aust 1996;164:282-4.

- *journals in electronic format / elektronički časopisi*

  Bacon DP, Garrett RM, Liotta CdrPL, Mays DE, Miller TE. Industrial hazards to military personnel. J Med CBR Def 2005-2006 [displayed 7 July 2005]. Available at http://www.jmedchemdef.org.

Books and other publications / knjige i ostale publikacije

- *personal author(s) / osoba kao autor*

  Flanagan RJ, Jones AL. Antidotes. New York (NY): Taylor and Francis Inc; 2001.

- *editor(s) as author / autor urednik*

  Wallace Hayes A, editor. Principles and Methods of Toxicology. 4th ed. London: Taylor and Francis; 2001.

  Wallace Hayes A, urednik. Principles and Methods of Toxicology. 4. izd. London: Taylor and Francis; 2001.

- *organization as author / organizacija kao autor*

  Environmental Protection Agency (EPA). Toxicology handbook. Rockville (MD): Government Institutes; 1986.

- *book chapter / poglavlje u knjizi*

  Phillips SJ, Whisnant JP. Hypertension and stroke. In: Laragh JH, Brenner BM, editors. Hypertension: pathophysiology, diagnosis, and management. 2nd ed. New York (NY): Raven Press; 1995. p. 465-78.

  Phillips SJ, Whisnant JP. Hypertension and stroke. U: Laragh JH, Brenner BM, urednici. Hypertension: pathophysiology, diagnosis, and management. 2. izd. New York (NY): Raven Press; 1995. str. 465-78.

- *proceedings / kongresni zbornici*

  Kimura J, Shibasaki H, editors. Recent advances in clinical neurophysiology. Proceedings of the 10th International Congress of EMG and Clinical Neurophysiology; 15-19 Oct 1995; Kyoto, Japan. Amsterdam: Elsevier; 1996.

  Kimura J, Shibasaki H, urednici. Recent advances in clinical neurophysiology. Proceedings of the 10th International Congress of EMG and Clinical Neurophysiology; 15.-19. listopada 1995.; Kyoto, Japan. Amsterdam: Elsevier; 1996.

- *paper in proceedings / radovi u zbornicima*

  Bengtsson S, Solheim BG. Enforcement of data protection, privacy and security in medical informatics. In: Lun KC, Degoulet P, Piemme TE, Rienhoff O, editors. MEDINFO 92. Proceedings of the 7th World Congress on Medical Informatics; 6-10 Sep 1992; Geneva, Switzerland. Amsterdam: North-Holland; 1992. p. 1561-5.

  Bengtsson S, Solheim BG. Enforcement of data protection, privacy and security in medical informatics. U: Lun KC, Degoulet P, Piemme TE, Rienhoff O, urednici. MEDINFO 92. Proceedings of the 7th World Congress on Medical Informatics;

6.-10. rujna 1992.; Ženeva, Švicarska. Amsterdam: North-Holland; 1992. str. 1561-5.

- *meeting abstract / sažetak sa sastanka*

Aas P, Myhrer T. Pretreatment and medical therapy of nerve-agent intoxication. In: CBMTS Industry IV. The Third World Congress on Chemical, Biological and Radiological Terrorism; 17-23 Sep 2005; Dubrovnik, Croatia. Technical Program. p. 20. Abstract No. 1.

Aas P, Myhrer T. Pretreatment and medical therapy of nerve-agent intoxication. U: CBMTS Industry IV. The Third World Congress on Chemical, Biological and Radiological Terrorism; 17.-23. rujna 2005.; Dubrovnik. Tehnički program. str. 20. Sažetak br. 1.

- *thesis / diplomski ili magistarski rad odnosno disertacija*

Macan J. Senzibilizacija dišnog sustava i biološke karakteristike prašinskih grinja u općem i radnom okolišu [Sensitization of the respiratory system and biological characteristics of dust mites in general and occupational environment; in Croatian]. [PhD thesis]. Zagreb: Medical School, University of Zagreb; 2002.

Macan J. Senzibilizacija dišnog sustava i biološke karakteristike prašinskih grinja u općem i radnom okolišu [disertacija]. Zagreb: Medicinski fakultet Sveučilišta u Zagrebu; 2002.

Electronic material / elektronički materijali

- *documents and materials in electronic format/ dokumenti i materijali u elektroničkome obliku*

Anderson SC, Poulsen KB. Anderson's electronic atlas of hematology [CD-ROM]. Philadelphia (PA): Lippincott Williams & Wilkins; 2002.

- web sites / web stranice

World Health Organization (WHO). Environmental Health Criteria 25 [displayed 23 November 2005]. Available at *http://www.inchem.org/documents/ehc/ehc/ehc25.htm*.

World Health Organization (WHO). Environmental Health Criteria 25 [pristup 23. studenoga 2005.]. Dostupno na *http://www.inchem.org/documents/ehc/ehc/ehc25.htm*.