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Ectoine as a promising protective agent in humans and animals

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Ectoine is a compatible water molecule-binding solute (osmoprotectant) produced by several bacterial species in response to osmotic stress and unfavourable environmental conditions. This amino acid derivative can accumulate inside cells at high concentrations without interfering with natural processes and can protect the cell against radiation or osmotic stress. This brief review presents the current state of knowledge about the effects of ectoine on animals and focuses on its practical use for enzyme stabilisation, human skin protection, anti-inflammatory treatment, inhibitory effects in neurodegenerative diseases, and other therapeutic potential in human or veterinary medicine.

KEY WORDS: *cell protection; compatible solute; macromolecular stability; osmoprotectant*

Various organisms have developed different mechanisms to protect themselves against stress caused by environmental factors such as drought or extreme temperatures. Bacteria, especially those living in extreme environments, have the ability to produce compounds of small molecular weight known as compatible solutes or osmoprotectants, which do not interfere with cellular processes. They can accumulate to high concentrations and prevent cell dehydration (1). According to the structure, compatible solutes belong to the following groups: sugars (sucrose, trehalose), polyols (sorbitol, glycerol, mannitol, mannosyl-glyceramide, mannosyl-glycerol), *N*-acetylated diamino acids (*N*-acetylglutaminylglutamine amide), betaines (betaine, glycine, and deritatives), and amino acids and derivatives (proline, glutamate, glutamine, alanine, ectoine, and hydroxyectoine).

These substances strongly bind water molecules and stabilise macromolecules. Although their mechanism of action is not entirely known, there are several hypotheses explaining biophysical principles by which they act. The most widely accepted hypothesis is the "preferential exclusion model", according to which osmoprotectants do not interact directly with the macromolecule in an aqueous solution but are repelled to the bulk region, increasing macromolecule's hydration and thereby preventing its denaturation (2-4).

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid; for structure see Figure 1a) is a water-binding zwitterionic amino acid derivative (142,156 Da), first isolated from *Ectothiorhodospira halochloris* (5). It is also produced and accumulated by other, mainly aerobic, chemoheterotrophic and halophilic bacteria, such as alpha- and gammaproteobacteria and *Actinobacteridae*, in which it stabilises cell membranes, enzymes, and nucleic acids at extreme temperatures or higher salt concentrations (6). Ectoine synthesis from its precursor, L-aspartate-β-semialdehyde is catalysed by ectoine ABC enzymes, including diaminobutyric acid (DABA) acetyltransferase (ectA), DABA aminotransferase (ectB), and ectoine synthase (ectC) (7). Genes encoding these enzymes are organised in either ectABC or ectABC-ask operons (8).

A number of biophysical studies show that ectoine binds water molecules even better than some other osmoprotectants such as glycerol (9, 10) and that it is well tolerated by humans, animals, and various cell cultures. In fact, ectoine retains strong hydration properties even at high NaCl concentrations (11).

The aim of this brief review is to present the current state of knowledge about the effects of ectoine on animals and discuss its practical use in enzyme stabilisation, human skin protection, anti-inflammatory treatment, prevention of neurodegenerative diseases, and other therapeutic potentials in human or veterinary medicine.

Skin protection

Ectoine has widely been used in cosmetic anti-ageing and moisturising creams to improve skin resistance to surfactants in skin cleansing solutions (12). It is an effective long-term moisturiser that prevents dehydration of the epidermis, even superior to the well-known membrane

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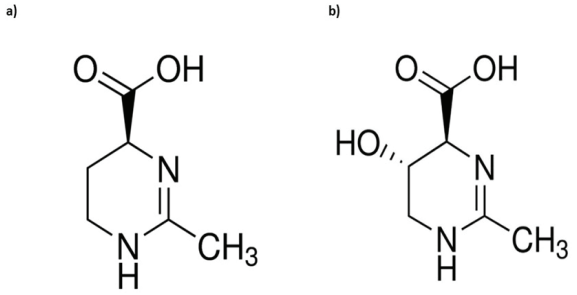

Figure 1 *Structural variants: ectoine (a) and hydroxyectoine (b)*

stabiliser phosphatidylcholine. Some authors suggest that at relatively low concentrations (up to 500 μmol L^{-1}) it can also be used as a whitening agent because of its inhibitory effect on melanin synthesis in B16-F0 and A2058 melanoma cell lines and on mushroom and cellular tyrosinase activities (13). Ectoine also alleviates skin inflammation and is currently recommended for the treatment of moderate atopic dermatitis (14).

In addition, ectoine strongly absorbs ultraviolet (UV) radiation and protects DNA from breaking down in various cell types (15; 16). Grether-Beck et al. (17) reported that ectoine reduced UV-A-induced ceramide signalling response in human keratinocytes, while Buenger et al. (16) reported protective effects against UV radiation in immunocytes and Langerhans cells.

Stabilising enzyme activity

A number of studies have indicated that ectoine increases the stability of enzymes maintaining hydration and thereby reducing protein susceptibility to denaturation. Interestingly, this amino acid derivative does not interact directly with protein surfaces but rather it forms a molecular net holding water molecules close to the macromolecule (10, 18, 19). Moreover, Roychoudhury et al. (20) have shown that ectoine, like other compatible solutes, strengthens intramolecular interactions essential for protein stability. Ectoine reduces denaturation of enzymes induced by a rapid change of temperature. It prolongs the activity of lactate dehydrogenase (LDH) and phosphofructokinase, enzymes that are normally sensitive to freeze-thawing, heating, and freeze-drying (21). It also increases the stability of phytase, ribonuclease-A, and polymerase of the double-stranded DNA at elevated temperature (22, 23).

Tenne et al. (24) found that ectoine derivative hydroxyectoine (for structure see Figure 1b) is even superior to ectoine in protecting against elevated temperatures.

Ectoine has also been reported to protect macromolecules against proteolytic agents. For example, zymogens chymotrypsinogen and trypsinogen were resistant to proteolysis by enteropeptidases (25). Another study showed that antibodies treated with ectoine were less susceptible to proteolytic degradation by pepsin (26).

In addition, ectoine can inhibit HIV replication (23) and can also stabilise retroviral vectors for gene therapy, which may be a useful property, because these vectors usually lose their infectivity during long-term storage and transport (27).

Cell protection

Various environmental stressors such as heat or toxic chemicals may impair the cell membrane function and result in cell dehydration and denaturation. Harishchandra et al. (28) propose that ectoine increases cell membrane fluidity to cope with extreme conditions such as high temperature or osmotic pressure. A recent biophysical study by Zacchai et al. (10) showed that ectoine is excluded from the hydration layer at the membrane surface and does not affect membrane molecular dynamics. Moreover, improved hydration of the cell surface thanks to ectoine increases intermolecular spacing and boosts the mobility of the lipid head groups in the cell membrane (Figure 2) (29).

Different types of cells subjected to high temperature tend to produce chaperone proteins such as heat shock proteins (Hsp), which repair misfolded peptides. Ectoine seems to affect their synthesis and is speculated to act as a chaperone molecule itself (30). Buommino et al. (31) reported that heat-stressed keratinocytes incubated with ectoine had higher levels of Hsp70 and inhibited the production of pro-inflammatory signals.

Some studies indicate that ectoine shows promising properties against the detrimental effects of some toxic compounds. In a study by Graf et al. (12) ectoine-treated human erythrocytes were more resistant to membrane-damaging sodium dodecyl sulphate detergent than untreated cells. Our recent study (32) has shown that ectoine has a potential to block pore-forming toxins, as the isolated bovine erythrocytes treated with ectoine turned out to be less sensitive to staphylococcal alpha-haemolysin. Interestingly, the toxin monomers preincubated with ectoine were less cytotoxic than those added to the cell suspension simultaneously with ectoine, which suggests that ectoine blocks the unfolding of the toxin monomers and thus prevents the formation of transmembrane pores in the cell membrane. These findings suggest that ectoine may be useful in protecting erythrocytes from staphylococcal haemolysins. Little is known, however, about ectoine protective effects against toxins produced by other bacterial species, mycotoxins, and toxic proteins found in animal venom. Future research should answer whether the osmoprotectant could be an effective antidote.

Alleviation of inflammatory reaction

Ectoine effectively alleviates inflammation, such as the experimentally induced colitis in rats (33) or nanoparticle-induced neutrophilic lung inflammation (34). It can also mitigate inflammatory reactions in the lung epithelial cells after inhalation of carbonaceous nanoparticles in mice. However, this effect was not observed when lung inflammation was induced by bacterial lipopolysaccharide (35). Peuschel et al. (36) reported that inhalational exposure

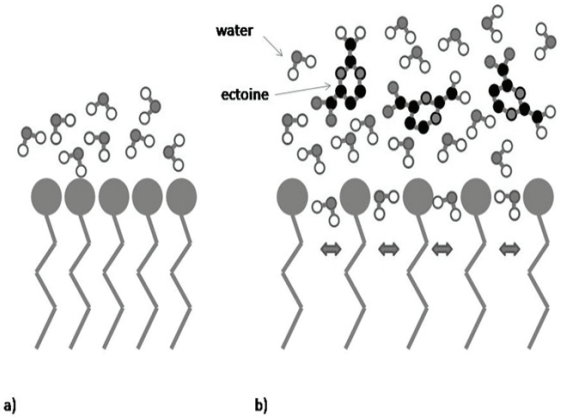

Figure 2 *Influence of water molecules alone (a) and aqueous solution of ectoine (b) on lipid monolayer. Ectoine increases the distance between lipid molecules and improves membrane fluidity.*

to carbon nanoparticles in mice resulted in the accumulation of ceramides in lipid rafts of cell membranes, activation of epidermal growth factor receptor (EGFR), and lung inflammation. Animals additionally treated with ectoine, however, showed milder allergic reactions because ectoine seems to have inhibited ceramide-mediated EGFR phosphorylation. Another study showed that ectoine and hydroxyectoine enhance lung surfactants, which suggests their potential use as supporting factors in inhalation therapy (37).

Ectoine was also found to protect ileal mucosa against ischaemia and reperfusion injury, which are common complications after the small bowel transplantation (38, 39). Alleviating the inflammatory reaction is related to the improvement of intestinal barrier and reduction of cytokine production (33).

Nasal spray and air drops containing ectoine are the new approaches to treating allergic rhinitis, rhinoconjunctivitis, and dry eye syndrome (29). Some studies showed that products containing ectoine may be a better alternative to other products, as they reduce ocular and nasal symptoms with no side effects (40-42). Promising effects such as reduction of nasal airway obstruction and crust formation were also observed in patients with rhinitis sicca anterior treated with nasal spray containing ectoine alone or in combination with dexpanthenol (43). Moreover, a recent study has shown that a mouth and throat spray containing ectoine can be very effective in the treatment of acute pharyngitis and laryngitis (44).

Cryoprotection

Compatible solutes such as ectoine and hydroxyectoine are effective agents in cryopreservation. Bissoyi and Pramanik (45) reported it to be an efficient additive in cryoprotective media for mononuclear cells isolated from human umbilical cord blood. Their 10 % foetal bovine serum medium containing a combination of hydroxyl ethyl starch, ectoine, and co-enzyme Q10 yielded the highest, 93 % viability of mononuclear cells. Sun et al. (46) reported that both ectoine and hydroxyectoine protected human endothelial cell line HPMEC-ST1.6R or mesenchymal stem cells and suggested that they may be used in cryobiology as an alternative to the toxic dimethyl sulphoxide (DMSO). One study on rats (47) showed that adding hydroxyectoine to the histidine-tryptophan-ketoglutarate solution reduces the cold ischaemic preservation injury of livers donated after cardiac death and used for transplantation. Ectoine was also successfully used as a cryoprotectant of human erythrocytes, as it prevented slow-freezing cell damage (48).

Protection against neurodegenerative diseases

Some pathological processes like amyloid formation and aggregation induce neurodegenerative diseases. Ectoine was found to prevent amyloid formation and delay the onset and progression of Alzheimer's disease (49). Other studies have shown that this amino acid derivative inhibits insulin amyloid formation and interacts with prion aggregation responsible for transmissible spongiform encephalopathies (50-52).

Protection of invertebrates against environmental stressors

Little is known about how ectoine acts in invertebrates. In our earlier studies (53-55) ectoine showed protective effects in *Daphnia magna* subjected to high temperature by inhibiting heat shock protein Hsp70, catalase activity, and NO radicals. Moreover, ectoine-treated *Daphnia magna* were more resistant to toxic disinfectants formaldehyde and hydrogen peroxide than untreated controls, as manifested through significantly lower mortality rates, better swimming activity, and better physiological parameters such as heart rate and thoracic limb activity (56). In practice, it could be used as a stabilising agent during the transport of some edible species sensitive to different forms of stress.

CONCLUSION

As ectoine is a potent protective agent against different forms of stress without toxic side effects, its current use in biotechnology, cosmetics, and medicine, leaves a lot of room for many innovative applications. However, since the reviewed literature covers a wide range of studies of different complexity, from isolated macromolecules and cell cultures to various animal species, the protective effects of ectoine observed in simple systems may fail in more complex ones. Moreover, ectoine inactivates some molecules; therefore its use may be limited due to possible interactions with other pharmaceuticals.

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Obećavajuća zaštitna svojstva ektoina u ljudi i životinja

Ektoin je kompatibilni osmolit (osmoprotektant) koji proizvodi više bakterijskih vrsta kao odgovor na osmotski stres i nepovoljne uvjete u okolišu. Ovaj se derivat aminokiseline može nakupiti i dosegnuti visoke razine u samoj stanici a da pritom ne ometa prirodne stanične procese. Usto štiti stanicu od zračenja ili osmotskoga stresa. Svrha je ovoga članka dati kratak pregled dosadašnjih spoznaja o djelovanju ektoina u životinja, s posebnim osvrtom na praktičnu primjenu ovoga osmoprotektanta u stabilizaciji enzima, zaštiti kože u ljudi, protuupalnoga liječenja, sprječavanja ili usporavanja neurodegenerativnih bolesti te u ostalim oblicima liječenja ljudi i životinja.

KLJUČNE RIJEČI: *kompatibilni osmolit; osmoprotektant; stabilnost makromolekula; zaštita stanica*

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Genotoxic effects of the carbamate insecticide Pirimor-50® in *Vicia faba* root tip meristems and human lymphocyte culture after direct application and treatment with its metabolic extracts

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The aim of the study was to evaluate genotoxic effects of Pirimor-50®, a pirimicarb-based formulation (50 % active ingredient), in human lymphocyte cultures and *Vicia faba* root meristems. Furthermore, the objective was to examine a combined influence of insecticide treatment with mammalian microsomal S9 and vegetal S10 metabolic fractions or S10 mix metabolic transformation extracts (after *Vicia faba* primary roots treatment with Pirimor-50®). We used sister chromatid exchange assay-SCE and measured cell cycle progression and proliferation (proportion of M_1-M_3 metaphases and replication index ratio-RI). Two processes were used for plant promutagen activation: *in vivo* activation-Pirimor-50® was applied for 4 h to the plant and then S10 mix was added to lymphocytes; and, *in vitro* activation-lymphocytes were treated with Pirimor-50® and S10 or S9 for 2 h. Direct treatment induced significantly higher SCE frequencies in meristems at 0.01 mg mL^{-1}. In lymphocytes, significantly higher SCE was at 1 mg mL^{-1} with decrease in RI and M_1-M_3 metaphase proportions at 0.5 mg mL^{-1} and cell division stop at 2.5 mg mL^{1}. S10 mix lymphocyte treatment showed significantly elevated SCE values at 2-2.5 mg mL^{-1}, with cell death at 3 mg mL^{-1}. Lymphocyte treatment with Pirimor-50® together with S9 or S10 showed slightly elevated SCE frequency but had a significant influence on RI decrease, with lowest values in S9 treatment. Since no data are available on the genotoxicity of Pirimor-50®, this study is one of the first to evaluate and compare its direct effect in two bioassays, animal and vegetal, and also the effect of plant and animal metabolism on its genotoxic potential.

KEY WORDS: *cellular proliferation kinetics; plant and animal promutagen activation; replication index; sister chromatid exchange*

Industrial and agricultural workers worldwide unintentionally get exposed to pesticide poisoning every year (1, 2). Epidemiological studies on pesticides have demonstrated that these can cause cancer in non-target species, including humans. The risk assessment of cytotoxic/genotoxic effects due to direct or indirect exposure to pesticides has become a major concern to public health because of their widespread use in households and the industry. Carbamates are a large group of synthetic pesticides extensively applied in modern agriculture as insecticides, fungicides, herbicides, nematicides, and/or sprout inhibitors (3). Although various experimental data have provided evidence that pesticides can possess genotoxic properties in animals and *in vitro* test systems after acute and chronic exposure, information about the genotoxic effects of some carbamates, like Pirimor-50®, are still limited and inconsistent (4).

Pirimor-50® is a *N*-methyl carbamate insecticide in which pirimicarb (2-dimethylamino-5,6-dimethylpyrimidin-4-yl dimethylcarbamate) is the active ingredient. Pirimor-50® represents a commercial formulation widely used in Mexico (5) and Croatia (6) for aphid control in a broad range of crops, including vegetable, cereal, and orchard crops (7). The WHO has classified it (8, 9) as "moderately hazardous" (class II) and USEPA as a compound with possible carcinogenic potential in humans (Classes II-III) (10).

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Pirimicarb is transported systemically through the plant and acts by contact, ingestion, and inhalation (11). Its main mechanism of action is the inhibition of acetylcholinesterase activity (12). This mechanism has been reported in mice, rats, and dogs but also in pesticide workers exposed for a longer period (plasma and erythrocyte cholinesterase inhibition) (9). The main property of pirimicarb is its rapid and extensive absorption in the organism, after which its intensive biotransformation begins. It results in 24 metabolites, 17 of which have been identified, some with toxic effect. Besides possible metabolite toxicity, risk assessment is also difficult because pesticides are generally not used as a single active ingredient but rather as a complex commercial formulation. Formulated products, in addition to the active component, contain different solvents and adjuvants, some of which have been reported to induce damage (7, 13-16). Hence, additional toxic effects exerted by adjuvants must also be taken into consideration for risk assessment. Accordingly, workers and the environment are exposed to a simultaneous action of the active ingredient and a variety of other chemicals contained in the formulated product.

Although widely used, with 23 formulated products containing pirimicarb as an active ingredient registered worldwide, studies on the genotoxic effects of pirimicarb and some commercial formulations are scarce (11, 17-18) and there are few cytogenetic studies. Pirimicarb has been reported as non-genotoxic in bacteria, yeast, fungi, and mammalian cells (19). However, positive results were found in mouse lymphoma cells L5178Y in the presence of metabolic activation (11) and in *Drosophila* (20). Furthermore, in human lymphocyte cultures, elevated levels of DNA single-strand breaks were identified by the use of comet assay (4). An increase in the frequency of chromosomal aberrations and sister chromatid exchange (SCE) in Chinese hamster ovary (CHO-K1) cells was observed by Soloneski and Larramendy (7). In addition, an increase in the micronuclei frequency in erythrocytes in *Rana arenarum* and in the fish *Cnesterodon decemmaculatus* exposed to Aficida® (50 % pirimicarb) was observed by Vera-Candioti *et al*. (17-18). Patton Flow® (50 % pirimicarb) also induced DNA single-strand breaks and cytotoxicity on *Cnesterodon decemmaculatus* (21). In individuals occupationally exposed to Pirimor-50®, Pilinskaia (22) found a significant increase in chromosomal aberrations in peripheral lymphocytes.

The metabolites of pesticides produced by plants and animals can act as promutagens, which is why it is necessary to evaluate the vegetal/animal metabolite after exposure to insecticides. When working with human lymphocyte culture, it is necessary to use in parallel the S9 rat liver enzymatic fraction in order to obtain data on the metabolites that would normally be found after the biotransformation of a chemical in the human body. In case of plant exposure, *Vicia faba* is considered a good plant system for treatment since it is metabolically active and contains the S10 enzymatic fraction. As such, it is a sensitive and effective plant to study the effects of pesticide metabolites on the genome stability using the SCE assay, in the plant itself, or on other cell lines with promutagen extracts from the treated *Vicia faba* roots (23, 24).

Although other commercial products as Aficida® and Patton Flow®, both pirimicarb-based pesticides (50 %) like Pirimor-50®, have been found capable of inducing genotoxic damage (17-18, 21, 25), no data/studies are available on the genotoxicity of Pirimor-50® as a pirimicarb-based formulation. Since major pesticides' commercial formulation showed more toxic effects than their active principles, it is important to examine the effect of the formulation on the metabolism in living organisms and to compare the results with the active compound (26). This is even more so since one of the formulations, Pirimor G, showed similar cell viability in three different cell line types, with no effect on the activation of different apoptotic or necrotic pathways, while the active ingredient showed significantly elevated levels of enzymes involved in these pathways (26).

Although nowadays most commonly used cytogenetic assays are micronucleus test and chromosomal aberration assay for *in vitro* and *in vivo* chemical tests, the SCE assay can serve not just as an indicator of genotoxic effects in cells but also as a biomarker of exposure and repair. It does so by reporting the changes in the frequency of reciprocal exchange of DNA segments between sister chromatids at identical loci, indicating a possible destabilisation of the cell genome, persistent damage, and the ability of cells to repair the possible (even persistent) damage. Although this assay has been removed from the OECD guideline list for mutagenicity testing assays on chemicals in 2013 (27), it is still widely used (more than 54 publications in 2016 already, *Web of Science*) due to its specificity and the information that other cytogenetic tests cannot offer. This is true when there is no chromosomal breakage or loss but only otherwise invisible exchanges that can make the genome more unstable and possibly result in a future loss or breakage of the chromosome.

The first aim of this study was to examine the influence of Pirimor-50® alone, as a formulated carbamate insecticide (50 % pirimicarb), on a possible genotoxic effect (measured as SCE frequency) caused in the *Vicia faba* root tip meristems and human lymphocyte cultures in a wide range of concentrations.

The second aim was to examine the *in vivo* and *in vitro* transformation of the Pirimor-50®. Plant and animal metabolites of the pesticide were applied to lymphocytes to estimate their influence on genotoxic effects, cell cycle progression, and proliferation measured as a proportion of the first (M_1), second (M_2), and third (M_3) metaphases, as well as the RI. This study is one of the first to evaluate and compare not only the direct effect of Pirimor-50® in two bioassays, animal and vegetal but also the effect of plant and animal drug metabolism on its genotoxic potential.

MATERIALS AND METHODS

Chemicals

RPMI medium 1640 with L-glutamine and phytohemagglutinin were purchased from Gibco of Mexico; Pirimor-50® (pirimicarb CAS number 23103-98-2) was provided by Zeneca of Mexico. The following chemicals were purchased from Sigma Chemical, St. Louis MO, USA: mitomycin C (MMC, CAS number 50-07-7), cyclophosphamide (CAS number 6055-19-2), dimethyl sulfoxide (DMSO, CAS No. 67-85-5), bromodeoxyuridine (BrdU, CAS number 59-14-3), uridine (Urd, CAS number 58-96-8), fluorodeoxyuridine (FdU, CAS number 3094-09-5), Giemsa (CAS number 51811-82), ethanol (purity 99.2 %, CAS number 6415-5), methanol (purity 99.8 %, CAS number 6756-1), acetic acid (purity 99.7 %, CAS number 5323-26-45), sodium phosphate monobasic (NaH_2PO_4, CAS number 7558-80-7), sodium phosphate dibasic (Na_2HPO_4, CAS number 7758-79-4), and sodium chloride (NaCl, CAS number 7647-14-5). Colchicine (CAS number 64-86-8) and potassium chloride (KCl, CAS number 222425) were purchased from Merck of Mexico.

Direct treatments without metabolic activation

Treatment of Vicia faba *root tips*

To verify the effect of Pirimor-50® on the root tip meristems of the *Vicia faba* (var. minor) chromosomes, SCEs were scored. Once the seeds germinated and reached 2-3 cm, root tips were put into a solution containing 100 μmol L^{-1} bromodeoxiuridine (BrdU), 0.1 μmol L^{-1} fluorodeoxiuridine (FdU), and 5 μmol L^{-1} uridine (Urd) for one replicative 20-h cycle at 20 °C in the dark. Afterwards, the root tips were treated for 4 h with Pirimor-50® at following concentrations: 0.005, 0.01, 0.015, 0.02, 0.025, 0.05, 0.075, and 0.1 mg mL^{-1}, all concentrations dissolved in distilled water. A fresh solution containing BrdU, FdU, and Urd was applied for a second replicative 20-h cycle.

The treatments were performed at 20 °C in the dark. Two experiments were run for each concentration. Two millimetres of the root tips were cut and treated with colchicine (0.05 %) for 3 h in the dark, then stained using the Feulgen differential technique described by Tempelaar et al. (28) and modified by Gómez-Arroyo and Villalobos-Pietrini (29).

Treatment of human lymphocyte cultures

When DMSO was used as a solvent, in all cases its final concentration was ≤1 %. Two experiments were carried out for each treatment using 3 mL of RPMI medium 1640 with L-glutamine and 0.2 mL of phytohemagglutinin added to 0.5 mL of a healthy donor's blood in a culture flask. The cultures were incubated at 37 °C for 72 h. After 24 h, BrdU was added to the culture medium at a final concentration of 5 μg mL^{-1}. In the experiments without metabolic activation, cultures were directly exposed to 0.05, 0.1, 0.25, 0.5, 0.75, 1, 1.5, 2, and 2.5 mg mL^{-1} of Pirimor-50®. Mitomycin C (400 ng mL^{-1}) was used as a positive control. Pirimor-50® was dissolved in 1 % DMSO, concentration that did not affect the SCE frequency according to our previous results (not shown here) and to the results of other studies (30-32). Preliminary experiments were performed in order to identify the optimal concentrations of Pirimor-50® that did not cause cell death. The cultures were incubated for additional 48 h at 37 °C, and 0.1 mL of colchicine (5 μmol L^{-1}) was added 2 h prior to the end of cell culture. Metaphase cells were harvested by centrifugation, treated with 0.075 mol L^{-1} of KCl for 20 min, and fixed in methanol-acetic acid (3:1). Slides were stained using the fluorescence-plus-Giemsa technique (33). In addition to the study of SCE, a BrdU differential staining technique was used to assay the effect of Pirimor-50® on cell replication. For evaluation of cytokinetics, the proportion of the first (M_1), second (M_2), and third (M_3) metaphases was obtained from 100 consecutive mitoses for each treatment, and the RI was calculated as follows: $RI=1M_1+2M_2+3M_3/100$ (34). To avoid bias, the slides were scored blinded.

The blood from three donors was used separately in different experiments. Because the SCE baseline frequencies among the donors were not significantly different, the influence of the inter-individual variability was negligible in response to the mitogens, culture conditions, or blood samples, as suggested by Morgan and Crossen (35), and Speit et al. (36). Samples were taken from three healthy male donors, nonsmokers and nondrinkers, with an average age of 30 years. The donors did not use any medication in six months prior to sampling and did not undergo any radiation/diagnostic treatment in the same period. This study was performed in accordance with the principles stated in the Declaration of Helsinki. All participants were informed regarding the study, accepted the idea of taking part in it, and they subsequently signed a document in which they agreed.

Treatments with metabolic activation

In vivo *activation. Treatment of human lymphocyte cultures with extracts of* Vicia faba *roots exposed to Pirimor-50®*

Vicia faba (var. minor) seeds were germinated between two cotton layers soaked in tap water. When the primary roots reached a length of 4-6 cm, they were immersed in Pirimor-50® at 0.5, 1, 1.5, 2, 2.5, and 3 mg mL^{-1} in a water solution at 20 °C in the dark for 4 h. The concentrations were chosen based on preliminary experiments that examined the phytotoxicity of the insecticide. The positive control roots were exposed to 3.6 mg mL^{-1} of ethanol (0.1 mol L^{-1}) for 4 h at 20 °C. Ethanol is a promutagen in *Vicia faba*, which increases the SCE frequency (27, 37-39).

The negative control was handled under the same experimental conditions, but the roots of *Vicia faba* were immersed in distilled water to obtain the S10 fraction.

After treatment, the roots were rinsed three times with distilled water and cut at approximately 2 cm from the primary root tips. The roots were macerated and homogenised at 4 °C in 0.1 mol L^{-1} sodium-phosphate buffer, pH 7.4. The ratio of the buffer solution in millilitres (2.0-2.5) to the fresh weight of the root cuttings in grams (2.0-2.5) was 1:1 (23). The homogenised roots were centrifuged for 15 min at 10,000 x *g* and 4 °C. The supernatant was sterilised using Millipore filters (0.45 μm pore size) and immediately used to treat human lymphocytes in culture. The total protein concentration in these extracts was determined using the Bio-Rad method (40). Protein concentration was constant from one experiment to another, with values between 4.2-5.0 μg μL^{-1}.

In vitro *activation. Treatment using* in vitro *promutagen activation by* Vicia faba *S10 mix*

Lymphocytes cultured for 48 h were exposed to Pirimor-50® at 0.5, 1, 1.5, 2, and 2.5 mg mL^{-1} (concentrations chosen in preliminary experiments) for 2 h in the dark at 37 °C with and without simultaneously adding the metabolic activation S10 mix (cultures were stationary for the first hour and gently shaken for the second hour). After treatment, cells were rinsed twice in 0.9 % sodium chloride and incubated for 24 h in an RPMI medium containing BrdU at a final concentration of 5 μg mL^{-1}. Colchicine (0.1 mL, 5 μmol L^{-1}) was added 70 h after starting the culture, and the same harvesting and staining procedures were performed as described above.

The metabolic activation system with the S10 mixture was prepared from the microsomal S10 fraction at a 1:9 ratio (v/v) with the following compounds: 8 mmol L^{-1} $MgCl_2$, 3.3 mmol L^{-1} KCl, 5 mmol L^{-1} glucose-6 phosphate, 4 mmol L^{-1} NADP and NAD, and 0.1 mol L^{-1} Na_2HPO_4-NaH_2PO_4 at pH 7.4. The 48 h cultures were incubated for 2 h at 37 °C with 500 μL of activation system and several concentrations of the insecticide. As a positive control, 3.6 mg mL^{-1} ethanol (0.1 mol L^{-1}) was used (23-24, 37, 39).

In vitro *activation. Treatment using* in vitro *promutagen activation by mammalian S9 mix*

The microsomal S9 fraction from rat liver in a commercial form (Mol Tox, from Molecular Toxicology Inc USA) was prepared according to Ames *et al*. (41) and Frantz and Malling (42).

The S9 mixture was prepared with 1 mL of S9 extract and NADPH generator system. It contained sterile cofactors: 0.2 mL (0.4 mol L^{-1}) $MgCl_2$, 5 mL of pH 7.4 phosphate buffer (0.2 mol L^{-1} Na_2HPO_4 and NaH_2PO_4), 0.4 mL NADP (0.1 molL^{-1}) and 0.05 mL of glucose-6-phosphate (1 mol L^{-1}), and 3.35 mL of distilled water; all of the reagents were freshly prepared. Similarly to direct treatments, BrdU was added after 24 h, and 500 μL of the S9 mixture and Pirimor-50® at concentrations 0.5-2.5 mg L^{-1} were added 48 h later and cultured for 2 h (cultures were stationary for the first hour and gently shaken for the second hour). After each treatment, cell cultures were washed twice with 0.9 % sodium chloride and incubated for 24 h with RPMI medium containing 100 μL of BrdU (0.4 mg mL^{-1}). The positive control was 50 μL (40 μmol L^{-1}) cyclophosphamide (CP) with and without the S9 mix, and the negative controls used BrdU (5 μg mL^{-1}) and DMSO (1 %).

Statistical analysis

To quantify the SCE frequencies, 25 well-spread metaphase cells at the second division were scored for each concentration (27). Two experiments were performed, and the results obtained were compared using Student's *t*-test. SCEs were statistically analysed using an ANOVA assay to determine significant differences among the treated groups. When a significant F value ($p<0.0001$) was found, a Newman-Keuls multiple comparison test was applied to identify groups with significant differences at $p<0.001$ when compared with controls. A chi squared (χ^2) test was used for RI. The parts of the decomposed χ^2 were used to compare the values of M_1, M_2, and M_3.

RESULTS

Direct treatments without metabolic activation

Treatment of Vicia faba *root tips*

Considering the SCE frequencies in *Vicia faba* root tip meristematic cells, a concentration-dependent response was observed between 0.005 mg mL^{-1} and 0.025 mg mL^{-1}. Besides the first concentration of 0.005 mg mL^{-1} of Pirimor-50®, all other were significantly higher ($p<0.001$) than the control frequency. However, three of the highest concentrations showed a decrease in the SCE frequency when compared to other concentrations (Table 1).

Treatment of human lymphocyte cultures

Table 2 showed the averages of two experiments in which Pirimor-50® was added directly to the human lymphocyte culture. Although the SCE frequency in the treated samples was higher than in the negative control, only the concentrations 1-2 mg mL^{-1} showed a significant difference from the control values ($p<0.001$). At 2.5 mg mL^{-1}, the detected cells were only in the M_1 fraction and so the SCE frequency could not be determined.

Cell kinetics and the RI are also listed in Table 2. Concentrations of Pirimor-50® starting at 0.5 mg mL^{-1} increased the M_1 and M_2 cell fractions but significantly decreased the M_3 fraction. Treatments produced significant inhibition of the cell cycle progression compared with the corresponding negative control values ($p<0.001$) starting

Table 1 *Sister chromatid exchanges induced by Pirimor-50® in* Vicia faba *root tip meristems*[a]

(mg mL^{-1})	X̄	±	S.E.
Control	26.20	±	0.89
0.005	32.84	±	1.21
0.010	37.10*	±	1.13
0.015	41.18*	±	1.21
0.020	42.06*	±	1.03
0.025	61.48*	±	1.33
0.050	59.75*	±	1.56
0.075	53.87*	±	1.27
0.100	40.26*	±	1.36

[a]*n=50 metaphase cells in two experiments.*
**Significant differences among controls and each treated group were found by analysis of variance F=95.55, p value is <0.0001, and therefore the Newman-Keuls multiple comparisons test was applied,* p<*0.001*

from 0.5 mg mL^{-1}. The RI diminished strongly as the concentration increased to 2.5 mg mL^{-1}, and only the M_1 fraction was observed at this concentration.

As expected, data revealed significant differences in the SCE frequency, cell kinetics, and RI between the negative and positive control (treated with MMC), ($p \leq 0.001$).

Treatments with metabolic activation

In vivo *activation. Treatment of human lymphocyte cultures with extracts of* Vicia faba *roots exposed to Pirimor-50®*

The *in vivo* activation by S10 *Vicia faba* mix is shown in Table 3. Extracts of the roots taken after 4 h insecticide treatment were applied to 24 h lymphocyte cultures. After 48 h of treatment, a concentration-dependent increase in the SCE frequency was observed for all concentrations, except for 3 mg mL^{-1}, where all of the examined cells were dead. Significant differences from the control values were detected for concentrations 2 and 2.5 mg mL^{-1}.

When ethanol was added directly to lymphocyte cultures, the SCE frequency was not significantly affected (negative control). Likewise, extracts from the untreated roots (also the negative control) did not increase the SCE frequency. However, significant differences were observed when lymphocytes were treated with extracts of the *Vicia faba* roots plus ethanol 3.6 mg mL^{-1} (the positive control) (Table 3).

The metabolites of Pirimor-50® contained in the *Vicia faba* root extracts applied to lymphocyte cultures did not produce significant effects on cell kinetics or RI (Table 3), but at 3 mg mL^{-1} we observed only dead cells.

In vitro *activation*

Pirimor-50® treatment with in vitro *promutagen activation by mammalian S9 mix and* Vicia faba *S10 mix*

As shown in Table 4, no significant increase in SCE frequencies was observed when the insecticide was applied directly for 2 h to lymphocyte cultures neither in the presence of animal (S9) or vegetal (S10) metabolic fractions, although the values were slightly higher than in the negative control. The M_3 cells and RI diminished significantly without and with S9 metabolic activation at all concentrations. Significant differences in the SCE frequency were observed with the positive control [we have added cyclophosphamide (CP) to the S9 mammalian metabolic fraction; and ethanol to the S10 *Vicia faba*

Table 2 *Sister chromatid exchanges induction and effects on cell kinetics (M_1, M_2, and M_3 cells) and replication index (RI) by direct treatments of Pirimor-50® in human lymphocyte cultures*[a]

		X̄±S.E.	M_1	M_2	M_3	% RI[b]
Negative control		4.79 ± 0.35	26	38	36	2.10
MMC (400 ng mL^{-1}) (positive control)		15.36 ± 0.65*	51	31	18**	1.67**
Lymphocyte cultures directly treated with Pirimor-50® (mg mL^{-1})	0.05	6.72 ± 0.57	26	33	41	2.15
	0.10	7.02 ± 0.51	26	35	39	2.13
	0.25	6.62 ± 0.38	25	39	36	2.11
	0.50	7.08 ± 0.52	49	40	11**	1.62**
	0.75	6.96 ± 0.45	50	40	10**	1.60**
	1.00	9.62 ± 0.57*	45	45	10**	1.65**
	1.50	9.50 ± 0.60*	59	35	06**	1.47**
	2.00	9.44 ± 0.71*	55	43	02**	1.47**
	2.50	Second division metaphases were not observed	100	0	0	1.00**

[a]*n=50 metaphase cells in two experiments;* [b] *Replication index, n= 200 consecutive metaphases. *Significant differences among controls and each treated group were obtained by analysis of variance F =29.02 the p value is < 0.0001, and therefore the Newman-Keuls multiple comparison test was applied p<0.001; **Significant with* χ^2, p<*0.05.*

Table 3 *Sister chromatid exchanges induction and effects on cell kinetics (M_1, M_2, and M_3 cells) and replication index (RI) by Pirimor-50® with* in vivo *metabolic activation by* Vicia faba *in human lymphocyte cultures*[a]

		X̄±S.E.	M_1	M_2	M_3	% RI[b]
Negative control		4.79±0.35	26	38	36	2.10
Lymphocyte cultures + *V. faba* extracts, untreated (negative control)		4.57±0.35	20	37	43	2.23
Lymphocyte cultures + ethanol (5 µg mL^{-1}) (negative control)		4.35±0.21	28	39	38	2.20
Lymphocyte cultures + ethanol 3600 mg L^{-1} + *V. faba* extracts (positive control)		8.96*±0.53	17	37	46	2.29
Lymphocyte cultures + *V. faba* extracts from treatment with Pirimor-50® (mg mL^{-1})	0.5	5.30±0.41	15	29	56	2.41
	1.0	5.42±0.58	12	33	55	2.43
	1.5	6.24±0.51	25	25	50	3.41
	2.0	7.56±0.52*	13	35	52	3.08
	2.5	9.54±0.53*	16	33	51	2.35
	3.0	Cellular death [c]				

[a]*n=50 metaphase cells in two experiments;* [b]*Replication index, n=200 consecutive metaphases; *Significant differences among controls and each treated group were obtained by analysis of variance; F=17.90 the p value is <0.0001, and therefore the Newman-Keuls multiple comparison test was applied;* p*<0.001*.

metabolic fraction]. Combined treatment did not change SCE significantly, influencing only proliferation kinetics and diminishing RI in the sequence: Pirimor-50®+S10mix> Pirimor-50®> Pirimor-50®+S9mix.

DISCUSSION

Pirimor-50® is the most widely used commercial formulation for aphid control. Aficida® and Patton Flow®, have been found capable of inducing genotoxic damage (17-18, 21, 25) in water living organisms, but no data is available on the genotoxicity of Pirimor-50® as a pirimicarb-based formulation in human lymphocytes and *Vicia faba*. In the only one study with Pirimor G formulation, Mesnage et al (26) studied the cytotoxic effects on three different types of human cell lines, HepG2, HEK293, and JEG3. They discovered that the formulation does not produce the same cytotoxic effect as the active ingredient alone (almost no difference in cell viability and no activation of Adenylate kinase activity or Caspases 3/7 activity). The present study is one of the first to evaluate and compare not only the direct effect of Pirimor-50® in two bioassays, animal and vegetal, but also the effect of plant and animal drug metabolism on its genotoxic potential.

Although the evaluated concentrations of Pirimor-50® in this study are expected to be rare in the environment, perhaps only observed when specific events occur (e.g. direct application), we cannot rule out the possibility that organisms and occupationally exposed human workers could be exposed accidentally to such high concentrations. We also need to point out that plants are exposed to these concentrations, as pirimicarb is registered for use as a pesticide to control a large variety of chewing and sucking insects on a wide range of crops in many countries at concentrations that go up to 0.2-2.5 mg mL^{-1} (48), and this range was covered in our experiments.

Direct treatments with Pirimor-50® increased the SCE frequency efficiently in *Vicia faba* under laboratory conditions. Although the increase in SCE frequencies positively correlated with concentrations, these frequencies decreased at the highest concentrations (Table 1), with no alteration of the mitotic index (data not showed). Such an alteration could be explained by the presence of the cytotoxic potential exerted by the formulation and inhibitory effects of the concentrations tested due to the alterations in cell kinetics, as has been demonstrated in other assays (21). Although the mitotic index of *Vicia faba* was not altered, there is a possibility that SCE frequencies diminished through a damaged cell elimination pathway or by a damage repair process upon increasing pesticide exposure. On the other side, we cannot rule out the possibility that the metabolic system of *Vicia faba* can participate in the detoxification process and also have an impact on the DNA damage frequency and damaged cell elimination. Vera-Candioti *et al*. (21) have found similar results but also no experimental evidence to explain this particular finding. Therefore, further experiments should be conducted to elucidate whether this observation is related to any of the above-mentioned possibilities or whether it is the result of several independent processes occurring simultaneously during metabolism and cell repair and elimination.

In direct treatments with Pirimor-50® in lymphocyte cultures for 48 h (Table 2), we have used the concentrations up to 3 mg mL^{-1} in order to cover the entire range to which individuals can be exposed in direct contact. A positive significant SCE response was obtained for 1-2 mg mL^{-1} concentrations but SCE frequencies were more or less similar for these concentrations. These results disagree with those reported by the USEPA (49) in which no induction of chromosomal aberrations on *in vitro* human lymphocytes

treated with a direct application of pirimicarb was observed. However, they are also in agreement with previous findings depicting the genotoxic cytotoxic potential of pirimicarb through the induction of SCE, chromosomal aberrations and cell cycle delay in CHO-K1 cells (7), and chromosomal damage in human lymphocytes *in vivo* (22), or *in vitro* (4). It should be highlighted that the behaviour found in human lymphocytes is similar to CHO-K1 cells treated with Aficida® and pirimicarb in the study by Soloneski and Larramendy (7).

The increase in the SCE frequency observed after direct exposure could suggest that high concentrations of Pirimor-50® can cause DNA strand breaks. Other authors have also noted that pirimicarb induces genotoxicity through chromosomal damage and DNA single-strand breaks both *in vivo* and *in vitro* (4, 7, 17-18, 22). It has been observed that pirimicarb can bind to DNA by intercalation (50). This mode of action could explain the results found in this study and also the decrease in RI that showed a significant effect on the concentration lower than the one where significantly higher SCE frequencies were found (starting from 0.5 mg mL^{-1}). Due to all these facts, a possible clastogenic effect of the insecticide could be suggested as already mentioned in the study by Soloneski et al. (51).

The results obtained in this study from the genotoxic and cytotoxic assays indicate that there are differences between the two bioassays employed concerning their sensitivity to Pirimor-50®. According to Soloneski et al. (51), this observation could be explained by the nature of each bioassay. Different assays can give different results depending on the test agent used and the bioassay employed, highlighting the importance of the use of more than one bioassay to determine the damage induced for a genotoxic agent.

On the other side, when *Vicia faba* extracts, obtained after treatment of *Vicia faba* roots for 4 h with different concentrations of the insecticide, were applied to lymphocyte cultures, concentrations of 2 and 2.5 mg mL^{-1} of Pirimor-50® caused a concentration-dependent response in the SCE frequency, and cellular death was induced at 3 mg mL^{-1}. No significant differences were found in cell kinetics and RI. The products obtained from the *in vivo* promutagen activation of Pirimor-50® by *Vicia faba* applied to lymphocyte cultures were capable of increasing the SCE frequency, which means that this compound acted directly and indirectly. However, toxicity diminished with plant metabolism, possibly indicating that certain detoxification mechanisms are involved (52-53). The same detoxification was observed with the thiocarbamate herbicides molinate and butylate in the presence of the S10 fraction (38).

Comparing both plant activation systems, *in vivo* and *in vitro*, significant SCE frequencies were only observed *in vivo*, and these differences could be related to the exposure time of lymphocytes and/or to active metabolites. In the *in vivo* activation experiments, treatments lasted 48 h, while exposure lasted only 2 h for the *in vitro* activation. The latter

Table 4 *Sister chromatid exchanges induction and effects on cell kinetics (M_1, M_2 and M_3 cells) and replication index (RI) by Pirimor-50® without and with* in vitro *animal and plant metabolic activation in human lymphocyte cultures*[a]

Concentration	Without metabolic activation			With S9 animal metabolic activation			With S10 plant metabolic activation		
	SCE / metaphase $\bar{X}$±S.E.	Metaphases M_1 M_2 M_3	RI[b]	SCE / metaphase $\bar{X}$±S.E.	Metaphases M_1 M_2 M_3	RI[b]	SCE / metaphase $\bar{X}$±S.E.	Metaphases M_1 M_2 M_3	RI[b]
Control	4.98±0.30	42 39 19	1.77	5.86±0.34	41 38 21	1.80	4.84±0.31	48 38 14	1.66
DMSO	6.10±0.51	42 37 21	1.79	5.95±0.42	47 33 20	1.73	6.56±0.52	43 34 23	1.80
CP	6.59±0.34	43 39 18	1.75	41.19±2.14*	40 40 20	1.80	7.40±0.34	50 33 17	1.67
Ethanol	6.01±0.35	47 39 14	1.67	7.96±0.47	48 37 15	1.67	9.10±0.25*	44 35 17	1.77
Pirimor-50® (mg mL^{-1}) 0.5	6.16±0.37	54 38 08**	1.54**	6.47±0.48	58 34 08**	1.50**	5.32±0.29	48 37 15	1.67
1.0	6.83±0.31	57 34 09**	1.52**	6.26±0.39	65 26 09**	1.44**	6.00±0.37	51 34 15	1.64
1.5	6.33±0.36	49 41 10**	1.61**	5.94±0.33	63 29 08**	1.45**	6.96±0.25	50 33 17	1.67
2.0	6.42±0.25	47 44 09**	1.62**	6.70±0.79	58 34 08**	1.50**	6.54±0.30	57 29 14	1.57
2.5	7.39±0.30	55 35 10**	1.55**	7.74±0.84	60 31 09**	1.49**	6.40±0.41	58 39 14	1.65

[a]*n=50 metaphase cells in two experiments;* [b]*Replication index, n=200 consecutive metaphases;* **Significant differences among controls and each treated group were obtained by analysis of variance; F=29.02 the p value is < 0.0001, and therefore the Newman-Keuls multiple comparison test was applied* $p<0.001$*;* ***Significant with* χ^2*,* $p<0.05$

time period may not have been sufficient for the cells to metabolise the insecticide. On the other hand, this may have been due to enzyme inactivation because direct contact of the insecticide with the S10 fraction could have inhibited the insecticide metabolism.

In plant metabolism, peroxidases are among the most important enzymes involved in the oxidative transformation of xenobiotics (54); peroxidases catalyse two categories of oxidative reactions in plant cells, the peroxidative reaction requiring H_2O_2 and the oxidative reaction using molecular oxygen (55). Calderón-Segura et al. (38) described the phytotoxic effect of the thiocarbamic herbicide butylate in *Vicia faba*. Cell death by this herbicide is caused by the absence of metabolism, and several other agents have been described to lower plant cell culture viability (56).

In the *in vitro* animal (S9)/vegetal (S10) metabolism assay, the SCE frequency was not significantly increased, but cell kinetics were affected with 2 h of treatment with Pirimor-50® both without and with the presence of the S9 fraction (Table 4). Both methods significantly decreased the number of M_3 cells, and the insecticide induced a cell cycle delay and significant differences in RI. This cell cycle arrest may be an adaptive process in which a surveillance mechanism delays the cell cycle when DNA lesions occur. The ability of cells to delay their cell cycle in order for the repair to take place is well known (57-58).

Several investigations have proven that commercial formulations have the ability to induce DNA damage (15, 59-62). For pirimicarb and its formulations, the results revealed, depending upon the endpoint employed, that the damage induced by the latter is, in general, greater than that produced by the pure pesticide. Unfortunately, manufacturers have not made the identity of the components present in excipient formulations available. However, the deleterious effect/s of the adjuvants/s present within the commercial formulation should neither be discarded nor underestimated.

It has been suggested that the damage induced by pirimicarb could be mostly related to the effect of desmethyl pirimicarb and/or desmethylformamido pirimicarb metabolites (11), two toxicologically relevant metabolites. So far, the mechanism/s by which N,N-dimethylcarbamate and their carbamate metabolites exert genotoxicity has not been fully established (7). The main metabolic pathway involves the loss of carbamate moiety to produce a range of substituted hydroxypyrimidines, some of which are glucuronide conjugates. Unfortunately, in our study we did not have the possibility to examine the content of the plant extracts and evaluate the metabolites found inside, as well as the level of other oxidative and antioxidative substances.

Taken together, our findings suggest the importance of further studies on this type of pesticide in order to achieve a complete knowledge on its genetic toxicology. Future studies either *in vivo* or *in vitro* are required in order to clarify its mechanism of action.

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Genotoksični učinci karbamatnog insekticida Pirimor-50® na vršni meristem korijena biljke *Vicia faba* i kulturu ljudskih limfocita nakon izravne primjene i tretiranja njegovim metaboličkim ekstraktima

Cilj ovog ispitivanja bio je procijeniti genotoksične učinke insekticida Pirimor-50®, formulacije koja se temelji na pirimikarbu (50 %-tni aktivni sastojak), u kulturama ljudskih limfocita i meristemu korijena biljke *Vicia faba*. Nadalje, cilj je bio ispitati objedinjeni utjecaj tretiranja insekticidom i metaboličkom mješavinom S9 za kultivirane stanice sisavaca i mješavinom S10 za stanice biljaka ili mješavinom S10 transformacijskih ekstrakata (nakon tretiranja primarnih korijena biljke *Vicia faba* insekticidom Pirimor-50®). Korišten je test izmjena sestrinskih kromatida (*Sister Chromatid Exchange – SCE*) i mjerena je progresija i proliferacija staničnog ciklusa (kroz omjer M_1-M_3 metafaza i vrijednost replikacijskog indeksa – RI). Dva su procesa korištena za aktivaciju biljnog promutagena: *in vivo* aktivacija – Pirimor-50® primjenjivan je tijekom 4 h na biljci, a potom je mješavina S10 dodana limfocitima, i *in vitro* aktivacija – limfociti su 2 h tretirani insekticidom Pirimor-50® i mješavinom S10 ili S9. Izravno tretiranje proizvelo je značajno veću učestalost SCE-a u meristemu pri 0,01 mg mL^{-1}. U limfocitima je razina SCE-a značajno povećana pri 1 mg mL^{-1}, uz smanjenje RI-ja i omjera M_1-M_3 metafaza pri 0,5 mg mL^{-1}, uz zastoj stanične diobe pri 2,5 mg mL^{-1}. Tretiranje limfocita mješavinom S10 značajno je povisilo vrijednosti SCE-a pri 2-2,5 mg mL^{-1}, a stanična smrt nastupila je pri 3 mg mL^{-1}. Tretiranje limfocita insekticidom Pirimor-50®, zajedno sa S9 ili S10, pokazalo je nešto veću učestalost SCE-a, ali i značajniji utjecaj na povećanje RI-ja, pri čemu su najniže vrijednosti utvrđene nakon tretiranja mješavinom S9. S obzirom na to da nema podataka o genotoksičnosti insekticida Pirimor-50®, ovo je istraživanje među prvima koje pomoću dvaju testova, životinjskoga i biljnoga, ispituje i uspoređuje njegov izravan učinak, ali i učinak biljnog i životinjskog metabolizma na njegov genotoksični potencijal.

KLJUČNE RIJEČI**:** *aktivacija biljnog i životinjskog promutagena; izmjene sestrinskih kromatida; kinetika stanične proliferacije; replikacijski indeks*

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An alternative approach to studying the effects of ZnO nanoparticles in cultured human lymphocytes: combining electrochemistry and genotoxicity tests

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Nanoparticle use has increased radically raising concern about possible adverse effects in humans. Zinc oxide nanoparticles (ZnO NPs) are among the most common nanomaterials in consumer and medical products. Several studies indicate problems with their safe use. The aim of our study was to see at which levels ZnO NPs start to produce adverse cytogenetic effects in human lymphocytes as an early attempt toward establishing safety limits for ZnO NP exposure in humans. We assessed the genotoxic effects of low ZnO NP concentrations (1.0, 2.5, 5, and 7.5 μg mL^{-1}) in lymphocyte cultures over 14 days of exposure. We also tested whether low and high-density lymphocytes differed in their ability to accumulate ZnO NPs in these experimental conditions. Primary DNA damage (measured with the alkaline comet assay) increased with nanoparticle concentration in unseparated and high density lymphocytes. The same happened with the fragmentation of *TP53* (measured with the comet-FISH). Nanoparticle accumulation was significant only with the two highest concentrations, regardless of lymphocyte density. High-density lymphocytes had significantly more intracellular Zn^{2+} than light-density ones. Our results suggest that exposure to ZnO NPs in concentrations above 5 μg mL^{-1} increases cytogenetic damage and intracellular Zn^{2+} levels in lymphocytes.

KEY WORDS: *comet-FISH,* in vitro*; primary DNA damage;* TP53*; voltammetry*

Nanotechnology has penetrated every segment of human life. Its ever growing presence has raised concern over the health risks it may be posing (1). The existing approaches to risk assessment have been challenged by new nanoparticles that can reach otherwise inaccessible biological structures and induce unexpected biological behaviour due to their small size and large surface area. For example, nanoparticles can both stimulate and suppress the immune system (2).

Among the most common nanomaterials in consumer and medical products are zinc oxide nanoparticles (ZnO NPs). They are widely used as drug delivery vehicles, anticancer agents, components of the restorative dental and food packaging materials, and as cosmetic, antiseptic, and ultraviolet protection products (3, 4). Exposure to ZnO NPs has come into focus with recent reports that their use may result in safety issues. Research *in vitro* has demonstrated dose-dependent cytotoxic effects of ZnO NPs in mouse neural cells, mouse ascites cells, and human epithelial lung/bronchus (5), liver (6), cardiac microvascular endothelial (7), and kidney cells (8). These toxic effects were mediated by oxidative stress and inflammation induced by ZnO NPs, which had stimulated the release of pro-inflammatory cytokines IL-6 and TNF-α.

Oxidative stress and inflammation have also been observed in animal models. Ma-Hock et al. (9) reported toxic effects of inhaled ZnO NPs on the respiratory epithelium in rats. Wang et al. (10) reported a dose-dependent increase in acute cytotoxic effects on the stomach, liver, heart, and spleen tissues of orally treated mice. Zhao et al. (11) reported ZnO NPs developmental toxicity in embryo-larval zebrafish. So far, however, safety limits for human exposure to ZnO NPs have not been proposed.

Recently, there have been attempts to assess the risk of genotoxicity posed by nanomaterials with the comet assay (12, 13). Considering that ZnO NPs have been reported to adversely affect lymphocytes (14), white blood cells seem a model of choice for testing nanoparticle genotoxicity. In our study we opted for human lymphocyte cultures to see whether 14-day exposure to increasing concentrations of ZnO NPs would produce genotoxic effects and whether they would be concentration-dependent. We also wanted to see whether lymphocytes differed in their capacity to accumulate zinc after exposure to the same concentration of ZnO NPs. To do that we took a new approach that combines voltammetry to measure intracellular exposure

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with the methods measuring effects: the alkaline assay to determine general DNA damage and the comet-FISH assays to determine specific effects on the structural integrity and copy number of the *TP53* gene.

MATERIALS AND METHODS

By exposing lymphocyte cultures to different ZnO NP levels over 14 days we ensured that the cells would be exposed in all the phases of the cell cycle, which makes possible the direct contact between the genome and NPs. This is because certain types of NPs can interact with the DNA only during mitosis, when the nuclear membrane breaks down. Therefore, the extended exposure ensured maximum access to the genetic material (15), simulating exposure conditions *in vivo*.

We used the alkaline comet assay to determine the primary DNA damage in the lymphocytes. To identify specific effects on structural integrity and determine the number of the *TP53* gene copies and the corresponding centromeres on chromosome 17 we used the comet-fluorescence *in situ* hybridisation (comet-FISH) assay. The *TP53* gene product plays an important role in cell-cycle regulation, leading to its arrest as a response to DNA damage. Too much irreparable genome damage triggers the *TP53*-dependant pathway of apoptosis. The loss of the *TP53* gene, which is a known tumour suppressor gene, indicates increased risk of cancer development (16).

To determine ZnO accumulation we measured the effective levels of ZnO that penetrated cellular membrane by determining the levels of Zn^{2+} in treated cells with differential pulse anodic stripping voltammetry (DPASV) and high-resolution inductively coupled plasma mass spectrometry (HR-ICPMS). Anodic stripping voltammetry is the most widely used method for the estimation of metals in a variety of matrices. Its advantages are sensitivity [elements can be measured at part per trillion (ppt) concentrations], simultaneous determination of several elements, capability to distinguish between oxidation states of atomic ions, and usefulness for speciation studies (17). HR-ICPMS offers several other benefits. It is a highly sensitive method for most elements. Both major and trace elements can be detected simultaneously. HR-ICPMS can easily handle simple and complex sample matrices. It has extremely low detection limits, ranging from part per billion (ppb) to ppt. Despite the advantages of ICP sources compared to other ionisation sources, the analytical precision is limited by inherent instability of the ion signal and sample introduction devices. Instabilities may arise from either a change in energy transfer from the plasma to the sample or variation efficiency in the nebulisation and transportation of the sample (18).

ZnO nanoparticle preparation

Zinc oxide nanoparticles (ZnO NPs) with average particle size <35 nm were purchased from Sigma-Aldrich (St. Louis, MO, USA). To obtain treatment solution under aseptic conditions, the ZnO NP stock solution (50 wt. % in water) was further diluted in phosphate buffer solution (PBS), pH 7.4 (Sigma-Aldrich). Before each treatment, this solution was sonicated for 20 minutes to ensure dispersion and prevent deagglomeration and reagglomeration (19).

Blood sampling and lymphocyte collection

Peripheral blood was taken from a healthy male non-smoker donor aged 26 years with no history of chronic conditions. The blood was collected from the antecubital vein in heparin-coated vacutainers (Becton Dickenson, New Jersey, NJ, USA). We opted for the single-donor design to avoid donor variations in response and scattering of the results. The procedure was reviewed and approved by the ethics committee of the Institute for Medical Research and Occupational Health, and the donor gave his consent in writing.

Lymphocytes were isolated, and their *in vitro* cultures established following the procedure described elsewhere (20).

Culture treatment

Duplicate samples of lymphocyte cultures were incubated in a 5 % CO_2 HeraCell® 240 incubator (Kendro Laboratory Products, Vienna, Austria) at 37 °C for 14 days with solutions containing 1.0, 2.5, 5, or 7.5 μg mL^{-1} of ZnO NPs. We based this concentration choice on our preliminary experiments (data not shown). Negative control was treated with PBS. Positive control was treated with 8 μg mL^{-1} methyl methanesulphonate (MMS; Sigma-Aldrich) for the last 4 h of incubation. Figure 1 shows a detailed treatment algorithm along with the study design.

Viability testing

To determine cell viability, the lymphocytes were simultaneously stained with acridine orange and ethidium bromide (Sigma-Aldrich), 100 mg mL^{-1} of each (v/v 1:1), and analysed with a epifluorescence microscope Olympus BX 51 (Olympus, Tokyo, Japan) under 600× magnification. Hundred cells per each duplicate slide were counted to determine the percentages of viable, apoptotic, and necrotic cells (21).

Experimental design

We took two routes to assess primary DNA damage (Figure 1). The first was to collect the entire lymphocyte culture population treated with ZnO NPs on the 15th day. The cultures were centrifuged at 300 *g* for 5 min, and the cells washed twice in a fresh culture medium (RPMI 1640, Gibco, Paisley, UK). The resulting lymphocyte pellet was

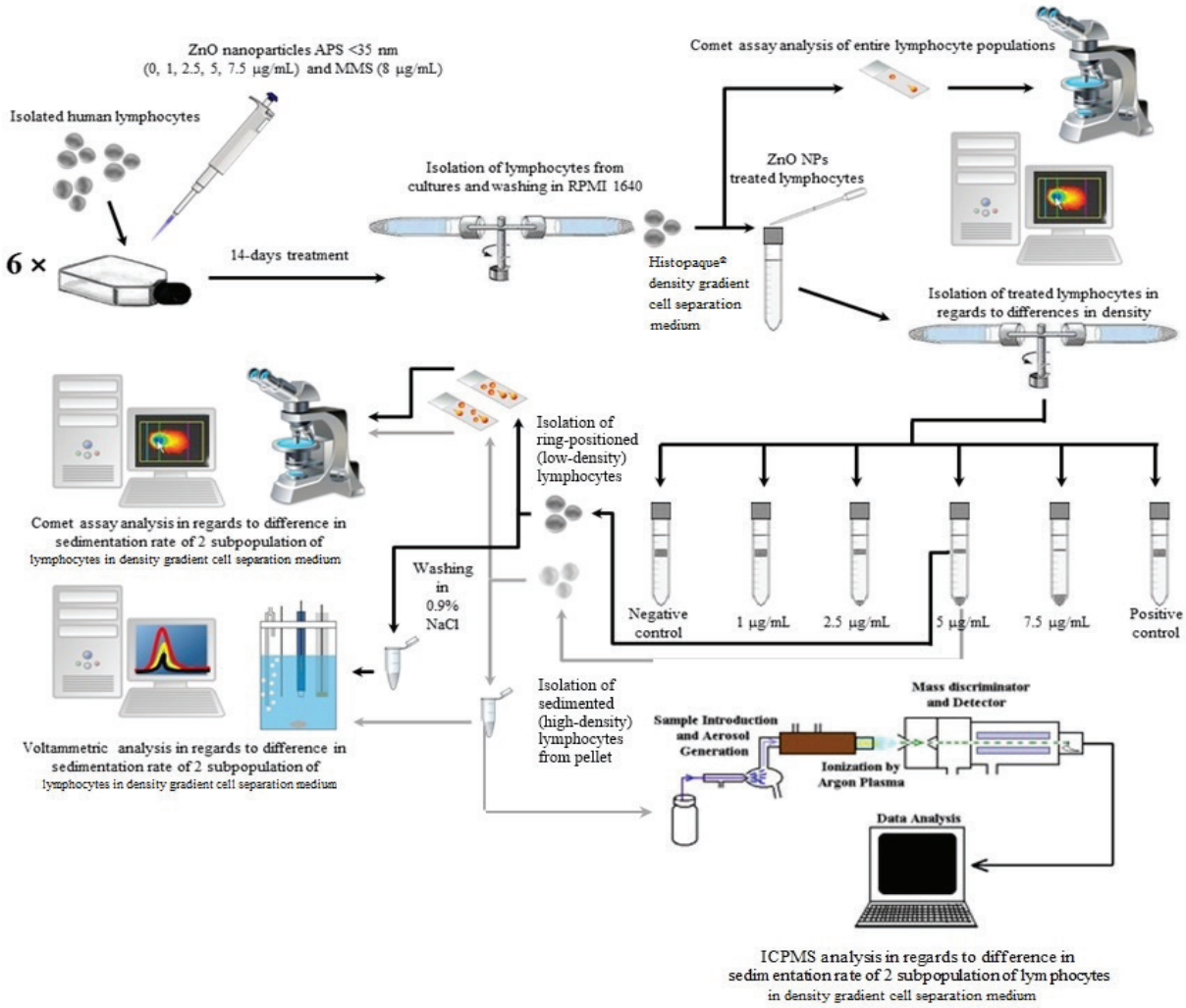

Figure 1 *Scheme of the experimental design*

diluted in saline (0.3 mL; 0.9 % NaCl w/w) and cell density determined. One microlitre of suspension containing 10^4 lymphocytes was mixed with 100 μL of 0.5 % low melting point agarose (Sigma-Aldrich) and placed on a precoated slide to prepare the microgels for the alkaline comet assay.

The second route was to collect lymphocyte cultures on day 15 (as in the first), centrifuge them at 300 *g* for 5 min, wash once and resuspend in RPMI 1640 (3 mL), layer on the Histopaque® 1077 reagent (3 mL, Sigma-Aldrich), and centrifuge at 400 *g* for 30 min to isolate lymphocytes. Isolation resulted in the separation of two lymphocyte populations. The low-density population formed a ring in the isolation medium, while the high-density population pelleted at the bottom of the tube (Figure 1). Each population was extracted separately, washed twice in RPMI 1640, and diluted in saline (0.3 mL). The slides for the comet assay were prepared according to the standard procedure (22). For each ZnO NP concentration tested, as well as for negative and positive controls we prepared four slides. Two were used for the alkaline comet assay and the other two for the comet-FISH assay.

Alkaline comet assay

For the alkaline comet assay we followed the procedure described by Singh et al. (22) with minor modifications. After a 15-minute denaturation followed electrophoresis at 0.7 V cm^{-1} and 300 mA for another 15 min. After electrophoresis, the slides were neutralised and stained with ethidium bromide. Scoring included 100 comets per each duplicate slide using the Comet Assay IV image analysis system (Perceptive Instruments, Suffolk, UK) coupled with epifluorescence microscope (Olympus BX 51) under 200× magnification. We looked for tail length and the percentage of DNA in tail (tail intensity) as the main DNA damage parameters.

Comet-FISH assay

The slides for the comet-FISH assay were processed according to the protocol described by Mladinić and Želježić (23). After electrophoresis and neutralisation, the slides were dehydrated in an ethanol series (70-100 %) and air dried. For each treatment we analysed 30 cells on each of the two duplicate slides using an Olympus AX70

epifluorescence microscope and CytoVision FISH software (Applied Imaging, Dornach, Germany). We recorded the position of the signals for the *TP53* gene and cen 17 in nucleoids and the number of copies. Then we scored the number of signals in the comet tail and head. The absence of the signals from both tail and head was considered a loss of heterozygosity (20).

DPASV and HR-ICPMS analysis of lymphocyte Zn^{2+} concentration

All chemicals used for the preparation of the samples were of high purity: ASTM type I ultra-pure water (Milli-Q; 18.2 MΩ cm, Millipore/Merck, Darmstadt, Germany), Trace SELECT® concentrated HNO_3 (Fluka, Milan, Italy), and sodium-acetate (NaAc; Merck). Aliquots of lymphocytes obtained as described above (Figure 1) were washed two more times in saline, centrifuged at 300 g for 5 min, and the pellet was resuspended in saline to obtain the final cell concentration of 10^5 cells mL^{-1}. Lymphocyte suspension (15 mL) was acidified with ultra-pure HNO_3 (150 µL; Trace SELECT®, Fluka) and left to rest overnight. The samples were then transferred to fluorinated ethylene propylene (FEP) bottles and UV-irradiated under a 250 W high-pressure Hg-lamp for another 24 h for the organic matter to decompose and transform all dissolved Zn into a specimen that can be measured with DPASV. As the working volume of the electrochemical cell used in DPASV is 15 mL, we needed to dilute the sample (~15 mL), so that it would not be used up for one analysis. The solution for the measurement was prepared by taking an aliquot of undiluted primary sample, adding 0.5 mL of 4 mol L^{-1} NaAc) to adjust pH around 4, and adding Milli-Q water to fill up the 15 mL volume. The blank sample containing HNO_3, NaAc, and Milli-Q water used for sample preparation was measured separately and found to contain 0.11 ng mL^{-1} of Zn^{2+}, most of which came from Milli-Q water. We therefore corrected our Zn^{2+} measurements in treated/control samples for the blank measurement, as we followed the same dilution procedure for all samples.

The concentration of Zn^{2+} was measured with DPASV as described by Vukosav et al. (24). Accumulation time was 1 min, and accumulation potential was -1.2 V. Figure 2 shows a typical set of the obtained voltammograms and the corresponding calibration plot (inset). For the measurement signal we used the peak Zn^{2+} voltammogram that we transformed using the 2nd derivative transformation (25). For Zn^{2+}control measurements we used the semi-quantitative HR-ICPMS (Element 2, Thermo Fisher Scientific, Bremen, Germany) (26).

Statistical analysis

Prior to statistical analysis we normalised all data ranges using base 10 log transformation. Differences in comet assay parameters and Zn^{2+} concentrations in lymphocytes were tested with the one-way ANOVA. The same method was used for low- and high-density lymphocytes. We also used the one-way ANOVA to test the differences in measured Zn^{2+} concentrations between DPASV and HR-ICPMS. To test the dependence of each cytogenetic endpoint on intracellular Zn^{2+} concentration and on ZnO concentration used in the treatment we used regression analysis. This method was also used to assess the correlation between the treatment ZnO NP concentrations and intracellular Zn^{2+} concentrations (determined with DPASV). All analyses were run on Statistica 12.0 software (StatSoft, Tulsa, OK, USA).

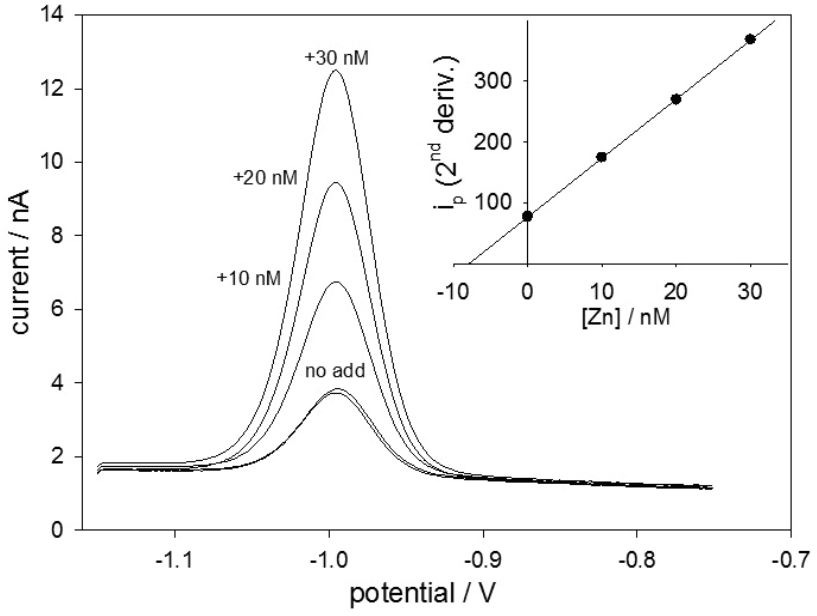

Figure 2 *Typical DPAS voltammograms of Zn^{2+} levels following the Tube 2,5, 5x standard addition method with a threefold dilution. Inset: standard addition plot from which Zn concentration was determined*

RESULTS

Zinc concentrations in treated lymphocytes

The 14-day treatment of lymphocyte cultures with ZnO NPs in the concentrations of 2.5, 5, and 7.5 μg mL^{-1} resulted in the separation of the lymphocytes into two populations of cells according to their relative density (Figure 3). Low-density lymphocytes formed a ring in Histopaque®, while high-density cells settled at the bottom, forming a pellet. This separation was not observed at ZnO NP concentration of 1 μg mL^{-1} or in negative control samples, all of which formed a ring. Even though the separation started with 2.5 μg mL^{-1}, the differences in intracellular Zn^{2+} concentrations between high-density cells and negative control or low-density cells were not significant (37.2±0.0 ng 10^{-5} cells *vs.* 13.8±4.9 ng 10^{-5} cells or *vs.* 19.5±1.7 ng 10^{-5} cells, respectively).

The accumulation of intracellular Zn^{2+} became significant with exposure to 5.0 and 7.5 μg mL^{-1} of ZnO NPs. In low-density lymphocytes it was significantly higher than in negative control (47.1±1.4 and 149.2±7.3 ng 10^{-5} cells, respectively), and in high-density lymphocytes it was significantly higher than in both negative control and low-density lymphocytes (207.2±3.4 and 430.2±8.1 ng 10^{-5} cells, respectively).

Both DPASV and HR-ICPMS used for the determination of intracellular Zn^{2+} levels showed very consistent and reproducible results over the entire range of concentrations and their values did not significantly differ (Figure 4). However, we considered the DPASV results more accurate.

Regression analysis showed a significant correlation between the applied ZnO NP concentrations and DPASV intracellular Zn^{2+} measurements.

Cell viability

Significant differences in the number of apoptotic cells started to show at 5 μg mL^{-1}, but only between high-density and negative control lymphocytes. This difference increased at 7.5 μg mL^{-1} and also became significant between high-density and low-density lymphocytes (Table 1).

Alkaline comet assay

We found no significant differences in the tail length and intensity between the unseparated ZnO NP-treated and negative control cells (first route; Table 2). In the separated cells, similar results were observed for low-density lymphocytes, except for tail intensity in the cells treated with 5 μg mL^{-1} of ZnO NPs, which increased significantly. High-density lymphocytes treated with the highest concentrations of ZnO NPs (5 and 7.5 μg mL^{-1}) had significantly greater tail length and intensity than negative control. Similar was the difference from low-density lymphocytes at 5 μg mL^{-1}, except for the tail length (Table 2).

Regression analysis showed a significant correlation between both the ZnO NP and Zn^{2+} concentrations and the comet tail length.

Comet-FISH analysis

Table 3 shows the fragmentation and deletion rate of the *TP53* gene and the corresponding chromosome 17 centromere. In the unseparated lymphocytes, a significant increase in *TP53* fragmentation was observed in samples treated with 2.5, 5, and 7.5 μg mL^{-1} of ZnO NPs compared to negative controls. ZnO NP treatment did not cause the loss of *TP53* heterozygosity.

In low-density lymphocytes significant *TP53* fragmentation was detected only in the sample exposed to 7.5 μg mL^{-1} of ZnO NPs.

The structural integrity of the *TP53* gene was more impaired in the high-density lymphocytes. Treatment with ZnO NPs concentrations of 2.5, 5, and 7.5 μg mL^{-1} resulted in significantly higher occurrence of *TP53* signals in the comet tail compared to negative controls. Fragmentation rates in high-density lymphocytes exposed to 2.5 and 5 μg mL^{-1} were also significantly higher than in low-density lymphocytes. The percentage of the fragmented centromere 17 copies in high-density cells became significantly higher only at 5 μg mL^{-1} but not at 7.5 μg mL^{-1}. We observed no significant differences in the deletion of the *TP53* copies between the treated and untreated or high and low-density groups.

Regression analysis showed a significant correlation between both the ZnO NP and Zn^{2+} concentrations and impaired structural integrity of the *TP53* gene (Table 3).

DISCUSSION

Our study is the first to investigate nanoparticle genotoxicity in lymphocyte populations separated by density due to different uptake of ZnO NPs. Unlike earlier studies (15, 27, 28), which used whole lymphocyte populations, ours is a novel approach that compares the effects of ZnO NPs by combining DPASV and HR-ICPMS with two comet assays.

The added value of our study was the testing of extended-term lymphocyte cultures instead of acute testing. By implementing this approach we ensured that the cells were exposed to ZnO NPs throughout the cell cycle, which makes possible the direct contact between genome and NPs.

Extended exposure to ZnO NPs led to significantly different results between the two approaches. When the unseparated lymphocytes were evaluated for viability, we found no significant differences between the treated and control cells, regardless of the concentration used (Table 1), but in the separated populations these differences became significant at concentrations above 5 μg mL^{-1}, and the proportion of apoptotic cells was significantly higher in high-density lymphocytes.

Table1 *Results of ethidium bromide/acridine orange staining of lymphocyte cultures treated with ZnO NPs for 14 days*

After-treatment procedure	ZnO treatment (μg mL^{-1})	Viable	Apoptotic (mean %±SD)	Necrotic
Unseparated lymphocytes	1	91.5±3.5	8.0±2.8	0.5±0.7
	2.5	90.0±1.4	8.5±2.1	1.5±0.7
	5	92.5±0.7	7.0±1.4	0.5±0.7
	7.5	88.0±2.8	8.5±2.1	3.5±0.7
	Negative control	93.0±1.4	7.0±1.4	0.0±0.0
	Positive control	74.6±5.1[1]	19.5±3.4[1]	5.9±1.7
Low-density lymphocytes separated in Histopaque®	1	88.5±0.7	11.5±0.7	0.0±0.0
	2.5	91.0±1.4	7.5±0.7	1.5±0.7
	5	89.0±1.4	10.0±2.8	1.0±1.4
	7.5	90.0±4.2	8.0±4.2	2.0±0.0
	Negative control	91.5±2.1	8.0±1.4	0.5±0.7
	Positive control	73.4±2.1[1]	21.9±4.0[1]	4.7±0.8
High-density lymphocytes separated in Histopaque®	1	-	-	-
	2.5	87.5±0.7	8.5±3.5	4.5±2.1
	5	82.0±1.4[1]	16.0±1.4[1]	2.0±0.0
	7.5	76.5±2.1[1,2]	20.0±7.1[1,2]	3.5±4.9
	Negative control	-	-	-
	Positive control	-	-	-

[1]statistically significant compared to negative control; [2]statistically significant compared to low-density cells. Null values mean that there were no high-density cells in the isolation medium, only low-density ones

Table 2 *Results of the alkaline comet assay in lymphocyte cultures treated with ZnO NPs for 14 days*

After-treatment procedure	ZnO treatment (μg mL^{-1})	Tail length (μm, mean±SD)	Tail intensity (% DNA, mean±SD)
Unseparated lymphocytes	1	23.7±5.1	2.6±6.0
	2.5	23.9±3.6	2.1±5.1
	5	23.9±4.4	3.4±6.6
	7.5	23.1±1.7	3.9±3.1
	Negative control	24.5±1.9	0.9±1.2
	Positive control	34.7±12.0[1]	10.6±4.9[1]
Low-density lymphocytes separated in Histopaque®	1	25.4±6.3	2.6±6.0
	2.5	26.3±3.1	0.9±1.2
	5	26.7±7.4	3.9±12.7[1]
	7.5	25.9±5.1	3.4±3.4
	Negative control	26.2±2.4	0.9±1.2
	Positive control	36.0±14.2[1]	12.7±4.6[1]
High-density lymphocytes separated in Histopaque®	1	-	-
	2.5	26.8±8.9	3.0±6.2
	5	39.4±26.3[1,2]	17.7±27.4[1]
	7.5	38.4±21.9[1,2]	10.7±16.4[1,2]
	Negative control	-	-
	Positive control	-	-

[1]statistically significant compared to negative control; [2]statistically significant compared to low-density cells. Null values mean that there were no high-density cells in the isolation medium, only low-density ones

Using DPASV and HR-ICPMS we clearly established that intracellular Zn^{2+} content in high-density cells was significantly higher than in both negative control and low-density cells exposed to the same ZnO NP concentrations (Figure 3) and that higher accumulation of Zn^{2+} triggered a greater cytotoxic effect. These findings correspond to the observations reviewed by Ivask et al. (27). Kim et al. (28) reported that ZnO NPs hindered mitochondrial function in rat alveolar epithelial cells *in vitro* by impairing transmembrane electron transport through increased inner membrane permeability. They also suggested that it was Zn^{+2} released from nanoparticles that contributed to the induction of oxidative stress. Sharma et al. (6) also proposed extensive reactive oxygen species formation as a contributor to apoptosis in a HepG2 cell line exposed to ZnO nanoparticles for 12 h. The exact mechanism of ZnO cytotoxicity, however, remains to be determined. Guo et al. (29) suggest that ZnO NPs trigger cell death by activating caspase-12 and by reducing the levels of caspase-9 and bcl-2 at the same time.

Differences in the observed ZnO NP cytotoxicity between our two approaches could be attributed to the composition of the lymphocyte suspensions used for viability testing. The lymphocyte population used in the first approach (without separation) contained cells which took up different amounts of ZnO NPs. Another important factor which might significantly affect both cyto- and genotoxicity findings are the intracellular concentrations of Zn^{2+} ions released from NPs. They might also vary between single cells, which further complicates the interpretation of our results.

Nevertheless, if the interpretation of the results were based only on the first approach, this could lead to a wrong conclusion, as no one could estimate the ratios between high and low-density cells or the extent of the oxidative stress which might have been caused by Zn^{2+} accumulation in individual cells. In other words, our results in these mixed populations might underestimate the real cyto- or genotoxicity caused by the treatment.

The advantage of the second approach (with separation) allowed us to distinguish between low-density lymphocytes, which exhibited lower cytotoxicity and accumulated lower amounts of ZnO and high-density ones.

Regardless of the separation, our study has shown that a significant increase in primary DNA damage started with exposure to 5 μg mL^{-1} of ZnO NPs (Table 1). Mu et al. (30) provided evidence on Zn release from ZnO NPs administered in the concentrations below 5.5 μg mL^{-1}. The level of dissolution they observed correlated with genome damage induced in different epithelial cell lines measured with the alkaline comet assay. Similar results have been reported by Demir et al. (31) for ZnO NPs of 50-80 nm in diameter. They detected genotoxic effects (specifically, oxidised bases in DNA) in human embryonic kidney fibroblasts and mouse embryonic fibroblasts at concentrations above 10 μg mL^{-1}. Zinc oxide NPs are capable of inducing primary DNA damage in many other human cell models *in vitro*: human epidermal cells (32), cervix carcinoma Hep-2 cells (33), bronchial epithelial BEAS-2B cells (34), epidermal keratinocytes (35), nasal mucosa cells (36), liver HepG2 cells (37), and hepatocytes (38). All these models showed dose-related effects on comet assay parameters. Our study confirms this dose-response relationship: tail length significantly correlated with ZnO NPs concentrations and intercellular Zn^{2+} concentrations. The difference, however, is that the cited studies evaluated genome damage induced by acute exposure, while ours measured the effects of longer-term exposure in lymphocytes separated by density,

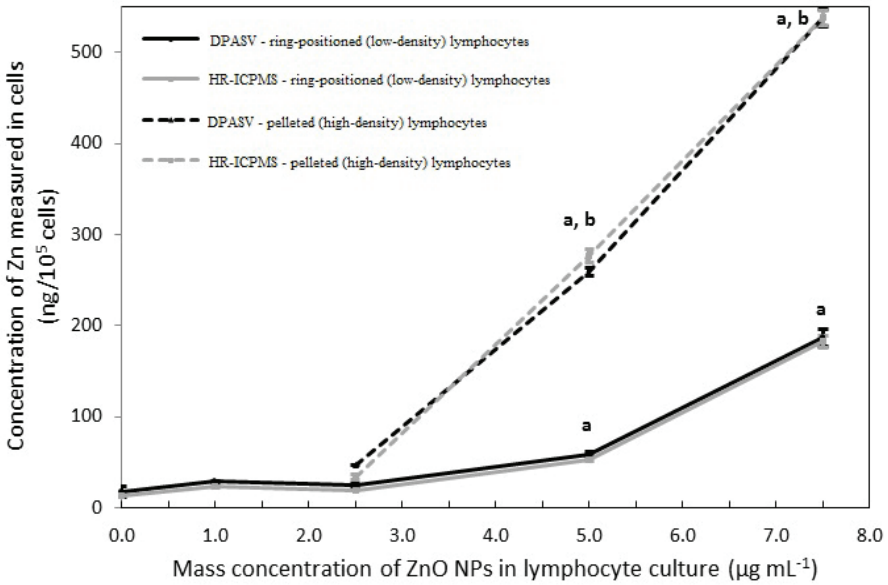

Figure 3 *Average Zn^{2+} concentrations in lymphocyte cultures treated with ZnO NPs for 14 days determined with the differential pulse anodic stripping voltammetry (DPASV) and high resolution inductively plasma mass spectrometry (HR-ICPMS).[a]significantly different from negative control; [b] significantly different from low-density lymphocytes ($p<0.05$)*

Table 3 *Comet-FISH assay-determined fragmentation and deletion of the TP53 gene and centromere of chromosome 17 in lymphocyte cultures treated with ZnO nanoparticles for 14 days*

After-treatment procedure	ZnO treatment (μg mL^{-1})	Fragmented copies		Deleted copies	
		TP53 gene	Cen 17	TP53 gene	Cen 17
		(mean %±SD)	(mean %±SD)	(mean %±SD)	(mean %±SD)
Unseparated lymphocytes	1	21.5±11.8	3.4±4.7	3.3±0.0	3.4±2.3
	2.5	36.6±4.7[1]	27.5±3.5[1]	4.2±1.2	0.0±0.0
	5	40.0±4.7[1]	21.6±2.3	3.8±0.6	0.8±1.2
	7.5	35.0±7.1[1]	13.4±9.4	4.2±1.2	1.7±0.0
	Negative control	13.3±0.0	5.0±2.4	2.5±1.1	0.8±1.2
	Positive control	49.7±8.5[1]	22.1±3.6[1]	10.3±3.6[1]	5.0±2.1[1]
Low-density lymphocytes separated in Histopaque®	1	15.0±2.4	3.3±4.7	3.3±0.0	3.3±2.3
	2.5	18.3±2.4	11.7±7.1	5.0±2.4	1.7±2.4
	5	20.0±4.7	16.7±0.0	5.0±2.4	2.5±3.5
	7.5	35.0±7.1[1]	18.3±2.4	6.7±0.0	2.5±1.2
	Negative control	13.3±0.0	5.0±2.4	2.5±1.1	0.8±1.2
	Positive control	48.1±6.2[1]	25.2±6.9[1]	8.0±2.6	4.6±2.0
High-density lymphocytes separated in Histopaque®	1	-	-	-	-
	2.5	38.3±11.8[1,2]	18.3±2.4	5.8±1.2	2.5±3.5
	5	48.3±2.4[1,2]	25.0±11.8[1]	5.0±0.0	3.3±0.0
	7.5	48.3±2.4[1]	21.7±2.4	5.8±1.8	3.3±4.7
	Negative control	-	-	-	-
	Positive control	-	-	-	-

[1]statistically significant compared to negative control; [2]statistically significant compared to low-density cells. Null values mean that there were no high-density cells in the isolation medium, only low-density ones

most likely due to different ZnO NPs uptake. In addition, the comet-FISH assay has revealed that ZnO NPs at concentrations above 2.5 μg mL^{-1} are capable of compromising primary DNA integrity (Table 3) in an intracellular concentration-dependent manner. The presence of ZnO NPs in cells may impair *TP53* transcription. This impairment makes the cells more susceptible to genotoxic effect (39).

We found a significant correlation between *TP53* fragmentation (primary damage) and both intracellular Zn^{2+} concentration and general primary DNA damage. It seems that the genotoxic action of ZnO NPs *per se* or via ROS induction randomly targets various genome regions rather than specific sites.

Genome integrity, and therefore functionality, was significantly affected by ZnO NPs in a concentration-dependent manner. This dependence, however, was not clear between the loss of *TP53* copies loss and the applied ZnO or intracellular Zn^{2+} concentrations.

Considering that the frequency of *TP53* deletions showed a limited and insignificant correlation with the frequency of *TP53* fragmentation, it is possible that the damage accumulated in the gene locus may be related to a later loss of gene copies, but this has to be studied further. This also suggests that gene fragmentation rate may be considered as a biomarker of exposure, while the deletion frequency should rather be considered as a biomarker of effect. Together with previous report by Ng et al. (40), who claim that an effective response to the genotoxic potential of ZnO NPs greatly depends on the integrity of the *TP53* gene, our findings speak in favour of exposure to ZnO NPs as a possible cancer risk factor.

Our approach based on the separation of two lymphocyte populations by density at treatment concentrations above 2.5 μg mL^{-1} and determination of intracellular Zn^{2+} with DPASV and HR-ICPMS showed that there was a significant difference in the uptake rate of ZnO NPs between lymphocytes. Gilbert et al. (41) have already evidenced that ZnO NPs enter cells undissolved. Particles are taken up through endocytosis, primarily via caveolae, and end up in intracellular vesicles (42). Once ZnO enters the cell, it tends to dissolve in aqueous media and convert to its biologically active form Zn^{2+} (43). Unlike caveolae, lysosomes contain zinc only in its ionic form, most likely due to their acidic content, which facilitates the dissolution of ZnO. Cytosolic zinc mostly binds to metalloproteins. Another route for Zn^{2+} uptake from extracellular environment to the cytosol is mediated by Zrt/Irt-like transmembrane proteins (44).

Our regression analysis has shown a strong correlation between ZnO concentrations and intracellular Zn^{2+} levels, especially at concentrations above 5 μg mL^{-1}. Differences in intracellular Zn^{2+} levels resulted in the separation of the lymphocytes by density, which allowed us to separately study the toxic effects in low and high-density populations.

This approach provided additional statistical power to the correlation analysis, whose aim was to test the relation between primary DNA lesions, loss of *TP53* integrity and heterozigocity, and intracellular Zn^{2+} concentrations. Differences in zinc accumulation between cultured cells of the same population have already been reported by Yoo et al. (45). The authors were reluctant to attribute them to the variations in molecular mechanisms involved in the endocytosis of NPs or to transmembrane zinc transporters but rather to the differences in relative surface charge of the nanoparticles. Cells take up positively charged ZnO NPs more efficiently. This explanation, however, does not apply for our study, because all our lymphocytes were exposed to the same nanoparticles and the ratio of positively charged particles should not vary within one culture. The differentiation rather suggests that there are differences in accumulation capacity between lymphocytes at ZnO NP concentrations above 2.5 μg mL^{-1}. As already mentioned, there are several pathways of Zn uptake in the cell, but there is a single way out: via the Zn^{2+}/H^{+} transporter ZnT-1. Yu et al. (46) reported that in human lung epithelial cells intracellular accumulation plateaued at ZnO levels ten times higher than used in our study due to the saturation of the uptake mechanisms. Our results suggest that high-density lymphocytes accumulated higher intracellular Zn^{2+} content with increasing concentrations of ZnO NPs. This finding suggests that mechanisms responsible for Zn^{2+} extrusion get saturated as early as 2.5 μg mL^{-1}, judging by significantly higher intracellular zinc concentrations compared to corresponding low-density lymphocytes (Figure 3).

The value of the comet assay in genotoxicity evaluation of materials with unknown biocompatibility was previously confirmed by Galić et al. (47). The three main parameters most commonly used to quantify DNA damage in the comet assay are the tail length, tail intensity, and tail moment (48). In our study, we assessed both tail length and tail intensity, and the obtained results suggest that they both adequately reflected the level of primary DNA damage. However, many literature sources consider tail intensity as more sensitive, especially at intense genotoxic events when the tail length reaches its maximum and plateaus (49). If ZnO can induce biologically significant primary DNA lesions that would become mutations or chromosome aberrations, tail intensity should exhibit a higher correlation to intracellular Zn^{2+} than tail length. We, however, observed the opposite. Tail length and *TP53* fragmentation correlated with intracellular Zn concentrations, while tail intensity did not. Even so, all highly depended on the migration of the DNA in the electric field during electrophoresis. The only endpoint that did not depend on electrophoresis was the deletion of *TP53*. It also did not significantly correlate either with ZnO NPs or Zn^{2+}.

All things considered, this study has not clarified the issue of the applicability of the comet assay as a widely used technique for assessing the risk of novel nanomaterials. As discussed earlier, ZnO NPs dissociate within the cell, which results in an increase in free intracellular Zn^{2+}. This ionic form of zinc is responsible for the observed adverse effects of ZnO in the cells. As a negatively charged macromolecule, DNA is an ideal target to released Zn^{2+}. These interactions may result in various types of primary DNA damage that are converted by alkaline electrophoresis into additional DNA breaks, which can be visualised and measured with the comet assay. Therefore, understanding Zn^{2+}/DNA interactions and their behaviour in the electric field is crucial for accurate interpretation of the obtained results.

The biological relevance of the obtained results strongly depends on accurate interpretation of the comet assays. In the electric field, denaturated DNA migrates toward the positively charged anode, while complexed Zn^{2+} would

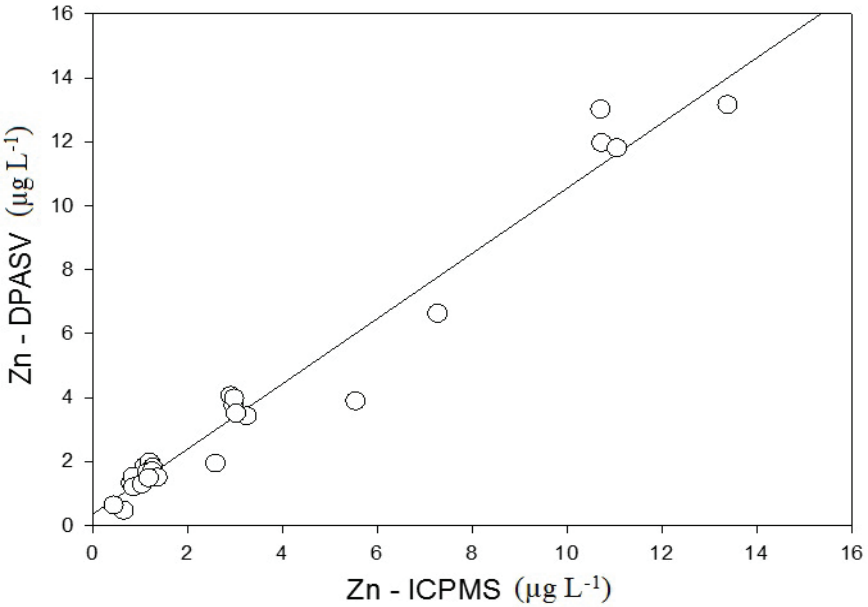

Figure 4 *Comparison of Zn^{2+} concentrations measured with the differential pulse anodic stripping voltammetry (DPASV) and high resolution inductively plasma mass spectrometry (HR-ICMS)*

migrate to the negatively charged cathode. The question remains whether and how complexed Zn^{2+} ions interfere with the ability of damaged DNA to migrate. Does the observed genotoxicity originate from biological interaction between Zn^{2+} and DNA *in situ* or is it an artefact resulting from the assay's limitations? Would complexed Zn^{2+} impair gene expression and stability *in situ*, or is it a consequence of exposing the complexed DNA to the electric field when Zn^{2+} induces strand breaks? It is difficult to answer this question, considering that most studies reported positive results of the alkaline comet assay but did not find oxidative DNA damage following exposure to NPs. As previous studies point, there are discrepancies between the endpoints that depend on comet assay electrophoresis and classical cytogenetic biomarkers (12). In designing protocols for the risk assessment of NP interactions with biological systems we should be aware of the concerns raised by Karlsson (12) and Doak et al. (50) that NPs may interfere with the comet assay outcomes. In spite of these limitations, our study has shown that combining electrochemical with genotoxicity methods can help to evaluate the cyto/genotoxicity of ZnO NPs, but further studies are needed to elucidate their toxicity profile.

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Alternativni pristup mjerenju koncentracije nanočestica ZnO u kulturama humanih limfocita - spoj elektrokemije i testova genotoksičnosti

S naglim porastom primjene nanočestica raste i mogućnost njihova štetna djelovanja u ljudi. Nanočestice cinkova oksida najčešći su oblik nanomaterijala u potrošačkim proizvodima i lijekovima. Nekoliko je istraživanja već upozorilo na probleme vezane uz njihovu sigurnu primjenu. Cilj je ovoga istraživanja bio utvrditi pri kojim razinama nanočestice ZnO počinju štetno citogenetski djelovati na humane limfocite i time otvoriti pitanje utvrđivanja graničnih razina za sigurnu primjenu nanočestica ZnO u ljudi. Stoga smo istražili genotoksične učinke niskih koncentracija nanočestica ZnO (1,0; 2,5; 5 i 7,5 μg mL^{-1}) izloživši kulture humanih limfocita njihovu djelovanju tijekom 14 dana. Uz to smo izmjerili razlikuju li se limfociti niske gustoće od onih visoke gustoće u sposobnosti akumuliranja nanočestica ZnO pri istim eksperimentalnim uvjetima. Primarno oštećenje DNA (izmjereno alkalnim komet-testom) povećalo se s rastom koncentracije nanočestica u limfocitima koje nismo razdvojili po gustoći te u limfocitima visoke gustoće. Slično smo povećanje zamijetili s fragmentacijom tumorskoga supresorskoga gena TP53 (izmjereno komet-FISH testom). Nakupljanje Zn^{2+} iona u stanicama bilo je značajno samo kod primjene dviju najviših koncentracija nanočestica ZnO, bez obzira na gustoću limfocita. Osim toga, limfociti visoke gustoće iskazali su i značajno više razine unutarstaničnoga Zn^{2+} od limfocita niske gustoće. Naši rezultati upozoravaju na to da se izlaganjem razinama nanočestica ZnO višima od 5 μg mL^{-1} značajno povisuju razine Zn^{2+} u limfocitima te se povećava oštećenje tih stanica i njihova genoma.

KLJUČNE RIJEČI: *komet-FISH test,* in vitro*; primarno oštećenje DNA;* TP53*; voltametrija*

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The sublethal effects of (2,4-Dichlorophenoxy) acetic acid (2,4-D) on narrow-clawed crayfish (*Astacus leptodactylus* Eschscholtz, 1823)

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2,4-D is a widely used phenoxy herbicide, potentially toxic to humans and biota. The objective of the present study was to reveal short term sublethal effects of 2,4-D on narrow-clawed freshwater crayfish (*Astacus leptodactylus* Eschscholtz, 1823), based on histology, total haemocyte counts, selected haemolymph parameters, and oxidative stress parameters. In the laboratory conditions crayfish specimens were exposed to 9 mg L^{-1} of 2,4-D for one week. Experiments were conducted under semi-static conditions in 20 L-capacity aquaria where 10 freshwater crayfish were stocked per aquarium. Exposure (experimental) and control groups were used and the experiments were repeated two times. No mortality and behavioural changes were recorded during the experiments. Total haemocyte counts decreased significantly, while haemolymph glucose levels increased (P<0.05), when compared to the control group. Haemolymph levels of calcium, chloride, sodium, potassium, magnesium, total protein, and lactate did not change. Exposure resulted with increased levels of malondialdehyde (MDA) only in hepatopancreas. However, results of gill FOX assay showed a significant decrease in oxidative stress parameters (P<0.05). MDA levels of gill and abdominal muscle tissues and FOX levels of hepatopancreas and abdominal muscle tissues did not change when compared to the control group. Significant histopathological alterations were observed both in hepatopancreas (multifocal deformations in tubule lumen) and gill tissue (melanisation of gill lamella). Exposure of crayfish even to a sublethal concentration of 2,4-D alters histopathology and lipid peroxidation due to stress. Biomarkers studied here seem to be useful for the assessment of adverse/toxic effects of pesticides on non-target, indicator aquatic organisms.

KEY WORDS: *haemolymph; herbicide; histology; oxidative stress; toxicity*

The first successful selective herbicide, 2,4-dichlorophenoxy acetic acid (2,4-D), was developed in 1946. It belongs to the phenoxy herbicide family and is still one of the systemic herbicides widely used to control many types of broadleaf weeds (1). It is used to control aquatic vegetation, in pasture and rangeland applications, in cultivated agriculture, forest management, home, and garden. It acts by sustaining high levels of the plant hormone auxin, which results in overstimulation of plant growth and death. In addition to this, it causes changes in the animal nervous system based on receptor interaction/interference of acetylcholine. Furthermore, it inhibits the acetyl cholinesterase (AChE) activity and increases the level of serotonin.

Available data on 2,4-D toxicity on aquatic organisms mostly rely on the reports of its acute toxic effects observed in different fish species. Oruc and Uner (4) studied the combined effects of azinphosmethyl and 2,4-D on *Oreochromis niloticus* to clarify the mode of its action on the cellular defence system. Farah et al. (5) studied stress behaviour and acute 96 h toxicity of three freshwater fish (*Heteropneustes fossilis*, *Clarias batrachus*, *Channa punctatus*) and estimated LC_{50} values as 81 m L^{-1}, 122 mg L^{-1}, and 107 mg L^{-1}. They also calculated 48 h LC_{50} in mosquito larvae (*Culex pipiens fatigans*) as 302 mg L^{-1}. Sarikaya and Yilmaz (6) established 96 h LC_{50} of 2,4-D on adult common carp as 63.24 mg L^{-1}. In their study, Sarikaya and Selvi (7) calculated the 48 h LC_{50} values for Nile tilapia (*O. niloticus* L.) larvae and adults as 28.23 mg L^{-1} and 86.90 mg L^{-1}, respectively.

Potential genotoxic effects of 2,4-D have been reported for the freshwater fish *Channa punctatus* using the micronucleus test (8); for catfish (*Clarias batrachus*) by studying micronuclei and erythrocyte alterations (9), and DNA degradation and apoptotic effects (10).

Detrimental effects of 2,4-D on the AChE activity were reported by Fonseca et al. (11), who studied 96 h effects of 1 and 10 mg L^{-1} 2,4-D on *Leporinus obtusidens*. The AChE

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activity of muscle tissue was reduced at both concentrations, as was the case at a concentration of 10 mg L^{-1} for brain tissue. Liver lactate and protein were significantly reduced after exposure to this herbicide. Plasma protein increased at both exposure concentrations. Catteneo et al. (12) reported an increased AChE activity in the brain and a decreased activity in muscle tissues after 600 mg L^{-1} to 700 mg L^{-1} 2,4-D exposure for 96 h. In the same study, plasma glucose levels increased and vacuolation of hepatocytes and changes in its arrangement cords were observed in animals exposed to 700 mg L^{-1} of 2,4-D.

Although the effects of 2,4-D were extensively studied in fish, very limited studies have been carried out in invertebrates. In a previous study, Benli et al. (2) estimated the 96 h LC_{50} for freshwater crayfish to be 32.6 mg L^{-1}. In addition, 2,4-D exposure has been linked with gonadal tumours in shellfish (1, 13-16). Limited information about the effects of this widely used herbicide 2,4-D on the aquatic invertebrate histology and biochemical biomarkers are available in the open literature.

The objective of the present study was to reveal short-term sublethal effects of 2,4-D on narrow-clawed freshwater crayfish (*Astacus leptodactylus* Eschscholtz, 1823), based on histology, total haemocyte counts, and measuring the levels of selected haemolymph parameters, as well as to evaluate the changes in basic oxidative stress parameters.

MATERIALS AND METHODS

Test organism

The narrow-clawed freshwater crayfish (*Astacus leptodactylus* Eschscholtz, 1823) was chosen as a representative of freshwater crustaceans, naturally distributed in water bodies around Eurasia (17-19). Crayfish specimens (N=60) were obtained from a local breeder during the inter-moult stage. Their average weight was 25.36±3.27 g and the length was 9.62±0.43 cm. Crayfish were placed randomly in control and experiment groups, each containing 30 individuals.

Acclimatisation period

Crayfish were allowed to acclimatise under laboratory conditions for two weeks. During this period, they were fed *ad libitum* daily with raw fish. The tanks were cleaned by siphoning twice and were aerated constantly. Water temperature was adjusted by thermostatic heaters to 20 ˚C. All experiments were performed according to the rules of "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (20).

Test chemical

2,4-dichlorophenoxy acetic acid (CAS Number: 94-75-7) was used in the experiments. It was obtained from the Insecticide Test Laboratory, Hacettepe University, Ankara Turkey. The pesticide was stored at +4°C before use.

Experimental design

After the two-week acclimatisation period, crayfish were transferred to test tanks, i.e. 25 L-capacity aquaria which contained 20 L of dechlorinated tap water. The composition of water was: dissolved oxygen 6.60±0.10 mg L^{-1}; pH=6.70±0.03; conductivity 0.205±0.01 mS cm^{-1}; total hardness 70 mg L^{-1} $CaCO_3$; calcium hardness 54 mg L^{-1} $CaCO_3$. Water temperature was adjusted by thermostatic heaters to 20 °C. During the experiments (except dosing) aquaria were constantly aerated.

Test concentration and exposure

The test concentration of 9 mg L^{-1} 2,4-D was selected as the 25 % of the 96 h LC_{50} value (32.6 mg L^{-1}), previously established for freshwater crayfish in a static bioassay test system (2). Under semi-static conditions, ten freshwater crayfish were stocked in each aquarium as a control and an exposure group. Exposure lasted for one week.

Haemolymph analysis

Following the exposure to 2,4-D, the haemolymph samples were taken from experimental animals. They were collected under ice anaesthesia on the basis of the second walking leg using a 2.5 mL disposable syringe. Briefly, first 0.9 mL of 4 % heparin was drawn into the syringe. Then 0.1 mL haemolymph was sampled from freshwater crayfish. Samples were used for determining the total haemocyte counts (THCs) and for haemolymph biochemical analyses. Total haemocyte counts were performed by modifications of the methods of Miller and Stanley (21), Evans (22), and Ward et al. (23). After haemolymph samples were centrifuged at 2500 *g*, 10 min at 4 ˚C, biochemical analyses were done by standard analytical techniques. Haemolymph Na^+, K^+, and Cl^- levels were measured by ISE module of an auto analyser that employed crown ether membrane electrodes for sodium and potassium and a molecular-oriented PVC membrane for chloride, which was specific for each ion of interest in the sample (24). Glucose concentrations were analysed by the hexokinase method which is an enzymatic UV test (25). Total protein, calcium, magnesium, and lactic acid were determined using a Beckman CX 7 autoanalyser (Beckman Coulter Inc., Diamond Diagnostics, USA)

Tissue analysis

After haemolymph sampling, crayfish were sacrificed immediately on ice anaesthesia for histological examination and tissue samples were collected for further analyses of oxidative stress parameters. All tissues were dissected and half of each gill, muscle, and hepatopancreas tissues were

stored for oxidative stress analysis at -80 °C. The rest of the tissues (the other half of the hepatopancreas, muscle, gill with antennal gland, gonads digestive tract, and heart) were directly fixed in Davidson's fixative (26).

Histological examination

After 24 h fixation in Davidson's fixative, tissues were transferred to 70 % ethyl alcohol. Routine histological tissue processing procedures were carried out as follows: dehydrated in alcohol series, cleared in xylene, embedded in paraffin. The paraffin blocks were sectioned with ThermoShandon 325 Finesse Rotary Microtome (Thermo Fisher Scientific, UK) and stained with hematoxylin and eosin (H&E). Slides were examined under a light microscope (Carl Zeiss, Germany), coupled with a camera.

Tissue MDA and ferrous oxidation assays

MDA levels in gills, muscles, and hepatopancreas were determined by the thiobarbituric acid (TBARS) assay (27). Differences in two absorbance measurements from the butanol phase were used as MDA values (nmol per 100 mg of tissue). Additionally, the ferrous oxidation (FOX) assay, which is based on the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) by hydrogen peroxide under acidic conditions was performed for the quantitative determination of low-level lipid hydroperoxides. Results for the FOX assay are given as HP (hydrogen peroxide) equivalents and calculated as nmol g^{-1} of wet tissue according to Hermes-Lima et al. (28).

Statistical analyses

The results are the means ± SD with two replicates. Analysis of data concerning the differences between groups was made using a non-parametric Mann Whitney-U Test. The critical significance level for the statistical tests performed was set at 0.05.

RESULTS AND DISCUSSION

Haemolymph analyses

Following one week exposure to 9 mg L^{-1} of 2,4-D, the values of total haemocyte counts (THCs) in *A. leptodactylus* decreased by 25 % as compared to the control group ($P<0.05$). The results are shown in Figure 1a. Although Le Moullac and Haffner (29) suggested the differences in haemocyte counts as non-specific, depending on the natural rhythms of the environment, and chemical and physico-chemical stress, they are generally accepted as reliable/good indicators of stress in crustaceans (29). In their previous studies, Le Moullac and Haffner (29), Smith and Johnston (30), Jussila et al. (31), Jussila et al. (32), and Smith and Johnston (30), reported similar findings for haemocyte counts in other crustaceans exposed to different toxicant and stress factors. For instance, Smith and Johnston (30) observed a significant decrease in THCs and phenoloxidase activity in common shrimp *Crangon crangon* following exposure to PCB 15 (polychlorinated biphenyl 15). Mello et al. (33) determined a decrease in THCs after exposure to 250 SC Sirus herbicide of pyrazosulfuron-ethyl 0.1 µg L^{-1}

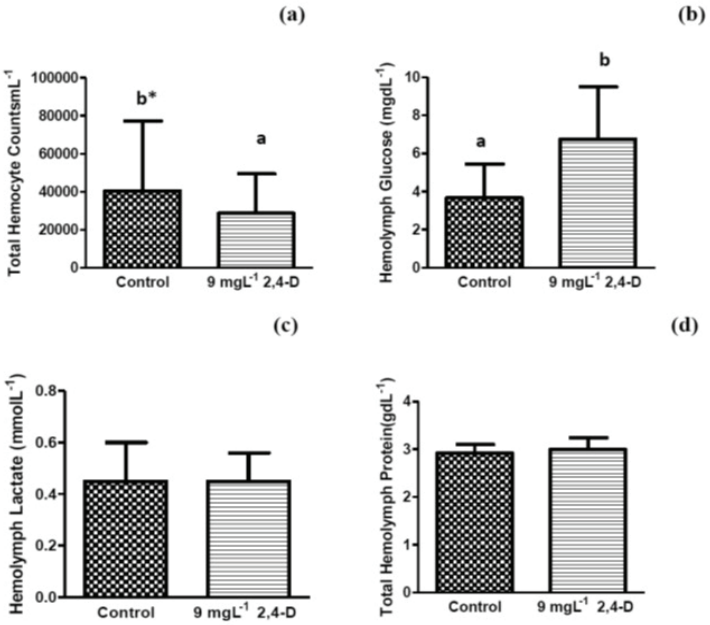

Figure 1 *a) Total haemocyte counts , b) haemolymph glucose, c) lactate, and d) total protein levels of freshwater crayfish after one-week exposure to 2,4-D. *Different small letters indicate significant difference between means (P<0.05), N=30 samples/group*

to 1000 µg L^{-1} for 96 h in *Litopenaus vannamei*. Qin et al. (34) also observed decreased THCs in freshwater crab *Sinopotamon henanense* after exposure to 58 mg L^{-1} and 116 mg L^{-1} Cd for 96 h.

In the present study, haemolymph glucose levels increased significantly after exposure to sublethal 2,4-D concentrations. The results are shown in Figure 1b. Toxicants and other environmental stressors can cause rapid hyperglycaemia in crustaceans just like in vertebrates. Crustaceans require a constant glucose supply for all organs and tissues (ex. muscle and brain). Glucose haemostasis is controlled by the hyperglycaemic hormone (CHH) produced by the X-organ-sinus-gland complex. Chang et al. (35) also found increased haemolymph glucose levels after 24 h exposure to 0.4 mg L^{-1} trichlorfon, an organophosphorus insecticide, in *M. rosenbergii.* High haemolymph glucose levels were also found after exposure to various pollutants such as cadmium, organic pollutants, naphthalene, and nitrite in red swamp crayfish, *Procambarus clarkii* (36), fiddler crab, *Uca pugilator* (37), *U. pugilator* (36), and narrow-clawed crayfish (*A. leptodactylus*) (38).

As seen in Figures 1c and 1d, haemolymph lactate and total protein levels were not altered significantly when compared to the control group. In decapods, lactate is the main product of anaerobic glycolysis; it usually increases in parallel with glucose level increase but slower. Contrary to our results, Bhavan and Geraldine (39) found increased haemolymph lactate levels in *Macrobrachium malcolmsonii* following exposure to endosulfan (10.6 ng L^{-1}, 16.0 ng L^{-1}, and 32.0 ng L^{-1}) for 21 days. Chang et al. (35) observed an increase after 24 h exposure to 0.4 mg L^{-1} organophosphorus insecticide trichlorfon in *M. rosenbergii.* Saladkova and Kholodkevich (40) reported protein level in crayfish haemolymph to be a reliable parameter for the physiological state. However, in our study no significant differences were found between the control and exposed groups. Haemocyanin is the predominant protein (80 % to 95 % of the total protein in the haemolymph) of decapod crustaceans (41) and known to be a biomarker of osmotic stress. Frontera et al. (42) reported unchanged total haemolymph protein values after exposure to 11.25 mg L^{-1} and 22.5 mg L^{-1} glyphosate for 50 days.

Our results on the elected haemolymph electrolytes in crayfish exposed to 9 mg L^{-1} 2,4-D were not significantly different when compared to the control group. Control levels measured for electrolytes were: calcium (36.36±1.29 mg dL^{-1}), chloride (129.26±3.00 mEq L^{-1}), sodium (193.20±3.59 mEq L^{-1}), potassium (4.56±0.16 mEq L^{-1}), and magnesium (4.83±0.25 mg dL^{-1}). Magnesium and calcium can be suggested as environmental monitoring biomarkers and indicators of crustacean health (43). In another study (35), haemolymph calcium, magnesium, and potassium concentrations were not significantly different among the prawns exposed to 0.1-0.3 mg L^{-1} trichlorfon (44); while potassium levels also showed no statistically significant differences in the prawns exposed to 0.4 mg L^{-1} of trichlorfon (35).

Histological analyses

Seven-day exposure to 2,4-D resulted with histological alterations in gill and hepatopancreas tissues of treated crayfish. However, there were no histological alterations observed in the control groups nor were there changes in other tissues of exposed crayfish, such as the tissues of antennal gland, gonads, muscle, heart, and digestive tract.

In gill tissues, melanisation (Figure 2) and hyperaemia were observed. In the hepatopancreas, multifocal deformation in tubule lumen was seen (Figure 3). Gills are a vital organ for aquatic organisms, which can play important roles in transporting respiratory gases, arranging osmoregulation and ion exchange, and can act as the first barrier for waterborne pollutants. Similar to our results, Bahavan and Geraldine (45) suggested abnormal gill tips in the prawn *Macrobrachium rosenbergii* exposed to 32 ng L^{-1} endosulfan. Chang et al. (35) observed structural alterations such as necrotic, hyperplasic, and clavate–globate lamellae with swelling, fusion, and increased mucus secretion in prawns after exposure to 0.4 mg L^{-1} trichlorfon for 24 h. The tubular structured organ, hepatopancreas of crustaceans is known to be analogous to the mammalian liver, which is also susceptible to xenobiotics such as pesticides. Bahavan and Geraldine (45) reported haemocyte accumulation in the haemocoel space, necrosis in tubules in the prawn *Macrobrachium rosenbergii* exposed to

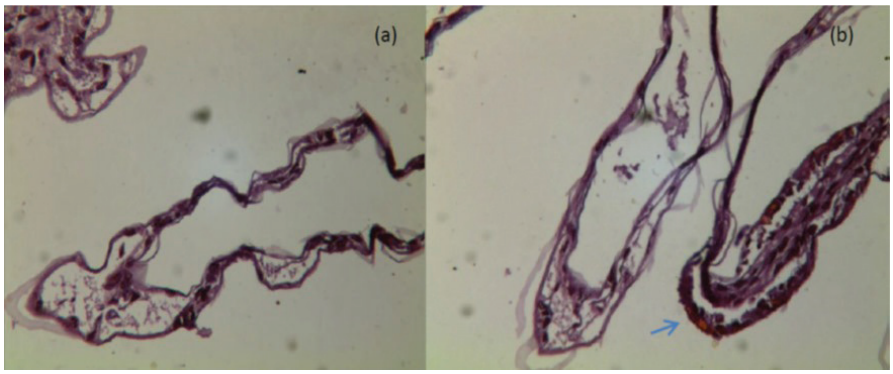

Figure 2 *Histological appearance of crayfish (*Astacus leptodactylus *Eschscholtz, 1823) gill lamella: (a) control (X100, H&E), (b) Melanisation in gill lamella of crayfish (arrow) (X100, H&E)*

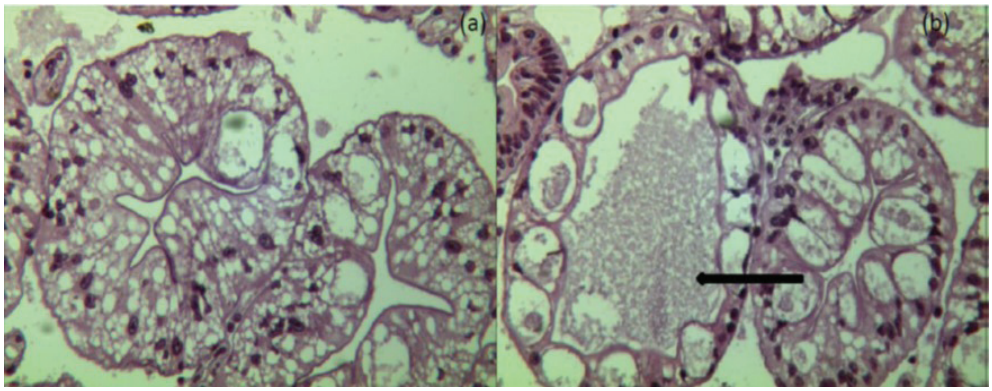

Figure 3 *Histology appearance of crayfish (*Astacus leptodactylus *Eschscholtz, 1823) hepatopancreas (a) control (X100, H&E) (b) multifocal hepatopancreas deformation in tubule lumen (arrow) (X100, H&E)*

32 ng L^{-1} endosulfan. Chaufan et al. (46) found epithelial disorganisation in hepatopancreas tubules. In addition, diameters and numbers of B cells increased after being fed with hexachlorobenzen-contaminated *Chlorella* for three days. As known (47), exposure of crustaceans to pesticides can also lead to histopathological changes such as the interstitial sinus haemocytic infiltration, thickened and/or separated necrotic cells from the basal laminae, melanisation and coagulation in the thickened basal laminae, abnormal lumen of the tubules, necrotic tubules containing tissue debris, and haemocytes can constitute a wall around the thickened basal laminae of the tubules. Such severe histopathological changes were not observed in study.

Biochemical analyses

The results of radical formation and their pertinent significance to crayfish health after one-week exposure to 9 mg L^{-1} 2,4-D were shown by MDA and FOX assays in hepatopancreas, abdomen muscle, and gill tissues of narrow-clawed crayfish, and are given in Figure 4. MDA (the end product of lipid peroxidation) levels in hepatopancreas tissues increased after exposure to 9 mg L^{-1} 2,4-D when compared to the control group (P<0.05). Similarly, Hua et al. (48) determined high MDA concentrations in the hepatopancreas of *Procambarus clarkii* after exposure to 0.01 µg L^{-1} deltamethrin for 6 h, a synthetic pyrethroid. Sarikaya et al. (49) reported decreased MDA levels in hepatopancreas after exposure to 5 µg L^{-1}, 10 µg L^{-1}, and 20 µg L^{-1} of fenitrothion for 24 h. Variation among the studies might be due to different mechanisms of toxic action of the three different pesticide groups. As depicted in Figure 5, the ferrous oxidation assay [FOXHP (hydrogen peroxide) equivalents] results for hepatopancreas and abdomen muscle did not change after exposure to sublethal 2,4-D. However, gill FOX assay results were significantly decreased after exposure to 2,4-D. The unchanged FOX assay results in hepatopancreas and abdomen muscle in the present study are in agreement with the results of Sarikaya et al. (47). Peroxidative effects observed in sublethal 2,4-D toxicity may cause damage in gills as an early response of cell/tissue damage seen in parallel with histology results.

In conclusion, the overall results of the present study showed that 2,4-D exposure at its sublethal concentration

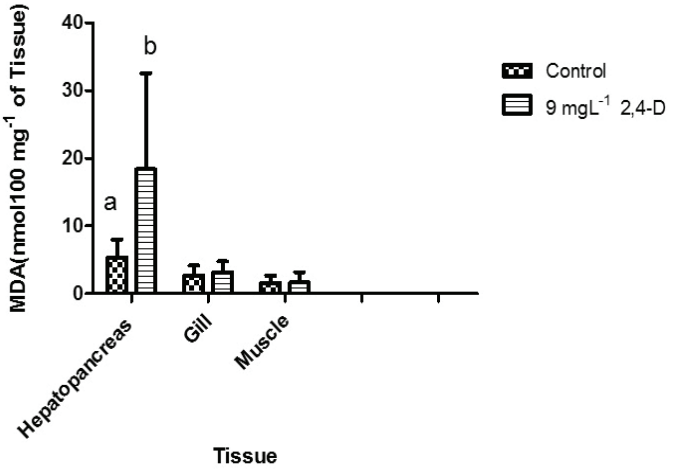

Figure 4 *MDA (nmol 100 mg^{-1} of tissue) (X±SD) levels of crayfish* Astacus leptodactylus *Eschscholtz, 1823) in hepatopancreas, abdomen muscle, and gill tissues after one-week exposure to 2,4-D. *Different small letters indicate significant difference between the means (*P*<0.05), N=30 samples/group*

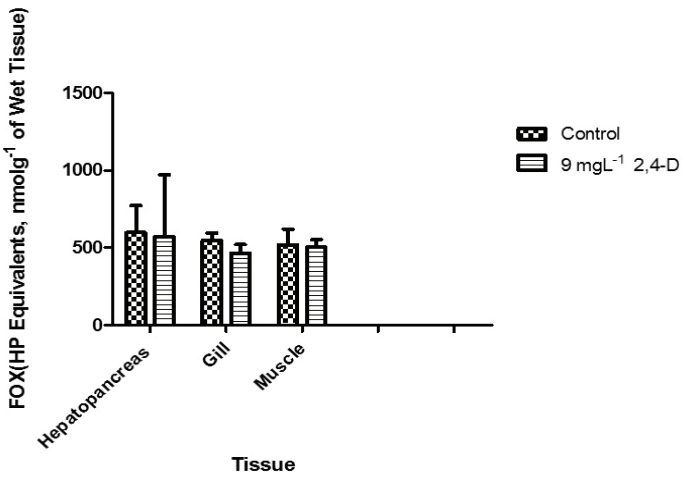

Figure 5 *The FOX (HP Equivalents, nmol g^{-1} of wet tissue) levels of crayfish* Astacus leptodactylus *Eschscholtz, 1823) in hepatopancreas, abdomen muscle, and gill tissues after one-week exposure to 2,4-D. *Different small letters indicate significant difference between the means (P<0.05), N=30 samples/group*

did not affect haemolymph electrolytes. However, haemolymph glucose (which is a well-established stress parameter) and total number of haemocytes (which points to an immune reaction) were altered after 2,4-D exposure. Histopathological alterations and oxidative stress were found in hepatopancreas and gill tissues. The obtained results indicate a strong response to tissue damage following treatment, manifested by histological findings, and increased levels of lipid peroxidation markers in crayfish. Such findings suggest that both histological methods and lipid peroxidation markers can be used as reliable ecotoxicological biomarkers: an "early warning system" for the survival of a species, as well as for environmental quality monitoring/protection. Future studies with different model or indicator aquatic species should focus on lipid peroxidation but also elaborate other suitable potential markers useful both in field biomonitoring and under controlled experimental conditions.

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Subletalni učinci 2,4-diklorofenoksi octene kiseline (2,4-D) na slatkovodnog uskoškarog raka (*Astacus leptodactylus* Eschscholtz, 1823)

2,4-D je fenoksi herbicid koji se upotrebljava diljem svijeta, a potencijalno je toksičan za ljude i biotu. Cilj ovoga istraživanja bio je ispitati kratkoročne subletalne učinke herbicida 2,4-D na slatkovodnog uskoškarog raka (*Astacus leptodactylus* Eschscholtz, 1823) proučavanjem histoloških promjena, ukupnog broja hemocita, odabranih hemolitičkih parametara i parametara oksidacijskoga stresa. Jedinke slatkovodnog raka izložene su u laboratorijskim uvjetima koncentraciji od 9 mg L^{-1} herbicida 2,4-D tijekom sedam dana. Eksperimenti su izvedeni u polustatičkim uvjetima u 20-litarskim akvarijima. U svakom akvariju držano je 10 jedinki rakova, podijeljenih u skupinu koja je bila izložena herbicidu i u kontrolnu skupinu, a eksperiment je ponovljen tri puta. Tijekom eksperimenata nisu zabilježene nikakve promjene u stopi smrtnosti ili u ponašanju životinja. Ukupan se broj hemocita značajno smanjio, a razine glukoze u hemolimfi povećale (P<0,05) u usporedbi s kontrolnom skupinom. Razine kalcija, klorida, kalija, magnezija, ukupnog proteina i laktata u hemolimfi nisu se promijenile. Izlaganje herbicidu povisilo je razine malondialdehida (MDA) u hepatopankreasu. Međutim, rezultati FOX-testa na škrgama pokazali su značajno smanjenje parametara oksidacijskoga stresa (P<0,05), za razliku od razina MDA izmjerenih FOX-testom u škrgama i abdominalnom mišićnom tkivu te u hepatopankreasu, koje se nisu promijenile. Uočene su značajne histopatološke promjene u tkivu hepatopankreasa (multifokalne deformacije tubularnog lumena) i škrga (melanizacija škržnih listića). Izlaganje rakova subletalnoj koncentraciji herbicida 2,4-D izazvalo je histopatološke promjene te potaknulo lipidnu peroksidaciju zbog stresa. Čini se da su biomarkeri koji su promatrani u ovom ispitivanju korisni za procjenu neželjenih učinaka pesticida na neciljne vodene indikatorske organizme.

KLJUČNE RIJEČI: *hemolimfa; herbicid; histologija; oksidacijski stres; toksičnost*

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Age and sex differences in genome damage between prepubertal and adult mice after exposure to ionising radiation

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The mechanisms that lead to sex and age differences in biological responses to exposure to ionising radiation and related health risks have still not been investigated to a satisfactory extent. The significance of sex hormones in the aetiology of radiogenic cancer types requires a better understanding of the mechanisms involved, especially during organism development. The aim of this study was to show age and sex differences in genome damage between prepubertal and adult mice after single exposure to gamma radiation. Genome damage was measured 24 h, 48 h, and 72 h after exposure of 3-week and 12-week old BALB/CJ mice to 8 Gy of gamma radiation using an *in vivo* micronucleus assay. There was a significantly higher genome damage in prepubertal than in adult animals of both sexes for all sampling times. Irradiation caused a higher frequency of micronuclei in males of both age groups. Our study confirms sex differences in the susceptibility to effects of ionising radiation in mice and is the first to show that such a difference occurs already at prepubertal age.

KEY WORDS: *gamma radiation; micronucleus; oestrogen; radiosensitivity; testosterone*

Radiation protection measures to be applied in cases of nuclear accidents and their link to sex or age differences are very limited (1). A recent report of the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) (2) pointed to the need for further investigations into the health risks to children caused by exposure to ionising radiation - both on human populations and animal models. However, in its evaluation of the current knowledge, UNSCEAR (2013) failed to include the interaction of ionising radiation with sex hormones during development and adulthood, as well as the impact of exposure to xenohormonally active agents on susceptibility to ionising radiation.

The mechanisms by which oestrogen may modify the response of an organism to ionising radiation have been reported only anecdotally; same as for testosterone (3-6). There is growing evidence regarding the impact of oestrogen and its receptors on carcinogenesis (7-10). So far, the biological effects of radiation via interaction with oestrogen receptors have rarely been investigated (4, 11, 12). The significance of interaction between ionising radiation and oestrogen receptors is clearly visible in the differences between mammary carcinogenesis mechanisms in the prepubertal period and in adulthood (13).

The radioprotective effect of phytoestrogens has been described in an animal model showing that the administration of genistein before irradiation significantly improves the survival of progenitor hematopoietic cells (14, 15). The mechanisms involved in modifying the effects of oestrogen on responses to ionising radiation, among others, include an increase in reactive oxygen species, gamma-H2Ax foci levels, and cell-cell signalling (16, 17). Similarly, recent data have shown an interaction between androgen receptors and ionising radiation (18, 19). A decrease in both testosterone and the sex hormone binding globulin has been reported in clinical studies, in men who underwent radiotherapy for rectal cancer (20).

There is no data on possible sex and age differences in genome damage after exposure to ionising radiation between prepuberty and adulthood. Prepuberty is a specific period of development during which complex micronenvironment settings are formed in an organism basically through a strong increase in sex hormones as a preparation phase for the final process of maturation (21). The effects of exposure during postnatal development, due to higher susceptibility of an organism to ionising radiation, may have lifelong consequences (22-24).

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Sex- and age-related differences in the effects of ionising radiation are affected by different levels of oestrogen receptors in tissues during prepuberty in comparison with adults, as reported in human adrenal tissue (25). Increased levels of oestrogen two months after exposure to ionising radiation were described in prepubertal mice (5).

Additionally, the Lifetime Attributable Risk (LAR) of cancer significantly differs during childhood and between sexes. For some radiogenic cancer types there is almost a two times higher risk between prepuberty and adulthood and two times higher risk in males than in females during prepuberty and adulthood (26). Such data show the need for more research into the mechanisms of radiocarcinogenesis in regard to the impact of sex hormones on cancer risks.

The aim of the current study was to investigate sex differences in genome damage in prepubertal and adult mice using an *in vivo* micronucleus assay (27). The main advantage of this method is that it requires a very small sample size, which enables a repeated sampling of the same animal and investigation of small animals during development. It has been shown to be a reliable method for biodosimetry (28). The dose was selected following similar studies in which the biological effects on the cellular level or DNA damage were investigated (14, 29-31).

MATERIALS AND METHODS

Animals

This study included 3-week-old and 12-week-old BALB/CJ mice obtained from a breeding colony of the Rudjer Boskovic Institute (Zagreb, Croatia). During the experiment, four animals were housed per cage. The bottom of the cage was covered with sawdust (Allspan®, Karlsruhe, Germany). Standard food for laboratory mice (4 RF 21 GLP Mucedola srl, Settimo Milanese, Italy) was used. All animals had free access to food and water. Animals were kept under standard conditions with a 12-h light/dark cycle, temperature of 22 °C, and 55 % humidity. All experiments were performed according to the ILAR Guide for the Care and Use of Laboratory Animals, Council Directive (#86/609/EEC) and Croatian Animal Protection Act (OG 135/06) and were approved by the Ethical Committee of the Ministry of Agriculture.

Radiation exposure

Each age and sex group consisted of eight animals. Animals received a single dose of 8 Gy (3,125 cGy s^{-1}) using X6MV photon irradiation (ONCOR linear accelerator, Siemens, Malvern, USA). One half of the dose was applied to the dorsal (PA, SSD=130 cm, bolus RW3 1cm) and the other half to the ventral side (AP, SSD=130 cm, bolus RW3 1 cm) of animals. Animals were sampled before irradiation, 24 h, 48 h, and 72 h after irradiation. During irradiation each animal was placed into a plexiglass cage.

In vivo *micronucleus assay*

The *in vivo* MN assay was performed 24 h, 48 h, and 72 h after exposure in 3-week-old mice, while in adult animals the analysis 72 h after irradiation was not performed due to the reduction of reticulocyte number. Peripheral blood was collected from the tail vein (5 μL per sample) from all animals. Blood smears were prepared on acridine orange (Sigma-Aldrich, St. Louis, USA) coated slides, covered with a coverslip, and analysed according to Hayashi et al. (27). The MN frequency was analysed in 2000 reticulocytes per sample. Analyses were performed by one scorer using a fluorescent microscope under 1000 x magnification (Olympus Provis AX70, Tokyo, Japan).

Statistics

Statistical analysis of data was performed using the Statistica 7.0 software package (StatSoft, Tulsa, USA). An independent sample t-test or one-way analysis of variance (ANOVA) followed by the Tukey *post-hoc* test were used to determine significant differences between the groups. The test value of $P<0.05$ was considered statistically significant.

RESULTS

Changes in the total number of micronucleated cells after irradiation in 3-week- and 12-week-old mice are shown in Figure 1A and 1B, respectively; while the distribution of cells according to the number of MN per cell is shown in Table 1.

Background levels before irradiation were 0.10 % MN in prepubertal males and 0.05 % MN in females. Background values were 0.10 % MN in adult males and 0.08 % MN in females. There was no significant difference between sex and age groups in MN frequencies. Cells with more than one MN were not detected (Table 1).

A significant increase in MN frequencies compared to basal values was observed after 24 h in both 3-week-old and adult mice as well as in both males and females but there were no significant differences between these two sexes (Figure 1).

The most pronounced increase in the incidence of micronucleated cells was observed for both age groups at 48 h after irradiation (Figure 1A and B). At this time, a significantly higher frequency of cells with MN was observed in 3-week-old and adult males than in female animals (indicated by an asterisk in Figure 1A and 1B) and the difference between males and females was more expressed in younger (~33 %) than in adult (~22 %) animals. Further, at 48 h after irradiation, in the same sex group, 3-week-old mice had a significantly higher incidence of cells with MN than 12-week-old mice (indicated by the sign

Table 1 *The incidence of mononuclear (1 MN), binuclear (2 MN), trinuclear (3 MN), and tetranuclear (4 MN) cells in reticulocytes from peripheral blood at 24 h, 48 h, and 72 h after irradiation*

Time	Distribution of cells according to the number of micronuclei (MN) per cell (%) (mean±SD)							
	Prepubertal mice (3-week-old)							
	1 MN		2 MN		3 MN		4 MN	
	Male	Female	Male	Female	Male	Female	Male	Female
0 h (control)	0.10±0.08[a]	0.05±0.06[a]	0	0	0	0	0	0
24 h	0.54±0.09[b]	0.54±0.11[b]	0	0	0	0	0	0
48 h	6.40±0.65[c]*#	4.60±0.57[c]*#	0.68±0.20[a]*#	0.1±0.07*	0	0.04±0.05	0	0
72 h	4.83±0.58[d]	5.30±0.68[c]	0.43±0.06[a]	0.15±0.19	0.07±0.11	0	0	0.03±0.05
	Adult mice (12-week-old)							
Time	1 MN		2 MN		3 MN		4 MN	
	Male	Female	Male	Female	Male	Female	Male	Female
0 h (control)	0.10±0.08[a]	0.08±0.10[a]	0	0	0	0	0	0
24 h	0.58±0.11[b]	0.42±0.13[b]	0	0	0	0	0	0
48 h	3.90±0.69[c]*#	2.92±0.36[c]*#	0.06±0.09*#	0.20±0.07*	0.02±0.04	0	0	0
72 h	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

([a, b, c, d])–significant differences between groups are indicated by different letters (p<0.05, Tukey post hoc *test); (*)–significant differences male* vs. *female mice (p<0.05, t-test); (#)–significant differences prepubertal* vs. *adult mice (p<0.05, t-test); n.a.–not available*

in Figure 1A and 1B). This age difference was more pronounced in male (~45 %) than in female (~35 %) mice.

After 72 h, in 3-week-old mice a significant decrease in cells with MN frequency was observed in males, while in females a slight increase was observed but the difference was not statistically significant (Figure 1A).

Besides determining the total number of micronucleated cells, the analysis of cell distribution according to the number of MN in the cell was performed. From Table 1, it can be seen that the incidence of cells with one MN for both age and sex groups generally follows the trend observed for the total number of micronucleated cells (Figure 1A and B). At 48 h, the incidence of cells with two MN was significantly higher in male than female 3-week-old mice (Table 1). Also, 3-week-old males had a significantly higher frequency of cells with two MN compared to adult male mice (Table 1). In 3-week-old mice, cells with three and three and four MN in one cell were detected at 48 h and 72 h after irradiation but their incidence was very low and was not strictly related with either sex (Table 1).

DISCUSSION

The exposure of general population to ionising radiation due to nuclear accidents such as Chernobyl and Fukushima showed that casualty management is still not satisfactory and that radiation protection plans for specifically susceptible subpopulations such as children according to age groups are still not available. Contrary to the historical approach to cancer risks after overexposure to ionising radiation, which is basically focused on the caused genome damage, current radiation biology is rapidly incorporating complex interactions between ionising radiation and the immune system, epigenetic modifications, sex hormones, and age at exposure. Such approach, however, demands additional investigations into the mechanisms underlying the detected age and sex differences in susceptibility to radiogenic cancers, which should give an insight into the causality of the involved complex pathways. For such studies, animal models as *in vitro* models that simulate developmental process are still not developed.

In order to investigate the differences in the radiosensitivity of prepubertal and adult organisms, in this study prepubertal, 3-week-old and adult mice of both sexes were irradiated with 8 Gy of gamma radiation. Such a high dose of radiation was selected in order to additionally validate the *in vivo* MN assay in regard to difference in haematopoietic potency between young and adult animals.

Ionising radiation caused a significantly higher frequency of MN in prepubertal animals than in adult animals of both sexes. Due to reticulocytopenia, which is already described in applied doses (31) in adult animals, it was not possible to measure the MN frequency 72 h after exposure. As in mice up to four weeks of age reticulocytes make up around 10 % of erythrocytes (32), reticulocytopenia

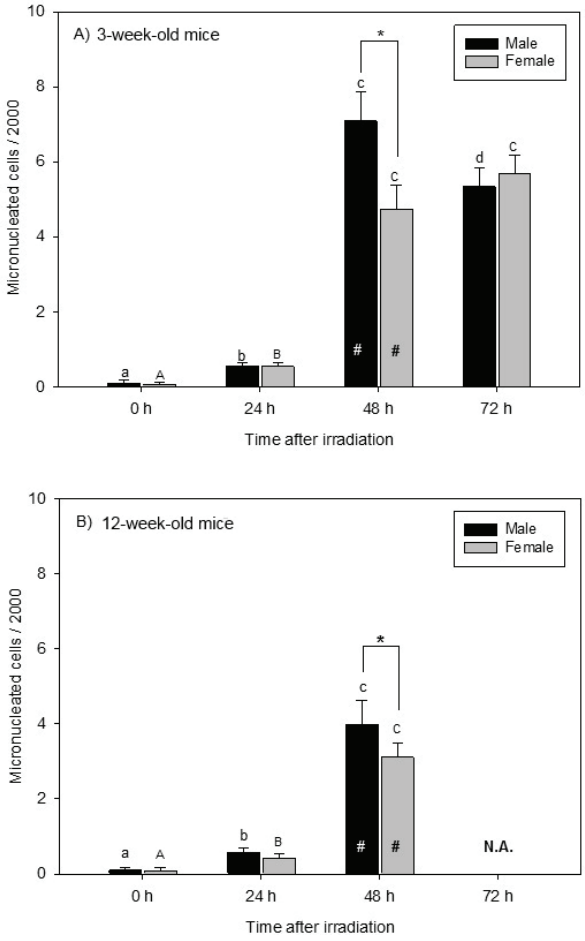

Figure 1 *Frequencies of micronucleated cells in 3-week-old (A) and 12-week-old (B) mice at 24, 48, and 72 h after irradiation. (Mean±SD). Significant differences (p<0.05, Tukey* post-hoc *test) between particular times are indicated by different small and capital letters above the error bars for male and female mice, respectively. Significant differences (p<0.05, t-test) between males and females are marked by an asterisk (*) and significant differences (p<0.05, t-test) between 3-week-old and 12-week-old mice at 48 h are marked by the sign (#) within the bars; N.A.-not available*

was less severe and the scoring in 3-week-old mice was possible. It is suggested that repopulation of reticulocytes may not have an impact on the results of MN frequencies as after irradiation the increase in the reticulocyte number is reported to be only after 72 h (33).

Our results are in concordance with historical data which shows that that the peak in the MN frequency has been observed 48 h after exposure to lower doses of 2 Gy or 3 Gy of gamma radiation (33, 34). This study also confirms the conclusions of previous studies that young animals are more susceptible to the effects of ionising radiation than adults (35-38). The same higher radiosensitivity has also been described in children exposed accidentally, either diagnostically or therapeutically (22, 39).

In our study, a significantly higher MN frequency in males of both age groups was detected. Similarly, although not statistically significant, lower MN frequencies in erythroblasts have been described in female adult mice than in adult male mice exposed to chronic gamma radiation (40). Acute exposure of mice to 5 Gy of X-rays caused different, tissue specific, levels of DNA strand breaks in female and male adult mice (41). In BALB/c mice after irradiation with 1 Gy X-rays, sex-specific changes in methylation were reported. Thus, significantly higher methylation levels were present in males than in females (11). Similarly, the difference in miRNA was also reported to be sex-specific (42). In mice exposed for two weeks to X-rays, adult male genome damage was higher than in females (0.05 and 0.10 Gy) (43).

This study is the first to show that sex differences are present even in prepubertal animals. The higher MN frequency in males than in females in both age groups is suggested to be attributed to the effects of oestrogen.

A possible mechanism by which oestrogen may cause a lower frequency of MN is the quiescence of stem cells (44) or enhanced DNA repair as shown in the case of pretreatment of animals with genistein before irradiation (45). Similarly, dietylstilbestrol, a xenoestrogen, is shown to suppress the proliferation of haematopoietic precursor cells and intensify DNA repair (46). However, in pregnant mice, a significantly increased genome damage in spontaneously dividing bone marrow cells occurred after a high increase in oestrogen and progesterone hormones and this phenomenon disappeared after delivery. It is therefore possible that oestrogen contributed to transient radiosensitivity (47).

Oestrogen as an endocrine, paracrine, and neuromodulator molecule has been shown to interact with the biological effects of ionising radiation. A different age-, sex- and tissue-related distribution of polymorphic oestrogen receptors (alpha, beta, GPR30) enables numerous possible interactions of oestrogen with biological pathways after action of environmental stressors (4). Thus, oestrogen may have radioprotective and radiosensitising effects depending on its level, tissue type, age, type of radiation, and dose.

Contrary to oestrogen, there is no data on the interaction between ionising radiation and pathways regulated by testosterone that may have an impact on radiogenic carcinogenesis. Data on testosterone and oestrogen levels during postnatal development of mice until adulthood are not available. In some organs, such as brain, oestrogen receptor distribution fluctuates during prepubertal period and the impact of the same oestrogen levels differs due to its different half-life (48). Additionally, letrozole, an aromatase inhibitor, is shown to increase radiosensitivity of cancer cells (49). As aromatase inhibitors such as letrozole cause an increase in testosterone, it could be indirectly concluded that testosterone causes radiosensitivity (50).

In both boys and girls, the increase in testosterone and oestrogen levels starts in prepuberty and is simultaneous with a significant reduction in the hormone binding protein, whose level is reduced by half from prepuberty to puberty

(51, 52). In humans, girls have higher levels of 17beta-testosteorne and estradiol during prepuberty, while in boys the estradiol level can be undetectable (53). Thus, changes in the hormonal status are present even before the first clinical signs of puberty (54), which points to the significance of the prepubertal period in the orchestration of physiological conditions for sex differences in response to the environment before puberty, including ionising radiation.

The investigation of the effects of oestrogen and testosterone on the biological effects of radiation is of great importance for (a) the inclusion of prepuberty and puberty group differences in the radiation protection legislation; (b) development of radioprotective xenoestrogen substances whose application would be age- and sex-adjusted; and (c) yielding a contribution to oncology for better effects in cases of combined hormonal and radiotherapy.

In summary, exposure to 8 Gy of gamma-radiation caused significantly increased levels of MN frequency - significantly higher in prepubertal and adult male animals. Further investigations into the mechanisms by which age and sex differences in responses to exposure to ionising radiation set in should include analyses or correlations between oestrogen and testosterone levels, haematopoiesis dynamics, levels of oestrogen, and androgen receptors in bone marrow stem cells and genome damage.

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Dobne i spolne razlike u oštećenju genoma između pretpubertetskih i odraslih miševa nakon izlaganja ionizirajućemu zračenju

Mehanizmi koji uzrokuju spolne i dobne razlike u biološkim odgovorima na izloženost ionizirajućemu zračenju i s tim u vezi zdravstvene rizike još nisu dovoljno ispitani. Kako bi se spoznao značaj spolnih hormona u etiologiji zračenjem izazvanih vrsta tumora, potrebno je bolje poznavanje mehanizama koji su uključeni u taj proces, osobito tijekom razvojne faze organizma. Cilj ovoga istraživanja bio je prikazati dobne i spolne razlike u oštećenju genoma između pretpubertetskih i odraslih miševa nakon jednokratnoga izlaganja gama-zračenju. Primjenom *in vivo* mikronukleus-testa izmjereno je oštećenje genoma nastalo 24 sata, 48 sati i 72 sata nakon izlaganja BALB/CJ miševa, starih tri tjedna i dvanaest tjedana, dozi gama zračenja od 8 Gy. U svim vremenskim točkama mjerenja uočeno je značajnije veće oštećenje genoma u pretpubertetskih u odnosu na odrasle jedinke obaju spolova. Zračenje je uzrokovalo veću učestalost mikronukleusa u muških jedinki u objema dobnim skupinama. Dobiveni rezultati potvrđuju postojanje spolnih razlika u osjetljivosti na učinke ionizirajućega zračenja u miševa, a ovo je prvo istraživanje rezultati kojega pokazuju da do takvih razlika dolazi već u pretpubertetskoj dobi.

KLJUČNE RIJEČI: *estrogen; gama zračenje; mikronukleus; radioosjetljivost; testosteron*

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Protective effects of quercetin and vitamin C against nicotine-induced toxicity in the blood of Wistar rats

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Nicotine is a potential inducer of oxidative stress, through which it can damage numerous biological molecules. The aim of our study was to investigate the prooxidative effects of nicotine and protective (additive or synergistic) effects of quercetin and vitamin C in the blood of experimental animals, to determine whether the combination of these antioxidants might be beneficial for clinical purposes. Wistar albino rats were receiving intraperitoneal nicotine injection (0.75 mg kg^{-1} per day) or saline (control group) or nicotine plus quercetin (40 mg kg^{-1} per day) and vitamin C (100 mg kg^{-1} per day) for three consecutive days. On day 4, we determined their blood lipid profile, liver enzymes, oxidative stress parameters, and antioxidative system parameters. Compared to untreated control, nicotine significantly increased total cholesterol, LDL-cholesterol, triglycerides, liver enzymes (alanine transaminase, aspartate transaminase, and lactate dehydrogenase) and oxidative stress parameters (superoxide anion, hydrogen peroxide, and lipid peroxide) and decreased HDL-cholesterol, glutathione, and superoxide dismutase/catalase activity. Quercetin + vitamin C reversed these values significantly compared to the nicotine alone group. Our results confirm that nicotine has significant prooxidative effects that may disrupt the redox balance and show that the quercetin + vitamin C combination supports antioxidant defence mechanisms with strong haematoprotective activity against nicotine-induced toxicity. In practical terms, this means that a diet rich in vitamin C and quercetin could prevent nicotine-induced toxicity and could also be useful in the supportive care of people exposed to nicotine.

KEY WORDS: *antioxidant defence; erythrocytes; lipid profile; oxidative damage; reactive oxygen species; tobacco*

Nicotine, the primary addictive component of cigarette smoke, is rapidly absorbed by the circulatory system and most of it is metabolised in the liver (1). Previous *in vivo* studies have shown that chronic use of nicotine disrupts the balance between the prooxidants and antioxidants in the circulation of experimental rats (2). Nicotine triggers the production of free radicals and reactive oxygen species (ROS), including superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$), which overwhelm the antioxidative defence system (AOS) and eventually generate oxidative stress (3-6).

Red blood cells are a good indicator of oxidative stress, as their components, haemoglobin and polyunsaturated fatty acids, are susceptible to it (7), which is why they contain antioxidants for efficient defence. These components are non-enzymatic [such as vitamins C and E, and glutathione (GSH)] and enzymatic [superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px)] (8). The defence system can be improved by the consumption of fruits and vegetables rich in antioxidants, including vitamin C and flavonoids - polyphenolic compounds common in fruits, vegetables, and some types of tea and wine (9).

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One such flavonoid found in abundance in red kidney beans, grapes, red wine, apples, tea, onions, and broccoli is quercetin. Its consumption in everyday diet is too low for a clinically meaningful protective effect but it can easily be increased with quercetin-rich food and supplements (10), whereas its bioavailability increases if consumed with other flavonoids, vitamins C and E, or catechins (11-13).

Considering that Muthukumaran et al. (14) have already demonstrated the antioxidant properties of quercetin alone against nicotine-induced oxidative stress in rats, the aim of our study was to see how effective the combination of quercetin and vitamin C could be, especially as this combination has never been studied before. By establishing conclusive evidence of the combination's effectiveness against nicotine-induced toxicity, we hoped to see whether this combination could find clinical application. Our secondary aim was to get an insight into the possible underlying mechanisms of (inter)action between nicotine and the combination of antioxidants.

MATERIALS AND METHODS

Chemicals

All reagents and chemicals used in this study were of analytical grade or higher purity, obtained from Sigma

Chemical Co. (St. Louis, MO, USA) and Merck (Darmstadt, Germany). The solutions were prepared with double-distilled water.

Experimental design

All the experimental procedures were approved by the University Ethics Committee for Animal Experimentation. We used 18 male albino *Wistar* rats, about 2 months old, weighing 200–220 g to exclude possible protective effects of oestradiol against provoked oxidative stress (15, 16). Before the experiments began, the animals were housed in plastic cages under standard laboratory conditions (temperature 22±2 °C; 12:12 h light:dark cycle) for at least one week to adapt. Tap water and commercial standard rodent laboratory diet were available *ad libitum* for the duration of the experiments. The rats were randomly divided into three groups (n=6 per group) and treated for three days, as follows: the control group was receiving intraperitoneal (*i.p.*) injections of isotonic saline [0.1 mL kg^{-1} body weight (b.w.)]; the nicotine group was receiving nicotine only [0.75 mg kg^{-1} (b.w.) per day *i.p.*]; and the nicotine + quercetin + vitamin C group was receiving nicotine as described above with quercetin [40 mg kg^{-1} (b.w.) per day *i.p.*] and vitamin C [100 mg kg^{-1} (b.w.) per day *i.p.*]. These doses were based on literature data (4, 17-21) and correspond to the human daily dietary intake of quercetin and vitamin C enriched food and the daily nicotine intake in people who smoke 10-20 cigarettes per day (2, 4).

Twenty-four hours after the last dose, the animals were anaesthetised with ether and decapitated. Blood samples for all analyses were collected on the day of sacrifice between 8 a. m. and 10 a. m. to avoid variations related to the circadian rhythm.

Analytical procedures

For the medium we selected rat blood as the best substrate. The blood samples were collected in tubes with anticoagulant (K-EDTA) and without one. Biochemical parameters were measured on the day of sacrifice. Plasma for the other analyses was separated by centrifugation at 1000 *g* (+4 °C) for 10 min. The erythrocytes were washed three times with an equal volume of cold saline (0.9 %, *v/v*). One millilitre of the washed erythrocytes was lysed with 3 mL of dH_2O (1:3, *v/v*) at 0 °C for 30 min. All samples were extracted from the lysates and stored at -20 °C for no longer than 7 days. ROS analysis was completed within 1–2 h after extraction. Lipid peroxides (LPO) and GSH were measured within 6 h after extraction. Antioxidant enzyme activity in erythrocyte lysate was measured following the method described by McCord and Fridovich (22).

Biochemical analysis

Blood samples in non-anticoagulant tubes were centrifuged at 1000 *g* (+4 °C) for 15 min to obtain serum for measurements. We analysed the serum for total cholesterol, high-density lipoprotein cholesterol (HDLC), low-density lipoprotein cholesterol (LDLC), triglycerides (TG) (expressed in mmol L^{-1}), and enzyme activity [alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH, expressed in U L^{-1})] with a C 8000 Architect autoanalyser (Wiesbaden, Germany) using standard diagnostic colourimetric kits (Abbott Laboratories, Abbott Park, IL, USA).

Oxidative stress parameters

Erythrocyte ROS concentrations were determined as follows: half the volume of 3 mol L^{-1} perchloric acid and 2 volumes of 20 mmol L^{-1} EDTA were added to one volume of the lysate. After extraction on ice and centrifugation on 1000 *g* (+4 °C) for 10 min, the extracts were neutralised with 2 mol L^{-1} of K_2CO_3. The spectrophotometric determination of $O_2^{\bullet-}$ was based on the reduction of nitroblue tetrazolium in the presence of $O_2^{\bullet-}$ (23). The determination of H_2O_2 was based on the oxidation of phenol red in the presence of horseradish peroxidase as catalyst (24). These concentrations are expressed in μmol L^{-1} of erythrocytes.

Lipid peroxides in the haemolysate were determined using the method described by Ohkawa et al., (25), based on the reaction of lipid peroxidation products (malondialdehydes) with thiobarbituric acid reactive substances (TBARS). Haemolysate samples were extracted with 28 % trichloroacetic acid and centrifuged at 1000 *g* for 10 min. For colour reaction we added 1 % TBA and incubated the mixture at 90 °C for 15 min. The absorbance was measured with a UV/Visible spectrophotometer (Jenway 6105, Bibby Scientific Limited, Staffordshire, UK). These results are expressed in μmol L^{-1} erythrocytes.

Determination of GSH and antioxidant enzymes

For GSH determination we used the method of Beutler (26), based on the oxidation of GSH with DTNB (5.5 ’-dithiobis-2-nitrobenzoic acid). Its concentrations are expressed in μmol L^{-1} of erythrocytes.

Superoxide dismutase (EC 1.15.1.1) activity was determined using the method described by Marklund and Marklund (27). This method is based on pyrogallol oxidation by $O_2^{\bullet-}$ and its dismutation by SOD. The enzyme activity is expressed as U mL^{-1} of erythrocytes.

For CAT (EC 1.11.1.6) activity measurements we used the method of Beutler (28). The method is based on the rate of H_2O_2 degradation mediated by CAT measured spectrophotometrically at 230 nm in 1 mol L^{-1} Tris-HCl solution (with 5 mmol L^{-1} EDTA, pH 8.0). Absorbance was measured with the Jenway 6105 UV/Visible spectrophotometer and the enzyme activity is expressed in U mL^{-1} of erythrocytes.

Statistical analysis

All analyses were done using the SPSS for Windows, version 13.0 (SPSS Inc., Chicago, IL, USA). All results are expressed as mean±SEM and evaluated using the one-way analysis of variance (ANOVA) test. If there was a significant difference between the groups after applying ANOVA, these groups were compared using Dunnett's multiple comparison tests (control with each treated group). We set statistical significance at $p \leq 0.05$ for all comparisons.

RESULTS AND DISCUSSION

Our findings have confirmed our expectations based on earlier reports about prooxidative effects of nicotine and the antioxidative effects of quercetin and vitamin C (12-14).

Oxidative stress parameters

Erythrocyte concentrations of $O_2^{\bullet -}$, H_2O_2, and LPO were significantly higher in the nicotine than control group. Co-administration of quercetin and vitamin C greatly reversed nicotine-induced changes in these oxidative stress parameters (Figure 1).

These findings confirm earlier conclusions that nicotine exerts its toxic effects through higher ROS production (3-6, 29). In normal conditions, $O_2^{\bullet -}$ is transformed to H_2O_2 by SOD. If its production increases over the SOD buffering capacity, $O_2^{\bullet -}$ can react with NO to form peroxynitrite ($ONOO^-$). Furthermore, in the presence of iron, $O_2^{\bullet -}$ and H_2O_2 can react (Haber-Weiss reaction) to form a highly reactive hydroxyl radical ($^{\bullet}OH$), which can also be produced by peroxynitrite decomposition. Hydroxyl radicals oxidise polyunsaturated fatty acids in biological membranes and induce the formation of LPO (8). Hence the significantly increased LPO levels in our erythrocytes (Figure 1).

Biochemical test results

Since increased LPO disrupts the normal function or destroys erythrocyte membranes and causes a leakage of the cytoplasmic marker such as LDH (indicator of cell and tissue damage) into circulation (30), we expected to find elevated values of this parameter as well as enhanced activity of AST and ALT, based on earlier findings in blood and various organs (14, 29, 31-34). Indeed, treatment with nicotine significantly increased LDH levels and the activities of ALT, AST compared to control (Table 1). Quercetin and vitamin C reversed these changes toward the control ones and minimised the adverse effects of nicotine (Table 1).

Our study has also demonstrated that by interfering with the metabolism of lipoproteins, nicotine causes a significant increase in total cholesterol, LDLC, and TG levels, whereas the level of HDLC significantly drops (Table 2). This is in line with the study by Sharif et al. (33), who have reported adverse nicotine effects on the lipid profile of adult male mice (*Mus musculus*). Once again, quercetin and vitamin C co-administration have shown their strong protective effects against nicotine toxicity by restoring lipid levels to near normal values (Table 2).

GSH and antioxidant enzymes

The nicotine group showed a significant drop in GSH and antioxidant enzyme activities compared to control, and the treatment with quercetin and vitamin C again reversed these effects (Figure 2).

Table 1 *Effects of quercetin and vitamin C on hepatic marker enzyme activities in the serum of nicotine-treated rats (values are expressed as means±SEM of 6 animals)*

Parameters	Experimental groups		
	Control	Nicotine	Nicotine + QN + Vit C
ALT (U L^{-1})	47.5±1.6	83.2±2.1*	57.4±2.2**
AST (U L^{-1})	148.2±5.8	184.3±5.2*	142.6±4.1**
LDH (U L^{-1})	625±22.3	1238±65.2*	840±42.3**

*QN: quercetin; Vit C: vitamin C; ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDH: lactate dehydrogenase. *significantly different from control (p<0.05); **significantly different from nicotine group (p<0.05)*

Table 2 *Effects of quercetin and vitamin C on lipid profile in the serum of nicotine-treated rats (values are expressed as means±SEM of 6 animals)*

Parameters	Experimental groups		
	Control	Nicotine	Nicotine + QN + Vit C
TC (mmol L^{-1})	1.14±0.06	1.56±0.07*	0.95±0.06**
HDLC (mmol L^{-1})	0.82±0.02	0.58±0.03*	0.86±0.05**
LDLC (mmol L^{-1})	0.11±0.01	0.37±0.03*	0.20±0.03**
TG (mmol L^{-1})	0.18±0.02	0.41±0.04*	0.24±0.05**

*QN: quercetin; Vit C: vitamin C; TC: total cholesterol; HDLC: high density lipoprotein cholesterol; LDLC: low density lipoprotein cholesterol; TG: triglicerides. *significantly different from control (p<0.05); **significantly different from nicotine group (p<0.05)*

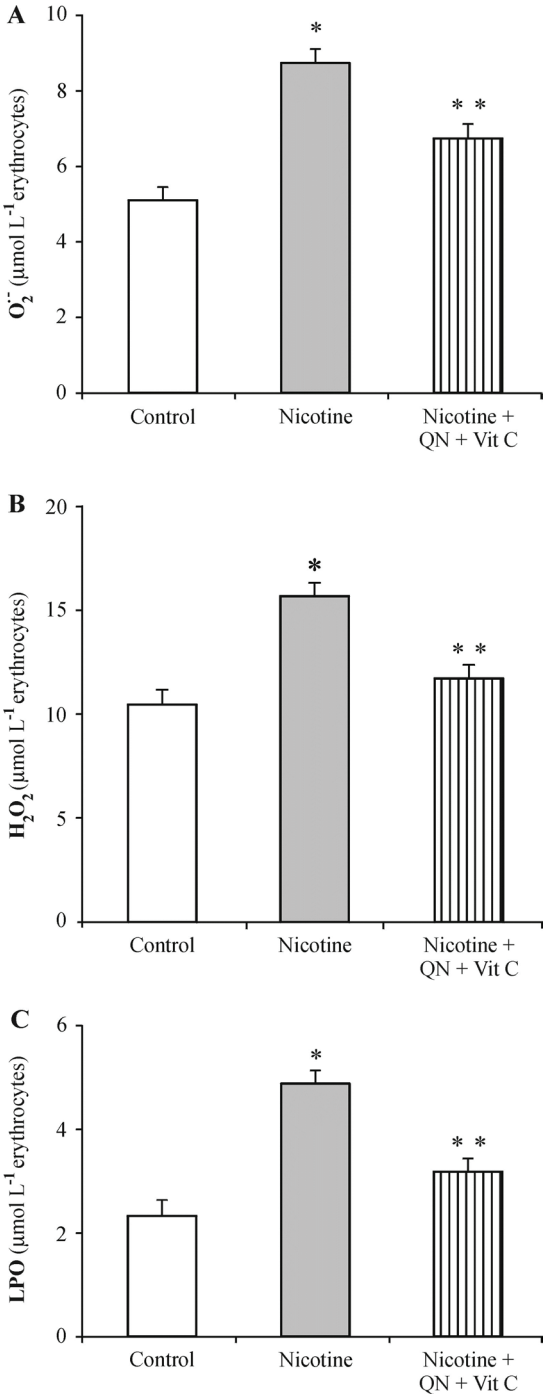

Figure 1 *Effects of quercetin and vitamin C on oxidative stress parameters ($O_2^{\bullet-}$, H_2O_2 and LPO) in the blood of nicotine-treated rats (values are expressed as means±SEM of 6 animals). QN: quercetin; Vit C: vitamin C; $O_2^{\bullet-}$: superoxide anion radical; H_2O_2: hydrogen peroxide; LPO: lipid peroxides. *significantly different from control (p<0.05); **significantly different from nicotine group (p<0.05)*

The decreased levels of GSH in nicotine-treated rats indicate that GSH protects membrane lipids from oxidation by scavenging free radicals, passing from reduced to oxidized form (GSSG) (14, 31, 35). Oxidative stress in erythrocytes induced by nicotine can inactivate SOD and CAT proteins or affect their synthesis *de novo*, which consequently reduces their activity. Lower SOD activity is due to its reaction with $O_2^{\bullet-}$ to form H_2O_2 and O_2. H_2O_2 is also a substrate for CAT, whose role is to degrade H_2O_2 into H_2O and O_2 (8), thereby decreasing its activity as we observed in our study. Other studies (31, 35) have also demonstrated the depletion of SOD and CAT in some organs of nicotine-treated rats.

As for the protective effects of the quercetin plus vitamin C combination, they seem to protect phospholipid bilayers by non-covalent bonding, enhancing this way the regeneration of endogenous antioxidants (12). Our results confirm the protective effects of quercetin and vitamin C against nicotine-induced changes in both enzymatic and non-enzymatic antioxidants due to either direct scavenging of ROS or induction of antioxidant enzymes.

There are some limitations to our study: the protective effects of the quercetin plus vitamin C combination was evaluated against nicotine alone, as only one of the many components of tobacco. Therefore, our findings can not be interpreted as simply as to claim that quercetin plus vitamin C would provide the same level of protection against tobacco products in general. Furthermore, we have not compared the protective effects of this combination with quercetin alone, which would probably give a better insight into the mechanisms of their interaction (additive or synergistic). In addition, further research should look into the effects of higher quercetin doses and whether these effects may become prooxidant, as suggested by Heeba and Mahmoud (36). Future research should also be able to elucidate the exact molecular mechanisms of quercetin and vitamin C protection against nicotine or other alkaloids and drugs.

Our findings, however, have practical implications. The combination of quercetin and vitamin C in the selected doses (40 mg kg^{-1} b.w. and 100 mg kg^{-1} b.w., respectively) seems to sufficiently reinforce the antioxidative defences and diminish the adverse effects of nicotine for us to recommend the adapted doses in persons exposed to nicotine, tobacco smokers in particular.

Conflict of interest

The authors declared that there are no conflicts of interest.

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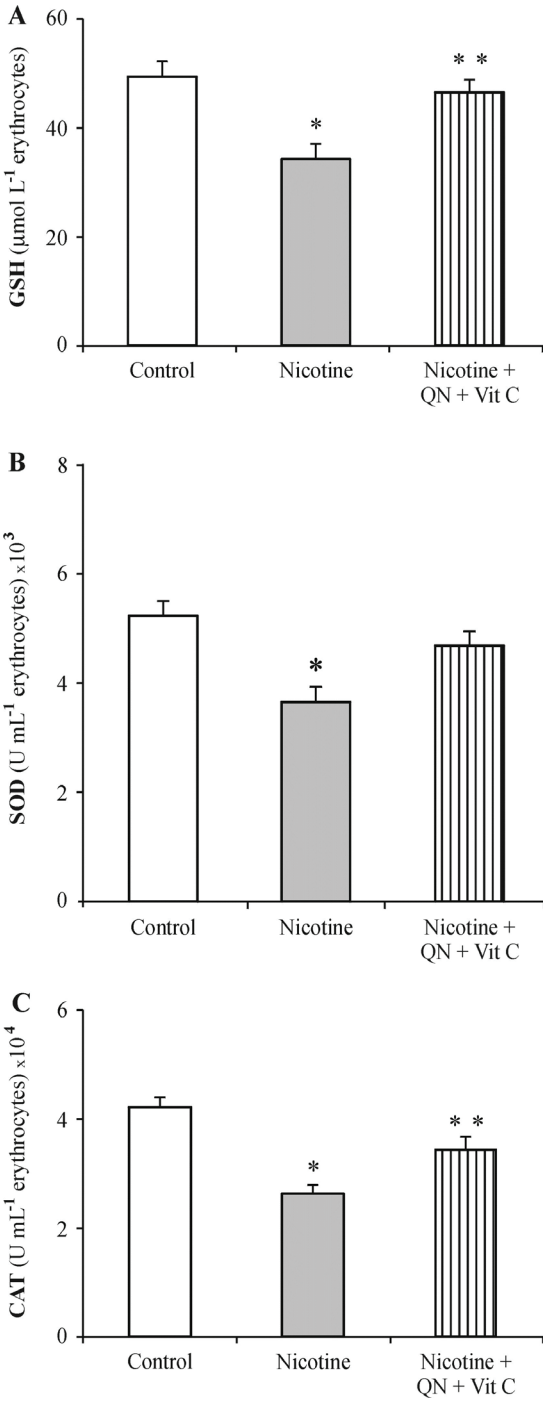

Figure 2 *Effects of quercetin and vitamin C on the antioxidative defense system (GSH, SOD, and CAT) in the blood of nicotine-treated rats (values are expressed as means±SEM of 6 animals). QN: quercetin; Vit C: vitamin C; GSH: reduced glutathione; SOD: superoxide dismutase; CAT: catalase. *significantly different from control (p<0.05); **significantly different from nicotine group (p<0.05)*

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Zaštitno djelovanje kvercetina i vitamina C protiv nikotinom izazvane toksičnosti u krvi Wistar štakora

Nikotin je potencijalni induktor oksidacijskoga stresa, preko kojega može oštetiti brojne biološke molekule. Cilj našega istraživanja bio je ispitati prooksidacijsko djelovanje nikotina i zaštitno (aditivno ili sinergističko) djelovanje kvercetina i vitamina C u krvi eksperimentalnih životinja te utvrditi može li kombinacija tih antioksidansa biti korisna u kliničke svrhe. *Wistar albino* štakori primali su intraperitonealno injekciju nikotina (0,75 mg kg^{-1} po danu) ili fiziološke otopine (kontrolna skupina) ili nikotina s kvercetinom (40 mg kg^{-1} po danu) i vitaminom C (100 mg kg^{-1} po danu) tri uzastopna dana. Četvrtoga dana odredili smo lipidni profil u krvi, jetrene enzime, parametre oksidacijskoga stresa i antioksidacijskoga sustava. U usporedbi s netretiranom kontrolnom skupinom, nikotin je značajno povećao ukupni kolesterol, LDL-kolesterol, trigliceride, jetrene enzime (alanin transaminaze, aspartat transaminaze i laktat dehidrogenaze) i parametre oksidacijskoga stresa (superoksid anion, vodikov peroksid i lipidne perokside), a smanjio HDL-kolesterol, glutation i aktivnosti superoksid dismutaze/katalaze. Kvercetin i vitamin C značajno su utjecali na te vrijednosti u odnosu na skupinu samo s nikotinom. Naši rezultati potvrdili su značajno prooksidacijsko djelovanje nikotina koje može poremetiti redoks ravnotežu i pokazuje da kombinacija kvercetina i vitamina C podržava antioksidacijske obrambene mehanizme s jakim hematoprotekcijskim aktivnostima protiv nikotinom izazvane toksičnosti. Možemo zaključiti da prehrana bogata kvercetinom i vitaminom C može koristiti kao prevencija nikotinom inducirane toksičnosti te da kombinacija tih dvaju antioksidansa može biti korisna u kliničkom oporavku ljudi izloženih nikotinu.

KLJUČNE RIJEČI: *antioksidacijska obrana; duhan; eritrociti; lipidni profil; oksidacijska oštećenja; reaktivne vrste kisika*

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Association between the *TP53* and *CYP2E1*5B* gene polymorphisms and non-small cell lung cancer

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Non-small cell lung cancer (NSCLC) is the most common form of lung cancer. Genetic polymorphisms in tumour suppressor genes and genes encoding xenobiotic metabolising enzymes alter the activity of their corresponding enzymes and are important individual susceptibility factors for NSCLC. Because of the lack of information in literature, the aim of our study was to investigate the role of the tumour suppressor gene *TP53* (Arg72Pro) and the xenobiotic metabolising *CYP2E1*5B* gene polymorphisms on the risk of NSCLC development. The study population consisted of 172 patients and 172 controls (156 men and 16 women in each group). Genetic polymorphisms were determined with real-time polymerase chain reaction (PCR) and PCR restriction fragment length polymorphism (PCR-RFLP). Multivariate analysis showed a significant association with NSCLC for the combination between the *TP53* codon72 Arg/Pro and the Pro/Pro genotypes (OR 2.21, 95 % CI 1.39–3.51; p=0.001). We also analysed whether combinations of these gene variants with *GSTM1*, *GSTT1*, *GSTP1* exon 5 (Ile105Val), and *GSTP1* exon 6 (Ala114Val) gene polymorphisms were associated with the NSCLC risk. A significant increase in the risk was observed for the following combinations: *TP53* codon72 variant with *GSTM1* null (OR 2.22, 95 % CI 1.23-4.04; p=0.009), *GSTT1* null (OR 2.98, 95 % CI 1.49-5.94; p=0.002), and *GSTP1* (Ala114Val) variant genotypes (OR 3.38, 95 % CI 1.54-7.41; p=0.002). Further studies with larger samples are needed to verify these findings.

KEY WORDS: *carcinogen metabolism;* CYP2E1*; genetic polymorphism; GST; NSCLC*

Lung cancer is the most common cancer type in the world, which affects men in particular. It is also the leading cause of death among cancers (1). Histologically, about 80 % of lung cancers are non-small cell lung carcinomas (NSCLC). The inbuilt phase I and II biotransformation enzyme systems inactivate environmental carcinogens, especially those present in tobacco smoke, depending on the regulating polymorphisms. Epidemiological studies show that genetic differences and interactions among genetic variants might modify lung cancer susceptibility (2, 3). Therefore, it is important to identify individual genetic and acquired factors that modify lung cancer risk in order to develop preventive strategies based on this information. This is particularly true for identifying polymorphisms of tumour suppressor genes and xenobiotic metabolising enzyme genes. The *TP53* tumour suppressor gene is an essential regulator of the cell-cycle arrest, DNA repair, and apoptosis (4). An important single nucleotide polymorphism detected in *TP53* is Arg72Pro. This allele has been shown to decrease the activity of the corresponding protein, which is crucial for the apoptotic function of TP53 (5, 6). Recent studies have reported the association between this polymorphism and lung cancer (7-11).

CYP2E1 is an important phase I biotransformation enzyme that transforms *N*-nitrosamines, vinyl chloride, and benzene in tobacco smoke into mutagenic and carcinogenic metabolites (12). In NSCLC patients it has been found to be overexpressed (13). However, its *CYP2E1*5B* allele has been shown to reduce enzyme activity and microsomal oxidation capacity (14, 15).

The complex pathways of (pro)carcinogen metabolism and the possible interactions between the genes encoding enzymes and TP53 protein clearly indicate the need for their combined assessment as predisposing factors for NSCLC development. However, current findings on the overall impact of *TP53*, *CYP2E1*, and *GST* polymorphisms on NSCLC are limited and controversial (16-18). The aim of this study was to learn more about these impacts by investigating: a) the association between NSCLC risk and *TP53* (Arg72Pro) and *CYP2E1*5B* polymorphisms either alone or in combination and b) possible gene interactions as risk modifiers for NSCLC by combining *TP53* (Arg72Pro) and *CYP2E1*5B* polymorphisms with phase II enzyme coding gene polymorphisms *GSTM1*, *GST1*, *GSTP1* (Ile105Val) (rs1695), and *GSTP1* (Ala114Val)

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(rs1138272) that we have previously genotyped in NSCLC patients (19).

PARTICIPANTS AND METHODS

This case-control study included 344 participants, 172 of whom were histologically confirmed NSCLC patients and 172 were matching controls by gender and mean age (±3 years). All NSCLC patients were enrolled at the Ataturk Pulmonary Diseases and Thoracic Surgery Hospital from February 2002 to November 2005. Their clinical data were collected from medical records. The staging and histological sub-typing of the lung carcinomas followed the international staging system for lung cancer and World Health Organisation histological classification of lung tumours (20). All other lung cancer types were excluded from the study.

Each patient had an interview to answer a detailed questionnaire about sociodemographic and history of smoking information. Pack years were calculated by multiplying packs smoked per day with years as a smoker.

The control group was recruited at a local healthcare centre and consisted of volunteers who were informed about the study during their routine health check-ups. The exclusion criteria were history of malignancy, pregnancy, chronic diseases, and diagnosed cancer of any type. Controls also completed a detailed questionnaire about smoking habits, work, and health history. The category of former smokers included NSCLC patients who had quit smoking at least one year before diagnosis and controls who had quit smoking at least one year before the interview. All participants were Caucasian and gave informed consent to participate in writing. The study was approved by the Medical Ethics Board of the Ataturk Pulmonary Diseases and Thoracic Surgery Hospital.

Genomic DNA samples for genetic polymorphism analysis were obtained from the blood drawn into coded 10 mL heparinised tubes and isolated with a DNA purification kit according to the manufacturer's instructions (Promega, Fitchburg, WI, USA). Isolated DNA was kept at -20 °C until use.

The *TP53* (Arg72Pro) polymorphism (rs1042522) was identified using the real-time polymerase chain reaction (PCR) as described by Talseth et al. (21). The *CYP2E1*5B* (RsaI/PstI, rs3813867/rs2031920) polymorphism was identified with the PCR-restriction fragment length polymorphism (PCR-RFLP) method described by Hayashi et al. (15) and confirmed by real-time PCR as described by Choi et al. (22).

Laboratory personnel were not informed about the source of DNA samples. For quality control we randomly selected 10 % of the samples, and the repeated genotyping procedures gave 100 % concordance. All genotyping data were independently reviewed by two authors.

Statistical analysis

For the comparison of mean age and pack-years between cases and controls we used Student's *t*-test. Multivariate logistic regression was used to calculate odds ratios (OR) between genotypes and NSCLC and 95 % confidence intervals adjusted for age, gender, and smoking status. Gene-smoking interaction adjusted for age was tested on the multiplicative scale by entering product terms in the multivariate logistic models. *P* values below 0.05 were considered significant. For statistical analysis we used the SPSS 15.0 software package (SPSS Inc., Chicago, IL, USA).

RESULTS

Table 1 shows the demographic information for all participants. NSCLC patients had significantly higher values of pack years smoked than controls (56 and 15 respectively) ($p<0.05$).

Table 2 shows the distribution of the *TP53* and *CYP2E1* genotypes and adjusted ORs for NSCLC. When calculating the ORs, we took wild-type or positive genotypes as reference. As we did not detect the homozygous mutant genotype (**5B/*5B*) (c2/c2) of *CYP2E1* in either group, we combined heterozygous and homozygous genotypes of other polymorphisms for the statistical analysis throughout the study and have presented the results accordingly. The

Table 1 *Participants' demographics*

	NSCLC patients (n=172)	Controls (n=172)
Age (years)		
Mean	56	53
Range	26-75	28-78
Gender, n (%)		
Male	156 (91)	156 (91)
Mean age	57	52
Age range	34-75	28-78
Female	16 (9)	16 (9)
Mean	55	65
Age range	26-70	32-76
Smoking, n (%)		
Current smoker	102 (59)	96 (56)
Former smoker	55 (32)	22 (13)
Non smoker	15 (9)	54 (31)
Pack years, mean*	56	15
Histological type of NSCLC, n (%)		
SCC	65 (38)	
AC	59 (34)	
UNSCLC	48 (28)	

**includes current and former smokers (ever smokers)*
SCC: squamous cell carcinoma, AC: adenocarcinoma, UNSCLC: unspecified non-small cell lung cancer

TP53 (Arg72Pro) polymorphism was in agreement with the Hardy-Weinberg equilibrium in the controls but not in the NSCLC patients.

We found a significant association between the *TP53* codon72 heterozygous (Arg/Pro) genotype and the risk of NSCLC (OR 2.87; 95 % CI 1.70-4.85; *p*=0.001). The association was not significant between *TP53* codon72 mutant (Pro/Pro) genotype and NSCLC (OR 1.34, 95 % CI 0.52-3.49; *p*=0.546), possibly due to the low number of Pro/Pro genotype carriers in both the NSCLC (n=13) and control group (n=15). When we combined the *TP53* codon72 Arg/Pro and Pro/Pro genotypes, we found a significant association between NSCLC risk and the genotypes containing variant allele, as shown in Table 2. The analysis of NSCLC subtypes showed a significant risk of squamous cell carcinoma and unspecified NSCLC in patients carrying the variant (Pro) allele of the *TP53* codon72 (OR 3.50; 95 % CI 1.76–6.99; *p*=0.001 and OR 3.22, 95 % CI 1.49–6.95; *p*=0.003, respectively) but not of adenocarcinoma (1.34, 95 % CI 0.72-2.51; *p*=0.360).

Table 3 shows no significant difference between *TP53* and *CYP2E1* gene interactions and the risk of NSCLC (top results). We then looked further to find if these variant genotypes would be associated with increased NSCLC risk if combined with the *GSTM1*, *GSTT1*, and *GSTP1* polymorphisms from our earlier study (19) and found a significant association for *TP53* codon72 Arg/Pro and Pro/Pro in combination with the *GSTM1* null genotypes. A similar significant association with NSCLC risk was found for the combinations of *TP53* codon72 Arg/Pro and Pro/Pro with the *GSTT1* null and the *GSTP1* (Ala114Val) genotypes (Table 3).

In contrast, the combination of *TP53* codon72 with the *GSTP1* (Ile105Val) variant was not significantly associated with the NSCLC risk, nor were the *CYP2E1* genotype combinations with *GSTM1*, *GSTT1*, *GSTP1* (Ile105Val) and *GSTP1* (Ala114Val) genotypes (Table 3).

We did not analyse the risk of histological subtypes with combined genotypes because of the small number of subjects in each subgroup.

As for the multivariate analysis of gene interactions with cigarette smoking, we found no significant association with the NSCLC risk (data not shown).

DISCUSSION

This is the first study demonstrating the association between *TP53* (Arg72Pro) polymorphism and the NSCLC risk in a Turkish population. Individuals carrying this variant had a 2.2 times higher risk than controls. *TP53* polymorphisms have widely been studied in NSCLC, but the results are contradictory. Three studies reported a significantly increased risk (23-25), one (26) reported an insignificant risk increase, and three (9, 17, 27) reported no significant association between *TP53* codon72 polymorphism and NSCLC. Along with Liu et al. (23), Yang et al. (24), and Loginov et al. (25), we, however, believe to be on the right track, since this *TP53* variant has lower activity in apoptosis and its association with an increased NSCLC risk is plausible.

Studies investigating the effect of the *CYP2E1*5B* polymorphism on NSCLC risk also reported inconsistent results (17, 28-32).

After the analysis of NSCLC subtypes, we found that the variant (Pro) allele carriers of *TP53* codon72 were significantly associated with the risk of SCC but not AC. Tan et al. (18) also reported an association with SCC, but Liu et al. (23) reported a significantly increased risk of AC

Table 2 *NSCLC risk association with TP53 and CYP2E1 polymorphisms*

Genotype	Controls, n (%)	NSCLC patients, n (%)	OR* (95%CI)	p value
TP53				
Arg/Arg	88 (51.16)	57 (33.14)	1	
Arg/Pro+Pro/Pro	84 (48.84)	115 (66.86)	2.21 (1.39-3.51)	**0.001**
CYP2E1				
*1A/*1A (c1/c1)	162 (94.19)	165 (95.93)	1	
*1A/*5B (c1/c2)	10 (5.81)	7 (4.07)	0.81 (0.28-2.31)	0.695
TP53	**Controls, n (%)**	**SCC patients, n (%)**	**OR* (95%CI)**	***p* value**
Arg/Arg	88 (51.16)	18 (27.69)	1	
Arg/Pro+Pro/Pro	84 (48.84)	47 (72.31)	3.50 (1.76-6.99)	**0.001**
TP53	**Controls, n (%)**	**AC patients, n (%)**	**OR* (95%CI)**	***p* value**
Arg/Arg	88 (51.16)	26 (44.07)	1	
Arg/Pro+Pro/Pro	84 (48.84)	33 (55.93)	1.34 (0.72-2.51)	0.360
TP53	**Controls, n (%)**	**UNSCLC patients, n (%)**	**OR* (95%CI)**	***p* value**
Arg/Arg	88 (51.16)	13 (27.08)	1	
Arg/Pro+Pro/Pro	84 (48.84)	35 (72.92)	3.22 (1.49-6.95)	**0.003**

**OR-odds ratio (95 % confidence interval) and* p *values obtained by multivariate logistic regression adjusted for age, gender, and smoking. Wild-type and positive genotypes were taken as reference*

Table 3 *NSCLC risk association with TP53, CYP2E1, and GST genotype combinations*

Genotype combinations	Controls (n)	NSCLC patients (n)	OR* (95 %CI)	*p* value
TP53, CYP2E1*5B				
Arg/Arg, *1A/*1A	82	53	1	
Arg/Pro, Pro/Pro, *1A/*5B	4	3	1.16 (0.25-5.39)	0.849
TP53, GSTM1				
Arg/Arg, positive	43	29	1	
Arg/Pro, Pro/Pro, null	48	72	2.22 (1.23-4.04)	**0.009**
Arg/Pro, null	40	63	2.35 (1.18-4.71)	**0.015**
Pro/Pro, null	8	9	1.55 (0.49-4.94)	0.455
TP53, GSTT1				
Arg/Arg, positive	72	43	1	
Arg/Pro, Pro/Pro, null	18	32	2.98 (1.49-5.94)	**0.002**
Arg/Pro, null	15	24	2.31 (1.04-5.13)	**0.040**
Pro/Pro, null	4	8	3.55 (0.92-3.69)	0.065
TP53, GSTP1 (Ile105Val)				
Arg/Arg, Ile/Ile	51	36	1	
Arg/Pro, Pro/Pro, Ile/Val, Val/Val	36	41	1.61 (0.87-2.99)	0.129
TP53, GSTP1 (Ala114Val)				
Arg/Arg, Ala/Ala	76	45	1	
Arg/Pro, Pro/Pro, Ala/Val, Val/Val	12	24	3.38 (1.54-7.41)	**0.002**
CYP2E1*5B, GSTM1				
*1A/*1A, positive	76	66	1	
*1A/*5B, null	7	1	0.16 (0.20-1.37)	0.095
CYP2E1*5B, GSTT1				
*1A/*1A, positive	130	121	1	
*1A/*5B, null	2	2	1.07 (0.15-7.75)	0.943
CYP2E1*5B, GSTP1 (Ile105Val)				
*1A/*1A, Ile/Ile	93	105	1	
*1A/*5B, Ile/Val, Val/Val	4	2	0.44 (0.79-2.47)	0.353
CYP2E1*5B, GSTP1 (Ala114Val)				
*1A/*1A, Ala/Ala	139	130	1	
*1A/*5B, Ala/Val, Val/Val	1	1	1.07 (0.07-17.27)	0.962

**OR-odds ratio (95 % confidence interval) and* p *values obtained by multivariate logistic regression adjusted for age, gender, and smoking. Wild-type and positive genotypes were taken as reference*

but not of SCC. These inconsistent findings need to be clarified in larger future studies.

Our analysis of the potential effect of gene interactions on NSCLC risk was motivated by the fact that cancer is a complex, multigene process and that individual susceptibility to cancer varies. The association between NSCLC risk and the *TP53* (Arg72Pro) polymorphism in combination with genes encoding enzymes involved in tobacco-specific carcinogen metabolism has not been well studied so far. In our study, the combination of the *TP53* codon72 and the *CYP2E1*5B* variants was not significantly associated with NSCLC. In Asian populations, the *CYP2E1*5B* polymorphism was found to be associated with lower NSCLC risk (32). Although this variant allele was relatively rare in our Caucasian participants compared to Asian populations, it may have countered the risk increased by the *TP53* codon72 variant, because their combination was not associated with NSCLC. However, we cannot exclude the possibility that the observed findings are pure chance.

We have also analysed the interactions between *TP53* and *CYP2E1* gene polymorphisms and the GST gene (*GSTM1*, *GSTT1* and *GSTP1)* polymorphisms that we had previously determined in the same patients in our 2012 study (19) to see whether they may affect the NSCLC risk. Our results confirm the findings of Klinchid et al. (33), who found a significant association between the *TP53* codon72 variant and *GSTM1* null combination and NSCLC. The highest risk of NSCLC was associated with the *TP53* codon72 variant plus *GSTP1* (Ala114Val) variant combination (OR=3.38). This combination increases the

risk of NSCLC 1.5 times over either variant alone. These findings are consistent with the effect of the mutant *TP53*, which impairs the apoptosis of transforming tumour cells, while lower GST variant activity insufficiently detoxifies carcinogenic chemicals.

What may limit the interpretation of our findings is a relatively small sample size, yet they clearly point that the *TP53* (Arg72Pro) polymorphism alone and in combination with *GSTM1*, *GSTT1*, or *GSTP1* (Ala114Val) may increase the risk of NSCLC, the last combination in particular. Further studies with larger samples are needed to verify these findings.

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Conflict of interest

The authors declare none.

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Povezanost polimorfizama gena *TP53* i *CYP2E1*5B* s nemikrocelularnim karcinomom pluća

Nemikrocelularni karcinom pluća (engl. *non-small cell lung cancer – NSCLC*) najčešći je oblik karcinoma pluća. Polimorfizimi gena za supresiju tumora i gena koji kodiraju enzime za razgradnju ksenobiotika mijenjaju aktivnost tih gena te značajno utječu na individualnu sklonost nastanku *NSCLC*-a. Budući da u literaturi nema dovoljno saznanja o njihovu utjecaju na nastanak bolesti, cilj je ovoga ispitivanja bio utvrditi povezanost polimorfizama tumorskoga supresorskoga gena *TP53* (Arg72Pro), odnosno gena *CYP2E1*5B* za metabolizam ksenobiotika, s rizikom od *NSCLC*-a. Ispitivanje je obuhvatilo 172 bolesnika s *NSCLC*-om i 172 kontrolna ispitanika (po 156 muškaraca i 16 žena u svakoj skupini). Genski su polimorfizmi utvrđeni polimeraznom lančanom reakcijom u stvarnom vremenu (engl. *real-time polymerase chain reaction – RT-PCR*) te kombinacijom *PCR*-a s metodom cijepanja DNA restrikcijskim enzimima (engl. *restriction fragment length polymorphism – PCR-RFLP*). Multivarijantna analiza upozorava na značajnu povezanost kombinacije *TP53* kodon72 Arg/Pro i Pro/Pro genotipova s *NSCLC*-om (OR 2,21; 95 % CI 1,39-3,51; p=0,001). Također je analizirana povezanost kombinacija ovih genskih varijanta s polimorfizmima gena glutation S-tranferaze *GSTM1*, *GSTT1*, *GSTP1* ekson 5 (Ile105Val) i *GSTP1* ekson 6 (Ala114Val) s rizikom od *NSCLC*-a te utvrđeno značajno povećanje rizika kod sljedećih kombinacija: *TP53* kodon72 s *GSTM1* nula (OR 2,22; 95 % CI 1,23-4,04; p=0,009), *GSTT1* nula (OR 2,98; 95 % CI 1,49-5,94; p=0,002) i *GSTP1* (Ala114Val) (OR 3,38; 95 % CI 1,54-7,41; p=0,002). Da bi se potvrdili ovi preliminarni rezultati, potrebna su istraživanja na većim uzorcima.

KLJUČNE RIJEČI: CYP2E1*; genski polimorfizam;* GST*; metabolizam kancerogena; NSCLC*

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Micronuclei and other nuclear anomalies in buccal epithelial cells of children with chronic kidney disease

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The objective of this study was to reveal the likely genomic instability in children with chronic kidney disease (CKD) using micronucleus (MN) assay on buccal epithelial cells (BEC). We investigated the frequencies of micronuclei and other nuclear anomalies, such as nuclear buds, binucleated cells, condensed chromatin, and karyorrhectic and pyknotic cells in BEC. Children with CKD were grouped as follows: children in the pre-dialysis (PreD) stage (N=17), children on regular haemodialysis (HD) (N=14), and children who have undergone transplantation (Tx) (N=17). As a control group, twenty age- and gender-matched healthy children were selected. The MN frequency in BEC of all groups of children with CKD was significantly elevated (5- to 7-fold) as compared to the control group ($p<0.001$). In contrast, the frequencies of nuclear buds were not significantly higher in the study groups compared to the control group. The frequencies of binucleated cells and condensed chromatin cells were significantly higher in all subgroups of children with CKD relative to the control group ($p<0.001$). Our results show that the BEC of pediatric PreD, HD, and Tx patients with CKD display increased cytogenetic, cytokinetic, and cytotoxic effects. They also point to the sensitivity and usefulness of the BEC MN assay in the assessment of genetic susceptibility of patients with CKD.

KEY WORDS: *buccal micronucleus assay; genotoxicity; dialysis; renal transplantation; nuclear anomalies*

Chronic kidney disease (CKD) is a major public health concern (1). Recent reviews indicate that the adverse health-related outcomes of CKD and/or end-stage renal disease (ESRD) are characterised by high incidences of cardiovascular morbidity and mortality (2), liver damage due to viral infections (3), chronic inflammation (4), oxidative stress (5), and an increased incidence of cancer (6, 7).

Extensive research is still required to clarify the mechanisms underlying these outcomes of CKD and treatments like haemodialysis and transplantation. The causes, development, and treatment of CKD in paediatric patients differ from those in adult patients (8). Long periods of haemodialysis treatment have been linked to DNA damage, mainly due to oxidative stress (9). In adult patients with CKD, increased cancer incidence and cytogenetic effects in peripheral lymphocytes have been observed (7). In addition, the patient's susceptibility to neoplastic disorders increased as a consequence of prolonged immunosuppression after transplantation (10).

Latency periods in most types of cancer are usually 15-20 years or longer, and conducting prospective epidemiological studies over long time periods is not always practical. Therefore, it is necessary to use the known biomarkers of DNA damage resulting from genetic instability to predict cancer risks and to identify high-risk individuals (11). Cytogenetic biomarkers are the most frequently used end points in human studies. In addition to peripheral blood lymphocytes, measurements of MN frequencies as well as other nuclear anomalies in buccal epithelial cells are increasingly used in molecular epidemiologic studies to investigate the impact of nutrition, lifestyle factors, genotoxic exposures, and genotype on DNA damage and cell death (12-14).

Studies from our laboratory (15, 16) have recently demonstrated significant increases in MN frequencies and basal DNA damage (determined by comet assay) in peripheral blood lymphocytes of children with CKD.

To the best of our knowledge, no genotoxicity studies of buccal cells of children with CKD have been reported so far. Therefore, we decided to investigate the frequencies of MN and other nuclear anomalies, such as nuclear buds (NBs), binucleated cells (BN), condensed chromatin (CC), karyorrhectic (KR), and pyknotic (PK) cells, in buccal epithelial cells (BEC) in children with CKD. There were

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three groups: children in the pre-dialysis (PreD) stage, children on regular hemodialysis (HD), and children who have had transplantation (Tx).

This study aims to reveal the possible genomic instability of CKD alone and treatment-related genotoxic and other effects (i.e. cytokinetic defects and cell death) in children with CKD using a non-invasive method: micronucleus assay on buccal tissue.

SUBJECTS AND METHODS

Study population

The study group consisted of 48 children (age range: 2-19 years) diagnosed with CKD. All of them were also involved in our previous studies (15, 16), where the causes of primary kidney disease and medication(s) received were described in more detail. Of these children, 17 (9 males and 8 females) were in the pre-dialysis stage, 14 (6 males and 8 females) were on regular haemodialysis, and 17 (10 males and 7 females) had kidney transplantation.

The study groups were: the children with blood creatinine levels higher than 1 mg dL^{-1} and glomerular filtration rates (GFR) in the range of 15-90 mL min^{-1} were selected for PD group. HD group consisted of children who underwent regular haemodialysis treatment for four hours three times per week. Tx group consisted of children who underwent kidney transplantation and were under immunosuppressant treatment, as described in our previous study (16). The duration of being diagnosed with PreD, HD, and Tx, expressed in months (±SD) was 41.35 ±38.83, 29.75±27.68, and 22.35±19.43, respectively.

For the purpose of the study, we selected non-smokers who had never consumed alcohol and who were without any malignancies. The matched control group consisted of 20 healthy children (11 males and 9 females; age range 4-18 years) who were free from medication and infection, as confirmed by face-to-face interviews with their parents.

This study was approved by the local ethical committee of the Başkent University, Faculty of Medicine (03/07/2007-07/39). Furthermore, the parents of all children gave their informed consent prior to involving their children in the study. Detailed questionnaire forms completed by the parents included age, height, weight, and education, cigarette smoking and environmental tobacco smoke (ETS) exposure, recent diagnostic X-ray examination (three months prior to the sampling), recent vaccination, sports activities of the children, and the use of vitamin supplementations. Moreover, for those children who were diagnosed with kidney disease, information regarding the kidney disease type, parameters, grade, treatment type, medication, and viral infection were completed by their physicians. The causes of primary kidney disease and medication received among children have also been described in detail elsewhere (15, 16).

Biological sampling

The sampling of buccal epithelial cells from both the CKD and control groups was done between 16 Jul 2008 and 29 Jul 2009. For each subject, we simultaneously collected BEC for the MN-assay and blood samples which had been used for the purposes of other genotoxicity studies. Thus, data on genotoxicity endpoints (e.g. micronucleus and comet) in peripheral lymphocytes and routine biochemical parameters of the same populations have been published elsewhere (15, 16).

Buccal epithelial cells were obtained by scraping both sides of the inside of the cheeks using pre-moistened wooden tongue depressors after the subjects had rinsed their mouths with water.

The collected cells were first smeared directly onto pre-cleaned and pre-moistened microscope slides and were then left to dry. Within the same day, the slides were transferred to the laboratory where they were further processed.

MN assay in buccal epithelial cells

Preparations of buccal epithelial cells were fixed in 80 % methanol (Merck, Germany) and air-dried. Slides were stained by the Feulgen reagent (pararosaniline, Merck, Germany) and then counterstained with Fast Green (Merck, Germany) (17, 18).

Microscopy analyses were performed using a light microscope (Zeiss Axioscope 2, Goettingen, Germany) at 400x magnification and confirmed at 1000x magnification. The coded slides were evaluated blindly by a single scorer.

The identification criteria of MN and other nuclear anomalies**,** previously described by Thomas et al. (19), were followed**.** For each subject, a total of 2000 differentiated and basal cells were analy**s**ed for the presence of the MN frequency and micronucleated cell and other nuclear anomaly (nuclear buds, condensed chromatin, binucleated, karyorrhectic and pyknotic cells) frequencies. Karyolytic cells were not scored. Basal and differentiated cell frequencies were not evaluated. The frequency of each parameter has been expressed as per thousand (‰).

Statistical analysis

Data were analysed using SPSS for Windows, version 11.5 (SPSS Inc., Chicago, IL, United States). The Shapiro-Wilk test was used to determine whether the distributions of continuous variables were normal. The Levene test was used to evaluate the homogeneity of variances. The data for continuous variables were expressed as the means ± standard deviations (SD) or medians (min.-max.), and for nominal variables as the number of cases and/or percentage (%). Regarding the number of independent groups, the mean differences were compared by Student's t test or One-Way ANOVA. Where applicable, the Mann-Whitney U or Kruskal-Wallis tests were applied to compare the median values. When the *p*-values (from One-Way ANOVA or

Kruskal-Wallis test statistics) were statistically significant, a *post-hoc* Tukey or Conover's multiple comparison test was applied to determine which groups differed. Where applicable, nominal data were evaluated by the Pearson Chi-square or Fisher's exact test.

Degrees of association between continuous variables were evaluated using the Spearman's rank correlation test. Correlation analyses were performed to explore the association (if any) between buccal MN frequencies in the study subjects and corresponding lymphocyte MN frequencies and routine biochemical parameters data from recent publications (15, 16). Stepwise linear regression analyses were used to define predictive factors and dependent variables (e.g. MN frequency in BEC, micronucleated cell frequency in BEC), nuclear buds, binucleated cells, condensed chromatin cells, karyorrhectic and pyknotic cells). For those dependent variables that were not normally distributed, log-transformation was applied via multiple linear regression analyses. Any variable determined by univariate tests to be statistically significant and clinically important was accepted as a candidate for the multivariable model. After adjusting for factors, such as age, gender, body mass index, smoking, X-ray exposure, drug usage, and sports activities, multiple linear regression analyses were performed to determine whether there were any statistically significant differences among the study groups. Coefficients of regression and 95 % confidence intervals were calculated for all independent variables. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

The demographic characteristics of the children with CKD and control group have already been reported in our previously published studies (15, 16). Considering that the present study and both of the previously mentioned ones were conducted simultaneously, here we briefly report the most important results which could help the reader to better understand the results of the MN assay on BEC.

As reported previously (15, 16), age, gender, and duration of sports activities among the study groups did not significantly vary. X-ray exposure three months prior to the sampling was significantly higher in CKD subgroups. None of them had a dental radiography examination. Vaccinations within a year were significantly more frequent in CKD subgroups, except HD *vs.* control group. Exposure to environmental tobacco smoke was significantly lower in CKD subjects, and differences in CKD subgroups *vs.* control group were statistically significant ($p<0.05$). The Tx group exhibited the highest BMI values, and these values were significantly different from the control and HD groups ($p<0.05$). In each CKD subgroup, either the MN frequency or the micronucleated cell frequency in BEC was significantly (5- to 7- fold) elevated in comparison with the control group ($p<0.001$; Table 1).

Cell anomalies also revealed differences between the groups of children studied. The frequency of nuclear buds in the study group was not significantly different from the control group (Table 1) even though the children in the HD subgroup demonstrated a 23 % increase in this parameter. Binucleated cell frequencies were significantly higher in all CKD subgroups relative to the control group ($p<0.001$; Table 1). The % increases for binucleated cells were 79 %, 56 %, and 86 % in the Pre-D, HD, and Tx subgroups, respectively.

Table 1 *MN and other nuclear anomalies (‰) (Mean±SD) in buccal epithelial cells of children with CKD and control group*

N	Children with CKD				Control group
	TS	PreD	HD	Tx	
	48	17	14	17	20
MN frequency in BEC (‰)	8.34±7.33	8.32±8.54	9.57±7.61	7.35±5.95	1.45±1.30*
Micronucleated cell frequency in BEC (‰)	5.36±3.99	5.09±4.22	5.79±3.93	5.29±4.03	1.03±0.95*
Nuclear buds (‰)	0.33±0.43	0.26±0.31	0.43±0.58	0.30±0.40	0.33±0.29
Binucleated cells (‰)	30.66±9.20	31.32±7.29	27.43±7.99	32.65±11.39	17.55±5.25*
Condensed chromatin cells (‰)	20.93±8.49	24.18±12.12	20.36±6.17	18.15±3.77	8.75±3.70*
Karyorrhectic cells (‰)	8.23±5.70	9.00±5.34	11.21±6.94	5.00±2.91***	3.25±1.97**
Pyknotic cells (‰)	24.41±14.83	29.18±16.82	29.04±11.28	15.82±11.76***	14.55±6.17**

Abbreviations: *TS=total study group of children with disease and under treatment; PreD=children in predialysis; HD=children in hemodialysis; Tx=children who underwent renal transplantation.* *p*<0.001; control group* vs. *TS, PreD, HD, and Tx.* **p*<0.05; control group* vs. *TS, PreD and HD.* ***p*<0.05; Tx* vs. *PreD and HD*

Table 2 *Results on the multiple linear regression analysis of MN frequency and micronucleated cell frequency in BEC of study subjects (patients+controls, N=68)*

	PreD	HD	Tx
MN frequency (R^2=0.617)	1.418	1.573	1.474
p	0.000	0.002	0.001
B (95 % CIs)	(0.683 to 2.152)	(0.612 to 2.534)	(0.665 to 2.283)
Micronucleated cell frequency (R^2=0.634)	1.271	1.489	1.421
p	0.000	0.001	0.000
B (95 % CIs)	(0.625 to 1.917)	(0.644 to 2.333)	(0.710 to 2.132)

Independent variables: *Tx group, HD group, PreD group, age, gender, BMI, environmental tobacco smoke exposure, sports activity (hours), use of vitamin (yes/no), X-ray exposure, antihypertensive drugs, erythropoietin, osteoporosis drugs, antibiotics, iron drugs, phosex.* ***Abbreviations:*** *CIs=confidence intervals, B=regression coefficient (slope)*

Significantly increased frequencies of condensed chromatin were found in each subgroup of children with CKD ($p<0.001$; Table 1).

Frequencies of karyorrhectic and pyknotic cells were significantly higher in the PreD and HD subgroups compared to the control group ($p<0.05$; Table 1). However, the frequencies of karyorrhectic and pyknotic cells in the Tx group were significantly lower than those in the PreD and HD groups ($p<0.05$; Table 1), and exhibited no significant differences relative to the control group.

Regression analysis indicates that every patient subgroup exhibited significant increases in buccal MN frequencies as well as micronucleated cell frequencies compared to control values ($p<0.01$; Table 2). This increase in the MN frequency was the highest for the HD subgroup (B=1.573, $p=0.002$), followed by the Tx (B=1.474, $p=0.001$) and PreD (B=1.418, $p=0.000$) subgroups, respectively. The same trend was observed with regard to BEC micronucleated cell frequencies (Table 2).

A PreD or HD status significantly influenced condensed chromatin and pyknotic cell frequencies in BEC ($p<0.01$; Table 3), whereas a Tx status significantly influenced condensed chromatin cell frequencies and binucleated cells in BEC ($p< 0.01$, Table 3). The effect on the frequencies of condensed chromatin cells was the highest in the PreD group (B=18.471, $p<0.00001$), followed by the HD (B=15.045, $p<0.00001$) and Tx (B=12.154, $p=0.001$) groups, respectively (Table 3), while the effect on the frequencies of pyknotic cells was the highest in the HD group (B=20.353, $p=0.003$), followed by the PreD group (B=16.993, $p=0.001$) (Table 3). A significant effect on the frequencies of binucleated cells was only observed for the Tx group (B=12.915, p=0.007) (Table 3).

Diagnostic X-ray examination had a significant negative effect on the frequencies of condensed chromatin (B=-7.735 $p<0.00001$) and a positive significant effect on the frequencies of nuclear buds (B=0.272, $p<0.05$). Other independent variables, such as age, gender, BMI, ETS exposure, sports activity (hours), vitamin usage (yes/no), routine biochemical parameters and medication usage, had no significant effect on MN frequencies or nuclear anomalies (Tables 2 and 3). When patients and control subjects were taken as a whole, correlation analysis showed significant differences regarding the MN frequencies in BEC and routine biochemical parameters, such as creatinine, BUN, parathyroid hormone, ferritin, albumin, uric acid (r=0.653, r=0.616, r=0.600, r=0.534, r=-0.450, r=0.436, respectively; $p<0.00001$), homocysteine, triglycerides, CRP, total protein, haematocrit, calcium and total cholesterol levels (r=0.408, r=0.365, r=0.336, r=-0.306, r=-0.302, r=-0.292, r=0.254, respectively; $p<0.05$). Similar correlations were obtained with regard to micronucleated cell frequencies in BEC.

Correlation analyses showed significant differences between the frequencies of nuclear anomalies in BEC and routine biochemical parameters. There was a significant correlation between the nuclear bud frequencies and ALT (r=0.387, $p<0.05$). The frequency of binucleated cells was significantly correlated to triglycerides (r=0.300, $p<0.00001$), creatinine, BUN, total cholesterol, CRP, ferritin, homocysteine, parathyroid hormone, and uric acid levels (r=0.445, r=0.260, r=0.361, r=0.265, r=0.342, r=0.253, r=0.426, r=0.268, respectively; $p<0.05$). Significant correlations were also obtained between the condensed chromatin cell frequency and creatinine, BUN, triglycerides, and parathyroid hormone levels (r=0.523, r=0.542, r=0.595, r=0.609, respectively; $p<0.00001$) as well as total cholesterol, albumin, ferritin, homocysteine, alkaline phosphatase, and uric acid levels (r=0.292, r=-0.247, r=0.410, r=0.403, r=0.340, r=0.348, respectively; $p<0.05$). Significant correlations between karyorrhectic cell frequencies and creatinine, BUN, and triglyceride levels (r=0.555, r=0.549, r=0.595, respectively; $p<0.00001$), as well as albumin, ferritin, homocysteine, parathyroid hormone, and potassium levels (r=-0.313, r=0.263, r=0.361, r=0.369, r=0.299, respectively; $p<0.05$) were also found. Pyknotic cell frequencies also correlated with creatinine, BUN, iron, parathyroid hormone, and sodium levels (r=0.374, r=0.385, r=0.318, r=0.260, r=-0.293, respectively; p<0.05).

There was a significant correlation between the MN frequency in BEC and the MN frequency in peripheral lymphocytes (in the whole study population; r=0.609; $p=0.000$). A similar significant correlation was found with regard to the micronucleated cell frequency in BEC (r=0.613; $p<0.00001$).

DISCUSSION

Recent data from the Turkish Society of Nephrology (20) reported 1643 haemodialyses, 2325 peritoneal dialyses, and 546 transplantations in children in our country. The increasing numbers of CKD patients and concurrently developing secondary diseases (especially cancer) indicate the importance of understanding this chronic disease, both in Turkey and all over the world. Children with CKD have an increased incidence of cancer (21), which is about ten times more frequent compared to healthy children of the same age (22). Thus, long-term survival and preventive strategies are crucial for children who have CKD and are undergoing treatment (23). Immunosuppressive drugs used to prevent graft rejection in recipients of kidney transplants and/or prolonged haemodialysis treatments have been linked to DNA damage and malignancies (8, 24).

Previous studies on chromosomal and/or DNA damage resulting from CKD and its treatment modes were based on adults. The biomarkers used in these studies were micronuclei in peripheral blood lymphocytes (PBL) (7, 25-27) and BEC (9), chromosome aberrations and sister chromatid exchanges (28, 29), and DNA strand breaks (comet assay) (26-28, 30, 31). The only reports regarding children with CKD were our recent studies using the MN and comet assays in PBL of children (15, 16).

Although PBL are generally accepted as appropriate surrogate tissue for biomarker studies, exfoliated epithelial cells, such as buccal, nasal, or urothelial cells constitute an alternative option, especially because the latter can be obtained with non-invasive methods. Over 90 % of cancers occur in epithelial tissues, and, in many cases, epithelial tissues are the actual targets of carcinogens (32). The time required for BEC cells to migrate from the oral cavity to the basal layer is approximately 1-3 weeks (33). There are no definitive data regarding the cell turnover rates of buccal cells that influence a patient's disease status and/or the effects of several treatment chemicals. Nonetheless, we used this tissue to determine the short-term effects of exposures and/or disease-treatment status, in part because it is the most easily accessible tissue (particularly for children).

Recent meta-analysis results (34) suggest that the MN frequency in BEC can be used in the prescreening and follow-up of precancerous oral lesions and may reflect the chromosomal instability of other organs. Increased buccal MN frequencies have also been observed in small-sized studies on patients with chronic diseases, with Alzheimer's disease and Down syndrome (34). Scoring MN and other nuclear anomalies such as nuclear buds (for DNA damage), binucleated cells (for cytokinetic defects), condensed chromatin, and karyorrhectic and pyknotic (for cell death) cells in BEC, known as the cytome approach, has also been found to be associated with Down syndrome and Alzheimer's disease (19). Though there have been a limited number of studies on nuclear anomalies other than the MN frequency, it has been suggested that the cytome approach may improve the predictive value of the MN assay in BEC (34).

Therefore, using the cytome approach in BEC, the monitoring of children with CKD alone and treatment-related effects can be of particular significance.

In the present study, cytogenetic, cytokinetic, and cytotoxic (cell death) effects in the BEC of children

Table 3 *Results on the multiple linear regression analysis of other nuclear anomaly frequencies in BEC of study subjects (patients+controls, N=68)*

	PreD	HD	Tx	X-ray exposure
Nuclear buds (R^2=0.279)	-0.203	-0.298	-0.322	0.272
p	0.162	0.143	0.072	0.021
B (95 % CIs)	(-0.490 to 0.084)	(-0.699 to 0.104)	(-0.674 to 0.030)	(0.042 to 0.502)
Binucleated cells (R^2=0.531)	7.748	7.052	12.915	-0.513
p	0.110	0.199	0.007	0.857
B (95 % CIs)	(-1.831 to 17.327)	(-3.867 to 17.972)	(3.750 to 22.080)	(-6.216 to 5.189)
Condensed chromatin cells (R^2=0.582)	18.471	15.045	12.154	-7.735
p	0.000	0.000	0.001	0.000
B (95 % CIs)	(12.932 to 24.010)	(8.320 to 21.770)	(5.352 to 18.955)	(-11.837 to-3.634)
Karyorrhectic cells (R^2=0.488)	3.010	3.425	-1.589	-0.448
p	0.180	0.265	0.480	0.746
B (95 % CIs)	(-1.432 to 7.452)	(-2.670 to 9.520)	(-6.068 to 2.890)	(-3.208 to 2.311)
Pyknotic cells (R^2=0.384)	16.993	20.353	4.994	-6.988
p	0.001	0.003	0.373	0.073
B (95 % CIs)	(7.485 to 26.500)	(7.250 to 33.456)	(-6.167 to 15.156)	(-14.652 to 0.675)

Independent variables: *Tx group, HD group, PreD group, age, gender, BMI, environmental tobacco smoke exposure, sports activity (hours), use of vitamin (yes/no), X-ray exposure, antihypertensive drugs, osteoporosis drugs, antibiotics, shohl solution, other drugs, triglycerides, parathyroid hormone.* ***Abbreviations:*** *CIs=confidence intervals, B=regression coefficient (slope)*

diagnosed with CKD were clearly observed in the PreD stage and during treatment (HD and Tx), in agreement with our recent studies on peripheral lymphocytes (15, 16). In the scientific literature, we could not find any study in which the BEC MN assay has been used for children with CKD. In fact, only Roth et al. (9) have used the BEC MN assay in adults with CKD. This study reported higher MN frequencies for HD patients, which supports the findings of our study. These authors also indicated that the duration of treatment was a significant factor (9).

In our study, a cell death effect was shown in the PreD and HD groups, while in the Tx group only condensed chromatin was observed (Table 1). Karyorrhectic and pyknotic cell frequencies were not different for the Tx group compared to control group children. This might be because, compared to the PreD and HD groups, most of the CKD biochemical parameters improved in the Tx-group children (15). Uremic toxins, reactive carbonyl compounds, long-term haemodialysis treatment and the accumulation of advanced glycation end-products (AGEs) may be responsible for the effects observed in the PreD and HD groups, due to their suppression of DNA repair (35). Because the cellular anomalies reflecting the cytokinetic and cytotoxic effects in BEC were not comprehensively determined via the mechanistic approach, our interpretation of the data could be rather speculative.

Nuclear buds are considered to be a biomarker for the elimination of amplified DNA and/or DNA repair complexes and thus thought to play an important role in tumour progression (36). In our study, nuclear buds were the only parameter that showed no significant differences between patients and controls. A similar result was also found for nuclear buds in the PBL MN assay performed in the same subjects (16). Therefore, it is possible to link CKD and treatment statuses in children to genome damage. In the same subjects, the MN frequencies in lymphocytes correlated with those in BEC suggesting that the assessments of BEC and lymphocytes may complement each other. Considering the The HUman MicroNucleus project on eXfoLiated buccal cells ($HUMN_{XL}$) project directions (37), more studies could be useful to prove the relationship between lymphocytes and BEC and to determine whether BEC may be used to predict the risk of cancer.

Considering that involvement in the PreD, HD, or Tx subgroups was the most significant factor linked to increased MN frequencies, as well as nuclear anomalies in the BEC of CKD children, an observation that supports the link between genetic instability and the development and treatment of this chronic disease is evident. Additionally, the significant associations observed among the biochemical parameters of CKD and BEC assay parameters further support this association. Also, a higher frequency of binucleated BEC observed in the present study and lower nuclear division index (NDI) in PBL (16) in the Tx group could be a signal of genome instability and cytokinetic defects that need further clarification.

It is known that several factors, such as age, gender, smoking, alcohol intake, and dietary habits, may contribute to spontaneous MN levels in exfoliated buccal cells (14). However, due to the limitations of the associated studies, the most important confounding factors have not yet been determined. These issues have recently been addressed in two review articles (14, 38).

The consistencies in age and gender between the study and control groups and the participation of non-smokers in the present study eliminated the most crucial of these confounding factors.

From a clinical point of view, diagnostic X-ray exposure in children and adults with CKD unavoidably occurred during their medical surveillance. Of course, this issue was also acknowledged by the clinician authors of our study. In the available literature, there are no cytogenetic effect studies on CKD which presented the X-ray exposure data of their populations. In the present study, 41.6 % of children with CKD had an X-ray exposure history (mostly chest radiography) in the last three months prior to the biological sampling. Therefore, as explained in the statistical analysis section, multiple regression analysis was performed after adjusting for factors such as X-ray exposure, age, gender, body mass index, smoking, drug usage, and sports activities (Tables 2, 3). Accordingly, X-ray exposure issue seemed to have no apparent confounding effect on buccal MN frequencies. Similar results have also been demonstrated in peripheral lymphocytes of the same children with CKD (15, 16). On the other hand, the X-ray exposure effect on nuclear anomalies other than MN, a positive X-ray effect, was only found for the formation of nuclear buds in BEC, while negative X-ray effects were found for condensed chromatin cells in BEC. The previously published studies that have investigated directly the effects of several radiographic and cephalographic techniques in dental practices (e.g. panoramic dental radiography, lateral cephalography, post anterior cephalography, and full-mouth X-ray) on the target tissue BEC collected ten days after X-ray exposure, which is the likely maximal rate of MN occurrence, have linked X-ray exposure to cytotoxicity but not genotoxicity determined as MN frequencies (39-42). In all these studies, an X-ray dose was between 0.003 mSv and 0.02 mSv (1-4). Choi (2011) compared the panoramic radiography for oral examination (0.014-0.024) to the effective doses of chest radiography (0.06-0.25 mSv in two views) and looked at variability (43). Therefore, the type, dose, target tissue, target tissue turnover of X-ray exposure, and time after exposure are crucial parameters. From this point of view, the interpretation of the X-ray exposure effect as a confounding factor in biomonitoring studies is quite difficult and providing detailed information and statistical analysis could be the appropriate approach, as we have done in this study. Obviously, the association between the ionising radiation effects and outcomes of buccal micronucleus assay has to be more extensively evaluated in future biomonitoring studies, in contrast with

micronucleus assay on lymphocytes, for which this association has nowadays been fairly well established (44).

From several previously published studies (45-49), including the one conducted in our laboratory, the 46 buccal MN frequencies reported for healthy children ranged from 0.10 to 4.03 ‰. The mean MN frequency for our control subjects (1.45 ‰) fell in that range. However, in our previous study, the background level variability observed in the buccal cells of healthy subjects (49) confirmed the need for method standardisation, which is an initiative of the $HUMN_{XL}$ project (37). When frequencies of nuclear anomalies of condensed chromatin and karyorrhectic and pyknotic cells were compared in different healthy children populations, large variabilities were observed. While some of them (40, 50), including our study, had much higher values, others (51) had lower values than the levels obtained in the study by Thomas et al. (52). The causes of this variability may, in part, reflect genetic and non-specific exposure differences, or in part, technical factors and differences in the sample preparation and interpretation of the scoring criteria between laboratories. Therefore, it was suggested that an automated scoring system should be developed to overcome this issue (53).

CONCLUSION

Increased cytogenetic, cytokinetic, and cytotoxic effects have been found in the buccal epithelial cells of children with CKD who underwent different treatment modes. Both the disease and treatment of CKD contribute to increased cytogenetic, cytokinetic, and cytotoxic effects on buccal epithelial cells of children. We believe that our data will add to the validation studies in the scope of the $HUMN_{XL}$ Project (37). Finally, they point to the sensitivity and usefulness of the BEC MN assay in the assessment of genetic susceptibility of patients with CKD.

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Declaration of interest

The authors declare that they have no competing interests.

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Mikronukleusi i ostala citogenetička oštećenja u epitelnim stanicama bukalne sluznice djece koja boluju od kroničnih bubrežnih bolesti

Cilj ovoga istraživanja bio je utvrditi moguću genomsku nestabilnost u djece koja boluju od kroničnih bubrežnih bolesti (KBB). Korišten je mikronukleus (MN) test na epitelnim stanicama bukalne sluznice. Ispitivali smo učestalost mikronukleusa i ostalih citogenetičkih oštećenja poput jezgrinih pupova, binuklearnih stanica, kondenziranoga kromatina, kariorektičnih i piknotičnih stanica u stanicama bukalne sluznice. Djeca s KBB-om bila su podijeljena u sljedeće skupine: djeca u fazi prije dijalize (PreD) (N=17), djeca koja su redovito na hemodijalizi (HD) (N=14) i djeca s transplantiranim bubregom (Tx) (N=17). Kontrolnu skupinu činila su zdrava djeca iste dobi i spola. Učestalost MN-a u stanicama bukalne sluznice u svim skupinama djece s KBB-om bila je značajno povećana (petero- do sedmerostruko) u usporedbi s kontrolnom skupinom (p<0,001). Suprotno tome, učestalost jezgrinih pupova nije bila značajnije veća u promatranoj skupini u usporedbi s kontrolnom skupinom. Učestalost binuklearnih stanica i kondenziranoga kromatina bila je značajnije veća u svim podskupinama djece s KBB-om u odnosu na kontrolnu skupinu (p<0,001). Naši rezultati upućuju ne samo na povećanu razinu citogenetičkih, citokinetičkih i citotoksičnih promjena u stanicama bukalne sluznice u djece PreD, HD i Tx skupina nego i na osjetljivost i korisnost MN-testa na stanicama bukalne sluznice u procjeni genetičke osjetljivosti pacijenata koji boluju od KBB-a.

KLJUČNE RIJEČI: *citogenetička oštećenja, dijaliza; genotoksičnost; mikronukleus-test na stanicama bukalne sluznice; transplantacija bubrega*

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Air sampling by pumping through a filter: effects of air flow rate, concentration, and decay of airborne substances

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This paper tackles the issue of interpreting the number of airborne particles adsorbed on a filter through which a certain volume of sampled air has been pumped. This number is equal to the product of the pumped volume and particle concentration in air, but only if the concentration is constant over time and if there is no substance decomposition on the filter during sampling. If this is not the case, one must take into account the inconstancy of the concentration and the decay law for a given substance, which is complicated even further if the flow rate through the filter is not constant. In this paper, we develop a formalism which considers all of these factors, resulting in a single, compact expression of general applicability. The use of this expression is exemplified by addressing a case of sampling airborne radioactive matter, where the decay law is already well known. This law is combined with three experimentally observed time dependences of the flow rate and two models for the time dependence of the particle concentration. We also discuss the implications of these calculations for certain other situations of interest to environmental studies.

KEY WORDS: *air sampling filters; particle concentration; radioactivity; substance decomposition*

Air sampling for the purpose of determining the concentration (n) of an airborne substance is frequently carried out by pumping air through a sampling filter. The filter is subsequently subjected to an appropriate analysis and n should then be calculated from observables such as the volume (V) of the pumped air and the number (N_F) of the substance atoms or molecules adsorbed on the filter. The formula $n=N_F/V$ is valid only if n is constant during the sampling and the substance remains stable after it has been deposited onto the filter, which is not always the case (e.g., because of a radioactive decay or a spontaneous chemical decomposition). Generally speaking, one often has to develop a specific model for interpreting the relation between n, N_F, and V rather than rely on the abovementioned simple ratio. For instance, a common outcome of such modelling is the correction to the calculated n (which is assumed to be constant) of a radioactive substance that has the half-life $T_{1/2}$ of the order of the sampling time T_S (1). The situation may become even more complicated if the flow rate φ through the sampling filter also changes over time t, as demonstrated in the case of an exponentially decreasing $\varphi(t)$ (2). Hence, inconstant $n(t)$ and/or $\varphi(t)$, as well as the decay of substances on the filter, cause deviations from $N_F=nV$, which should not be neglected.

In this paper, we develop a formalism that accounts for the interpretation of $N_F(T_S)$, which is N_F at the end of a sampling at $t=T_S$, in situations when a substance of interest decays over time, while either (or both) $n(t)$ and $\varphi(t)$ may be inconstant during the sampling. The profile of $\varphi(t)$ is determined by the characteristics of a particular sampling system (e.g., reduction of filter porosity as the adsorption progresses, changes in meteorological conditions or in the functioning of the pumping system, etc.) and should be available experimentally by using appropriate flow-rate meters. The decay law can be inferred by understanding the general properties of a substance of interest. The issue of $n(t)$ is less straightforward and is a subject of appropriate modelling with regard to other relevant information. Bearing the above in mind, we derived a single expression that can be used in every case where the inputs to it ($\varphi(t)$, $n(t)$, and the decay law for a substance) are available from separate considerations.

In order to illustrate the applications of the mentioned expression, we focus on investigations of radioactive matter suspended in air. This is a case where the decay law is well known and $n(t)$ can be modelled to fit with certain realistic situations. Selected forms of $\varphi(t)$ (i.e., constant, exponentially decreasing, and linearly decreasing) – which have all been observed experimentally – are addressed together with certain profiles of $n(t)$. We first turn to constant $n(t)$, which is a good approximation in the routine monitoring of the presence of radionuclides known to maintain a fairly constant concentration over prolonged time periods (e.g., cosmogenic ^{7}Be with $T_{1/2}$=53.4 days). Then we consider a sudden increase of $n(t)$ in a short period of time, followed by an exponential decrease of $n(t)$. This situation may simulate an accident where radionuclides (many of which short-lived) were emitted into the atmosphere over a short time and their presence in air is afterwards reduced

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primarily by radioactive decay. Other similar applications of the formalism are also discussed.

The same approach can be used for other airborne substances decomposable under certain conditions – such as pesticides (3) or nitrogen oxides (4) – or in cases where strong variations in $n(t)$ are suspected, for other reasons, even if a studied substance does not decay. While the inputs to the model depend on a particular situation, the calculation method is the same as in the examples we discuss.

Derivation of the model

Setting up the model

In numerous cases, reduction of the number N of given particles (e.g., unstable atoms or molecules) from the initial number $N_0=N(t=0)$ follows the rate equation

$$\frac{dN}{dt} = -\lambda N^{\mu} \qquad [1]$$

where $\lambda>0$ is a constant and $\mu\geq 1$ is an integer. The solution of Eq. [1] is given by $N(t)=N_0Y(t)$, where

$$Y(t)=e^{-\lambda t},\ \mu=1, \qquad [2]$$

or

$$Y(t) = \left[1+(\mu-1)N_0^{\mu-1}t\right]^{\frac{1}{1-\mu}},\quad \mu>1 \qquad [3]$$

If air containing particles of a concentration n is pumped through a filter at a rate φ, the number N_F of the particles adsorbed on the filter obeys

$$\frac{dN_F}{dt} = -\lambda N_F^{\mu} + n\phi \qquad [4]$$

While the term $-\lambda N_F^{\mu}$ describes the decrease of N_F due to the decay given by Eq. [1], the term $n\varphi$ accounts for the increase of N_F due to new particles being adsorbed on the filter by the pumping. We assume that the sampling commences at $t=0$ and ends at $t=T_S$ when the number of particles on the filter equals $N_F(T_S)$. In order to determine this number, the filter is processed further by applying appropriate analytical methods, and our goal is to find an expression that relates $N_F(T_S)$, $n(t)$, and $\varphi(t)$ in general terms.

Derivation of the expression for $N_F(T_S)$

A sketch of a $\varphi(0\leq t\leq T_S)$ is given in Fig. 1, the total area under the curve being

$$V = \int_0^{T_S} \phi(t)dt \qquad [5]$$

We subdivide the sampling time using equidistant points $t_0=0,t_1,\ldots,t_k,\ldots,t_n,T_S$. This fragmentises V into volumes $V_0,V_1,\ldots,V_k,\ldots,V_n$ such that $V_k\approx\varphi(t_k)\Delta t$ is pumped during the time interval $\Delta t=t_{k+1}-t_k$. The adsorbed number of particles corresponding to this interval is $N_{F,k}(t_k)\approx n(t_k)V_k$. As outlined

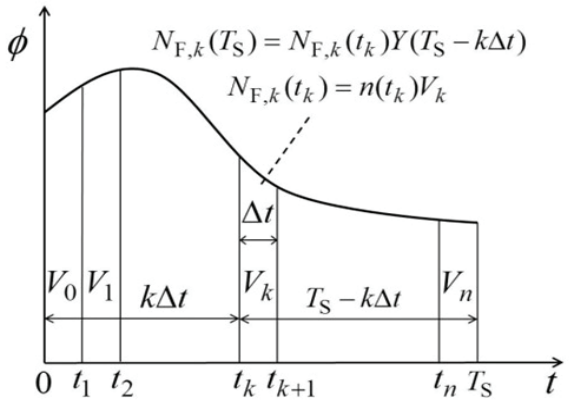

Figure 1 *Sketch of a time-dependent φ, where the elements required for understanding our derivation of* $N_F(T_S)$ *are shown*

in Fig. 1, because the time difference between T_S and t_k equals $T_S-k\Delta t$, and since $N(t)=N_0Y(t)$ applies, the approximate contribution $N_{F,k}(T_S)$ of the volume V_k to final $N_F(T_S)$ is

$$N_{F,k}(T_S) \approx N_{F,k}(t_k)Y(T_S - k\Delta t) = n(t_k)\phi(t_k)Y(T_S - k\Delta t)\Delta t\,. \qquad [6]$$

By summing up all $N_{F,k}(T_S)$, we obtain

$$N_F(T_S) \approx \sum_{k=0}^{n} N_{F,k}(T_S) \approx \sum_{k=0}^{n} n(t_k)\phi(t_k)Y(T_S - k\Delta t)\Delta t \qquad [7]$$

The exact solution is obtained in the continuum limit ($n\to\infty$, $\Delta t\to dt$, $k\Delta t\to t$) where the sum becomes

$$N_F(T_S) = \int_0^{T_S} n(t)\phi(t)Y(T_S - t)dt \qquad [8]$$

In Eq. [8], $N_F(T_S)$ and $\varphi(t)$ can be found experimentally, $Y(t)$ can be inferred by knowing the properties of a substance studied, and $n(t)$ has to be modelled with regard to other available information. Hence, one assesses $n(t)$ – which is central in environmental studies – by comparing experimental indicators and chosen models. If both $Y(t)$ and $n(t)$ are constant, Eq. [8] gives $N_F(T_S)=nV$, which also holds for Eq. [4].

Investigations of airborne radioactive matter: selected examples

Radioactive decay follows a first-order ($\mu=1$) rate equation the solution of which is given by Eq. [2], the half-life being related to λ by $T_{1/2}=\ln 2/\lambda$. Once a sampling of air, outlined above, has been completed, one determines the activity $A_F(T_S)=\lambda N_F(T_S)$ of the filter. Since the activity concentration in air is yielded by $a(t)=\lambda n(t)$, we use this and Eq. [2] to write Eq. [8] as

$$A_F(T_S) = e^{-\lambda T_S}\int_{\alpha}^{\beta} a(t)\phi(t)e^{\lambda t}dt \qquad [9]$$

The integration limits α and β may differ from 0 and T_S if $a(t)$ is not non-zero during the entire sampling period. We emphasise that Eq. [9] includes a time-dependent $a(t)$, which, to our knowledge, has not been considered in similar

approaches. So far, only a constant activity concentration has been assumed, together with either a constant (1) or exponentially decreasing (2) $\varphi(t)$. Below, we exemplify the use of Eq. [9] via six selected combinations of $\varphi(t)$ and $a(t)$.

Example 1: both a(t) *and* φ(t) *are constant*

For constant $\varphi(t)=\varphi(t=0)=\varphi_0$ and $a(t)=a_0$, we set $\alpha=0$ and $\beta=T_S$ in Eq. [9] to obtain

$$A_F(T_S) = a_0\phi_0 \frac{1-e^{-\lambda T_S}}{\lambda}, \qquad [10]$$

which is the same result as in (1) and it is used to find a_0 when $T_{1/2}$ is smaller than or comparable to T_S. A good example of an application of Eq. [10] is the routine monitoring of ^{7}Be ($T_{1/2}$=53.4 days) when T_S is about a few weeks (5-7). If $T_{1/2}>>T_S$, Eq. [10] is reduced to $A_F(T_S)=a_0\varphi_0T_S=a_0V$, which is used in the monitoring of common long-lived radionuclides in air; e.g., ^{210}Pb with $T_{1/2}$=20.4 years (8), and in other experimental realisations where the above inequality is satisfied (9-13).

Example 2: a(t) *is constant and* φ(t) *decreases exponentially*

It may occur that $\varphi(t)$ changes over time, especially if a large amount of air is sampled (causing the reduction of filter porosity) or there are variations in the performance of a sampler due to either extrinsic or intrinsic causes. One possible form of $\varphi(t)$ is

$$\varphi(t)=\varphi_0e^{-\gamma t} \qquad [11]$$

(where $\gamma>0$), which was observed (2) for samplings over one week, with $\varphi_0\approx750$ m^{3}h^{-1} and $\gamma\approx0.01$ s^{-1}. If we take $a(t)=a_0$, $\alpha=0$, $\beta=T_S$, and use (11) for $\varphi(t)$, Eq. [9] yields

$$A_F(T_S) = \frac{a_0}{\lambda}\left[\left(\phi_0+\frac{\phi_1}{\lambda}\right)\left(1-e^{-\lambda T_S}\right)-\phi_1T_S\right], \qquad [12]$$

This is the same result as that obtained in (7), where only this particular situation for $a(t)$ and $\varphi(t)$ was considered. Furthermore, they used an alternative calculation method, i.e., they solved Eq. [4].

Example 3: a(t) *is constant while* φ(t) *decreases linearly*

An exponential drop is not the only experimentally observed decrease of $\varphi(t)$. For our sampling system (a high-volume sampler operating with chlorinated polyvinylchloride filters), we mainly observed

$$\varphi(t)=\varphi_0-\varphi_1t \qquad [13]$$

(where $\varphi_0,\varphi_1>0$), with typically $\varphi_0\approx750$ m^3 h^{-1}, $\varphi_1\approx2$ m^3 h^{-2}, and $T_S\approx150$-350 h. By again assuming that $a(t)=a_0$, $\alpha=0$, $\beta=T_S$ in Eq. [9], and using (13) for $\varphi(t)$, we obtain

$$A_F(T_S) = \frac{a_0}{\lambda}\left[\left(\phi_0+\frac{\phi_1}{\lambda}\right)\left(1-e^{-\lambda T_S}\right)-\phi_1T_S\right], \qquad [14]$$

which is a result that has not been derived by other authors. Together with previously known results from (10) and (12), the above expression completes the formulae that relate $A_F(T_S)$ and $a(t)=a_0$ for the profiles of $\varphi(t)$ that have, to our knowledge, been reported so far for the sampling of air in radioactivity studies.

Example 4: φ(t) *is constant and* a(t) *decreases exponentially*

The approximation of a constant $a(t)$ is obviously not always applicable. Among numerous possible forms of inconstant $a(t)$, we address one that may simulate an accident in which one or more radionuclides (each with its own λ) were released into the atmosphere during a short time interval around some $t=T_0$, which has been followed by an exponential decrease of the corresponding activity concentration in air because of the decay of the emitted radionuclides. An appropriate expression for describing this scenario is

$$a(t)=a_me^{-\lambda(t-T_0)},\ t>T_0 \qquad [15]$$

accounting for a decrease of $a(t)$ from the maximum value $a_m=a(T_0)$. Note that the decay in the air [reflecting the decrease of $n(t)$] is treated separately from the decay on the filter, the latter being accounted for in Eqs. [8] and [9] via the function $Y(t)=\exp(-\lambda t)$. Since T_0 is known, as well as λ of a given radionuclide, in order to reconstruct $a(t)$, one should find a relation between $A_F(T_S)$ and a_m.

There are two possible situations with regard to the sampling period ($0\le t\le T_S$) and T_0, which is depicted in Fig. 2. The first possibility is described by the curve labelled 1 (where $T_0=T_{0,1}\ge0$), for which the emission occurs during the sampling. In the second case (the curve labelled 2, with $T_0=T_{0,2}<0$), sampling starts after emission has occurred.

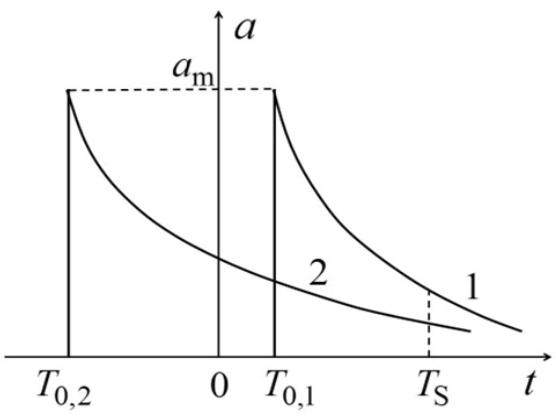

Figure 2 *Sudden increase of* a(t)*, followed by a decrease due to the decay of the emitted radionuclide. Two curves labelled 1 and 2 depict two possible situations with regard to the emission time and sampling period, see the text for details*

We first consider the case of $\varphi(t)=\varphi(t=0)=\varphi_0$. For $T_0\geq 0$ (curve 1 in Fig. 2), we combine Eqs. [9] and [15] by setting $\alpha=T_0$ and $\beta=T_S$, which results in

$$A_F(T_S) = a_m\phi_0 e^{-\lambda(T_S-T_0)}(T_S-T_0). \quad [16]$$

When $T_0<0$ (curve 2 in Fig. 2), we set $\alpha=0$ and $\beta=T_S$ to obtain

$$A_F(T_S) = a_m\phi_0 e^{-\lambda(T_S+|T_0|)}T_S. \quad [17]$$

Example 5: both a*(t) and* φ*(t) decrease exponentially*

As before, we extend the calculation of $A_F(T_S)$ for $a(t)$ given by Eq. [15] to the experimentally observed inconstant forms of $\varphi(t)$. We first consider an exponentially decreasing $\varphi(t)$ given by Eq. [11]. Using the same procedure as above, we obtain

$$A_F(T_S) = a_m\phi_0 e^{-\lambda(T_S-T_0)}\frac{e^{-\gamma T_0}-e^{-\gamma T_S}}{\gamma} \quad [18]$$

for $T_0\geq 0$, and

$$A_F(T_S) = a_m\phi_0 e^{-\lambda(T_S+|T_0|)}\frac{1-e^{-\gamma T_S}}{\gamma} \quad [19]$$

for $T_0<0$.

Example 6: a*(t) decreases exponentially and* φ*(t) decreases linearly*

We conclude the examples of applications of the presented formalism by turning to the combination of linear $\varphi(t)=\varphi_0-\varphi_1 t$ and $a(t)$ given by Eq. [15]. By the same method as in the above two examples, we find

$$A_F(T_S) = a_m e^{-\lambda(T_S-T_0)}\left[\phi_0(T_S-T_0)-\frac{\phi_1}{2}\left(T_S^2-T_0^2\right)\right] \quad [20]$$

for $T_0\geq 0$, and

$$A_F(T_S) = a_m e^{-\lambda(T_S+|T_0|)}\left[\phi_0 T_S-\frac{\phi_1}{2}T_S^2\right] \quad [21]$$

for $T_0<0$.

An example of the experimental use of the model

We complete this paper by addressing the practical use of the presented approach. Since there has been no recent accidental situation that would lead to a modelling similar to that in Examples 4, 5, and 6, we turn to data available from routine monitoring of common radionuclides in air. In Fig. 3, we first demonstrate typical $\varphi(t)$ data for our high-volume sampler, where it is rather clear that the $\varphi(t)$ in our case decreases linearly.

One of the main sources of radioactivity in air is ^{7}Be with $T_{1/2}$=53.4, which is a naturally occurring radionuclide with a constant presence due to its cosmogenic origin. The sampling under consideration was carried out for T_S=223.23 h, and $\varphi(t)$ was decreasing linearly with φ_0=762±18 m^3 h^{-1}

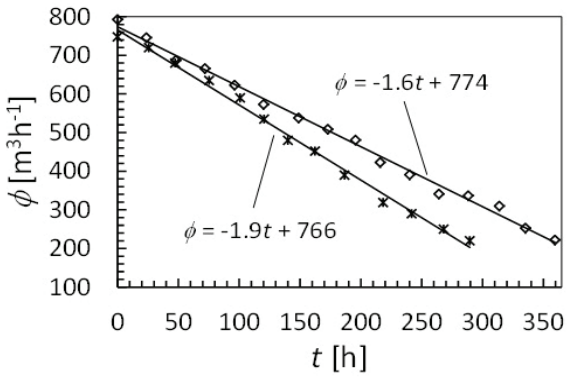

Figure 3 *Two typical experimental* φ*(t) for our sampling system, obviously exhibiting a linear dependence*

and φ_1=2.24±012 m^{3}h^{-2}; hence, the pumped air volume was $V=\varphi_0 T_S=\varphi_1 T_S^2$=58478.4 m^3. Because of a relatively short T_S and the absence of any unusual conditions during the sampling, it is reasonable to assume that the concentration of ^{7}Be in air was fairly constant and that $a(t)=a_0$ and Eq. [14] apply.

By means of gamma-ray spectrometry, we measured $a_F(T_S)=A_F(T_S)/V$=3.713±0.010 m Bq m^{-3}, whereas Eq. [14] yields a_0=3.750±0.011 m Bq m^{-3} (in $a_F(T_S)$ and a_0, the uncertainties of φ_0, φ_1, as well as those arising from gamma–ray spectrometry, are taken into account). Their relative difference $[a_0-a_F(T_S)]/a_F(T_S=0.01)$ is rather small but is nevertheless statistically significant. The reason is that the uncertainties δ_F and δ_0 and of $a_F(T_S)$ and a_0, respectively, are small enough that the two results do not overlap. Namely, $[a_0-a_F(T_S)]/[\delta_0+\delta_F]$=1.79 is larger than unity and the two results are consequently distinguishable. This should not be disregarded if analytical accuracy is pursued in the spirit of quality assurance procedures (14).

Outlook

The above six examples of the use of Eq. [9] cover the combinations of three experimentally observed $\varphi(t)$ dependences and two profiles of $a(t)$ that can be linked to certain situations of practical relevance. Owing to the generality of Eq. [9] and, implicitly, of Eq. [8], these calculations can be extended further using either analytical or numerical integration.

An interesting phenomenon to mention is the gradual increase of $n(t)$ until it reaches a maximum and then continues to decrease gradually, which is also a viable scenario in environmental studies. Mathematically, one could in this case model $n(t)$ by a number of peaked functions such as symmetric Gaussian, Lorentzian, or parabolic functions, various similar non–symmetric functions, etc., each containing two or more parameters that define their shapes. Since the success of the presented formalism depends on reducing the number of adjustable parameters, it is desirable to put some effort in fixing as many parameters as possible. For instance, in our examples

3-6, the fixed parameter is T_0 which is assumed to be known *a priori*, and the full profile of $a(t)$ is then obtained by finding only one unknown parameter, that is, a_m. Hence, our formalism provides a versatile and robust tool but it requires careful approach in every particular situation.

Developing these calculations, i.e. implementing formulas such as the ones observed by this paper into, for instance, a comprehensive urban air monitoring network, would constitute the next logical step, as other authors have proposed (15).

CONCLUSIONS

Generally, interpreting the number N_F of particles adsorbed on a filter through which a certain volume V of air was pumped is no easy task. This complexity stems from possible time (t) dependences of the quantities that contribute to N_F, these being the concentration n of an airborne substance of interest, the flow rate φ through the sampling filter, and the decay law $Y(t)$ if the substance is decomposable. In numerous cases, the expression $N_F=nV$, where n is assumed to be constant, oversimplifies the actual situation. We have developed a formalism that links N_F to a time dependent $n(t)$, $\varphi(t)$, and $Y(t)$, which results in a compact single expression of general applicability. Applications of the expression were exemplified by addressing airborne radioactive matter, for which $Y(t)$ is well known. Three experimentally reported profiles of $\varphi(t)$ were combined with two modelled $n(t)$ dependences and these examples both demonstrate the validity of the expression, possibly offering solutions for certain other, potentially important problems.

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Uzorkovanje zraka pumpanjem kroz filtar: učinci brzine strujanja i koncentracije zraka i raspadanja spojeva

Tema je ovoga rada problem s brojem lebdećih čestica apsorbiranih na filtar kroz koji je pumpan i time uzorkovan određeni volumen zraka. Taj broj jednak je umnošku uzorkovanog volumena i koncentracije čestica samo ako je protok stalan u vremenu i nema raspada tvari na filtru tijekom uzorkovanja. U protivnom se mora uzeti u obzir zakon raspada za određenu tvar, što se još više komplicira ako protok kroz filtar nije konstantan. Razvili smo formalizam koji uključuje sve ove čimbenike, što je rezultiralo jednim kompaktnim izrazom opće primjenjivosti. Uporaba ovoga izraza demonstrirana je na slučaju uzorkovanja radioaktivnih tvari u zraku, za koje je zakon radioaktivnoga raspada dobro poznat. Taj je zakon kombiniran s trima eksperimentalno opaženim vremenskim ovisnostima protoka i dvama modelima za vremensku ovisnost koncentracije čestica. Također je diskutirana primjena tih izračuna u nekim drugim interesnim situacijama u istraživanjima okoliša.

KLJUČNE RIJEČI: *uzorkovanje zraka; koncentracija čestica; protok; radioaktivnost; raspad tvari*

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Radon measurements in well and spring water of the Tuzla area, Bosnia and Herzegovina

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Investigations of natural radioactivity in water, air, and soil are conducted frequently and routinely. Exposure to high concentrations of natural radioactive radon gas can cause irradiation of respiratory organs, which can lead to lung cancer. This paper presents measurements of radon activity concentrations in dug wells and natural springs of the Tuzla area (Bosnia and Herzegovina), which ranged from 214 to 3702 mBq L^{-1}. Our results have shown that the radon activity concentration did not exceed the EU reference level for radon in drinking water (100 Bq L^{-1}).

KEY WORDS: *annual effective dose; drinking water; natural radon radiation; pH value; radon activity concentration; temperature*

Groundwater is favoured as a source of drinking water in many countries. It is often thought to be cleaner or easier to treat compared to surface water and as a result many wells have been either dug or drilled (1). Natural radioactivity in drinking water causes internal human exposure caused by the decay of radionuclides taken into the body through ingestion and/or inhalation indirectly when they are incorporated into the human food chain (2). Groundwater that runs through rocks also contains considerable amounts of dissolved radioactive isotopes, especially radon (^{222}Rn in particular) (3). Radon is the only naturally produced radioactive gas. It originates from the breakdown of uranium in soil, rock, and water. ^{222}Rn is an odourless, radioactive noble gas that originates from the decay of radium (^{226}Ra) and has a half-life of 3.8 days. Both radon and radium are part of the uranium (^{238}U) decay series. Together with the thorium (^{232}Th), actinium (^{235}U) and potassium (^{40}K) decay series, it forms the natural decay series (4). Households that rely on water from bedrock wells are at higher risk due to elevated radon levels in the air. Radon gas dissolved in water can be released into indoor air through normal household activities such as showering, dishwashing, and laundry.

Radon circulates by two basic processes, diffusion, and forced flow. Diffusion occurs inevitably, even though its extent may be limited; therefore, diffusive migration sets a lower limit on the transport of radon. Forced flow depends on pressure gradients, which may or may not be present in a given situation. An important property of radon is its solubility in water; therefore it can be transported across larger distances through soil (5).

An elevated level of radon ingested through water is believed to lead to cancer, primarily of the stomach. The dominant health concern with radon, however, is the lung cancer risk created when radon gas escapes water and is inhaled (5). Ingested radon diffuses into the tissues of the stomach and small intestine. From there it enters the bloodstream and is carried throughout the body. The majority of ingested radon is thought to be exhaled when the blood flow carries it to the lungs. Based on a National Academy of Science Report, the Environmental Protection Agency (EPA) estimates that radon in drinking water causes about 168 cancer deaths per year: 89 % from lung cancer and 11 % from stomach cancer (6).

The Environmental Protection Agency has proposed 11.1 Bq L^{-1} as the maximum contamination level (MCL) for radon in drinking water (7). In 2013, the European Union adopted Directive EC2013/51/EURATOM laying down requirements for protecting the health of the general public with regard to radioactive substances in water intended for human consumption (8). According to this Directive, Member States may set a level for radon that must not be exceeded, having in mind that the level set can exceed 100 Bq L^{-1} but not 1000 Bq L^{-1}. Bosnia and Herzegovina currently does not have any regulations concerning the radon level in drinking water.

An important parameter of water is relative acidity or alkalinity, which is determined by measuring the pH value. The pH value of water is affected by different chemical reactions with different minerals of ground and rocks, but also with the atmosphere. Radium, the immediate parent of radon is readily soluble in low pH water. Also pH is one of

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the most important parameters influencing uranium content and its mobility. Low pH value is the most important water parameter linked to high radium concentration (9). The temperature of the water is also a very important parameter because it affects the development dynamics of many physical, chemical and biochemical processes. In general, increasing the temperature of water also increases the unfolding speed of certain chemical and biochemical reactions and decreases the solubility of oxygen and other gases. An increasing water temperature also decreases the solubility of radon in water (10).

Monitoring of natural radioactivity in drinking water is important in exposure assessments of to radiation by water consumption. The presence of radionuclides in drinking water can cause health hazards to humans as a result of internal radiation exposure from direct ingestion and absorption in human tissues. The aim of this paper was to investigate the ^{222}Rn concentrations in water samples collected from selected wells and springs and study the relationship between the radon concentration and temperature and pH values. This study also presents the estimated annual effective radon ingestion dose. The measurements presented herein are the first of its kind done according to the AlphaGUARD method in the wider region of Tuzla, Bosnia and Herzegovina (Figure 1).

MATERIALS AND METHODS

Study area

The City of Tuzla is located in the Jala basin, in northeast Bosnia. Dug wells and springs are very widespread here. Most dug wells can be found in private yards in Tuzla's suburban areas mostly at elevations greater than that of the central city area. The people inhabiting the area have grown accustomed to using water from wells on a daily basis for different purposes and even for drinking. The groundwater is drawn by a pump or using containers such as buckets.

In the areas investigated here, the depth of the dug wells ranged from 6 to 18 m. In areas without dug wells, water samples were taken from natural springs, which are publicly available in the Tuzla area . Water samples for this research were taken from more "popular" water springs often used for drinking instead of the water from the city water supply system. The samples were collected at 13 sites with dug wells and 6 sites with natural springs (Figure 1); in mineral springs: Tuzlanski Kiseljak, Miladije Voda 1, Petrak, Slavinovići, Mosnik, and Miladije Voda 2; and dug wells: Solina 1, Solina 2, Solina 3, Brđani, Dokanj, Breške, Svojtina, Avdibašići, Hidani, Simin Han, Mandići, Čaklovići, and Dolovi.

High radon concentrations sometimes also occur in association with pegmatites, as well as gneisses and vulcanites rich in quartz and feldspar. The radon concentration in groundwater from bedrock is low in areas where uranium concentrations in the rocks are low (diorite, gabbro and basic vulcanites, limestone, sandstone, and shale) (5). The area researched mainly comprised tertiary originated sediment formations, predominantly limestone and sand, with a presence of lignite and coal deposits.

The natural spring water contained various minerals, such as salts and sulphur compounds, or calcium and magnesium. Minerals dissolve in water as they move through underground rocks. Natural springs such as Miladije Voda 1, Petrak, Slavinovići, and Miladije Voda 2 contained sulphur, while Tuzlanski Kiseljak contained a

Figure 1 *A map of the sampling locations*

large amount of magnesium. The natural spring water from Mosnik did not contain either sulphur or magnesium.

Sampling

The main precondition for achieving precision in radon activity concentration measurements in water is appropriate sampling. In particular, radon losses on the way between sampling and the measurement have to be minimized. During the measurements, the method of direct transfer of samples was used. For the direct transfer of samples, plastic syringes with maximum volumes of 100 mL were used. Further, the sample was injected directly from the syringe into the top opening of the degassed vessels. We also took care that the time between the sampling and measurement be as short as possible.

Experimental design

For measurements of the radon activity concentration in water, the AquaKIT measuring system in combination with the AlphaGUARD PQ2000PRO and AlphaPUMP electronic pumping unit (Genitron Instruments, Germany) was used. Measurements were performed by AlphaGUARD, which in addition to continuous measurements of radon concentration in air and water, can also measure basic meteorological parameters such as temperature, barometric pressure, and humidity range. The measuring range of this device for radon activity concentration is from 2 to 2×10^6 Bq m^{-3}, while temperature measurements can range from -10 °C to 50 °C. AlphaGUARD's radon detector is based on a design-optimized pulse ionization chamber. The cylinder ionization chamber of the AlphaGUARD has an active volume of 0.56 L, whereas its metallic interior has a potential of +750 V when the instrument is turned on.

The AlphaGUARD was placed in diffusion mode with changes in measurement interval at 10 min. Radon penetrates into the inside of the appliance-ionisation chamber through a glass fibre filter, where α-particles resulting from radioactive decay ionize the air contained within. The electrical pulses generated in the chamber correspond to the number of atoms of decaying radon. The measurement results are ultimately visible on the display (11, 12).

The AlphaPUMP is a battery operated electronically powered manual pumping unit for gases. It can be gradually adjusted from 0.03 to 1 L per minute. An internal aqua-stop filter prevents liquid material from entering the system (13).

The AquaKIT is a measuring system for directly measuring the concentration of radon gas in water. AquaKIT is used for all waters, including waste waters and highly salted waters. The glass vessels of th AquaKIT provide a hermetically enclosed volume of radon ejected from the water sample, as well as enable a quick exchange of samples, which prevents incorrect measurements resulting from leaks. The sample is injected directly from the syringe into the upper opening of the degassed vessels. In a closed circulation, radon gas is ejected from the water sample with the help of the AlphaPUMP. With the AlphaGUARD PQ2000PRO, whose ionization chamber is also part of the gas cycle, the radon concentration in the system is determined and stored.

Table 1 *Geographic coordinates of the sampling locations*

No.	Locality	Type of source	Latitude (N)	Longitude (E)	Elevation (m)
1	Tuzlanski Kiseljak	mineral spring	44°31'18"	18°33'15"	198
2	Miladije Voda 1	mineral spring	44°31'30"	18°31'00"	230
3	Petrak	mineral spring	44°32'48"	18°31'23"	194
4	Slavinovići	mineral spring	44°31'46"	18°43'37"	260
5	Mosnik	mineral spring	44°32'01"	18°40'20"	272
6	Miladije Voda 2	mineral spring	44°31'30"	18°31'00"	230
7	Solina 1	dug well	44°34'15"	18°42'01"	299
8	Solina 2	dug well	44°34'15"	18°41'59"	299
9	Solina 3	dug well	44°34'14"	18°41'59"	297
10	Brđani	dug well	44°34'19"	18°41'47"	289
11	Dokanj	dug well	44°35'40"	18°41'04"	337
12	Breške	dug well	44°38'08"	18°38'41"	340
13	Svojtina	dug well	44°33'47"	18°42'14"	312
14	Avdibašići	dug well	44°35'04"	18°44'32"	414
15	Hidani	dug well	44°35'08"	18°43'49"	373
16	Simin Han	dug well	44°32'46"	18°43'38"	280
17	Mandići	dug well	44°33'30"	18°40'14"	277
18	Čaklovići	dug well	44°30'55"	18°46'25"	356
19	Dolovi	dug well	44°34'03"	18°40'22"	418

For the determination of radon concentrations in the water samples, the following equation was used:

$$C_{water} = \frac{C_{air} \times \left(\frac{V_{System} - V_{Sample}}{V_{Sample}} + k_{^{222}Rn} \right) - C_0}{1000} \quad [1]$$

where: C_{water} is the radon concentration in water (Bq L^{-1}); C_{air} is the radon concentration in air after expelling the radon (Bq m^{-3}); C_0 is the radon concentration in the measuring equipment before sampling (Bq m^{-3}); V_{system} is the interior volume of the measurement equipment (1.122 mL); V_{sample} is the volume of the water sample (mL); $k_{^{222}Rn}$ is the radon diffusion coefficient (14).

Depending on the temperature diffusion coefficient, radon was calculated from the following equation:

$$k_{^{222}Rn} = 0.106 + 0.405e^{-0.052t} \quad [2]$$

where: $k_{^{222}Rn}$ is the diffusion coefficient of radon and t is water temperature (°C) (15).

Based on the results of the measurements, the annual effective ingestion dose of radon was calculated using:

$$H = C_{water} \cdot D \cdot G \cdot T \quad [3]$$

where: H is the annual effective ingestion dose for radon (μSv y^{-1}); C_{water} is the radon activity concentration in water (mBq L^{-1}); D is the ingesting dose conversion factor for radon (3.5 nSv Bq^{-1}); G is the water consumption per day; T is the duration of consumption (16).

In order to calculate the annual effective dose for radon obtained by ingesting water from the investigated water samples, we used an annual intake of water of 2 L per day, which corresponds to the consumption of population over 17 years of age (17).

RESULTS AND DISCUSSION

Table 1 shows the geographic coordinates of the sampling locations, while Table 2 shows the temperature values of the investigated water samples and the calculated diffusion coefficients. The pH values and radon activity concentration in air before the start of measurement and after expelling the radon from the water samples are also shown in Table 2. The radon activity concentration in the air after expulsion from the water samples ranged within 38-350 Bq m^{-3} and 22-155 Bq m^{-3} for dug wells and natural springs, respectively.

The radon activity concentration in the water ranged 214-3702 mBq L^{-1} in general, and for dug wells alone 385-3702 mBq L^{-1}, with the highest concentration found at locality Solina 3 (No. 9, Table 2, Figure 2) and the lowest at Dolovi (No. 19, Table 2, Figure 2). These locations are not geographically far apart, but they are at different elevations: Dolovi are approximately 100 m higher than Solina 3. Also, the depth of the well at Solina 3 was much greater than at Dolovi. Furthermore, location Solina 3 is accommodated in a plain area where soils with ruinous limestone prevail and may in some places even be clayey.

The activity concentration of radon in water samples from natural springs was in the range from 214 to 1607 mBq L^{-1}. The highest value was determined at locality Mosnik (No. 5, Table 2, Figure 2) and the lowest at Miladije Voda 2 (No. 6, Table 2, Figure 2). As obvious, Mosnik had a much higher activity concentration in comparison to other mineral springs. Mosnik is located near an urban part of Tuzla, at a higher elevation than the other investigated natural springs. Its water comes from bedrock built mainly of quartz sand.

The radon activity concentrations for dug wells obtained in this study are much lower than, for instance, those

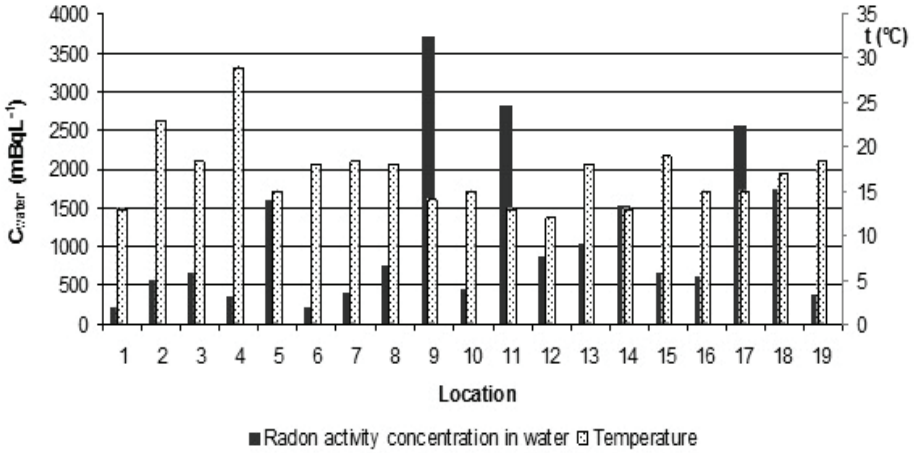

Figure 2 *The radon activity concentration (C_{water}) and the temperature (t) of the investigated water samples*

Table 2 *The values of the radon activity concentration in the air before the start of measurement (C_0) and after expelling radon from the water samples (C_{air}), pH value, temperature, diffusion coefficient, and radon activity concentration*

No.	Locality	Type of source	Depth of dug well (m)	t (°C)	pH	k_{222Rn}	C_0 (Bq m^{-3})	C_{air} (Bq m^{-3})	C_{water} (mBq L^{-1})
1	Tuzlanski Kiseljak	mineral spring	-	13	6.3	0.31	14	24	239
2	Miladije Voda 1	mineral spring	-	23	8.5	0.23	20	55	555
3	Petrak	mineral spring	-	18.5	8.5	0.26	11	64	660
4	Slavinovići	mineral spring	-	29	8.3	0.19	14	35	350
5	Mosnik	mineral spring	-	15	7.3	0.29	22	155	1607
6	Miladije Voda 2	mineral spring	-	18	6.3	0.26	17	22	214
7	Solina 1	dug well	13	18.5	7.2	0.26	19	41	411
8	Solina 2	dug well	12	18	7.3	0.26	10	73	755
9	Solina 3	dug well	18	14	7.3	0.3	20	350	3702
10	Brđani	dug well	9	15	7.6	0.29	10	45	463
11	Dokanj	dug well	16	13	7.5	0.31	27	272	2837
12	Breške	dug well	10	12	7.1	0.32	27	86	880
13	Svojtina	dug well	12	18	7.1	0.26	27	102	1042
14	Avdibašići	dug well	11	13	7	0.31	20	148	1538
15	Hidani	dug well	9	19	7.6	0.26	24	65	657
16	Simin Han	dug well	10	15	7.2	0.29	14	60	617
17	Mandići	dug well	14	15	7.4	0.29	12	245	2563
18	Čaklovići	dug well	11	17	7.3	0.27	15	169	1758
19	Dolovi	dug well	6	18.5	7.2	0.26	13	38	385
mineral spring range				13-29	6.3-8.5	0.19-0.31	11-22	22-155	214-1607
dug well range			6-18	12-19	7-7.6	0.26-0.32	10-27	38-350	385-3702
mineral spring				19.4±5.8	7.4±1.1	0.26±0.04	16.3±4.1	59±50	604±522
dug well			11.6±3.2	15.8±2.4	7.3±0.2	0.28±0.02	18.3±6.5	130±101	1354±1069

observed in Croatia; 4.0-17.4 Bq L^{-1} (18) and 4.4-17.6 Bq L^{-1} (19). In comparison with radon concentrations in bottled tap water from Serbia, the values were not very different (20). The highest radon activity concentration in our study is lower than any of the ^{222}Rn concentrations observed in drinking water in Bulgaria (21). Furthermore, in comparison with the EU reference level, as well as those in Russia (120 Bq L^{-1}), Norway (500 Bq L^{-1}), and Finland (1000 Bq L^{-1}), the values obtained in this study were much lower (22, 23).

The lowest temperature value in the tested samples was 12 °C, at Breške (No. 12, Table 2) with a diffusion coefficient of 0.32, whereas the highest temperature in the tested samples was 29 °C, at Slavinovići (No. 4, Table 2) with a corresponding diffusion coefficient of 0.19. Figure 2 shows the radon activity concentration in water as a function of the temperature of the investigated water samples. Contrary to expectations, no dependence between drinking water temperature and radon concentration was detected (Figure 3). Radon activity concentration and temperature had a negative weak correlation (r=-0.43). The correlation coefficients between radon and temperature for dug wells and mineral springs were -0.50 and -0.28, respectively.

The pH value varied from 7.0 to 7.6 for the wells, and 6.3-8.5 for the natural springs. The results in Table 2 and Figure 4 indicate that the radon activity concentration was independent from pH value of water. Radon activity concentration and pH value of water had no correlation (r=0.01) and very weak correlation coefficients were obtained (Figure 5).

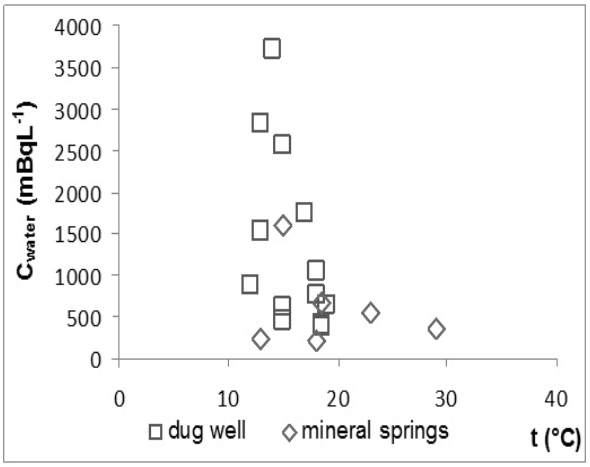

Figure 3 *The radon activity concentration (C_{water}) as a function of the temperature (t) of the water samples*

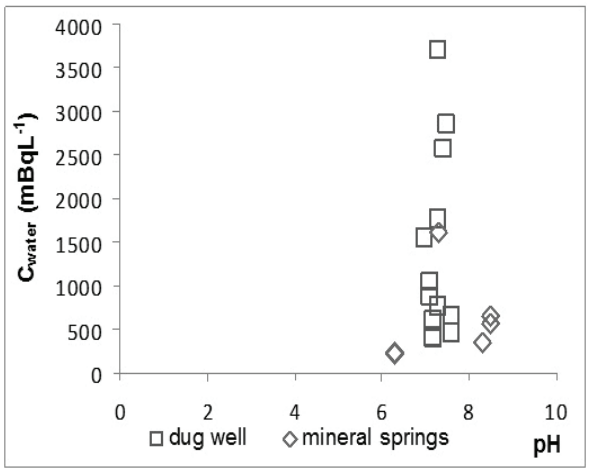

Figure 5 *The radon activity concentration (C_{water}) as a function of the pH value of the water samples*

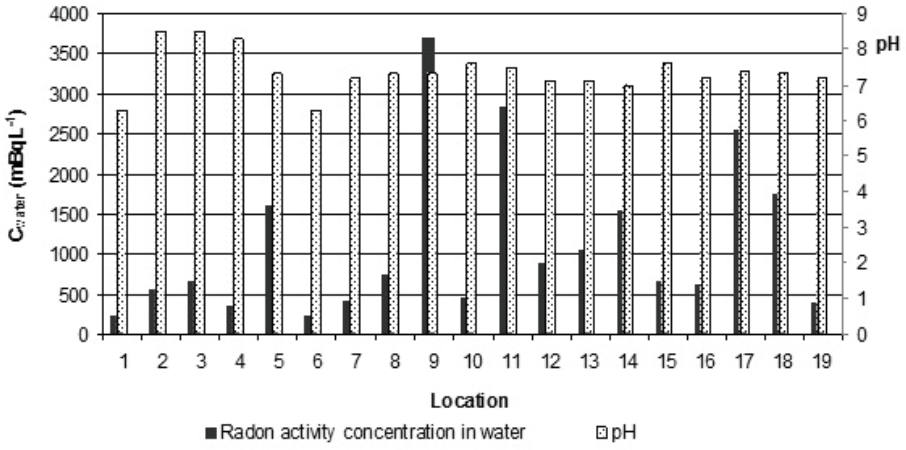

Figure 4 *The radon activity concentration (C_{water}) and the pH value of the investigated water samples*

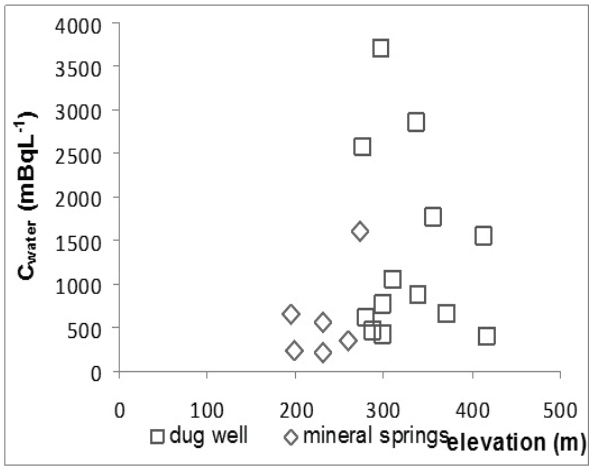

Figure 6 *The radon activity concentration (C_{water}) as a function of the elevation of the sampling locations*

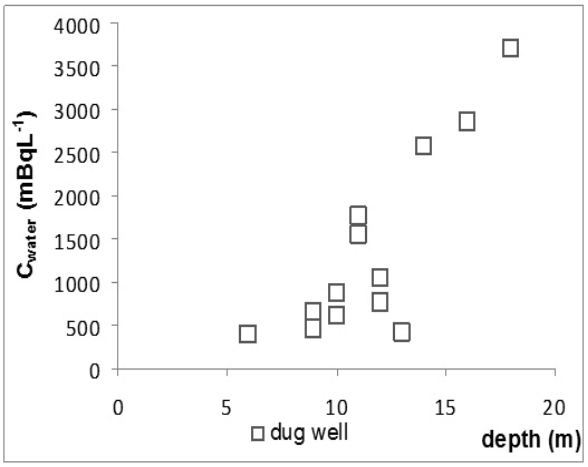

Figure 7 *The radon activity concentration (C_{water}) as a function of the dug well depth*

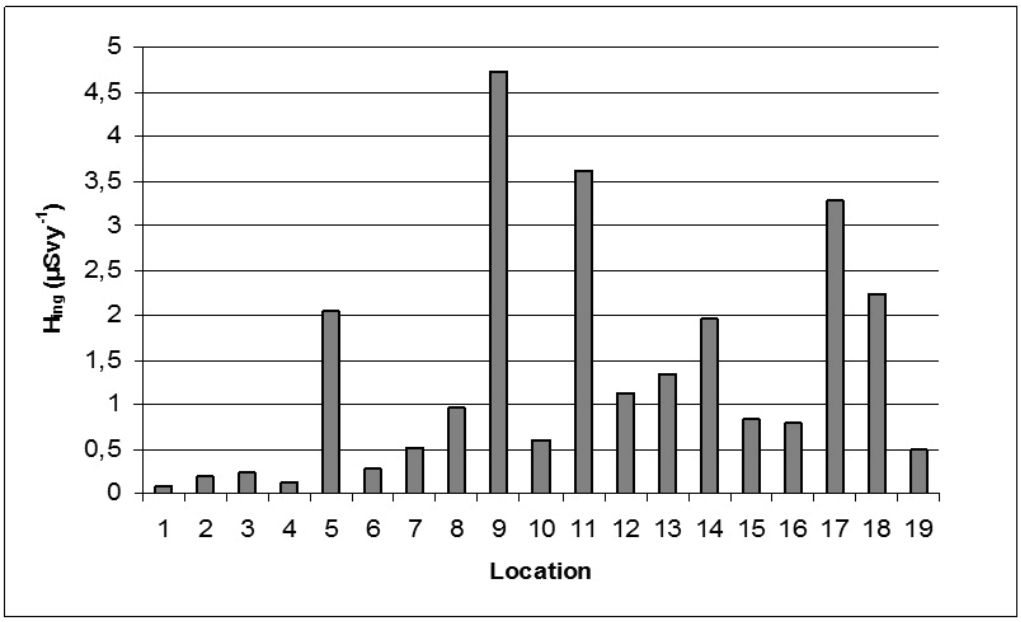

Figure 8 *The annual effective ingestion dose for radon at the researched location*

According to the elevation of the sampling location, a moderate negative correlation was found for mineral spring water and a very weak one for dug well water (Figure 6).

The depth of every well was measured and related to the obtained activity concentrations (Table 2). As can be seen from Figure 7, a strong positive correlation between the radon activity concentration and the depth of the dug wells was determined (r=0.84). The radon activity concentration for water samples increased with the depth of the dug well.

The estimated annual effective dose received by the population as a result of ingesting the water was in the range 0.08-4.72 μSv y^{-1} (Figure 8), which is within the same range as for Novi Sad City, Serbia and lower than for dug well water in Croatia (6, 18-20). The range does not exceed the reference level of 0.1 mSv y^{-1} recommended by the World Health Organization (WHO) (24).

CONCLUSIONS

The radon activity concentrations in Tuzla's drinking waters were below the reference level recommended by the EU (100 Bq L^{-1}). The results presented in this paper have shown that the radon activity concentration did not depend on pH value and temperature of water, but most likely did depend on depth. What is perhaps most important in this, the annual ingestion dose for the investigated water samples was proven to be below the proposed reference level (24).

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Mjerenje koncentracije radona u bunarskoj i izvorskoj vodi na području Tuzle, Bosna i Hercegovina

Istraživanja o koncentraciji aktivnosti radona u vodi, zraku i tlu dobro su znana. Izlaganje visokim razinama koncentracije aktivnosti radona uzrokuje ozračivanje dišnih organa, što može dovesti do raka pluća. U ovom su radu predstavljeni rezultati istraživanja o koncentraciji aktivnosti radona u vodi za piće uzorkovanoj iz bunara i prirodnih izvora na tuzlanskome području. Mjerenja su provedena radon mjernim sustavom AlphaGUARD. Razine koncentracije aktivnosti radona na istraživanim lokalitetima bile su od 214 do 3702 mBq L^{-1}. Dobiveni rezultati pokazali su da je koncentracija radona u istraživanim uzorcima bunarske i izvorske vode niža od 100 Bq L^{-1}, što je razina koncentracije aktivnosti radona u vodi za piće koju je predložila Europska komisija.

KLJUČNE RIJEČI*: godišnja efektivna doza; voda za piće; prirodna radijacija radona; pH vrijednost, koncentracija aktivnosti radona; temperatura*

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Detection of heavy metals in common vegetables at Varaždin City Market, Croatia

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The present study was aimed at the estimation of heavy metal content in vegetables sold at the city market of one of the densely populated Croatian cities, Varaždin, and to establish the relationship between their levels and possible sources of contamination. Twenty-eight samples of the most common diet vegetables (red and white potato, onion, carrot, common bean, lettuce, and cabbage) were randomly bought at the market in September and October 2013. Using the atomic absorption spectrometry method, concentrations of nine heavy metals (As, Cd, Cr, Cu, Hg, Mn, Ni, Pb, and Zn) were measured in the selected samples. The results showed that, in five out of 28 samples analysed, six concentrations exceeded the maximum levels provided for in the regulations: five for Pb and one for Cd. Maximum regulated levels for Pb were exceeded in two samples of red potato, two samples of common bean, and one sample of carrot (17.9 %), and for Cd in a sample of red potato (3.6 %). In conclusion, the cause of the overstepping of the maximum levels for Pb and Cd in the vegetables analysed was most likely the contaminated soil. The possible sources of soil contamination include traffic, nearby industry, floodwaters of rivers and streams, and the use of pesticides and fertilisers in agricultural production.

KEY WORDS: *cadmium; lead; maximum permitted level; food analysis; atomic absorption spectrometry*

Soil is a multifunctional good with many important roles: ecological regulation, filtration of water, universal buffering, regulation of climate, as a source of biodiversity, and, most importantly, in supplying plants with water, air and nutrients, therefore enabling the production of organic compounds in the process of photosynthesis (1, 2). All heavy metals are naturally present in the soil; however, elevated levels most frequently occur due to contamination, which can be of anthropogenic or natural origin. Anthropogenic activities making major contributions to soil contamination include industry, mining, waste management, traffic, agriculture, artificial fertilisers, metal-based pesticides, municipal sewage wastes, and irrigation (1, 3, 4). Some activities cause mobilisation of heavy metals, increasing their circulation through soil, water, and air and their transfer to the human food chain (5). The contamination of agricultural soil is of special concern (3, 5, 6). In Croatia, regulations define the maximum permitted levels of heavy metals in agricultural soil (7, 8).

The concentration of heavy metals in plants depends on the species, cultivar, growth stage, and organ of the plant, the concentration and bioavailability of heavy metals in the soil (strongly influenced by soil pH), and environmental conditions (9-12). Vegetables, an important dietary source of essential nutrients, may also contain elevated concentrations of heavy metals due to high transfer into the harvested organ (4).

Heavy metals may translocate to the shoots (e.g. Cd, Fe, Zn) or accumulate in roots (e.g. As, Cr, and Pb), and the concentrations of heavy metals are generally the highest in roots, followed by stem, leaves, and fruits (13-15).

For living organisms, heavy metals can be essential (e.g., Zn, Cu, Mn, Cr, Ni), utilised in small quantities in their metabolism and maintained at optimal levels through homeostasis, or non-essential and toxic (e.g., Cd, Pb, Hg, As) (11, 16, 17). In higher plants, essential micronutrients include Fe, Mn, B, Zn, Cu, Mo, Cl, and Ni, and non-essential As, Cd, Hg, and Pb (18). Essential heavy metals in plants are involved in many important processes, including redox processes, functions of metalloenzymes and metalloproteins, photosynthesis, respiration, expression, and regulation of genes, synthesis of proteins and plant defence mechanisms (19). The concentrations necessary for plant growth (critical deficiency concentrations) and toxic concentrations (critical toxicity concentrations) vary considerably depending on the plant species, plant age and concentration of other elements (18). For some, the line between micronutrient and phytotoxic substance is very thin (1, 11).

Regarding bioavailability for plant uptake, heavy metals can be bioavailable (e.g. As, Cd, Cu, Ni, Zn), moderately bioavailable (Mn), or least bioavailable (Cr, Pb) (20). Uptake of metals is influenced by solubility of the metal, complexation and chelation reactions, pH of soil solution, and concentration of metals (11). In alkaline soils, the

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availability of Fe, Cu, Mn, and Zn is very low, while in acid soils, concentrations of Al and Mn may rise to toxic levels and negatively influence plant growth (21).

The toxic effect of heavy metals, both essential and non-essential, depends on the type of target organism, the conditions of intake, the availability and concentration of the heavy metal and the kinetics of uptake, while the mechanisms of heavy-metal ion toxicity include the blocking of the functional group of biomolecules, displacing the essential metal ion in biomolecules, and modification of the active conformation of biomolecules (22). Heavy metals may affect cellular oxidation state, lipid peroxidation, breaking of DNA strands, protein expression and folding, degradation in proteasome, protein interactions, cell cycle and apoptosis; they are persistent and toxic, and they accumulate through the food chain (17, 23-25).

Since plants take up heavy metals, due to absorption from both polluted soil and environment, monitoring of their levels in vegetables is essential for preventing excessive build-up of metals in the food chain.

The aim of this study was to investigate the heavy-metal content of common diet vegetables sold at the market in Varaždin, Croatia. Vegetable samples were collected based on dietary preferences common in continental Croatia (lettuce, cabbage, red and white potato, onion, carrot and common bean). The presence of nine heavy metals – arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), manganese (Mn), nickel (Ni), lead (Pb), and zinc (Zn) was estimated.

MATERIALS AND METHODS

Study location

Varaždin was selected as one of the densely populated cities of Croatia, characterised by different industrial and commercial enterprises as potential sources of pollution, and relatively heavy traffic around and within the city. At the same time, large-scale vegetable production is conducted in this area, largely to supply the main city market. The climate in Varaždin region is continental. The mean annual air temperature is 10.2 °C and mean monthly air temperature ranges from -3.7 °C in January to 26.0 °C in July (26). Also, the mean annual precipitation is 843.1 mm and mean monthly precipitation ranges from 38.9 mm in January to 96.5 mm in June (26).

Vegetable samples

In September and October 2013, 28 samples of seven vegetables presumed to be most common in the diet of the continental region of Croatia were bought at 11 different sales locations at the city market in Varaždin: lettuce (*Lactuca sativa* L., fam. *Asteraceae*) cultivar "crystal", cabbage (*Brassica oleracea* L. var. *capitata* L., fam. *Brassicaceae*) white or green cultivar, carrot (*Daucus carota* L. subsp. *sativus* (Hoffm.) Schübl. & G. Martens, fam. *Apiaceae*), onion (*Allium cepa* L., fam. *Amaryllidaceae*), common bean (*Phaseolus vulgaris* L., fam. *Fabaceae*), and potato (*Solanum tuberosum* L., fam. *Solanaceae*).

Sample preparation for laboratory analysis

Samples were treated identically as during their preparation for human consumption. After washing with running water, fresh vegetable samples were weighed on a technical scale, chopped into smaller pieces, and air-dried at room temperature. Once dry, the samples were weighed again on a technical scale and ground.

Extraction of heavy metals soluble in a mixture of nitric and perchloric acids

Heavy metals were extracted from plant tissues into a solution. To 1 g of each sample (measured with the Kern ABJ 220-4M analytical scale) placed in a 50 mL laboratory glass, 10 mL of 65.0 % HNO_3 (p.a., Kemika, Zagreb, Croatia) was added. The samples were digested in a water bath at 50 °C until the release of gaseous NO_2 stopped. Afterwards, 3 mL of 70.0 % $HClO_4$ (p.a., Kemika, Zagreb, Croatia) was added to the samples, which were then once again digested. The cooled samples were filtered through a filter paper (70 mm diameter, blue ribbon, Munktell – Grade 391) into volumetric flasks and diluted with deionised water (deionised with Millipore DirectQ 3 Water Purification System, Molsheim, France) up to a total volume of 50 mL. For the purpose of heavy metal detection, the samples were further diluted with deionised water in the proportions 1:5, 1:10, and 1:100.

Atomic absorption spectrometry (AAS)

The concentrations of heavy metals in the vegetable samples were detected with atomic absorption spectrometry (AAS). The presence of As, Cd, Cr, Ni, and Pb was measured by graphite-furnace AAS (detection limits of 0.05 μg L^{-1}, 0.002 μg L^{-1}, 0.004 μg L^{-1}, 0.07 μg L^{-1}, and 0.05 μg L^{-1}, respectively) and the presence of Cu, Mn, and Zn by air-acetylene flame AAS (detection limit of 0.0015 μg L^{-1} for all three heavy metals), both using a Perkin Elmer AAnalyst 800 atomic absorption spectrometer (PerkinElmer, Inc., Shelton, Connecticut, USA). The presence of Hg was measured by hydride-generation technique (detection limit of 0.009 μg L^{-1}) using a Perkin Elmer AAnalyst 800 atomic absorption spectrometer coupled with a Flow Injection for Atomic Spectroscopy (FIAS) 100 system (PerkinElmer, Inc., Shelton, Connecticut, USA). Hollow cathode lamps (HCL) were used as sources of radiation for detection of Cd, Cr, Cu, Mn, Ni, Pb, and Zn, while electrodeless discharge lamps (EDL) were used for detection of As and Hg. For each heavy metal determined, the respective atomic spectroscopy standard, grade pure, was used as standard reference material (PerkinElmer, Inc., Shelton, Connecticut, USA). In all the

vegetable samples, measurement of each heavy metal was performed once.

Maximum levels for heavy metals in vegetables

The results of the AAS analysis were compared with current regulations – i.e. Commission Regulation (EC) No 1881/2006 with amendments – which define the maximum levels for Cd and Pb in vegetables, while the maximum levels for As, Cr, Cu, Hg, Mn, Ni, and Zn in vegetables are not defined (27, 28). For Cd, the maximum levels in vegetables (mg kg^{-1} of wet weight) are defined as follows: in leaf vegetables 0.20; in stem vegetables, root vegetables, and peeled potatoes 0.10; in vegetables and fruit excluding leaf vegetables, stem vegetables, root vegetables and potatoes 0.050 (27, 28). For Pb, the maximum levels in vegetables (mg kg^{-1} of wet weight) are defined as follows: in *Brassica* vegetables and leaf vegetables 0.30; in vegetables including peeled potatoes and excluding *Brassica* vegetables and leaf vegetables 0.10; in legumes 0.20 (27, 28). Since the current regulations do not define the maximum levels for As and Hg in vegetables, the results of analysis were compared with the maximum permitted levels for As and Hg defined in a previous regulation (29). For As, the maximum permitted level in fruit and vegetables (mg kg^{-1}) was defined as 0.3 (29). For Hg, the maximum permitted levels in vegetables (mg kg^{-1}) were defined as follows: in leaf vegetables and peeled potato 0.05; in other fruit and vegetables 0.2 (29).

Critical deficiency and toxicity concentrations in vegetables

The concentrations of essential heavy metals (Cu, Mn, Ni, Zn) detected in the vegetables were compared with critical deficiency and toxicity concentrations (19), and the concentrations of non-essential heavy metals were compared with toxicity thresholds (As, Cd, Cr, Pb) (15) and limit for phytotoxic effects (Hg) (30). However, these concentrations were not available for all the metals analysed. In higher plants, the critical deficiency concentrations in dry weight are as follows: for Cu 1-5 mg kg^{-1} (in vegetative organs), for Mn 10-20 mg kg^{-1} (in leaves) and for Zn below 15-20 mg kg^{-1} (in leaves) (19). For Ni, the adequate range is from 0.01 to above 10 mg kg^{-1} (19). The critical toxicity concentrations in dry weight are as follows: for Cu above 20-30 mg kg^{-1} (in leaves of crops), for Mn 200-5300 mg kg^{-1} (in shoots), for Ni above 10-50 mg kg^{-1} (in crops), and for Zn from 100 to above 300 mg kg^{-1} (in leaves of crops) (19). The concentrations of non-essential As, Cd, Cr, and Pb were compared with plant toxicity thresholds of 20, 5-10, 1-2, and 10-20 mg kg^{-1}, respectively (15). The concentrations of Hg were compared with a limit for phytotoxic effects in food crops of 1 mg kg^{-1} (30).

RESULTS

In order to allow comparison with both regulations and deficiency and toxicity concentrations, thresholds and limits in plants, the results are presented as concentrations of fresh (Figure 1, Figure 2) and dry weight (Table 1).

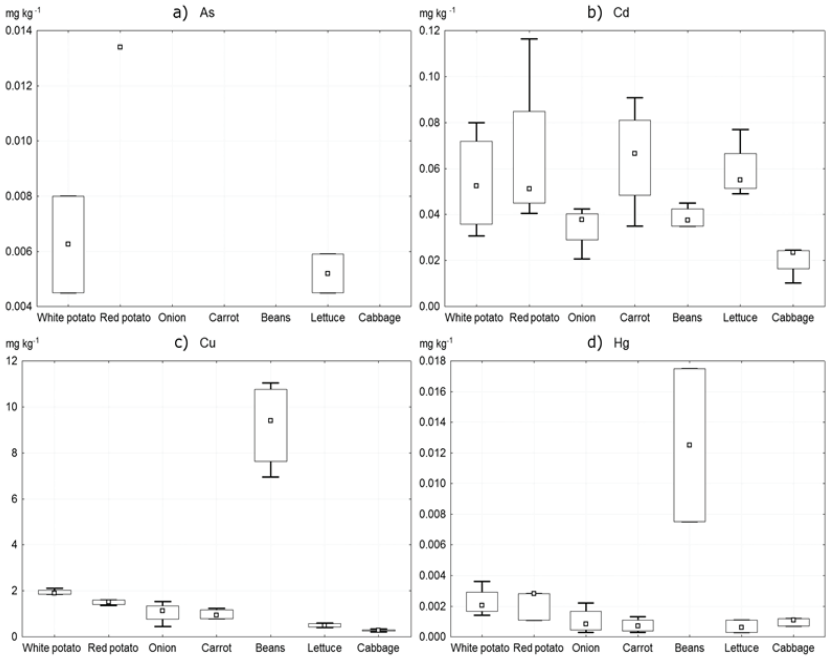

Figure 1 *Concentrations of a) arsenic (As), b) cadmium (Cd), c) copper (Cu) and d) mercury (Hg) in vegetable samples (fresh weight) collected at Varaždin market*

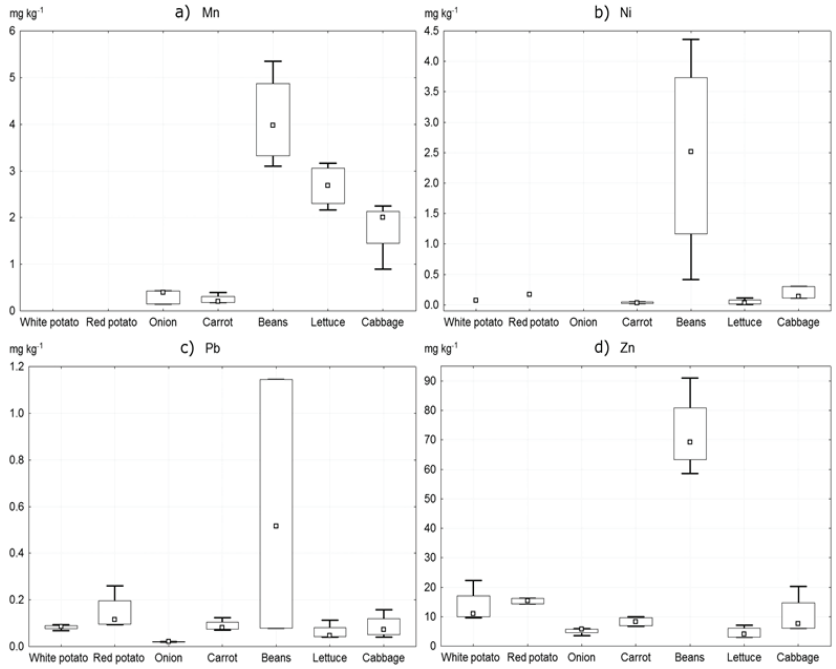

Figure 2 *Concentrations of a) manganese (Mn), b) nickel (Ni), c) lead (Pb) and d) zinc (Zn) in vegetable samples (fresh weight) collected at Varaždin market*

The concentrations of As in fresh weight for five samples ranged between 0.005 and 0.013 mg kg^{-1}, with the highest concentration detected in a sample of red potato (Figure 1a) at sales location OPG 6. The concentrations of Cd ranged between 0.01 and 0.12 mg kg^{-1}, with the highest concentration detected in a sample of red potato (Figure 1b) at sales location OPG 4. The concentrations of Cu ranged between 0.2 and 11.1 mg kg^{-1}, with the highest concentration detected in a sample of beans (Figure 1c) at sales location OPG 4. The concentrations of Hg in 24 samples ranged between 0.0003 and 0.0175 mg kg^{-1}, with the highest concentration detected in a sample of beans (Figure 1d) at sales location OPG 3. The concentrations of Mn in 19 samples ranged between 0.2 and 5.4 mg kg^{-1}, with the highest concentration detected in a sample of beans (Figure 2a) at sales location OPG 7. The concentrations of Ni in 15 samples ranged between 0.01 and 4.36 mg kg^{-1}, with the highest concentration detected in a sample of beans (Figure 2b) at sales location OPG 7. The concentrations of Pb in 26 samples ranged between 0.02 and 1.15 mg kg^{-1}, with the highest concentration detected in a sample of beans (Figure 2c) at sales location OPG 2. The concentrations of Zn ranged between 3.0 and 91.0 mg kg^{-1}, with the highest concentration detected in a sample of beans (Figure 2d) at sales location OPG 3. The heavy-metal content was below the detection limit of the instrument as follows: for As in 23 samples (82.1 %), for Cr in all samples analysed (100 %), for Hg in four samples (14.3 %), for Mn in nine samples (32.1 %), for Ni in 13 samples (46.4 %) and for Pb in two samples (7.1 %). The presence of Cd, Cu, and Zn was detectable in all 28 samples analysed.

Overstepping the maximum level defined in the current regulations (27, 28) was detected for Cd in one sample (3.6 %), and for Pb in five samples (17.9 %) – more precisely: for both Cd (0.12 mg kg^{-1}) and Pb (0.26 mg kg^{-1}) in a sample of red potato; and for Pb alone in a sample of red potato (0.13 mg kg^{-1}), in a sample of carrot (0.12 mg kg^{-1}), and in two samples of beans (1.15 mg kg^{-1} and 0.52 mg kg^{-1}) (Fig. 1b, Fig. 2c). Since the maximum levels for As, Hg, Cu, Mn, Ni, and Zn are not defined in current regulations, no possible transgression could be determined. When the detected concentrations of As and Hg were compared with the maximum permitted levels defined in the previous regulation (29), no transgression was observed.

In comparison with critical deficiency and critical toxicity concentrations (19) of essential heavy metals in higher plants (in dry weight), the concentrations of Cu (2.1-12.3 mg kg^{-1}) were either within the critical deficiency concentration range (in all cabbage samples and one onion sample) or above it, and below the critical toxicity concentrations in all samples (Table 1). The concentrations of Mn (1.2-62.3 mg kg^{-1}) were above the critical deficiency concentration range in lettuce and one cabbage sample, within the range in one cabbage sample, and below it in potatoes, onion, carrot, beans, and two samples of cabbage, and below the critical toxicity concentrations in all samples. The concentrations of Ni (0.15-4.36 mg kg^{-1}) were within the adequate range and below the critical toxicity concentrations in all samples. The concentrations of Zn (28.0-198.0 mg kg^{-1}) were above the critical deficiency concentration range in all samples and within the critical toxicity concentration range in two samples of lettuce and one sample of cabbage, and below the critical toxicity range

Table 1 *Concentrations of As, Cd, Cr, Cu, Hg, Mn, Ni, Pb, and Zn detected in vegetable samples (dry weight) collected at Varaždin market. dl=detection limit of instrument*

	Arithmetic mean (mg kg^{-1} of dry weight)±standard deviation (mg kg^{-1}) Range (minimum - maximum) (mg kg^{-1})								
	As	**Cd**	**Cr**	**Cu**	**Hg**	**Mn**	**Ni**	**Pb**	**Zn**
White potato	0.027±0.011	0.23±0.09	<dl	8.2±0.5	0.0098±0.0041	<dl	0.31*	0.36±0.04	57.4±25.1
	0.019-0.034	0.13-0.34		7.9-9.0	0.0060-0.0155			0.30-0.40	41.0-94.5
Red potato	0.053*	0.26±0.14	<dl	5.9±0.4	0.0088±0.0038	<dl	0.67*	0.58±0.31	60.4±4.2
		0.16-0.46		5.4-6.4	0.0045-0.0110			0.37-1.03	56.5-64.0
Onion	<dl	0.28±0.08	<dl	8.5±3.6	0.0084±0.0069	2.6±1.2	<dl	0.17±0.01	41.9±9.3
		0.17-0.34		3.6-12.3	0.0025-0.0180	1.2-3.5		0.16-0.18	28.0-47.0
Carrot	<dl	0.49±0.18	<dl	7.4±1.7	0.0054±0.0033	1.9±0.8	0.27±0.17	0.68±0.18	63.3±12.1
		0.27-0.69		6.0-9.4	0.0020-0.0095	1.4-3.0	0.15-0.39	0.54-0.94	51.0-75.5
Beans	<dl	0.04±0.00	<dl	9.2±1.9	0.0125±0.0050	4.1±1.0	2.45±1.68	0.25±0.23	72.0±13.7
		0.04-0.05		7.0-11.1	0.0075-0.0175	3.1-5.4	0.42-4.36	0.08-0.52	58.5-91.0
Lettuce	0.102±0.020	1.16±0.24	<dl	9.8±1.7	0.0127±0.0081	52.7±9.0	0.87±0.90	1.22±0.67	90.3±38.4
	0.088-0.116	0.97-1.52		8.1-11.8	0.0055-0.0215	42.7-62.3	0.15-2.15	0.82-2.22	59.5-139.5
Cabbage	<dl	0.20±0.07	<dl	2.7±0.5	0.0097±0.0028	17.4±5.9	1.81±1.0	0.83±0.49	101.3±65.9
		0.10-0.24		2.1-3.4	0.0065-0.0115	8.7-21.9	1.11-2.95	0.40-1.53	59.5-198.0
Total	0.062±0.040	0.38±0.37	<dl	7.4±2.8	0.0094±0.0050	16.4±20.6	1.35±1.29	0.61±0.47	69.5±33.6
	0.019-0.116	0.04-1.52		2.1-12.3	0.0020-0.0215	1.2-62.3	0.15-4.36	0.08-2.22	28.0-198.0

**Only one sample above detection limit*

in all other samples. In comparison with plant toxicity thresholds (15) and the limit of phytotoxic effects (30) for non-essential heavy metals, the concentrations of As (0.019-0.116 mg kg^{-1}), Cd (0.04-1.52 mg kg^{-1}), Cr (below detection limit), and Pb (0.08-2.22 mg kg^{-1}) were below the respective plant toxicity thresholds, and the concentrations of Hg (0.0020-0.0215 mg kg^{-1}) were below the limit of phytotoxic effects in food crops (Table 1).

DISCUSSION

To summarise, the concentrations of Cd and Pb exceeded the maximum levels stipulated in the regulations (27, 28) in five of the 28 samples of vegetables analysed. In these five samples, six detected concentrations were above the maximum levels: one for Cd (Figure 1b) and five for Pb (Figure 2c).

Lončarić et al. (31), for example, investigated heavy metals (Fe, Mn, Zn, Cu, Ni, Mo, Cd, Pb, and Cr) in 29 soil and 147 vegetable samples from vegetable gardens in the urban areas of Osijek and its surroundings. A very close relationship was established between high Pb soil concentrations and exceedingly high concentrations of Pb in vegetables (detected in 19 of the 147 samples): a high number of such samples was detected in root vegetables (12 of 46). Although no exceedingly high concentration of Cd in soil was recorded, exceedingly high concentrations of Cd were detected in vegetables at three localities. Next, Puntarić et al. (32) detected concentrations of heavy metals (Pb, Cd, As, and Hg) in 100 samples of leafy vegetables, including lettuce (22 samples) and white cabbage (11 samples), and 16 samples of soil in gardens in the urban area of Zagreb. The concentrations of heavy metals exceeded the regulated maximum permitted levels in nine vegetable samples (9 %): for Pb in three samples, for Cd in one sample, and for Hg in five samples. The findings from Osijek and Zagreb both support the results of our study. In the vegetable samples from the Zagreb area, high Hg content was also observed, which was not the case in the vegetable samples from Varaždin. The authors hypothesised that elevated Hg content in eastern Zagreb might be due to thermal-heating plant and a battery factory, while in northern Zagreb culprits might be crematorium and the dominant winds which seem to transport contaminants from the industrial eastern parts to the residential northern parts of the city (32). Furthermore, Vitali et al. (33) compared the concentrations of heavy metals (Cd, Pb, Hg, As, Zn, and Cu) in white cabbage and potato samples in and out of war-affected regions in Croatia. No significant differences were found between the areas affected by war and those unaffected. All cabbage samples were below the regulated levels for Cd and Pb, all potato samples were below the regulated levels for Cd, and eight (of 20) potato samples exceeded the regulated levels for Pb. These findings are similar to the results of our study, since all cabbage samples were below the maximum regulated levels for all heavy metals analysed, and the Pb content exceeded the maximum level in potato. Sapunar-Postruznik et al. (34) analysed data on the levels of Pb and Cd in foodstuffs (22 groups for Pb and 19 for Cd) available on the Croatian market during a five-year period (from 1988 to 1993). In the group of vegetables, the mean concentrations of Pb and Cd in 510 samples were 94 μg kg^{-1} and 24 μg kg^{-1}, respectively. In our study, the mean concentrations of Pb and Cd in vegetables were higher (140 and 50 μg kg^{-1}, respectively). Of note, the number of samples analysed in our study was considerably lower.

In 2013, Croatia became a Member State of the European Union (EU), and the Commission Regulation (EC) 1881/2006 on setting maximum levels for certain contaminants in foodstuffs (with amendments) became valid (27, 28), while the previous regulation, the Ordinance on maximum levels of certain contaminants in foodstuffs (35) was suspended. Further below, the information provided in the European Food Safety Authority (EFSA) reports on investigated heavy metals is discussed.

Although there are no official maximum levels permitted at the EU level for As, EFSA provided an estimate of daily dietary exposure to inorganic As in the European population to be between 0.09-0.45 μg kg^{-1} (minimum - maximum of lower bound, LB) and 0.24-1.37 μg kg^{-1} (minimum - maximum of upper bound, UB) b.w. (36). In comparison with EFSA's measurements of As content in vegetables, the mean concentrations of As (total) in our study were higher in red potato and similar in white potato (13 and 6 μg kg^{-1} compared with 1.6-7.2 μg kg^{-1}) and lower in lettuce (5 μg kg^{-1} compared with 6.9-10.8 μg kg^{-1}). Of note, in our study the concentration of As was below the limit of detection in all onion, carrot, bean, and cabbage samples (Figure 1a).

For Cd, a tolerable weekly intake (TWI) of 2.5 μg kg^{-1} b.w. has been established (37, 38). EFSA estimated the mean dietary exposure to Cd in the adult population to be between 1.9 and 3.0 μg kg^{-1} b.w. per week (37). In comparison with EFSA's measurements, the mean concentrations of Cd in our study were higher in white and red potato (0.05 and 0.06 mg kg^{-1} compared with 0.02 mg kg^{-1}), carrot (0.06 mg kg^{-1} compared with 0.02 mg kg^{-1}), beans (0.04 mg kg^{-1} compared with 0.01 mg kg^{-1}) and lettuce (0.06 mg kg^{-1} compared with 0.02 mg kg^{-1}), and almost the same in cabbage (0.02 mg kg^{-1} compared with 0.02 mg kg^{-1}) (Figure 1b). A comparison for onion was not possible. Also, EFSA found that 63 samples (2.6 %) of stem and root vegetables and no leaf vegetables (without spinach) exceeded the maximum level defined in the regulations (37). In our study, only one sample of red potato exceeded the maximum level for Cd (3.6 %).

For Hg, a TWI for methylmercury of 1.3 μg kg^{-1} b.w. and a TWI for inorganic mercury of 4 μg kg^{-1} b.w. have been established, both expressed as mercury, and no maximum levels for Hg in vegetables are currently established at the EU level (39). EFSA estimated the mean

dietary exposure to methylmercury in the overall European population between 0.06 and 1.57 μg kg^{-1} b.w. per week, and to inorganic Hg between 0.13 and 2.16 μg kg^{-1} b.w. per week (39). In comparison with EFSA's measurements of Hg content in vegetables, the mean concentrations of Hg (total) in our study were higher in white and red potato and beans (2.3, 2.2, and 12.5 μg kg^{-1} compared with 0.1-2.1 μg kg^{-1} for potatoes and potato products and 1.4-2.2 μg kg^{-1} for dried beans), and similar in onion, carrot, lettuce, and cabbage (1.1, 0.8, 0.7, and 1 μg kg^{-1}, respectively, compared with 0.1-2.3 μg kg^{-1} for bulb vegetables, 0.4-2.6 μg kg^{-1} for root vegetables, 0.5-3.8 μg kg^{-1} for leaf vegetables and 0.4-1.3 μg kg^{-1} for *Brassica* vegetables) (Figure 1d).

For Pb, EFSA concluded that the previously established PTWI of 25 μg kg^{-1} b.w. was no longer appropriate, due to critical endpoints for which no safe threshold could be established, and calculated benchmark doses and corresponding dietary intakes of 0.50 μg kg^{-1} b.w. per day for developmental neurotoxicity in children, and 1.50 μg kg^{-1} b.w. per day for cardiovascular and 0.63 μg kg^{-1} b.w. per day for nephrotoxic effects, both in adults (40). EFSA estimated the mean dietary exposure to Pb in the adult European population to be between 0.36 and 1.24 μg kg^{-1} b.w. per day (40). In comparison with EFSA's measurements of Pb content in vegetables, the mean concentrations of Pb in our study were higher in white and red potato, carrot, beans and cabbage (0.08, 0.15, 0.09, 0.58, and 0.09 mg kg^{-1}, respectively, compared with 0.02-0.03 mg kg^{-1} for potatoes other than peeled, 0.05-0.06 mg kg^{-1} for root vegetables, 0.02-0.04 mg kg^{-1} for legumes and 0.01-0.03 mg kg^{-1} for *Brassica* vegetables), and similar in lettuce (0.06 mg kg^{-1} compared with 0.05-0.06 mg kg^{-1}) (Figure 2c). A comparison for onion was not possible. EFSA found that the Pb content was above 1 mg kg^{-1} in 771 samples (40). In our study, five samples (two samples of red potato, one sample of carrot and two samples of beans) exceeded the maximum regulated level for Pb (17.9 %).

For Ni, a tolerable daily intake (TDI) of 2.8 μg kg^{-1} b.w. has been established, and no maximum levels are currently established for Ni in food at the EU level (41). EFSA estimated the mean chronic dietary exposure to Ni in the European population between 2.0 and 13.1 μg kg^{-1} b.w. per day (41). In comparison with EFSA's measurements of Ni content in vegetables, the mean concentrations of Ni in our study were higher in cabbage (190 μg kg^{-1} compared with 59-79 μg kg^{-1} for *Brassica* vegetables) and lower in carrot, beans, lettuce, and white and red potato (40, 2450, 40, 70, and 170 μg kg^{-1} compared with 160-170 μg kg^{-1} for carrots, 2900 μg kg^{-1} for dried beans, 110-120 μg kg^{-1} for leaf vegetables and 260-270 μg kg^{-1} for main-crop potatoes) (Figure 2b). Of note, in our study the Ni content was below the limit of detection in all onion samples.

For Cr^{3+}, a possible essential trace element for humans, a TDI of 300 μg kg^{-1} b.w. has been established, while no TDI has been established for toxic Cr^{6+}, and no maximum levels for Cr in food at the EU level are currently established (42, 43). EFSA estimated dietary exposure to Cr^{3+} in the European population between 0.6 and 5.9 μg kg^{-1} b.w. per day and to Cr^{6+} in drinking water between 0.7 and 159.1 ng kg^{-1} b.w. per day (43). The content of Cr in our study was below the detection limit in all samples analysed.

For Cu, an essential element for humans, a tolerable upper intake level of 5 mg per day has been established, and an adequate intake of 1.6 mg per day for men and 1.3 mg per day for women has been proposed, both in adults (44, 45). EFSA estimated dietary intake of Cu in the adult European population between 1.15 and 2.07 mg per day (45). In a Total Diet Study (TDS) in France, the mean concentrations of Cu (in fresh weight) were 0.853 mg kg^{-1} in potato-based products, 0.674 mg kg^{-1} in vegetables and 2.45 mg kg^{-1} in dried vegetables (46). In a TDS in the United Kingdom, the mean concentrations of Cu (unspecified whether in fresh or dry weight) were 0.580 mg kg^{-1} in green vegetables, 1.12 mg kg^{-1} in potatoes, and 0.808 mg kg^{-1} in other vegetables (47). In comparison, the mean concentrations of Cu in our study in white and red potato (1.9 and 1.5 mg kg^{-1}, respectively) were higher than in France and the United Kingdom, which might be attributed to the fact that the potatoes in our study were not peeled. The mean concentration of Cu in onion (1.1 mg kg^{-1}) was lower than in France and higher than in the United Kingdom. The mean concentrations of Cu in carrot (1.0 mg kg^{-1}) and beans (9.2 mg kg^{-1}) were higher than in France and the United Kingdom. High content of heavy metals in bean seeds is discussed further below. The mean concentration of Cu in lettuce (0.5 mg kg^{-1}) was lower than in France and similar to that in the United Kingdom. The mean concentration of Cu in cabbage (0.3 mg kg^{-1}) was lower than in France and the United Kingdom.

For Mn, a tolerable upper intake level has not been set, and an adequate intake of 3 mg per day in adults has been proposed (48, 49). EFSA estimated dietary intake of Mn in the adult European population between 2 and 6 mg per day (49). In a TDS in France, the mean concentrations of Mn were 1.03 mg kg^{-1} in potato-based products, 1.47 mg kg^{-1} in vegetables, and 4.40 mg kg^{-1} in dried vegetables (46). In a TDS in the United Kingdom, the mean concentrations of Mn were 2.06 mg kg^{-1} in green vegetables, 1.58 mg kg^{-1} in potatoes, and 1.54 mg kg^{-1} in other vegetables (47). In comparison, the content of Mn in our study was below the limit of detection in all potato samples. The mean concentrations of Mn in onion (0.3 mg kg^{-1}) and carrot (0.2 mg kg^{-1}) were lower than in France and the United Kingdom. The mean concentration of Mn in beans (4.1 mg kg^{-1}) was higher than the concentration of Mn in vegetables (but lower than the concentration of Mn in dried vegetables) in France and higher than in the United Kingdom. The mean concentration of Mn in lettuce (2.7 mg kg^{-1}) was higher than in France and the United Kingdom. The mean concentration of Mn in cabbage

(1.8 mg kg^{-1}) was higher than in France and lower than in the United Kingdom.

For Zn, a tolerable upper intake level of 25 mg per day has been recommended, and a population reference intake range from 7.5 to 12.7 mg per day in women, and from 9.4 to 16.3 mg per day in men, both in adults, has been established (50, 51). EFSA estimated dietary intake of Zn in the adult European population between 8.0 and 14.0 mg per day (51). In a TDS in France, the mean concentrations of Zn were 2.49 mg kg^{-1} in potato-based products, 2.57 mg kg^{-1} in vegetables, and 9.79 mg kg^{-1} in dried vegetables (46). In a TDS in the United Kingdom, the mean concentrations of Zn were 3.26 mg kg^{-1} in green vegetables, 3.66 mg kg^{-1} in potatoes and 2.62 mg kg^{-1} in other vegetables (47). In comparison, the mean concentrations of Zn in our study in white and red potato (13.5 and 15.3 mg kg^{-1}, respectively) were higher than in France and the United Kingdom. The higher Zn concentrations could be attributed to potato peel, since the potatoes in our study were not peeled. The mean concentrations of Zn in beans (72.0 mg kg^{-1}), onion (5.2 mg kg^{-1}), carrot (8.3 mg kg^{-1}), lettuce (4.6 mg kg^{-1}), and cabbage (10.4 mg kg^{-1}) were higher than in France and the United Kingdom.

Overall, in our study only one sample of red potato exceeded the maximum level for Cd (3.6 %). On the other hand, five samples (two samples of red potato, one sample of carrot, and two samples of beans) exceeded the maximum regulated level for Pb (17.9 %). Therefore, red potato could represent a source of elevated exposure to Cd, while red potato, beans, and carrot could represent a source of elevated exposure to Pb, in the local population. Since there is no safe threshold for several critical endpoints of Pb exposure, including developmental neurotoxicity in children and nephrotoxicity and cardiovascular effects in adults (40), the overstepping of the maximum level in 17.9 % of the samples could be of concern for the local population, especially in children. Such a high percentage of contaminated samples calls for caution and, further, more comprehensive analysis.

The concentration of As was detectable in only five samples, while the content of Cr was below the detection limit in all the samples analysed. Therefore, no conclusions can be drawn for As exposure, while vegetables probably do not contribute to Cr exposure in the local population.

Interestingly, bean samples contained the highest concentrations of Cu, Hg, Mn, Ni, Pb, and Zn (Figure 1, Figure 2). All vegetable samples were bought at a local market, and the samples of beans were bought from different sellers (OPG-2, OPG-3, OPG-4 and OPG-7). Unlike the other vegetables, beans were sold already dried and out of the pod. Of note, EFSA found relatively high Ni concentrations in "legumes, nuts, and oilseeds", and dried beans contained the highest mean concentrations of Ni (41). Most importantly, developing seeds store essential heavy metals, e.g. Zn, Cu, Mn, and Ni, and may accumulate minerals at concentrations several times higher than those required for cell functioning (52-53). Since non-essential metals may also be transported to seeds by same mechanisms (14), this could explain the high content of Pb and Hg in bean seeds. Therefore, beans could represent a source of exposure to these metals in the local population.

Regarding the essential heavy metals, the concentrations of Cu detected in our study were either within or above the critical deficiency range (19) and below the critical toxicity range in all samples (Table 1). The concentrations of Cu in all cabbage samples (2.1-3.4 mg kg^{-1}) and one onion sample (3.6 mg kg^{-1}) were within the critical deficiency range (1-5 mg kg^{-1}). Various soil factors, including pH, external Cu supply, high organic-matter content, N availability, and Zn supply could have influenced the Cu concentration in vegetables (19). The concentrations of Mn were above the critical deficiency range (10-20 mg kg^{-1}) in lettuce. However, in cabbage (8.7-21.9 mg kg^{-1}), one sample was above the range, two samples were within (19.5 and 19.6 mg kg^{-1}), and one was below, as well as in all potato samples (below the detection limit), onion (1.2-3.5 mg kg^{-1}), carrot (1.4-3.0 mg kg^{-1}), and beans (3.1-5.4 mg kg^{-1}) (Table 1). This could be explained by the fact that the critical deficiency concentration for Mn was defined in fully-developed leaves (19), while the content of Mn in our study was detected in other plant organs. The concentrations of Mn in our study were below the critical toxicity concentrations (19) in all samples. The concentrations of Ni were within the adequate range and below the critical toxicity range. The concentrations of Zn were above the critical deficiency range in all samples and below the critical toxicity range in most of the samples. In two samples of lettuce (102.0 and 139.5 mg kg^{-1}) and one sample of cabbage (198.0 mg kg^{-1}) the concentrations of Zn were within the critical toxicity range (100-300 mg kg^{-1}) (19). High Zn supply may lower the concentration of Mn in plants (19). However, of the three samples with high Zn content, only in the sample of cabbage with the highest Zn concentration (198.0 mg kg^{-1}) was the concentration of Mn (19.5 mg kg^{-1}) within the critical deficiency range. Regarding the non-essential heavy metals, the concentrations of As, Cd, Cr, and Pb were below the corresponding plant toxicity thresholds (15) and the concentrations of Hg were below the limit of phytotoxic effects in food crops (30).

The concentrations of heavy metals in plants largely depend on the concentrations of heavy metals in the soil (9, 10). In our study, the overstepping of maximum levels for Cd and Pb in vegetable samples was most likely due to soil contamination with these heavy metals. The possible sources of soil contamination with Cd and Pb are similar, and include floodwater in the lowlands along rivers and streams, atmospheric deposition, proximity of traffic and industry, and use of artificial fertilisers and pesticides in agriculture (1, 54-56). Since the vegetables were bought at Varaždin market from local sellers, they might have been grown in Northwestern Croatia. The content of heavy metals in soil was not evaluated in this study. However, in several

previous studies, authors have recorded increased concentrations of Cd and Pb in soils of the Varaždin area (54, 56-59).

The control of food contamination is crucial for the safety of consumers. A phytosanitary strategy has been created to set guidelines for the development of plant-health national policy in Croatia (60). Although various factors may contribute to elevated concentrations of heavy metals in food, soil contamination is one of the most important (9, 10). A programme for permanent monitoring of the soil in Croatia has been developed; however, data regarding the state of the soil has still not been collected (61, 62).

CONCLUSIONS

The concentrations of nine heavy metals (As, Cd, Cr, Cu, Hg, Mn, Ni, Pb, Zn) were detected in the seven vegetables presumed most common in the diet of the continental region of Croatia (red and white potato, onion, carrot, beans, lettuce, and cabbage), bought at Varaždin market in 2013, using the AAS method. On the basis of the results obtained, the following conclusions can be drawn. Maximum regulated levels for Pb and Cd were overstepped in five samples. For Pb, transgression was detected in five samples (17.9 %): two samples of red potato, one sample of carrot and two samples of beans. For Cd, transgression was detected in one sample of red potato (3.6 %), which also contained an excessive concentration of Pb. Increased concentrations of Pb and Cd in the samples of vegetables were most likely due to contaminated agricultural soil. The possible sources of soil contamination with Pb and Cd include use of fertilisers and pesticides, floods, atmospheric deposition, proximity of traffic and other sources of contamination. Accordingly, increased concentrations of Pb and Cd in vegetables have also been detected in comparable studies previously conducted in Croatia. It was also previously shown that: heavy metal content in soil influences the concentration of heavy metals in vegetables, vegetables majorly contribute to dietary exposure to Pb and Cd, and increased concentrations of Pb and Cd may negatively affect human health. Therefore, monitoring of the concentration of heavy metals in both food and soil is necessary.

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Određivanje teških metala u čestim vrstama povrća na Varaždinskoj gradskoj tržnici

Cilj istraživanja bio je procijeniti sadržaj teških metala u povrću dostupnom na gradskoj tržnici jednog od gušće naseljenih hrvatskih gradova, Varaždina, te ispitati povezanost koncentracija teških metala s mogućim izvorima onečišćenja. Ukupno 28 uzoraka često konzumiranog povrća (crveni i bijeli krumpir, luk, mrkva, grah, salata i kupus) nasumično je kupljeno na Varaždinskoj tržnici u rujnu i listopadu 2013. godine. Koncentracije devet teških metala (As, Cd, Cr, Cu, Hg, Mn, Ni, Pb i Zn) izmjerene su metodom atomske apsorpcijske spektrometrije. U pet od 28 ispitanih uzoraka povrća, šest je koncentracija teških metala bilo iznad najvećih dopuštenih količina: pet za Pb i jedna za Cd. Najveće dopuštene količine Pb prekoračene su u dva uzorka crvenog krumpira, dva uzorka graha i jednom uzorku mrkve (17,9 %), a Cd u jednom uzorku crvenog krumpira (3,6 %). Uzrok prekoračenja najvećih dopuštenih količina Pb i Cd u uzorcima povrća onečišćeno je tlo. Mogući uzroci onečišćenja tla uključuju promet, blizinu industrije, poplavne vode rijeka i potoka te upotrebu pesticida i mineralnih gnojiva u poljoprivrednoj proizvodnji.

KLJUČNE RIJEČI: *kadmij; olovo; najveće dopuštene količine; analiza hrane; atomska apsorpcijska spektrometrija*

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Testing the associations between different aspects of seafarers' employment contract and on-board internet access and their job and life satisfaction and health

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The aim of this study was to test for associations between different aspects of contract and on-board internet access and seafarers' satisfaction and health. Altogether 298 Croatian seafarers, all officers, employed on cargo ships, with a minimum work experience of two years with their current shipping company, participated in an online survey. The questionnaire included sociodemographic items, questions relating to their employment contract and internet access, and measures of job satisfaction, life satisfaction, mental health, and gastrointestinal and cardiovascular symptoms. Their job- and life-satisfaction levels were higher for shorter duration on board, favourable ratio of work to non-work days, and compliance with the employment contract regarding the changes to work and non-work days. Mental health differed likewise but only in relation to two aspects of the contract: on-board duration and compliance with the contract. The level of gastrointestinal symptoms was lower in cases of shorter on-board duration and compliance with the contract, and in seafarers who have free, unlimited internet access on board. Lower level of cardiovascular symptoms was found in seafarers with free, unlimited internet access on board. Our findings suggest that in promoting satisfaction and health in seafaring, attention should be given to reducing on-board duration, compliance with the contract, and internet accessibility on board.

KEY WORDS: *compliance with contract; gastrointestinal and cardiovascular symptoms; isolated working environment; mental health; on-board duration; ratio of work to non-work days*

Seafarers face many stressors, risks and challenges (1, 2) which may have impact on their physical and mental health. However, studies relating to health, morbidity, and mortality in seafarers [e.g. gastrointestinal disease mortality (3); cardiovascular disease mortality (4)] are often confounded by the "healthy worker effect", which is explained by self-selection and adaptation: seafarers who cannot adapt to work on board, as well as those who suffer from the effects of occupational stressors, leave the occupation. Still, Oldenburg et al. (5) show that, even if this effect is considered, it appears that cardiac risk factors occur slightly more frequently in seafarers than in the general population. Furthermore, data on suicides in seafarers (6) show that the mental health of seafarers in many cases continues to be very poor and often fatal. Therefore, studies on the role of different work characteristics in the explanation of physical and mental health, as well as the satisfaction of seafarers, are very important, from both the individual and organisational perspectives.

One inherent characteristic of seafaring is living and working in an isolated confined environment, away from home, with no opportunity to leave the workplace for a specified time period, which may act as a chronic work stressor (7). Indeed, long-term separation from home and family and social isolation on board are among the most frequently cited psychosocial stressors in seafaring (1, 6, 8-11). In this article we focus on two important work characteristics: seafarer's employment contract and internet access, which were shown to be important in describing Croatian seafarers' (dis)satisfaction (12). The study (12) shows that dissatisfaction with the contract stems from three aspects: 1) length of time on board, 2) unfavourable ratio of workdays to days off (e.g. 4-2 rather than 3-3), and 3) non-compliance with the contract regarding the stay on board and at home (irregularity of shifts). Even those participants with shorter duration on board (e.g. 2 months) and favourable contracts in terms of the balance between workdays and days off (e.g. 3-3 months), when describing sources of job dissatisfaction, see irregularity as one of those important negative work characteristics which "markedly violate leisure time at home, disable quality rest and family and social obligations, and also negatively affect financial planning". Unavailability and price of the internet was often cited as a source of dissatisfaction. Conversely, free internet access on board was described by many participants as one of the rare sources of satisfaction on board.

Since this study was descriptive and qualitative, we performed a quantitative study to test whether three aspects

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of the contract – (1) duration on board, 2) ratio between working and non-working days, and 3) compliance with the contract regarding the changes in working and free time – as well as the access to free internet on board, have significant effects on job satisfaction and life satisfaction. On the basis of the qualitative study we assumed that satisfaction with work and life would be higher in seafarers with shorter durations on board, favourable ratios between work and non-work days, regular shifts, and free access to the internet on board. Being physically away from home and family and living in an isolated shipping environment may lead to anxiety and depression (6, 8, 9). Therefore, we tested the effects of different aspects of the contract and internet access on mental health, assuming that better mental health would be found in seafarers with shorter and favourable contracts, regular shifts, and access to the internet on board. Finally, our objective was also to test these effects on physical health, where we focused on gastrointestinal and cardiovascular symptoms, since it has been shown that cardiovascular disease and gastrointestinal disease are among the major causes of morbidity and death in seafarers (13). Morbidity in seafarers is also explained by lifestyle factors, especially by smoking, alcohol use, malnutrition (unbalanced, high-fat diet), and lack of exercise on board (14, 15). Furthermore, work on board is organised in shifts (watch system), and disturbance of circadian rhythms imposed by shift work, especially night work, is recognised as an important factor in the development of gastrointestinal and cardiovascular diseases (16). We hypothesised, therefore, that levels of gastrointestinal and cardiovascular symptoms might also be affected by negative characteristics of the contract, such as duration of on-board stay, unfavourable ratio between working and off days, and irregularity of shifts. Furthermore, considering the importance of cyberspace communication on board with partners, family, and friends (12, 17, 18) for satisfaction, turnover, and retention in seafarers, we also hypothesised that non-accessibility of the internet on board may act as a chronic stressor which may lead to somatic stress reactions (19), i.e. negatively affect seafarers' health.

In spite of the importance of accessibility of the internet on board in promoting seafarers' satisfaction and retention, there is no data about its effects on the mental and physical health of seafarers. Furthermore, to our knowledge, there is no available study which has tested the effects of different aspects of seafarer's employment contract on satisfaction and health, while controlling the important characteristics which may confound these effects, such as nation and rank. Previous studies have shown that the perception of stressors on board, self-rated health measures, and psychological well-being and satisfaction, largely vary with culture or country, rank and profession, and the type of vessel (1, 20-22), largely because of cultural/rank differences in staying on board. Therefore, testing the impact of the contract on different outcome measures in a specific national group of Croatian seafarers may be an advantage of this study. In order to achieve further homogeneity of the sample, we focused only on officer ranks, employed in international cargo shipping. In doing so, we avoided the masking of the effects of the rank and type of maritime activity. We considered cargo ships a qualitatively different working environment compared to passenger or offshore vessels. Finally, given the labour fluctuations in seafaring, in order to examine the tested effects of work characteristics which differ among shipping companies, we limited our sample to those seafarers who had been employed at the same shipping company for a minimum of two years.

METHODS

Participants

The study included a total of 298 Croatian seafarers employed on cargo ships. The average age was 39.16 years (SD=9.98), length of service in the maritime sector 15.09 years (SD=9.71), and length of service with the current company 11.57 (SD=10.42).

The study included only higher-ranking officers (no ratings). Numbers and percentages of education level and job description are given in Table 1, as well as other relevant demographic characteristics. The largest numbers of participants worked on LNG and LPG ships, oil tankers, and oil products and container ships, while the rest of the sample worked on other cargo ships (see Table 1).

Procedure and Ethics

In order to reach Croatian seafarers employed on cargo ships, we contacted various organisations and groups which helped us with the advertising of the study on their web-pages and/or forwarding e-mail invitations to seafarers relating to their organisations (Croatian shipping companies, Croatian agencies for seafarers, the Seafarers Union of Croatia, educational institutions in Croatia involved in the training of seafarers, the Croatian web page *Pomorac.net,* LinkedIn groups of Croatian seafarers, etc.). Response-rate data is missing from our study for two reasons: (1) use of non-probabilistic sampling procedure (not possible to calculate the number of seafarers who got forwarded e-mail invitations from their company/agency/colleague or who saw the advertisement of the study on the web page of their agency/union etc.), (2) the lack of a population frame for Croatian officers on international cargo ships (employed in the current shipping organisation for a minimum of two years). The latest statistical data on Croatian seafarers show that Croatian seafarers make up approximately one percent of the total number of seafarers (23). In Croatia there are approximately 20,000 seafarers, of which 15,184 take part in international sea shipping (23). On the basis of national statistics (official statistics of seafarers in international shipping, by the Ministry of Maritime Affairs, Transport, and Infrastructure) for 2014, of which 58 % are in higher

Table 1 *Demographic characteristics*

		Mean	SD	Min	Max
Age		39.16	9.98	21	65
Service length – MS[1]		15.09	9.71	2	44
Service length – WO[2]		11.57	10.42	2	44
				N	%
Education level	High school			78	26.17
	College			147	49.32
	University			73	24.50
Job description	Engine officer			71	23.83
	Deck officer			120	40.27
	Chief engineer			40	13.42
	Commander			67	22.48
Type of ship/cargo	LNG[3] and LPG[4]			113	37.92
	Tankers (oil and oil products)			74	24.83
	Containers			61	20.47
	Bulk cargo			20	6.71
	Chemicals			13	4.36
	Ro-Ro (cargo on wheels)			9	3.02
	General cargo			5	1.68
	Combined cargo			3	1.01
Shipping organisation	Croatian			21	7.05
	Foreign			277	92.95
Marital status	Single			55	18.46
	Married / in domestic partnership			243	81.54
Number of children	0			94	31.54
	1			69	23.15
	2			108	36.24
	3 or more			27	9.06
Current location	Ship			110	36.91
	Home			188	63.09

Note: [1] = length of service in maritime sector (MS); [2] = length of service in current work organisation (WO); [3] = liquefied natural gas; [4] = liquefied petroleum gas

ranks (commanders, chief engineers, engine officers, and deck officers), our sample amounts to 3.4 % of the population of Croatian officers in international shipping (including foreign and national companies). However, there is no precise data for cargo ships only. Data on the Croatian maritime market (24) show that Croatian seafarers in international shipping were employed on tankers (45 %), dry cargo ships (20 %), LNG (15 %), offshore (10 %) and passenger ships (10 %). Overall, one of the disadvantages of our study is the limited generalisability of its results, which stems from the sample's questionable representativeness of the population of Croatian officers on cargo ships; therefore further studies on representative samples are strongly recommended. However, considering the fact that seafarers are a hard-to-reach population, and that the use of a non-probabilistic sample procedure increases confidence in anonymity for the participants, we consider the sample obtained to be good enough for investigating the tested effects.

The study was previously approved by the Ethics Committee of the Department of Psychology, University of Zadar. All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1975 Helsinki declaration and its later amendments. The study was conducted in 2014 using an online survey. At the time of participation, about 63 % of participants were at home, and about 37 % were at sea. Invitations to participate in the study included ethical requirements for informed consent, voluntary participation, and confidentiality of data and anonymity of participants. Data collected did not include any names, contact details or names of the shipping companies in which participants were employed. Contact details of researchers were

provided, and participants could contact a researcher with questions or comments. Feedback about the results of the study was guaranteed to participants on request.

Materials

The first part of the questionnaire (see Annex for whole questionnaire) was created by the authors and included basic demographic items and questions related to the contract and internet access. Demographic questions included: gender, age, years of service, education level, job description, type of cargo ship, marital status, children, and current location (all shown in Table 1 – except gender, since all participants were men).

Three questions regarding the contract were asked: (1) number of months on board according to the contract, (2) number of months at home according to the contract, and (3) compliance with the contract regarding the changes to ship and home periods. For the first two questions, the possible answers were: 1, 2, 3, 4, 5, 6, and more than 6. Numbers and percentages for exact durations of ship-home periods are given in Table 2. However, considering the huge range of answers, which were also unequally distributed, and in order to respond to our research objective, we used somewhat different variables: length of duration on board (divided into 2, 3, 4, and more than 4 months) and the ratio between ship and home period (operationalised as favourable and non-favourable) (see Table 3). For compliance with the contract, four categories of answers were provided: changes to the periods on board and at home are 1= always in compliance with the contract (N=39), 2=mostly in compliance with contract (N=193), 3=mostly not in compliance with the contract (N=50), 4=never in compliance with the contract (N=16). In further analysis we merged the categories always and mostly into one and named it "regular shifts", and mostly not and never into another named "irregular shifts".

Table 2 *Ship-home duration in months according to the contract*

Ship-home time (months)	N	%
2-2	37	12.42
3-3	113	37.92
4-4	31	10.40
5-5	1	0.34
6-6	6	2.01
2-1	1	0.34
3-1	1	0.34
3-2	11	3.69
4-2	45	15.10
4-3	29	9.73
5-2	1	0.34
5-3	7	2.35
6-2	1	0.34
6-3	3	1.01
6-4	1	0.34
6-5	3	1.00
>6-1	1	0.34
>6-3	1	0.34
>6-4	3	1.00
>6-5	1	0.34
>6-6	1	0.34

The question relating to internet access was »having free and unlimited access to the internet on board«, and the possible answers were dichotomous: yes and no.

Job satisfaction was assessed with the scale of Overall Job Satisfaction (25), consisting of five statements to which participants expressed their agreement on a scale from 1 to 5 (1 = strongly disagree; 5 = strongly agree). Examples of items: "I feel fairly satisfied with my present job", "Each day at work seems like it will never end" (reverse-coded item).

The Satisfaction with Life Scale (26-28) was used as a known, valid, and reliable measure of global cognitive judgments of one's life satisfaction. It consists of five items (e.g. "In most ways my life is close to my ideal", "If I could live my life over, I would change almost nothing"). The version with a 5-point scale (from 1=strongly disagree to 5=strongly agree) of answers (29) was used.

Mental health was measured by the five-item version of the Mental Health Inventory, MHI-5 (30), which assesses the frequency of five domains of mental health on a six-point scale (1 = all the time to 6 = never). These domains include anxiety ("How much of the time have you been a very nervous or anxious person?"), general positive affect ("How much of the time have you felt calm or peaceful?", "How much of the time have you been a happy person?"), depression ("How much of the time have you felt downhearted or blue?"), and behavioural/emotional control ("How often have you felt so down in the dumps that nothing could cheer you up?"). The MHI-5 is considered a brief measure of depressive symptoms and feelings of anxiety. The two general positive-affect items are reverse-coded and summed with the other three items to give a composite score on the scale, with higher scores indicating better overall mental health.

Gastrointestinal (digestive) symptoms and cardiovascular symptoms were measured by two subscales of the Physical Health Questionnaire section of the Shiftwork Survey, which was developed as a shorter version of the Standard Shiftwork Index (31). Each subscale consists of eight questions, with response options ranging from 1 = almost never to 4 = almost always. Examples of items: "How often do you suffer from constipation or diarrhoea?", "How often is your appetite disturbed?" (gastrointestinal symptoms); "Have you ever been aware of your heart beating irregularly?", "Do you suffer from shortness of breath when climbing the stairs normally?" (cardiovascular symptoms). A total score is computed for

Table 3 *Descriptive statistics for different aspects of contract and access to free, unlimited internet on board*

		N	%
Time on board	2 months	38	12.75
	3 months	125	41.49
	4 months	106	35.57
	5 months or more	29	9.73
Ratio between working and free days	Favourable (=)	188	63.09
	Unfavourable (≠)	110	36.91
Compliance with contract	Regular shifts	232	77.85
	Irregular shifts	66	22.15
Free unlimited internet access	No	134	44.29
	Yes	164	55.03

each scale by summing the individual scores, and a higher score on each scale is associated with poorer physical health.

All the scales used had good internal consistency in our sample, with alpha coefficients ranging from 0.82 to 0.90 (See Table 4 for a summary).

Statistics

In order to test the effects of different aspects of contract and internet access by parametric statistical analysis (ANOVA), we first checked the normality of our scale data. Visual inspection of histograms and skewness and kurtosis measures (Table 4) show that job- and life-satisfaction distributions and mental health are moderately negatively skewed. Mental-health distribution was also leptokurtic. The distributions of gastrointestinal and, especially, cardiovascular symptoms were positively skewed. These results are expected, since our participants are derived from a healthy population of seafarers who are subjected to regular medical examination. Moreover, one-way ANOVA is considered a robust test against the normality assumption, i.e. tolerates violations to its normality assumption rather well. Schmider et al. (32) showed that type error α and type error β remain constant under violation. However, in the case of the highly skewed distribution of cardiovascular symptoms, we transformed this variable by logarithmic method using Log(10) (33, 34) in order to apply ANOVAs.

We tested the effects of (1) the contract duration, (2) ratio between work and non-work days, (3) compliance with the contract, and (4) internet access on five dependent measures: job satisfaction, life satisfaction, mental health, gastrointestinal symptoms, and cardiovascular symptoms-log(10) (Tables 5-9). Before applying ANOVAs, we checked the assumption of homogeneity of variance using Levene's test, and in cases of violation of this assumption we used Welch's test instead of ANOVA. Furthermore, for duration effect we used a *post-hoc* test: Tukey after ANOVAs and Games Howell in cases of assumption of unequal variances.

RESULTS

Time on board had a significant effect on job satisfaction (Table 5), with the highest level of job satisfaction in the shortest duration on board (two months). The Tukey *post-hoc* test indicated that differences among the four categories

Table 4 *Descriptive statistics for job satisfaction, life satisfaction, mental health, gastrointestinal symptoms, and cardiovascular symptoms*

Scale (number of items)	Cronbach alpha	Mean	SD	Median	Theoretical range	Range	Skewness (SE)	Kurtosis (SE)
Job satisfaction (5)	.85	16.62	4.41	17	5-25	5-25	-0.56 (0.14)	-0.00 (0.28)
Life satisfaction (5)	.85	15.23	4.22	16	5-25	5-25	-0.29 (0.14)	-0.34 (0.28)
Mental Health (5)	.82	22.55	3.36	23	5-30	10-30	-0.55 (0.14)	0.75 (0.28)
Gastrointestinal symptoms (8)	.90	13.39	4.53	13	8-32	8-30	0.79 (0.14)	0.20 (0.28)
Cardiovascular symptoms (8)	.89	11.05	3.51	10	8-32	8-25	1.16 (0.14)	0.55 (0.28)
Cardiovascular symptoms^{t}		1.02	0.12	1		0.9-1.4	0.76 (0.14)	-0.60 (0.28)

Note: ^{t} – transformed as log(10)

Table 5 *Effects of different aspects of contract and internet access on job satisfaction*

		Mean	SD	df1 / df2	Levene (p)	F (p)
Time on board (months)	2	18.12	4.03	3/294	0.99 (0.399)	3.42 (0.018)*
	3	16.84	4.10			
	4	15.68	4.74			
	>4	17.19	4.43			
Ratio between working and free days	Favourable (=)	17.20	4.21	1/296	0.53 (0.466)	9.05 (0.003)**
	Unfavourable (≠)	15.63	4.59			
Compliance with the contract	Regular shifts	17.12	4.29	1/296	0.46 (0.498)	13.92 (0.000)***
	Irregular shifts	14.87	4.43			
Free unlimited internet access	No	16.50	4.18	1/296	1.86 (0.174)	0.19 (0.661)
	Yes	16.72	0.661			

*Note: *p<0.05; **p<0.01; ***p<0.001*

of on-board duration were significant only between two and four months (p<0.05). Job satisfaction also differed regarding the other two aspects of the contract. It was higher in the favourable ratio between working and free days, compared to the unfavourable, and in regular shifts, compared to irregular shifts.

All three aspects of the contract had a significant effect on life satisfaction (Table 6). Life satisfaction was, on average, higher in seafarers with shorter on-board duration, favourable ratio of working and free days, and regular shifts. The Games Howell *post-hoc* test showed that the category of the shortest duration (two months) differed significantly from all other duration categories (p<0.01). Contrary to expectation, access to the internet did not have an effect on either satisfaction measure (Tables 5 and 6), but in the case of life satisfaction the level of significance was rather close to 0.05.

Mental health significantly differed with regard to duration of time on board and compliance with the contract, with higher levels in the shortest on-board duration and contract compliance (Table 7). Mental-health level was the highest in the shortest on-board duration (two months), and

Table 6 *Effects of different aspects of contract and internet access on life satisfaction*

		Mean	SD	df1 / df2	Levene (p)	F (p)	Welch
Time on board (months)	2	17.68	3.02	3/294	2.77 (0.042)*		8.84 (0.000)***
	3	15.27	4.06				
	4	14.53	4.41				
	>4	14.45	4.51				
Ratio between working and free days	Favourable (=)	15.69	4.18	1/296	0.02 (0.876)	6.15 (0.014)*	
	Unfavourable (≠)	14.45	4.20				
Compliance with contract	Regular shifts	15.77	3.96	1/296	5.46 (0.020)*		15.49 (0.000)***
	Irregular shifts	13.33	4.57				
Free unlimited internet access	No	14.72	4.26	1/296	0.334 (0.564)	3.60 (0.060)	
	Yes	15.65	4.16				

*Note: *p<.05; ***p<.001*

Table 7 *Effects of different aspects of contract and internet access on mental health*

		Mean	SD	df1 / df2	Levene (p)	F (p)	Welch (p)
Time on board (months)	2	24.11	2.32	3/294	3.33 (0.020)		5.76 (0.001)**
	3	22.13	3.58				
	4	22.52	3.38				
	>4	22.45	3.00				
Ratio between working and free days	Favourable (=)	22.68	3.26	1/296	2.56 (0.111)	0.77 (0.381)	
	Unfavourable (≠)	22.33	3.53				
Compliance with the contract	Regular shifts	22.83	3.11	1/296	6.67 (0.010)*		5.74 (0.019)*
	Irregular shifts	21.56	3.98				
Free unlimited internet access	No	22.21	3.22	1/296	0.21 (.648)	2.417 (.121)	
	Yes	22.82	3.45				

*Note: *p<.05; **p<.01*

this level differed significantly, according to the Games Howell *post-hoc* test, from that of three months (p<0.01) and four months (p<0.05). Neither the ratio between working and free days nor access to the internet had a significant effect on mental health (Table 7).

Levels of gastrointestinal symptoms (Table 8) were significantly lower in shorter on-board duration (The Games Howell post-hoc test indicated a difference only between two and four months, p<0.05), and in seafarers with access to free and unlimited internet on board, compared to those without it. The ratio between working and free days did not have an effect on gastrointestinal symptoms, while the effect of compliance with the contract was close to significance (p=0.051).

Of the four tested effects, only the internet access had a significant effect on cardiovascular symptoms (Table 9), with a higher level of symptoms in seafarers who did not have free, unlimited access to the internet on board. Since a transformed variable of cardiovascular symptoms was used, we checked the transformed and original means of the two categories of the internet access to be sure that the direction of difference was the same (33).

Table 8 *Effects of different aspects of the employment contract and internet access on gastrointestinal symptoms*

		Mean	SD	df1 / df2	Levene (p)	F (p)	Welch (p)
Time on board (months)	2	12.05	3.07	3/294	6.67 (0.000)***		3.39 (0.021)*
	3	13.71	4.85				
	4	13.00	4.06				
	>4	15.17	5.71				
Ratio between working and free days	Favourable (=)	13.39	4.54	1/296	0.468 (0.494)	0.00 (0.996)	
	Unfavourable (≠)	13.39	4.53				
Compliance with the contract	Regular shifts	13.11	4.45	1/296	0.90 (0.344)	3.83 (0.050)	
	Irregular shifts	14.35	4.72				
Free unlimited internet access	No	14.13	4.85	1/296	3.53 (0.061)	6.71 (0.010)*	
	Yes	12.78	4.18				

*Note: *p<.05; ***p<.001*

DISCUSSION

The results of this study showed the importance of two specific psychosocial work characteristics – employment contract and the internet access – in the explanation of satisfaction and self-rated health measures in seafarers. More precisely, job and life satisfaction levels were higher for shorter duration on board, favourable ratio of work to non-work days, and compliance with the contract regarding the changes in work and non-work days (regular shifts). Mental health was, on average, higher for shorter contracts and regular shifts. Of the two physical-health measures, only gastrointestinal symptoms were affected by the contract characteristics. The level of gastrointestinal symptoms was lower in those seafarers with shorter duration on board, and in those working in regular shifts. Both measures of physical health differed with regard to the internet access. Lower levels of gastrointestinal and cardiovascular symptoms were found in seafarers who have free, unlimited internet access on board. Although not all, our hypotheses were largely confirmed. Surprisingly, although on the basis of the qualitative study (12) we expected that the internet accessibility would have significant effects on satisfaction measures, these effects trailed.

In interpreting the results obtained we have to bear in mind that our design was cross-sectional, so it is not possible to draw causal conclusions from it. For example, self-rated health measures are largely affected by other factors besides the tested work characteristics, such as lifestyle factors (14, 15). Furthermore, it is possible that some other work characteristic which we did not include relates to the tested characteristics of the employment contract and/or internet accessibility, so higher satisfaction and better health status may be the consequences of some other favourable characteristic(s) of the shipping work environment (e.g. better organisation of work on board, i.e. fewer working and more resting hours, more favourable opportunities for physical and social activities, healthier food on board, etc.). A further limitation of our cross-sectional design is a possible underestimation of satisfaction and health levels due to the healthy worker effect.

Another possible threat to the validity of the data stems from the fact that we could not monitor the identities and truthfulness of participants in our online survey. However, we do not have any reason to believe that our data was flawed in any way. Studies which compare traditional (paper-and-pencil) and online surveys have shown that the results obtained by internet surveys do not appear to be tainted by false data or repeat responders, and they are, so far, consistent with results from traditional methods (35, 36). Still, our data may suffer from the general disadvantages of self-report measures, such as social-desirability bias and unwillingness to reveal private details about one's feelings or attitudes regarding the job. However, we believe that, with the anonymous procedure for participating in the online survey, and with instructions and explanation relating to the study procedure being obtained from a neutral researcher, these effects should be minimised. On the other hand, it still might be possible that participants have overstated their self-reports in some direction. Furthermore, it should be emphasised that the self-reported measures of gastrointestinal and cardiovascular symptoms assess only some of the symptoms that are included in the used scales, which of course does not imply an objective diagnosis of gastrointestinal or cardiovascular disease. Further research should therefore check the associations found in this study

Table 9 *Effects of different aspects of contract and internet access on cardiovascular symptoms*

		Mean	SD	df1 / df2	Levene (p)	F (p)	Welch (p)
Time on board (months)	2	10.45	2.50	3/294	6.08 (0.001)**		0.63 (0.597)
	3	11.09	3.37				
	4	10.95	3.64				
	>4	12.00	4.60				
Ratio between working and free days	Favourable (=)	10.91	3.33	1/296	1.71 (0.192)	0.48 (0.488)	
	Unfavourable (≠)	11.27	3.80				
Compliance with the contract	Regular shifts	10.86	3.30	1/296	2.56 (0.110)	2.24 (.136)	
	Irregular shifts	11.70	4.14				
Free unlimited internet access	No	11.53	3.72	1/296	2.16 (0.143)	4.79 (0.029)*	
	Yes	10.65	3.29				

*Note: *p<.05; **p<.01*

by using objective parameters of health. Finally, the lack of data on the response rate, and the questionable representativeness of the target population, explained in the Method section, may also be an important limitation. More precisely, since the study relied on voluntary participation, the data may be biased, but we cannot test whether there is any systematic difference between our participants and non-participants.

In spite of the limitations noted, our design also had some advantages. Although the homogeneity of our sample (Croatian officers employed on cargo ships) limits the external validity, i.e. generalisability of our findings, we consider it an advantage which improves the internal validity, since we have avoided possible confounding effects of culture/nation, rank, and type of maritime activity. Further, we consider our total N to be relatively large, while considering seafarers a hard-to-reach population (7). Finally, we included only those seafarers who had been with the same company for a minimum of two years, since we believed that some duration was needed to be able to test the effects of work characteristics on the outcome measures. However, in spite of the rational reasons for this decision, in this way we avoided seafarers who had left their previous company (within the previous two years) for some reason. Unfavourable characteristics of the work contract and internet non-accessibility might be among their reasons.

Conclusions and practical implications

To our knowledge, this was the first study which tested the effects of different characteristics of the employment contract and internet accessibility on different measures of satisfaction and health of seafarers.

In spite of the fact that the significant effects obtained only show relations between more favourable work environments, in respect of the contract and internet accessibility and satisfaction and health measures, we consider these relations very important. Living and working in an isolated environment for lengthy periods of time, especially in situations of not knowing for sure when one will go home, and without internet accessibility, may act as strong psychosocial stressors in seafarers. Although the mechanisms of relations between a stressful environment and well-being and health measures may be explained by some other untested characteristic of the work environment, or by mediator variables, such as maladaptive coping strategies related to an unhealthy life style (15), or personal hardiness (7), this does not diminish the importance of the obtained associations. In fact, the results of this study can be considered a strong starting point for further research in the field, which should verify the associations obtained in this study by using more objective measures and representative samples.

Considering all mentioned limitations of this cross-sectional study, the practical implication that can be given on the basis of the obtained results is that in promoting satisfaction and health in seafaring, attention should be given to reducing on-board duration, compliance with the contract regarding working and non-working days, and internet accessibility on board. This primarily relates to managers of shipping companies who should be aware of the importance of work environments, in respect of the contract and internet accessibility, for seafarers' satisfaction and health. Reducing long on-board duration and improving regularity of shifts, as well as providing free and unlimited internet access on board, as primary level interventions of occupational stress management may be important for the overall satisfaction and health in seafarers. From the organisational perspective, promoting work-related well-being and health of seafarers may also have important repercussions for retention of seafarers and minimising organizational costs of turnover.

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Ethical approval

The study was approved by the Ethical Committee of the Department of Psychology, University in Zadar.

Declaration of authorship

AS and ZP contributed to the conception and design of the study, and to data acquisition. AS contributed to data analysis and to interpretation of data. AS and ZP drafted the manuscript.

Competing interests

Authors declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work; no other relationships or activities that could appear to have influenced the submitted work.

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Provjera povezanosti različitih aspekata ugovora i dostupnosti interneta na brodu sa zadovoljstvom poslom i životom te zdravljem pomoraca

Cilj ovoga istraživanja bio je provjeriti povezanosti između različitih aspekata ugovora i dostupnosti interneta na brodu sa zadovoljstvom i zdravljem pomoraca. *Online* upitnik ispunilo je 298 hrvatskih pomoraca časničkoga ranga, zaposlenih na teretnim brodovima s radnim iskustvom u trenutačnoj radnoj organizaciji od najmanje dvije godine. Upitnik je uz pitanja o osnovnim socio-demografskim karakteristikama uključivao pitanja vezana za radni ugovor i dostupnost interneta na brodu te mjere zadovoljstva poslom, zadovoljstva životom, psihičkog zdravlja, gastrointestinalnih i kardiovaskularnih simptoma. Prosječne razine zadovoljstva poslom i životom bile su veće kod kraćih ugovornih razdoblja na brodu, povoljnijeg omjera radnih i neradnih dana te poštovanja ugovora u pogledu izmjena radnih i neradnih dana. Razina psihičkoga zdravlja razlikovala se u istom smjeru, ali samo za dva aspekta ugovora: dužinu razdoblja na brodu i poštovanje ugovora. Razina gastrointestinalnih simptoma bila je manja kod pomoraca koji su na brodu provodili kraća razdoblja i kod kojih je poštovan ugovor te kod pomoraca s neograničenim pristupom internetu na brodu, kod kojih je utvrđena i niža razina kardiovaskularnih simptoma. U promicanju zadovoljstva i zdravlja pomoraca pažnja bi se trebala usmjeriti na smanjivanje ugovornih razdoblja na brodu, poštovanje ugovora te poboljšavanje dostupnosti interneta na brodu.

KLJUČNE RIJEČI: *gastrointestinalni i kardiovaskularni simptomi; izolirano radno okruženje; omjer radnih i neradnih dana; poštovanje ugovora; psihičko zdravlje*; *ugovorno razdoblje na brodu*

ANNEX: QUESTIONNAIRE

Please answer the following questions related to your demographic and work characteristics as accurately as possible.

Gender: Male / Female
Age (years)
Service length in maritime sector (years)
Service length in current shipping organisation (years)
Education level: 1) High school, 2) College, 3) University
Job description: 1) Engine Officer, 2) Deck Officer, 3) Chief engineer, 4) Commander
Type of cargo ship: 1) General cargo, 2) Tankers (oil and oil products), 3) Bulk cargo, 4) Containers, 5) LNG and LPG, 6) Chemicals, 7) Ro-Ro (cargo on wheels), 8) Combined cargo
Shipping organisation: 1) Croatian, 2) Foreign
Marital status: 1) Single, 2) Married / in domestic partnership
Children (number)
Current location: 1) On-board, 2) At home
On-board stay according to contract: 1, 2, 3, 4, 5, 6, more than 6 months
Home stay according to contract: 1, 2, 3, 4, 5, 6, more than 6 months
Compliance with contract regarding changes to ship and home periods: 1=always in compliance with contract; 2=mostly in compliance with contract; 3=mostly not in compliance with contract; 4=never in compliance with contract
Having free and unlimited access to the internet on board: 1) Yes, 2) No

The following items relate to your evaluation of your life and job. Using the scale below, indicate your level of agreement with each item by ticking the appropriate number.

1=strongly disagree; 2=mostly disagree;
3=neither agree nor disagree; 4=mostly agree;
5=strongly agree

1.	In most ways my life is close to my ideal.	1 2 3 4 5
2.	The conditions of my life are excellent.	1 2 3 4 5
3.	I am satisfied with my life.	1 2 3 4 5
4.	So far I have gotten the important things I want in life.	1 2 3 4 5
5.	If I could live my life over, I would change almost nothing.	1 2 3 4 5
6.	I feel fairly satisfied with my present job.	1 2 3 4 5
7.	Most days I am enthusiastic about my work.	1 2 3 4 5
8.	Each day at work seems like it will never end.	1 2 3 4 5
9.	I find real enjoyment in my work.	1 2 3 4 5
10.	I consider my job to be rather unpleasant.	1 2 3 4 5

For each following question indicate the number on the scale that best describes you during the past month.

1=all of the time; 2=most of the time;
3=a good bit of the time; 4=some of the time;
5=a little of the time; 6=none of the time / never

	How much of the time have you…	
1.	… been a very nervous or anxious person?	1 2 3 4 5 6
2.	… felt calm or peaceful?	1 2 3 4 5 6
3.	… been a happy person?	1 2 3 4 5 6
4.	… felt downhearted or blue?	1 2 3 4 5 6
5.	… felt so down in the dumps that nothing could cheer you up?	1 2 3 4 5 6

Please answer the following questions by indicating the appropriate number.

1=almost never; 2=quite seldom;
3=quite often; 4=almost always

	How often…				
1.	… is your appetite disturbed?	1	2	3	4
2.	… do you have to watch what you eat to avoid stomach upsets?	1	2	3	4
3.	… do you feel nauseous?	1	2	3	4
4.	… do you suffer from heartburn or stomach-ache?	1	2	3	4
5.	… do you complain of digestion difficulties?	1	2	3	4
6.	… do you suffer from bloated stomach or flatulence?	1	2	3	4
7.	… do you suffer from pain in your abdomen?	1	2	3	4
8.	… do you suffer from constipation or diarrhoea?	1	2	3	4
9.	… do you suffer from heart palpitations?	1	2	3	4
10.	… do you suffer from aches and pains in your chest?	1	2	3	4
11.	… do you suffer from dizziness?	1	2	3	4
12.	… do you suffer from sudden rushes of blood to your head?	1	2	3	4
13.	… do you suffer from shortness of breath when climbing the stairs normally?	1	2	3	4
14.	… have you been told that you have high blood pressure?	1	2	3	4
15.	… have you been aware of your heart beating irregularly?	1	2	3	4
16.	… do you feel "tight" in your chest?	1	2	3	4

Letter to the Editor

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Gastric decontamination in aluminium phosphide poisoning: a case against the use of water-based solutions

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Dear Editor-in-Chief,

We read with great interest the article entitled "A review of aluminium phosphide poisoning and a flowchart to treat it" written by Hashemi-Domeneh et al. (1) and recently published in the *Archives of Industrial Hygiene and Toxicology*. The authors mentioned performing a gastric lavage using a solution of potassium permanganate (1/10000/1 g per 10 L) and administering activated charcoal (1 g kg^{-1} in children and 50-100 g in adults) and sodium bicarbonate (two 44 meq vials [100 mL] per L) as options for gastrointestinal decontamination, claiming that these compounds can be effective at decreasing toxic phosphine (PH_3) production. However, a closer look at the list of references in the paper indicated that none of these protocols have yet been clinically studied.

What drew our attention is that these solutions are composed of water. Therefore, they can facilitate PH_3 gas release from aluminium phosphide (AlP) (2). To document this phenomenon, we added a 5 g tablet of Bhostoxin® into 50 mL solutions of sodium bicarbonate (44 meq), potassium permanganate (0.005 g; 1/10000 solution), activated charcoal (10 g), and castor oil (see Figure 1).

The other important points recently considered by scientists and which speak in favour of our argument include an exothermic reaction that follows potassium permanganate administration (3, 4), induction of haemolysis and methemoglobinemia due to the oxidizing properties of potassium permanganate (5, 6), inconceivability of PH_3 oxidation following administration of potassium permanganate considering that it is a hard nucleophile (7), and inefficiency of charcoal in AlP adsorption and prevention of PH_3 release (8). On the other hand, *in vitro* studies have proposed that liquid vegetable oils and paraffin are effective in the prevention of phosphine fumigation (9), which has been supported by a successful management of acute AlP poisoning in a case report as well as in an animal study (10, 11).

To conclude, solutions composed of water should not be used for gastric decontamination after acute AlP poisoning. Instead, using vegetable oils for gastric lavage or castor oil to inhibit greater PH_3 release as well as stimulation of luminal evacuation (2) could be practical. However, this idea has not been evaluated in a properly designed study. Instead of gastric lavage, we have the experience of giving 60 mL of castor oil orally for gastrointestinal decontamination in all of the cases of acute AlP toxicity presented to the three main university hospitals in Shiraz, Iran, within the last three years. This appears to be a good starting point. Hence, the evaluation of its efficacy in a randomized clinical trial should be the next step.

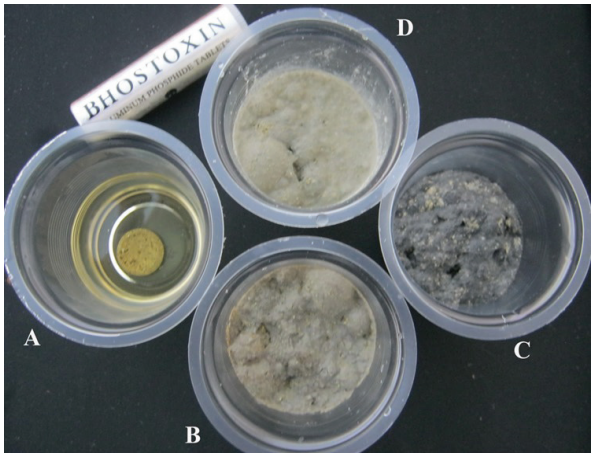

Figure 1 *Phosphine gas release from Bhostoxin® tablets in contact with water, regardless of compound. The release of PH_3 from the AlP tablets results in the formation of a greenish foamy layer on the surface of the solutions. The formation of PH_3 gas bubbles did not occur only in the castor oil container. A: castor oil, B: potassium permanganate solution, C: activated charcoal solution, D: sodium bicarbonate solution*

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IN MEMORIAM

Dr. sc. Spomenka Telišman
(1945.-2016.)

Dr. sc. Spomenka Telišman, umirovljena znanstvena savjetnica Instituta za medicinska istraživanja i medicinu rada, preminula je u Zagrebu, 3. studenoga 2016. godine. Bila je istaknuta znanstvenica s međunarodno priznatim dostignućima u području toksikologije metala, a osobito će biti zapamćena po istraživanjima vezanim uz učinke metala na reprodukcijsko zdravlje muškaraca.

Spomenka Telišman (rođena Pavičić) završila je osnovnu školu i gimnaziju u Zagrebu. Diplomirala je na Kemijskom odjelu Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu 1971. godine, a postdiplomski studij Analitičke kemije završila je 1974. godine. Na znanstvenom usavršavanju iz analitike i toksikologije metala u ljudi boravila je tijekom 1979. godine u Engleskoj *(Regional Toxicology Laboratory, Dudley Road Hospital, Birmingham; Trace Metal Unit, Department of Chemical Pathology and Human Metabolism, Southampton General Hospital, Southampton; Health and Safety Executive Occupational Medicine Laboratories, London*), gdje je imala prilike raditi s vodećim znanstvenicima u tom području, prof. Barbarom Clayton i dr. Trevorom Delvesom. Doktorirala je na Sveučilištu u Zagrebu 1983. godine obranivši doktorski rad pod naslovom „Djelovanje olova na endogeni karbonilhemoglobin". U zvanje znanstvene suradnice izabrana je 1984. godine, a znanstvenom savjetnicom u trajnom zvanju postala je 1998. godine.

Cijeli svoj radni vijek provela je u Institutu za medicinska istraživanja i medicinu rada gdje se 1971. godine zaposlila u Kliničko-toksikološkom laboratoriju (danas Jedinica za analitičku toksikologiju i mineralni metabolizam) baveći se istraživanjima u području zdravstvene ekologije i medicine rada do umirovljenja 2007. godine.

Njena je znanstvena aktivnost uključivala istraživanja vezana uz procjenu profesionalne i okolišne izloženosti ljudi metalima (osobito olovu i kadmiju) primjenom humanog biološkog monitoringa (engl. *human biomonitoring*, HBM), učinaka toksičnih i esencijalnih metala i njihovih interakcija na zdravlje ljudi, te izbor optimalnog reprezentativnog biološkog biljega (biomarkera) u procjeni opterećenosti organizma metalima (osobito olovu) i njihovih zdravstvenih učinaka. Kada je sredinom 1980-tih godina Institut pozvan na sudjelovanje u međunarodnom projektu WHO/UNEP-a pod nazivom "*Human Exposure Assessment Locations* (HEALs)", usmjerenom na procjenu ukupne izloženosti ljudi različitim onečišćenjima iz okoliša u različitim zemljama, dr. Telišman je, kao član grupe istraživača, sudjelovala u međunarodnoj pilot-studiji procjene izloženosti ljudi olovu i kadmiju "*Exposure Monitoring of Lead and Cadmium: An International Pilot Study within the WHO/ UNEP Human Exposure Assessment Location (HEAL) Programme*".

Pionirsku ulogu u nas i u svijetu dr. sc. Telišman imala je u istraživanjima učinaka olova i kadmija i njihovih interakcija s esencijalnim elementima na reprodukcijsko zdravlje muškaraca. Isticala je važnost istovremenog razmatranja kombiniranog utjecaja različitih toksičnih i esencijalnih elemenata, kao i drugih čimbenika čovjekova okoliša i/ili životnih navika. Značaj tih istraživanja prepoznat je i financijski podržan te je dr. Telišman vodila nekoliko domaćih i međunarodnih znanstvenoistraživačkih projekata. Izdvojila bih samo neke: "An Investigation of Lead Concentration in Mother's Milk in Relation to Blood Lead" (1981.-1983., *Commission of the European Communities),* "The Effect of Lead on the Reproductive System of Males" (1986.-1989., *U.S. Environmental Protection Agency*), "The Effect of Lead on the Reproduction Ability in Men" (1989.-1991., *International Lead Zinc Research Organization, ILZRO*), "Učinci metala na reprodukcijsko zdravlje muškaraca" (2002.-2006., MZOS).

Bila je članica Hrvatskog toksikološkog društva te Udruge hrvatskih djelatnika UN sustava (UHDUN) od njihova osnutka. Međunarodna komisija za medicinu rada (*International Commission on Occupational Health, ICOH*) primila je dr. Telišman u svoje članstvo 1988. godine, a od 1994. godine postaje članicom međunarodnog Znanstvenog odbora za toksikologiju metala (*Scientific Commitee on Metal Toxicology*, sjedište pri *ICOH*). Aktivno je sudjelovala u radu brojnih izvršnih odnosno znanstvenih odbora, predsjedavala sekcijama i bila pozvani predavač na nekoliko međunarodnih znanstvenih skupova u području medicine rada i toksikologije metala. Pri tome posebno treba istaknuti njeno višegodišnje članstvo u znanstvenom odboru serije međunarodnih sastanaka o teškim metalima (*International Conference on Heavy Metals in the Environment:* New Orleans 1987., Ženeva 1989., Edinburgh 1991. i Toronto 1993. godine).

Sudjelovala je u nastavi dodiplomskog i poslijediplomskog specijalističkog i doktorskog studija pri Medicinskom fakultetu Sveučilišta u Zagrebu, a svoje znanje i iskustvo prenosila je također kao mentor magistarskih i doktorskih radova, težeći izvrsnosti kako u znanstvenoistraživačkom tako i u stručnom radu.

Pozvani je autor ili koautor poglavlja u enciklopedijskim izdanjima (npr. *Toxicology: Effects of age, sex and other factors.* U: Stellman JM (ur.) Encyclopaedia of Occupational Health and Safety, Geneva: ILO 1988; *Reproductive and developmental toxicity of metals.* U: Nordberg GF, Fowler BA, Nordberg M, Friberg LT. (ur.) Handbook on the Toxicology of Metals, Amsterdam: APE 2007.).

Za svoj istaknuti znanstveni rad, dr. sc. Telišman nagrađena je godišnjom nagradom Instituta za medicinska istraživanja i medicinu rada 2007. godine za rad s najvećim znanstvenim odjekom. Njezin znanstveni članak "Semen quality and reproductive endocrine function in relation to biomarkers of lead, cadmium, zinc, and copper in men", objavljen 2000. godine u *Environmental Health Perspectives*, postigao je zapažen odjek u međunarodnoj znanstvenoj zajednici (71 citat do kraja 2006. godine prema WoS-u). Izvrsnost rezultata istraživanja prikazanih u spomenutom članku potvrđuje se i danas (do kraja 2016. godine citiran je više od 195 puta prema WoS-u).

Kao vrsni stručnjak u području toksikologije metala i njihovih učinaka na zdravlje, dr. sc. Spomenka Telišman svojim je postignućima zauzela važno mjesto u hrvatskoj i svjetskoj znanosti.

Alica Pizent

REPORT

V. hrvatski toksikološki kongres s međunarodnim sudjelovanjem
Poreč, 9.-12. listopada 2016.

Hrvatsko toksikološko društvo (HTD) organiziralo je V. hrvatski toksikološki kongres s međunarodnim sudjelovanjem (CROTOX 2016). Kongres je održan u hotelu Valamar Diamant u Poreču 9.-12. listopada 2016. pod pokroviteljstvom Instituta za medicinska istraživanja i medicinu rada i Hrvatskog zavoda za toksikologiju i antidoping te uz financijsku pomoć Hrvatske akademije znanosti i umjetnosti, Grada Zagreba i sponzora.

Rad CROTOX-a 2016 bio je podijeljen u nekoliko sekcija čije teme pokrivaju značajna područja toksikologije: *Drugs of Abuse and Antidotes, Exposure and Risk Assessment, Regulatory Toxicology, Food Toxicology, Genotoxicity, Toxicology of Metals and Nanotoxicology and Ecotoxicology*. Znanstveni odbor CROTOX-a 2016. odabrao je poznate znanstvenike za pozvane predavače koji su se ljubazno odazvali pozivu.

Osim pozvanih predavača predavanja su održali i predavači s kraćim izlaganjima kao i šest mladih znanstvenika kojima je Predsjedništvo HTD-a dodijelio nagrade za najbolje sažetke kako bi mogli sudjelovati na kongresu. Ti su mladi znanstvenici prema uvjetima za dodjelu nagrada održali predavanja o svom radu u trajanju od petnaest minuta. Predavanja svih šest mladih znanstvenika bila su tako dobra da se čini da je budućnost toksikologije u Hrvatskoj osigurana. Osim toga održana je i posterska sekcija na kojoj je prikazano 58 radova koji su bili razvrstani jednako kao i predavanja.

Sažeci svih radova tiskani su u Knjizi sažetaka kao dodatni broj časopisa Arhiva za higijenu rada i toksikologiju (Vol. 67/Suppl. 1). Svim sudionicima kongresa podijeljena je Knjiga sažetaka.

Na CROTOX-u 2016 bilo je 129 sudionika, 17 pozvanih predavača (šest iz Hrvatske i 11 iz inozemstva) i 15 predavača s kraćim izlaganjima (sedam iz Hrvatske i osam iz inozemstva). U toj su skupini i šest mladih znanstvenika (troje iz Hrvatske i troje iz inozemstva) koji su dobili nagradu Predsjedništva HTD-a za najbolji sažetak. Na kongresu CROTOX 2016 dodijeljena je i jedna nagrada Povjerenstva za odabir najboljeg postera.

Održavanje kongresa važno je zato što na kongresima znanstvenici imaju mogućnost prikazati rezultate svojih istraživanja i o njima raspraviti s drugim znanstvenicima. Osim toga na kongresima se upoznaju mladi znanstvenici međusobno kao i s eminentnim znanstvenicima. Međusobno poznavanje posebno je važno prigodom prijavljivanja bilo nacionalnih bilo međunarodnih projekata.

Smatram da je CROTOX 2016 bio vrlo uspješan kongres te će HTD s veseljem organizirati sljedeći kongres 2020. godine.

Predsjednica Hrvatskog toksikološkog društva:
dr. sc. Maja Peraica, dr. med., znanstv. savjetnica

ANNOUNCEMENT

Jedanaesti simpozij Hrvatskoga društva za zaštitu od zračenja s međunarodnim sudjelovanjem
„Etika i kultura u zaštiti od zračenja“
Osijek, Hotel Osijek, 5.-7. travnja 2017.

U organizaciji Hrvatskoga društva za zaštitu od zračenja (HDZZ), uz glavne suorganizatore: Odjel za fiziku Sveučilišta u Osijeku, Institut Ruđer Bošković, Institut za medicinska istraživanja i medicinu rada i Državni zavod za radiološku i nuklearnu sigurnost, u gradu Osijeku (u Hotelu Osijek) od 5. do 7. travnja 2017. održati će se Jedanaesti simpozij Hrvatskoga društva za zaštitu od zračenja s međunarodnim sudjelovanjem s naglaskom na etiku i kulturu u zaštiti od zračenja.

Ovim se simpozijem nastavlja dugogodišnja tradicija organiziranog okupljanja znanstvenika i stručnjaka različitih profila radi razmjene novih informacija i prikaza aktualnih saznanja na području zaštite od zračenja. Na skupovima koje organizira HDZZ tradicionalno sudjeluju znanstvenici i stručnjaci iz domaćih znanstvenoistraživačkih i državnih institucija, iz zdravstva i gospodarstva te sa sveučilišta, ali i inozemni predavači te izlagači.

Rad simpozija odvijat će se u okviru sljedećih tema: Opće teme u znanosti o zračenju i zaštiti od zračenja; Etika i kultura u zaštiti od zračenja; Zaštita od zračenja u medicini; Biološki učinci zračenja; Dozimetrija zračenja; Instrumentacija i mjerne tehnike; Radioekologija; Izloženost stanovništva zračenju; Radon; Neionizirajuća zračenja. Tijekom Simpozija održati će se i godišnja, ovoga puta i izborna skupština HDZZ-a.

Do 1. veljače 2017. kotizacija za članove HDZZ-a za sudjelovanje na skupu iznosi 1200 kn, a 1500 kn za ostale sudionike (nakon 1. veljače 2017. i na simpoziju 1500 kn za članove, odnosno 1800 kn za ostale). Plaćanjem kotizacije sudionik stječe pravo na sve tiskane materijale simpozija, Zbornik radova, koktel dobrodošlice, kavu ili čaj tijekom odmora i svečanu večeru. Prateća osoba plaća pola kotizacije. Svi sudionici simpozija s plaćenom kotizacijom dobit će potvrdu o sudjelovanju i odgovarajući broj bodova prema odluci Povjerenstva za trajno usavršavanje Hrvatske liječničke komore. Službeni jezici skupa su hrvatski i engleski (bez prevođenja).

Sve informacije o rezervaciji smještaja u hotelu Osijek, kao i sve ostale informacije o Simpoziju mogu se pronaći na poveznici *http://hdzz11.fizika.unios.hr/*

Svi koji žele sudjelovati u radu simpozija mogu se prijaviti elektroničkom poštom na adresu: *hdzz11@fizika.unios.hr* putem prijavnog obrasca koji je dostupan i na mrežnoj stranici Društva, na poveznici *http://www.hdzz.hr*. Prihvaćeni i u roku pristigli radovi bit će tiskani u *Zborniku radova* prije održavanja simpozija. Rok za predaju radova u konačnom obliku je 15. veljače 2017.

Znanstveni odbor simpozija vodi izv. prof. dr. sc. Vanja Radolić, a Organizacijski odbor vodi doc. dr. sc. Marina Poje Sovilj s Odjela za fiziku Sveučilišta u Osijeku (Trg Ljudevita Gaja 6, 31 000 Osijek) od kojih se mogu dobiti sve dodatne informacije o skupu (tel: +385 31 232 727, fax: +385 31 232 701 i e-adresa: *hdzz11@fizika.unios.hr*).

Marina Poje Sovilj
Vanja Radolić

CORRIGENDUM

Corrigendum

We have discovered a technical analytical error in our study ***Dzhambov AM, Dimitrova DD. Exposure-response relationship between traffic noise and the risk of stroke: a systematic review with meta-analysis. Arh Hig Rada Toksikol 2016;67(2):136-51 (DOI: 10.1515/aiht-2016-67-2751).*** When transforming categorical risk estimates into linear estimates for inclusion in the meta-analysis using the vwls and glst STATA commands, we did not adequately transpose the exposure levels before inputting them into the software. As a result, the pooled effect of road traffic noise, reported as RR=1.01 (0.96, 1.06), should read **RR=1.03 (0.87, 1.22)**, and the effect of air traffic noise, reported as RR=1.01 (1.00, 1.02), should read **RR=1.05 (1.00, 1.10)**. Even though this error entails minor corrections throughout the text, it does not affect the overall interpretation of the study results. The revised figures show that the pooled risk estimates for stroke were very similar to those previously reported by Vienneau et al. (see ref. 3 in the article) for ischaemic heart disease. We regret any inconvenience we may have caused to the readers and the publisher.

Angel Dzhambov and Donka Dimitrova

Editor's note on the corrigendum:

Considering that there were many minor corrections made throughout the article, we have decided to replace the entire online article with the corrected/updated version, which is available at our online publisher's website (https://www.degruyter.com/view/j/aiht.2016.67.issue-2/aiht-2016-67-2751/aiht-2016-67-2751.xml) and our Croatian repository website (http://hrcak.srce.hr/file/235677). We apologise to our printed version readers for being unable to provide the corrected version in print. Instead, we suggest that you visit the above links and retrieve the updated article.

Nevenka Kopjar, Editor in Chief