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Cover page:
A piece of parched land. Photo by Radojko Orlić taken at the bank of Lake Jarun in Zagreb. After a dry summer spell, this issue brings a shower of interesting new articles.
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Scientific Paper

EFFECTS OF ORAL AND INTRAPERITONEAL MAGNESIUM TREATMENT AGAINST CADMIUM-INDUCED OXIDATIVE STRESS IN PLASMA OF RATS

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Cadmium (Cd) has been recognised as one of the most important environmental and industrial pollutants, and up-to-date investigations have shown that one of the mechanisms of its toxicity is associated with the induction of oxidative stress. The aim of this study was to determine the connection between acute oral and intraperitoneal exposure to Cd and parameters indicative of oxidative stress in the plasma of rats, as well as to examine the potential protective effect of magnesium (Mg) in conditions of acute oral and intraperitoneal Cd poisoning.

The experiment was performed on male albino Wistar rats (n=40) randomly divided into control group, Cd_{or} group that received 30 mg kg^{-1} b.w. Cd by oral gavage, $Cd+Mg_{or}$ group that orally received 50 mg kg^{-1} b.w. Mg one hour before oral Cd, Cd_{ip} group that received 1.5 mg kg^{-1} b.w. Cd intraperitoneally, and $Cd+Mg_{ip}$ group that intraperitoneally received 3 mg kg^{-1} b.w. Mg 10 min before intraperitoneal Cd. The animals were sacrificed 24 h after treatment and the following parameters were measured: superoxide-dismutase activity, superoxide anion, total oxidative status, advanced oxidation protein products, and malondialdehyde.

All parameters of oxidative stress in rat plasma were negatively affected by Cd treatment with more pronounced negative effects after intraperitoneal treatment, with the exception of superoxide dismutase (SOD) activity. Although both oral and intraperitoneal Mg pretreatment had protective effects, more pronounced beneficial effects were observed after oral administration, since it managed to completely prevent Cd-induced changes in the investigated parameters. The observed results support the use of Mg as potential protective agent against toxic effects caused by Cd.

KEY WORDS: *bioelement supplementation, interactions between metals, mechanisms of toxicity*

Cadmium (Cd) has been recognised as one of the most important environmental and industrial pollutants of both natural and anthropogenic origin. The production and utilisation of Cd result in professional exposure to Cd, while food contamination and cigarette smoking contribute to exposure of the general population. It has also been estimated that exposure to this metal will increase in the decades to come (1). Cadmium has no uniform mechanism of toxicity (2-4). It has been reported that one of the mechanisms is disturbance of prooxidant-antioxidant balance in tissues, which results in increased levels of reactive oxygen species (ROS) and oxidative damage of macromolecules as reviewed by Matović et al. (5). This can lead to various pathological conditions in humans and animals, such as hepatic and renal dysfunction, testicular damage, respiratory disorders, and cancer (6-9).

There are limited experimental data pointing out Mg ability to reduce organ Cd accumulation and its beneficial effects against Cd-altered bioelement levels and parameters of oxidative stress (10-12). The wide safety margin between beneficial and toxic concentrations of Mg in tissues and biological fluids suggests that Mg may be used to prevent and treat Cd poisoning. This potential is of great importance, having in mind that the therapy of Cd poisoning has not yet been established. However, the exact mechanisms of Mg beneficial effects against Cd poisoning are not completely understood.

The aim of this study was to determine how Mg affects parameters of oxidative stress in the blood of rats acutely poisoned with Cd. For this purpose we measured plasma enzyme superoxide dismutase (SOD) activity, superoxide anion ($O_2^{\cdot-}$) content, total oxidative status (TOS), malondialdehyde (MDA), and advanced oxidation protein products (AOPP) levels. Two routes of Cd exposure were chosen on the basis of two possible scenarios of human exposure to Cd: oral (or) as a model for exposure via food and water, and intraperitoneal (ip) as a model for parenteral exposure, i.e. inhalation. Our previous study (13) has shown that oral pretreatment with Mg had beneficial effects on all parameters of oxidative stress in the liver, suggesting gastrointestinal tract (GIT) as an important site of Cd and Mg interactions. However, the beneficial effect of Mg ip treatment observed in that study showed that competition between Cd and Mg in GIT can not be the only explanation for the protective effects of Mg against Cd poisoning. In this study we investigated the effects of oral and intraperitoneal Mg on parameters of oxidative stress rat plasma 24 h after Cd treatment in order to better understand possible protective mechanisms of Mg pretreatment against Cd toxicity.

MATERIALS AND METHODS

The study protocol was approved by the Ethics Committee of the Military Medical Academy for animal experiments. Procedures involving animals and their care were in compliance with Guidelines for Animal Studies no. 282-12/2002.

Animals

The study included 40 male Wistar rats weighing 170 g to 240 g, which were obtained from the Military Medical Academy, Belgrade. The animals were housed in cages under standard laboratory conditions [temperature (22±2) ºC; relative humidity (50±10) %] with light and dark cycles exchanging every 12 h. They had free access to water and received standard pelleted diet mixture (Veterinary Institute "Subotica", Subotica, Serbia). The diet contained 2.4 mg g^{-1} Mg and 19.2 ng g^{-1} Cd (as determined in our laboratory).

Experimental design

The animals were randomised into five groups of eight rats: control group, Cd_{or}, $Cd+Mg_{or}$, Cd_{ip}, and $Cd+Mg_{ip}$ group. Oral and intraperitoneal doses of Cd producing toxic effects and Mg doses with possible protective effects were chosen on the basis of our previous experiments and literature data (10-15). For oral treatment, $CdCl_2$ and $Mg(CH_3COO)_2$ (Merck, Darmstadt, Germany) were dissolved in distilled water and the Cd_{or} group received a single Cd dose of 30 mg kg^{-1} b.w. by orogastric tube, while the $Cd+Mg_{or}$ group received a single Mg dose of 50 mg kg^{-1} b.w. one hour before receiving oral Cd. A solution for intraperitoneal treatment was obtained in isotonic saline and the total injected volume in the peritoneal cavity was 0.5 mL. Rats in the Cd_{ip} group were intraperitoneally injected a single Cd dose of 1.5 mg kg^{-1} b.w., while rats in the $Cd+Mg_{ip}$ group received a single Mg dose of 3 mg kg^{-1} b.w. 10 minutes before Cd injection. Control animals were unexposed to either Mg or Cd. The animals were killed under ether anaesthesia twenty-four hours after Cd poisoning and blood samples taken from the heart and collected into heparin tubes. One portion of collected blood was used for cadmium, magnesium, and zinc determination, while the other portion was centrifuged at 837x*g* for 15 min and various parameters of oxidative stress were determined in the obtained plasma.

Analytical procedures

All chemicals and reagents were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Luis, USA). Parameters of oxidative stress in blood plasma SOD activity, $O_2^{\cdot-}$, MDA, AOPP, and TOS were measured using an ILAB 300+ analyser (Instrumentation Laboratory, Lexington, Massachusetts).

Plasma SOD (16) was assayed according to the Misra and Fridovich method (17) in which inhibition of epinephrine auto-oxidation by SOD in the examined sample is recorded as an increase in absorbance at 505 nm for 3 min. One unit of SOD activity is defined as

the activity that inhibits auto-oxidation of adrenalin by 50 %. The rate of nitroblue tetrazolium reduction (NBT) was monitored at 515 nm every 15 s within a minute to measure the level of $O_2^{\cdot-}$(18). Lipid peroxidation (LPO) was evaluated by measuring MDA concentration according to a method described by Girroti et al. (19). This assay is based on the formation of a complex between thiobarbituric acid and MDA, which absorbs at 535 nm. AOPP levels, which indicate the level of ROS-mediated protein damage, were determined by the spectroscopic analysis of modified proteins at 340 nm (20) and expressed as chloramine-T equivalents (μmol L^{-1}). Total oxidative status was determined using the method described by Erel (21). The assay is based on the oxidation of ferrous ion to ferric ion in the presence of various oxidant species in acidic medium and the measurement of the formed ferric ion by xylenol orange.

For Cd, Mg, and Zn determination blood samples were mineralised with a mixture of HNO_3 and $HClO_4$ in a ratio 4:1, and Cd concentrations were measured by graphite furnace atomic absorption spectrophotometry (AAS, SpectrAA 220, GTA 110, Varian, Melbourne, Australia), while Mg and Zn concentrations were determined by flame atomic absorption spectrophotometry (AAS, apparatus GBC 932AA, Victoria, Australia). The accuracy and precision of analysis were established by the analysis of these elements in standard reference bovine liver from the National Bureau of Standards (NIST SRM 1577a bovine liver, National Institute of Standards and Technology, Gaithersburg, Maryland, USA).

Statistical analysis

For statistical analysis we used SPSS (version 11.5 for Windows) and MedCalc® 12.1.4. The one-sample Kolmogorov-Smirnov test showed that only Cd and Mg concentrations were normally distributed and for these parameters the results were presented as means±SD and statistical evaluation of the data was performed using a one-way analysis of variance (ANOVA). Other parameters were presented as medians and ranges and statistical evaluation was performed using Kruskal-Wallis nonparametric test followed by post-hoc Conover test for pairwise comparison. Furthermore, Spearman's correlation analysis was used to determine the relationship between SOD activity and Zn levels in blood. Statistical significance was defined as $P<0.05$.

RESULTS

SOD activity

Cadmium given both orally or intraperitoneally decreased SOD activity significantly compared to control values, while no significant difference was observed in SOD activity between the Cd_{or} and Cd_{ip} groups. Oral Mg co-treatment managed to prevent any significant changes in SOD activity since there were no significant difference between the $Cd+Mg_{or}$ group and control group, whereas *i.p.* Mg treatment resulted in higher SOD activity compared with the Cd_{ip} group although SOD activity was still significantly lower than in the control group (Table 1).

$O_2^{\cdot-}$ and TOS levels

Regardless of the route of administration, Cd induced a significant increase in both $O_2^{\cdot-}$ and TOS levels compared to controls, and this increase was much higher in the group that received Cd intraperitoneally for both parameters. Oral Mg managed to completely prevent changes in $O_2^{\cdot-}$ and TOS levels, keeping them within control range. Intraperitoneal Mg co-treatment induced a significant decrease in $O_2^{\cdot-}$ levels compared to the Cd_{ip} group, but could not maintain $O_2^{\cdot-}$ levels within control range. On the other hand, intraperitoneal Mg managed to prevent the increase in TOS levels, as control and $Cd+Mg_{ip}$. TOS levels did not differ significantly (Table 1).

MDA and AOPP levels

Both oral and intraperitoneal Cd treatment resulted in a significant increase in MDA and AOPP levels compared to controls, and these levels were significantly higher in the group treated intraperitoneally when compared to oral treatment. Oral administration of Mg in Cd-poisoned rats significantly decreased the level of both parameters to values observed in control group. Intraperitoneal administration showed the same positive effect on AOPP levels as oral, while MDA levels in the $Cd+Mg_{ip}$ group were significantly lower than in the Cd_{ip} group, but higher than in controls (Table 1).

Cd, Mg, and Zn concentrations

Table 2 shows that Cd blood levels in both intraperitoneally treated groups were significantly higher than in the control and orally treated groups. Oral Cd treatment did significantly affect blood Cd

Table 1 *The effect of Cd or Cd+Mg treatment on the superoxide dismutase (SOD) activity, superoxide anion ($O_2^{\cdot-}$) content, total oxidative status (TOS), malondialdehyde (MDA) and advansed oxidation protein products (AOPP) levels in plasma*

Parameter		Control	Cd_{or}	$Cd+Mg_{or}$	Cd_{ip}	$Cd+Mg_{ip}$
SOD / IU	Median	137[a]	130[b]	138[a]	123[b]	134[c]
	Range	131 to 139	127 to 134	135 to 139	119 to 133	127 to 137
$O^{\cdot-}_2$ / μmol min^{-1} L^{-1} red. NBT	Median	87.5[a]	107[b]	78[a]	284[c]	162[b]
	Range	60 to 99	101 to 128	62 to 96	234 to 370	110 to 190
TOS / μmol L^{-1}	Median	28.3[a]	49[b]	26.6[a]	68.3[c]	24[a]
	Range	15.6 to 39.8	41.6 to 63.2	21.4 to 44.4	46 to 83.8	22.6 to 34.4
MDA / μmol L^{-1}	Median	0.68[a]	1.14[b]	0.68[a]	1.82[c]	1.14[b]
	Range	0.46 to 0.93	0.93 to 1.69	0.51 to 0.93	1.36 to 1.93	1.06 to 1.44
AOPP / μmol L^{-1} of chloramine-T equivalents	Median	42.83[a]	43.78[b]	43.53[a]	45.47[b]	43.27[a]
	Range	41.06 to 43.22	43.28 to 54.94	42.28 to 44.39	43.11 to 47.39	42.22 to 44.56

Control group - non-treated animals, Cd_{or} group - rats intoxicated orally with 30 mg kg^{-1} b.w. Cd; $Cd+Mg_{or}$ group - rats given orally 30 mg kg^{-1} b.w. Cd and 50 mg kg^{-1} b.w. Mg one hour prior to Cd treatment; $Cd_{i.p.}$ group - rats intraperitoneally injected a single dose of 1.5 mg kg^{-1} b.w. Cd; $Cd+Mg_{i.p.}$ group - rats intraperitoneally injected a single dose of 3 mg kg^{-1} b.w. Mg 10 minutes prior to i.p. treatment with Cd. The values are expressed as medians and ranges and statistical evaluation was performed using Kruskal-Wallis nonparametric test followed by post-hoc Conover test for pairwise comparison. Means not sharing the same letter are significantly different ($P<0.05$).

levels, and neither did Cd+Mg co-treatment. Blood Mg was in the control range in all groups. Table 2 also shows changes in blood Zn of control and treated rats. A significant decrease was observed in both the Cd_{or} and Cd_{ip} groups compared to control, with no significant difference between these two particular groups. However, oral Mg administration managed to prevent Cd-induced changes in Zn levels.

DISCUSSION

All investigated parameters of oxidative stress in rat plasma were negatively affected by Cd treatment. These observed changes are in accordance with our previous results and results of other authors who have suggested that Cd toxicity is mediated by decrease in antioxidant enzymes, production of reactive oxygen species and lipid peroxidation (13, 14, 22, 23). However, more pronounced negative effects of Cd, except for SOD activity, were observed in rats that were treated intraperitoneally. This can be explained by significantly higher blood Cd levels in Cd_{ip} than in Cd_{or} group.

Our study shows that Cd influences plasma SOD activity in rats, but that this effect does not depend on the route of exposure. Similarly, no route-dependent changes in SOD activity were found in the liver of Cd-poisoned rats (13). The antagonism between Cd and Zn has been investigated for many decades, and it is known that Cd can replace Zn in many vital enzymatic reactions (24-26). This suggests that Cd may induce changes in SOD activity by interacting with Zn, which is present in the active centre of SOD isoenzymes beside Cu and Mg. Blood Zn levels in

Table 2 *The effect of Cd or Cd+Mg treatment on the Cd, Mg and Zn levels in plasma*

Parameter		Control	Cd_{or}	$Cd+Mg_{or}$	Cd_{ip}	$Cd+Mg_{ip}$
Cd / μg L^{-1}		11.38±3.57 [a]	25.37±4.50 [a]	31.86±9.02 [a]	77.42±35.61 [b]	123.38±26.24 [b]
Mg / mg L^{-1}		33.53±3.29 [a]	33.70±2.59 [a]	35.72±3.34 [a]	35.78±2.62 [a]	34.33±2.29 [a]
Zn / mg L^{-1}	Median	5.65[a]	4.95[b]	5.65[a]	5.23[b]	5.04[b]
	Range	5.42 to 7.6	4.03 to 6.15	5.17 to 6.51	4.95 to 5.68	4.22 to 5.5

Control group - non-treated animals, Cd_{or} group - rats intoxicated orally with 30 mg kg^{-1} b.w. Cd; $Cd+Mg_{or}$ group - rats given orally 30 mg kg^{-1} b.w. Cd and 50 mg kg^{-1} b.w. Mg one hour prior to Cd treatment; Cd_{ip} group-rats intraperitoneally injected a single dose of 1.5 mg kg^{-1} b.w. Cd; $Cd+Mg_{ip}$ group - rats intraperitoneally injected a single dose of 3 mg kg^{-1} b.w. Mg 10 minutes prior to i.p. treatment with Cd. The values for Cd and Mg are expressed as mean values ± S.D. while values for Zn are expressed as medians and ranges. Means not sharing the same letter are significantly different ($P<0.05$).

both the Cd_{or} and Cd_{ip} treated rats were significantly lower than control, but no significant difference was found between routes of exposure, which may explain similar levels of SOD activity in these groups. This suggests that mainly affects SOD through interaction with Zn. This finding is supported by Spearman's correlation analysis that revealed a positive correlation between blood Zn and SOD activity (ρ=0.339, *P*=0.035).

This study was focused on how Mg affects blood parameters of oxidative stress in rats acutely poisoned with Cd. The results show that oral Mg pre-treatment was effective in reducing this stress: SOD activity, $O_2^{\cdot-}$, TOS, MDA, and AOPP were all restored to control values. These results are in accordance with our previous investigations that demonstrated beneficial effects of Mg on parameters of oxidative stress in the liver (13, 27). This protective effect of oral Mg supplementation could be explained by Mg and Cd interactions in the gastrointestinal tract (GIT) where Mg competes with Cd for divalent metal transporters. Recent studies (28, 29) have shown that Mg absorption from the GIT happens through divalent cation channels TRPM 7 and TRPM 6 (transient receptor potential melastation-related 7 and 6), which have been demonstrated to be primarily Mg^{2+} channels, but are also implicated in Cd trafficking. Besides, Mg transporter protein-MagT2 can also be engaged in other divalent cation transport. Recent data (29) also suggested that ancient conserved domain protein 2 is a non-selective divalent cation transporter which favours transfer of divalent cations transport in case of Mg deficiency. Therefore, excessive Mg intake can significantly decrease Cd transport through these channels. However, this principle alone can not explain our results, as there was no significant difference in blood Cd levels between the Cd_{or} and $Cd+Mg_{or}$ groups. Concentration of Cd in these treated groups were even similar to those obtained in control group, but it should be pointed out that Cd was determined only 24 h after the treatment when both Cd and Mg were already distributed in the organism. The positive effects of Mg on SOD activity can be explained by the protective effect of Mg on Zn levels in blood since oral Mg administration counteracts Cd-induced Zn reduction in blood. One explanation for this phenomenon may be that Mg prevents Cd-induced extensive loss of Zn via urine, which was observed in our previous study on rabbits (30).

Intraperitoneal Mg treatment manifested beneficial effects on $O_2^{\cdot-}$ and TOS levels and subsequently on MDA and AOPP levels, while it did not exert any effect on SOD activity. The most pronounced changes were observed in TOS and AOPP levels: Mg_{ip} administration managed to completely remove Cd toxic effects on these parameters, restoring them to control values. Since Mg_{ip} treatment showed a beneficial effect on TOS [that includes reactive oxygen metabolites such as hydrogen peroxide (H_2O_2) and lipid hydroperoxides (LOOH)], but not on SOD activity, it can be hypothesised that Mg affects other antioxidant enzymes such as gluthatione peroxidase (GPX). This enzyme plays an important role in lipid hydroperoxide reduction to their corresponding alcohols and in the decomposition of hydrogen peroxide to water. Previous studies have shown that Cd adversely affects GPX activity in different tissues such as serum, liver, and kidney (31-33), which can result in elevated TOS levels, as observed in this study. On the other hand, Vernet et al. (34) found that Mg deficiency decreased GPX activity, suggesting a positive role of supplemental Mg. The beneficial effect of Mg_{ip} treatment on $O_2^{\cdot-}$ levels might also be explained by enhanced GPX activity and subsequent decrease in H_2O_2 levels. Having in mind that H_2O_2 is also a product of superoxide anion radical dismutation, its decrease can lead to lowered levels of $O_2^{\cdot-}$ as a substrate. Intraperitoneal Mg pretreatment totally reversed Cd-induced changes in AOPP levels and managed to counteract, at least partly, Cd-induced changes in MDA levels. Under the same experimental conditions, no effect on SOD activity was observed, perhaps as a consequence of unchanged blood Zn levels after intraperitoneal Mg supplementation.

Our findings show that both oral and intraperitoneal Mg pretreatment have a protective effect against Cd poisoning, but also indicate that the route of Mg administration affects interactions between Cd and Mg, as oral administration provides more beneficial effects. These differences can be explained by the fact that after oral treatment, Mg is present in the GIT where it can modify Cd absorption. Furthermore, oral Mg pretreatment in this study prevented blood Zn drop while intraperitoneal treatment did not.

CONCLUSION

The results of this study show that acute intraperitoneal administration of Cd has much more potent toxic effect than oral Cd administration on parameters of oxidative stress in rat plasma. Our

results also show that oral and intraperitoneal Mg pretreatment can have a beneficial effect on Cd-induced oxidative stress in rat plasma, but this effect was more pronounced after oral administration. As no specific therapy has been adopted for Cd poisoning, the observed beneficial effects of both oral and intraperitoneal Mg encourage further use of Mg as protection against the toxic effects caused by Cd.

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Sažetak

UTJECAJ ORALNOG I INTRAPERITONEALNOG TRETMANA MAGNEZIJEM NA OKSIDATIVNI STRES U PLAZMI ŠTAKORA TROVANIH KADMIJEM

Kadmij (Cd) jedan je od najvažnijih industrijskih onečišćivača i onečišćivača životne sredine. Dosadašnja su istraživanja pokazala da je jedan od mehanizama toksičnosti ovog metala nastanak oksidativnog stresa. Cilj ovog rada bio je ispitati utjecaj akutnog oralnog i intraperitonealnog trovanja kadmijem na parametre oksidativnog stresa u plazmi štakora i eventualni zaštitni efekt magnezija (Mg) u danim uvjetima.
Eksperiment je izveden na 40 mužjaka albino Wistar štakora podijeljenih u ove skupine: kontrolnu, Cd_{or} skupinu koja je primila 30 mg kg^{-1} t.m. Cd oralnom gavažom, $Cd+Mg_{or}$ skupinu koja je oralno tretirana s 50 mg kg^{-1} t.m. Mg jedan sat prije oralno apliciranog Cd, Cd_{ip} skupinu koja je primila 1,5 mg kg^{-1} t.m. Cd intraperitonealno i $Cd+Mg_{ip}$ skupinu koja je intraperitonealno tretirana s 3 mg kg^{-1} t.m. Mg 10 min prije intraperitonealno apliciranog Cd. Životinje su žrtvovane 24 h nakon tretmana i izmjereni su ovi parametri: aktivnost enzima superoksid dismutaze, koncentracije superoksidnog aniona, uznapredovalih produkata oksidacije proteina i malondialdehida.
Kadmij je negativno djelovao na sve ispitivane parametre oksidativnog stresa u plazmi štakora pri čemu su negativni efekti bili izraženiji nakon intraperitonealne primjene, s izuzetkom efekta na aktivnost enzima superoksid dismutaze (SOD). Iako je Mg pokazao pozitivan učinak i nakon oralne i nakon intraperitonealne primjene, izraženiji pozitivni efekti uočeni su nakon oralnog tretmana magnezijem. Rezultati ovog rada upućuju na mogućnost primjene Mg u prevenciji toksičnih efekata Cd.

KLJUČNE RIJEČI: *interakcije između metala, mehanizmi toksičnosti, suplementacija bioelementima*

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Scientific Paper

COMBINED EFFECTS OF CADMIUM AND DECABROMINATED DIPHENYL ETHER ON THYROID HORMONES IN RATS

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The aim of this study was to see how a mixture of cadmium (Cd) and decabrominated diphenyl ether (BDE209) affect thyroid function, namely thyroid-stimulating hormone (TSH), thyroxin (T4), free thyroxin (FT4), triiodothyronin (T3), and free triiodothyronin (FT3) in Wistar rats (eight per group) receiving either a single substance or their combination by gavage for 28 days. Three groups were receiving Cd alone in the doses of 2.5 mg kg^{-1}, 7.5 mg kg^{-1}, or 15 mg kg^{-1} b. w. a day, three groups were receiving BDE209 in the doses of 1000 mg kg^{-1}, 2000 mg kg^{-1}, or 4000 mg kg^{-1} b. w. a day, while nine groups were receiving different mixtures of Cd and BDE209 in these doses (3x3 design). The results have indicated that the Cd+BDE209 mixtures more potently disrupt thyroid hormone homeostasis than would be expected from these chemicals alone.

KEY TERMS: *BDE209, Cd, thyroid-stimulating hormone, thyroxin, triiodothyronin*

Chemical risk assessment procedures commonly rely on determining the effects of a single substance. Over the last decade, however, a number of well-designed studies have investigated the effects of multi-component mixtures (1-4). High-level exposure to cadmium (Cd) is usually a result of environmental contamination due to various anthropogenic activities (5, 6). Acute or chronic exposure to Cd can affect the liver, kidney, bone, and testes in humans and experimental animals (7-13). In addition, there is evidence that Cd can alter thyroid function (6, 14-17).

Flame retardants have also become very important environmental and occupational pollutants. Through history, BDE209 was thought to be released minimally into the environment during all phases of its use and not available biologically due to its large molecular size and low aqueous solubility. However, today it is a widespread environmental contaminant with evidence demonstrating its bioaccumulation and toxic potential (18-23). Recent studies with polybrominated diphenyl ethers (PBDEs) have shown that they disturb liver enzyme activity and thyroid hormone function (24-29), reduce epididymal sperm function (30), and may affect the nervous system and newborns (31, 32).

Cadmium and BDE209 are jointly present in the environmental media, food, biota, and human tissues

through several pathways as a result of emission from various sources. Combined exposure to Cd and organic pollutants may result in complex toxicity. In view of the importance of *in vivo* behaviour of contaminants concomitantly present in an organism, this study was aimed at determining the effects of mixtures of Cd and BDE209 on thyroid-stimulating hormone (TSH), thyroxin (T4), free thyroxin (FT4), triiodothyronin (T3), and free triiodothyronin (FT3) in rats.

MATERIALS AND METHODS

Experimental animals

Male albino Wistar rats weighing 200 g to 240 g were obtained from a disease-free stock bred at the Military Medical Academy in Belgrade, Serbia. The animals were housed in plastic cages with a plastic bottom and wire mesh top, in a climate-controlled facility with a constant 12-hour day and night cycle at 20 °C to 24 °C and relative humidity between 40 % and 60 %. The animals had free access to food and tap water throughout the study and were treated according to the guidelines for animal studies (no. 9667-1/2011) issued by the Academy's ethics committee.

After a quarantine period of 14 days, groups of eight animals each were receiving either a single substance or their combinations by gavage in a volume of 0.5 mL kg^{-1} b.w. per day for 28 days. Three groups were receiving Cd alone in the form of $CdCl_2$ x H_2O (Merck, Darmastadt, Germany) in the doses of 2.5 mg kg^{-1}, 7.5 mg kg^{-1}, or 15 mg kg^{-1} b. w. per day ($Cd_{2.5}$, $Cd_{7.5}$, and Cd_{15}, respectively) and three groups BDE 209 alone as a suspension in dimethyl sulphoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) in the doses of 1000 mg kg^{-1}, 2000 mg kg^{-1}, or 4000 mg kg^{-1} b. w. per day ($BDE209_{1000}$, $BDE209_{2000}$, and $BDE209_{4000}$, respectively). Cadmium doses were selected to reflect environmental to occupational exposure (7, 9, 16). The choice of BDE209 doses was based on literature data on its very low absorption rate of 0.3 % to 2 % (18, 19). The remaining nine groups were receiving combinations of Cd and BDE209 doses according to the 3x3 design.

Before gavage Cd salt and BDE209 were dissolved in DMSO.

Rats in the vehicle control group were receiving DMSO alone (DMSO group), while control animals were receiving saline (control group).

We did our best to minimise the suffering and the number of animals used. Throughout the study, the rats were continuously monitored for body weight, clinical signs such as changes in the skin, fur, eyes, mucous membranes, secretions and excretions, autonomic activity (lacrimation, piloerection, pupil size, unusual respiratory patterns), behaviour, and food and water intake. Signs of toxicity were monitored on a daily basis, while water and food intake were recorded weekly. After decapitation, blood was collected from the carotid arteries in glass tubes and then centrifuged at 3000×*g* for 30 min. The supernatant (serum) was transferred to polypropylene test tubes and stored at −70 °C until thyroid hormone analysis.

Hormone analysis

Serum samples were analysed for thyroid-stimulating hormone, thyroxin, triiodothyronin, and free triiodothyronin with commercial tests using a Roche Elecsys 2010 analyser (Roche Diagnostics, Mannheim, Germany). Values for T3 and T4 are expressed as nmol L^{-1}, for FT3 and FT4 as pmol L^{-1} while for TSH as mU L^{-1}. The analytical ranges were 0.005 mU L^{-1} to 100 mU L^{-1} for TSH, 0.30 nmol L^{-1} to 10 nmol L^{-1} for T3, 5.40 nmol L^{-1} to 320 nmol L^{-1} for T4, 0.40 pmol L^{-1} to 50 pmol L^{-1} for FT3, and 0.30 pmol L^{-1} to 100 pmol L^{-1} for FT4. Total precisions over these ranges (expressed as coefficients of variance) were 5.4 % for TSH, 4.8 % for T3, 3.3 % for T4, 2.2 % for FT3, and 2.7 % for FT4.

Statistical analysis

To establish significance of differences between the groups, we used one-way analysis of variance (ANOVA) and subsequently Tukey's paired comparisons or Fisher's least significant difference (LSD) as *post hoc* tests. The level of statistical significance for all tests was set at $p<0.05$. All data were analysed using the statistical package Statistica 7.0.

RESULTS

Slower body weight gain was the most common in groups treated with the combination of compounds (in eight out of nine groups), while Cd or BDE209 alone did not cause significant changes compared to controls (Table 1). However, water and food consumption did not differ significantly between animals receiving single compounds (Cd or BDE209) and animals receiving their combinations.

Table 1 *Body weight and food and water intake in Wistar rats orally exposed to BDE209, Cd, or their combinations for 28 days.*

	Body weight / g		Daily consumption	
Group	**Final**	**Gain**	**Food / g**	**Water / mL**
Control	312.50±5.01	115.7±9.04	40.75±1.50	73.25±5.32
DMSO	300.04±14.14	85.00±19.15	30.00±8.89	53.00±14.61
$Cd_{2.5}$	331.25±24.16	107.50±26.05	24.51±2.33a	45.66±2.37a
$Cd_{7.5}$	290.00±52.15	80.00±31.62	20.03±3.15a	36.35±3.08a,b
Cd_{15}	304.29±20.70	82.86±24.98a	17.51±3.38a,b	37.53±5.66a
$BDE209_{1000}$	317.50±19.08	113.80±16.85b	22.92±4.02a	44.58±12.58a
$BDE209_{2000}$	305.00±18.71	88.00±10.95a	25.00±5.47a	41.68±16.01a
$BDE209_{4000}$	291.67±48.75	84.00±25.10a	27.08±1.58a	43.77±4.15a,b
$Cd_{2.5}$+$BDE209_{1000}$	286.25±27.74	81.30±26.96a,c,d	21.56±4.72a	34.37±11.38a
$Cd_{7.5}$+$BDE209_{1000}$	258.33±41.19	20.00±1.41a,b,c,d	18.30±11.03a	30.45±15.28a,b
Cd_{15}+$BDE209_{1000}$	261.43±48.11	66.67±30.77a,d	23.84±5.55a	32.41±10.53a,b
$Cd_{2.5}$+$BDE209_{2000}$	243.33±30.11	26.00±8.94a,b,c,d	18.75±12.58a	26.25±18.33a
$Cd_{7.5}$+$BDE209_{2000}$	270.00±24.49	71.67±20.41a	22.08±5.49a	50.42±22.14
Cd_{15}+$BDE209_{2000}$	215.00±8.37	20.00±7.07a,b,c,d	16.48±7.69a	29.15±4.79a,b,d
$Cd_{2.5}$+$BDE209_{4000}$	250.01±38.47	22.50±8.25a,b,c,d	20.82±5.19a	45.85±4.79a
$Cd_{7.5}$+$BDE209_{4000}$	228.00±10.95	30.00±10.95a,b,c,d	19.57±4.17a	41.28±0.85a,b
Cd_{15}+$BDE209_{4000}$	232.00±21.68	57.50±23.63a,d	20.82±3.21a	33.75±11.15a

$Cd_{2.5}$, $Cd_{7.5}$, and Cd_{15}- receiving Cd alone in a daily dose of 2.5 mg kg^{-1}, 7.5 mg kg^{-1} or 15 mg kg^{-1} b. w., respectively
$BDE209_{1000}$, $BDE209_{2000}$, and $BDE209_{4000}$ - receiving BDE209 alone in a daily dose of 1000 mg kg^{-1}, 2000 mg kg^{-1}, or 4000 mg kg^{-1} b. w., respectively
a - significantly different from control; b - significantly different from the DMSO group; c - significantly different from the same Cd dose; d – significantly different from the same BDE209 dose (one way ANOVA and post-hoc *Fisher's Least Significant Difference (LSD) test, $p<0.05$).*

Food consumption was lower in all groups compared to control, and the Cd_{15} group also showed a significant decrease compared to the DMSO group (Table 1). Groups treated with combinations did not differ in food consumption from groups treated with Cd or BDE209 alone. Water consumption decreased in all groups compared to control, with the exception of the $Cd_{7.5}$+$BDE209_{2000}$ group. Compared to the DMSO group, only the $Cd_{7.5}$ and the $BDE209_{4000}$ groups showed lower water consumption, and the same effect was observed in the following four combination groups: $Cd_{7.5}$ or Cd_{15}+$BDE209_{1000}$, Cd_{15}+$BDE209_{2000}$ and $Cd_{7.5}$+$BDE209_{4000}$. The only statistically significant difference between the combination groups and corresponding single-compound groups was noticed for the Cd_{15}+$BDE209_{2000}$ *vs.* $BDE209_{2000}$ group; the first drank about 30 % less water than the $BDE209_{2000}$ group. Decreases in food and water intake of almost 50 % in some groups are in accordance with lower body weight gain. Except for food and water intake and body weight gain, there were no other clinical signs of poisoning.

All three Cd doses induced a significant decrease in T4 and FT4 levels *vs.* control, whereas 2.5 mg kg^{-1} Cd led to a decrease in FT3 and 15 mg kg^{-1} Cd to decrease in T3 and FT3 levels (Table 2). Significant differences from the DMSO group were seen in the $Cd_{2.5}$ (FT4), $Cd_{7.5}$ (FT3, T4), and Cd_{15} (FT3) groups. Similar to Cd alone, all three doses of BDE209 induced a significant decrease in T4 and FT4 levels *vs.* control. Compared to the DMSO group, a significant decrease in hormone levels, given in brackets, was observed in the $BDE209_{1000}$ (FT3, T4), $BDE209_{2000}$ (FT3, T4, FT4), and $BDE209_{4000}$ (T3) groups.

Combinations added to these effects, particularly in the groups receiving BDE209 (1000 mg kg^{-1} or 2000 mg kg^{-1}) plus Cd (all three doses). Cd+BDE209 (1000 mg kg^{-1}) significantly decreased T4 and FT4 levels compared to the groups given the corresponding doses of either Cd or BDE209. BDE209 (2000 mg kg^{-1}) plus Cd also significantly decreased T4 and FT4, and additionally T3 levels. We measured TSH levels as well, but they were all below the limit of detection (0.005 mU L^{-1}).

Statistical analysis of the ratio between T3 and T4 pointed out that it was significantly higher in almost all groups compared to control. In the $Cd_{7.5}$ group and in the groups receiving combinations $Cd_{2.5}$+$BDE209_{1000}$ and $Cd_{2.5}$+$BDE209_{2000}$ this ratio was also significantly higher than in the DMSO group. All Cd combinations with the highest dose of BDE209 also showed a significant increase in respect to treatment with corresponding dose of BDE209 alone. Expressed in percentages, the ratio between T3 and T4 varied from 5 % to 27 %, while in groups Cd+$BDE209_{4000}$ it varied from 10 % to 18 %, compared to the DMSO group.

DISCUSSION

Both pollutants, alone or in combination lowered thyroid hormone levels compared to control, but the combined effect was more pronounced. The combined effect on the levels of T3, FT3, T4, and FT4 may be viewed as additive. A number of studies have already shown that Cd alters thyroid function in experimental

Table 2 *Serum thyroid hormone levels in Wistar rats orally exposed to Cd, BDE209, or their combinations for 28 days*

	Thyroid hormone levels				Ratio
Group	**T3 / nmol L^{-1}**	**FT3 / pmol L^{-1}**	**T4 / nmol L^{-1}**	**FT4 / pmol L^{-1}**	**T3/T4**
Control	1.52±0.06	4.57±0.19	82.66±3.36	42.51±2.22	0.0183
DMSO	1.46±0.05	4.37±0.33	62.93±4.45a	32.85±4.18a	0.0232
$Cd_{2.5}$	1.40±0.13	3.29±0.24a	54.66±4.11a	27.88±3.15a,b	0.0256a
$Cd_{7.5}$	1.40±0.15	3.69±0.45b	47.43±9.04a,b	23.84±3.60a	0.0295a,b
Cd_{15}	1.34±0.10a	3.56±0.25a,b	54.90±4.40a	29.44±2.65a	0.0244a
$BDE209_{1000}$	1.39±0.06a	2.98±0.87a,b	53.84±5.51a,b	31.02±2.88a	0.0258a
$BDE209_{2000}$	1.44±0.09	4.72±0.73b	49.61±6.77a,b	25.31±3.31a,b	0.0290a
$BDE209_{4000}$	1.30±0.12a,b	4.38±0.79	61.85±8.02a	29.82±3.82a	0.0210
$Cd_{2.5}$+$BDE209_{1000}$	1.34±0.05a	3.33±0.17a	47.48±4.04a,b,c	24.15±3.00a,b,c,d	0.0282a,b
$Cd_{7.5}$+$BDE209_{1000}$	1.38±0.06a	4.01±0.20	64.48±4.12a,c,d	31.00±3.36a,b,c	0.0214
Cd_{15}+$BDE209_{1000}$	1.30±0.05a,b	3.22±0.31a,b	47.78±7.74a,b,c	24.32±2.28a,b,c,d	0.0272a
$Cd_{2.5}$+$BDE209_{2000}$	1.28±0.14a,b,c,d	4.09±0.50c	43.46±5.42a,b,c	22.31±2.49a,b,c	0.0295a,b
$Cd_{7.5}$+$BDE209_{2000}$	1.27±0.12a,b,c,d	3.81±0.53d	47.43±7.58a,c,d	25.32±3.62a,b	0.0268a
Cd_{15}+$BDE209_{2000}$	1.22±0.17a,b,c,d	3.83±0.33d	47.13±8.58a,b,c	24.29±3.27a,b,c	0.0259a
$Cd_{2.5}$+$BDE209_{4000}$	1.31±0.15a,b	3.94±0.49c	51.41±14.21a,b,d	28.11±5.22a,b	0.0255a,d
$Cd_{7.5}$+$BDE209_{4000}$	1.32±0.14a,b	4.09±0.26	48.11±4.60a,b	23.90±2.22a,d	0.0274a,d
Cd_{15}+$BDE209_{4000}$	1.43±0.10	4.52±0.53c	54.15±7.65a,b	29.03±4.15a	0.0264 a,d

$Cd_{2.5}$, $Cd_{7.5}$, and Cd_{15}- receiving Cd alone in a daily dose of 2.5 mg kg^{-1}, 7.5 mg kg^{-1} or 15 mg kg^{-1} b. w., respectively
$BDE209_{1000}$, $BDE209_{2000}$, and $BDE209_{4000}$ - receiving BDE209 alone in a daily dose of 1000 mg kg^{-1}, 2000 mg kg^{-1}, or 4000 mg kg^{-1} b. w., respectively
a - significantly different from control; b - significantly different from the DMSO group; c - significantly different from the same Cd dose; d – significantly different from the same BDE209 dose (one way ANOVA and post-hoc *Fisher's Least Significant Difference (LSD) test, $p<0.05$).*

animals (6, 7, 9, 14, 15-17, 33) and humans (34). Yoshizuka et al. (17) suggested that Cd accumulated in the mitochondria of thyroid follicular epithelial cells might disturb the oxidative phosphorylation of this organelle, lower energy supply, and therefore inhibit the synthesis and release of thyroid hormones. Similarly, Pilat-Marcinkiewicz et al. (16) observed a dose-dependent effect on the structure and function of thyroid follicular cells in rats. Apart from the studies suggesting that Cd interferes with the thyroid function at the glandular level, there are findings supporting the effects at the peripheral level by inhibiting the conversion of T4 to T3 (14, 35-38). Thyroid hormones are metabolised in peripheral tissue by deiodination, conjugation, deamination, and decarboxylation enzyme reactions, and any change in these metabolic pathways may significantly affect thyroid function at the cellular level (35-37). As deiodination of T4 to T3, which occurs mainly in the liver, depends on 5′-monodeiodinase activity (35, 37), hepatic dysfunction is likely to affect thyroid hormone levels (36), T3 in particular. However, thyroid hormone levels are mainly regulated by the hypothalamic-pituitary-thyroid axis and if this axis is affected by Cd, so will hormone release (39, 40).

Several reports have shown that a broad range of chemicals to which humans are routinely and inadvertently exposed can bind to thyroid receptors (TRs) and may produce complex effects on thyroid hormone signalling (41-43). Polybrominated diphenylethers, structurally related to BDE209, have been also recognised to bind to TRs and perhaps mimic thyroid hormones (42, 43). We may therefore assume that BDE209 binds to thyroid receptors rather than acts through the hypothalamic-pituitary axis. However, Zhou et al. (28) found that decaBDE caused no changes in any of the thyroid hormone levels (28). In contrast, Kim et al. (44) found significant increases in thyroid weight of pregnant rats exposed to high doses of BDE209 and significantly lower concentrations of T4 in F1 female offspring exposed to BDE209 on postnatal day 42. In a recent study, Lee et al. (22), found significantly lower total serum T3 concentrations, degenerated follicular epithelium, and hepatocyte hypertrophy and vacuolisation in newborn rats exposed to BDE209. These findings confirm that BDE209 might affect thyroid hormone synthesis and metabolism.

The combination of Cd and BDE209 toxicity has even more pronounced effects on T3 and T4, which can be explained by different mechanisms of their action. Although, we assumed additive response, future studies should look into the type of interactions between Cd and BDE209 at dose levels relevant for human exposure.

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Sažetak

KOMBINIRANO DJELOVANJE KADMIJA I DEKABROMIRANOG DIFENIL ETERA NA HORMONE ŠTITNJAČE U ŠTAKORA

Cilj ovoga istraživanja bio je utvrditi na koji način smjesa kadmija (Cd) i dekabromiranog difenil etera (BDE209) djeluje na aktivnost štitnjače. Određivane su aktivnosti stimulirajućega hormona štitnjače (TSH), tiroksina (T4), slobodnog tiroksina (FT4), trijodtironina (T3) te slobodnog trijodtironina (FT3) kao parametara koji upućuju na funkcionalnost štitnjače. Kao eksperimentalni testni sustav korišteni su Wistar štakori (n=8 po grupi), kojima je tijekom 28 dana dozirana pojedinačna tvar ili smjesa kadmija i dekabromiranog difeniletera. Životinje su bile podijeljene u tri grupe koje su primale tri različite doze kadmija: 2,5 mg kg^{-1}, 7,5 mg kg^{-1} i 15 mg kg^{-1} tjelesne težine po danu. Tri grupe životinja primale su tri različite doze BDE209: 1000 mg kg^{-1}, 2000 mg kg^{-1}, odnosno 4000 mg kg^{-1} tjelesne težine po danu. Preostale životinje bile su podijeljene u devet grupa kojima su bile dozirane različite koncentracije kadmija i dekabromiranog difenil etera (3x3 dizajn). Rezultati pokazuju da Cd+BDE209 smjesa u većoj mjeri remeti homeostazu hormona štitnjače u odnosu na pojedinačne spojeve.

KLJUČNE RIJEČI: *BDE209, Cd, stimulirajući hormon štitnjače, tiroksin, trijodtironin*

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Scientific Paper

PROTECTIVE EFFECTS OF DIETARY FIBRE AGAINST MANGANESE-INDUCED NEUROBEHAVIORAL ABERRATIONS IN RATS

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We tested the hypothesis that dietary fibre (DF) has protective effects against manganese (Mn)-induced neurotoxicity. Forty-eight one-month old Sprague-Dawley rats were randomly divided into six groups: control, 16 % DF, Mn (50 mg kg^{-1} body weight), Mn+ 4 % DF, Mn+ 8 % DF, and Mn+ 16 % DF. After oral administration of Mn (as $MnCl_2$) by intragastric tube during one month, we determined Mn concentrations in the blood, liver, cerebral cortex, and stool and tested neurobehavioral functions. Administration of Mn was associated with increased Mn concentration in the blood, liver, and cerebral cortex and increased Mn excretion in the stool. Aberrations in neurobehavioral performance included increases in escape latency and number of errors and decrease in step-down latency. Irrespective of the applied dose, the addition of DF in forage decreased tissue Mn concentrations and increased Mn excretion rate in the stool by 20 % to 35 %. All neurobehavioral aberrations were also improved. Our findings show that oral exposure to Mn may cause neurobehavioral abnormalities in adult rats that could be efficiently alleviated by concomitant supplementation of DF in animal feed.

KEY WORDS: *escape latency, neurobehavioral test performance, number of errors, oral exposure, step-down latency*

Manganese (Mn) is distributed widely in the air and water in a number of chemical and physical forms. It is very active and could be in 11 oxidation states in the geological environment. Among those, Mn^{2+} and Mn^{3+} are especially important for biological tissues (1). Generally, Mn^{2+} is more stable than Mn^{3+}.

Manganese is an essential trace nutrient in all forms of life. It is necessary for normal functioning of a variety of physiological processes that include protein, amino acid, carbohydrate, and lipid metabolism and can be found in various biological tissues. Manganese also plays an essential role in regulating bone and connective tissue growth, cellular energy, immune function, and blood clotting. As a cofactor for glial-specific glutamine synthetase, pyruvate carboxylase, superoxide dismutase (SOD), and other enzymes in the brain, Mn is involved in neurotransmitter synthesis and metabolism (2, 3).

For many years, however Mn has also been known to exert neurotoxic effects. It can enter the brain tissue by crossing the blood-brain barrier via several routes. Excessive chronic exposure to Mn can cause progressive and persistent neurodegenerative damage associated with numerous psychiatric and motor disturbances, resembling the idiopathic Parkinson's disease. The syndrome includes tremor, postural instability, bradykinesia, gait disturbance, rigidity, ataxia, and even cognitive deficits (1, 4). Among these

symptoms, cognitive deficits such as spatial working memory, reference memory, and learning capacity disorders have received less attention than motor function disorders. Josephs et al. (4) reported reduced learning capacity in occupationally Mn-exposed welders, and Schneider et al. (5) found that monkeys developed subtle deficits in spatial working memory after exposure to $MnSO_4$.

Exposure to airborne Mn pollution occurs through fumes at the workplace and outdoors. Epidemiological studies in occupationally exposed workers showed a significant positive correlation between lifetime cumulative or integrated Mn exposure and neurological dysfunction (1, 6-7). Chronic exposure to excessive Mn levels can lead to a variety of psychiatric and motor disturbances, so-called manganism. It can occur due to occupational exposure during Mn dioxide mining and grinding, ferri-manganese smelting and welding, dry-battery-factory production, Mn oxide production, and Mn salt production (7). Symptoms of manganism include cephalalgia, fatigue, sleep disturbances, sialorrhoea, adynamia, muscular pain and hypertonia, mask-like face, gait changes, reduced coordination, hallucinations, and mental irritability. Among these symptoms, cognitive deficits including disturbance of learning and memory are particularly dramatic. Bowler et al. (8) explored the relationship between Mn exposure and learning and memory in bridge welders. Bouchard et al. (9) conducted a follow-up study of alloy workers and concluded that Mn cumulative exposure could lead to learning and memory deficits. In spite of the criticism due to certain biases pointed out in the above mentioned reports, the fact remains that Bowler et al. (8) confirmed the correlation between abnormal neuropsychological findings and Mn exposure in welders in a dose-related manner.

In recent years, Mn in the form of 3-hydroxy-methyl cyclopentadienyl manganese (MMT) has replaced tetraethyl-lead in gasoline as an antiknock agent. This application of Mn has led to more serious ambient air pollution (10).

Manganese can be absorbed through the respiratory tract by inhalation; 'but most of the absorption is through the digestive tract, especially in chronic exposure. So far, studies of the intake and neurotoxicity of Mn have principally focused on its absorption through the respiratory tract, and less attention was paid to the absorption through the gastrointestinal tract, especially in population-based studies.

Dietary fibres (DF) are present in many plants and play an important role in the maintenance of human health (11-13). A copious intake of DF reduces the risk of numerous health disorders and diseases, such as hypertension, cardiovascular diseases, and colorectal cancer (11, 13, 14). Furthermore, DF consumption may improve serum lipid and cholesterol concentrations (15), blood glucose in diabetes (11), and possibly even the immune function. Sherry et al. (16) reported that soluble DF had an ability to alleviate endotoxin-induced sickness behaviour through the up-regulation of interleukin-4 (IL-4) and lymphocyte T helper (Th2) cell polarisation. All these reports identify the benefits of DF and suggest that DF can be considered the "seventh nutrient".

One of DF's benefits is that it can influence the absorption of metals such as cadmium (Cd), lead (Pb), mercury (Hg), and copper (Cu). More than twenty years ago, Drews et al. (17) reported the effect of DF on Cu, zinc (Zn), and magnesium (Mg) utilisation in adolescent boys. Recently, Yang et al. (18) reported that chlorella (whose main compound is DF) intake had effects on Cd^{2+}, Pb^{2+}, Hg^{2+} absorption; Shim et al. (19) reported the effects of chlorella intake on Cd metabolism in rats; and Ou et al. (20) found that wheat bran DF effectively bound three tested metal ions and provided protection against metal' toxicity. In the most recent paper, Callegaro et al. (21) reported that cereal bran supplements (a type of food from Brazil with DF as its main component) reduced the effects of Cd at lower concentrations in young rats, but was inefficient against higher Cd concentrations.

To sum up, literature data published so far suggest that DF could change the absorption of several metal ions and/or accelerate their excretion. However, reports on DF interaction with Mn ions are rare. This is why we wanted to test the hypothesis that DF had protective effects against Mn neurotoxicity in an animal model *in vivo* by exposing adult rats to different Mn concentrations with or without DF and testing their neurobehavioral performance.

MATERIALS AND METHODS

Chemicals

Manganese chloride ($MnCl_2$, purchased from Chemical Reagent Company of Chinese Medicine Group, Shanghai, China) was used as test chemical. Soybean DF (purchased from Yunhai International

Trade Limited Company (Tianjin, China) is composed in main portion of total dietary fibres (dry basis ≥65 %); other ingredients are moisture (≤8 %), ash (≤7 %), protein (≤20 %), and fat (≤1 %). No toxic substance was detected (such as other heavy metals). Its quality was controlled by the Bureau of Product Quality Supervision of the Shandong Province, China. Nitric acid (purchased from Development Center of Kemio Chemical Reagent, Tianjin, China) was chromatographic pure grade.

Animals

Forty-eight three-week-old male Sprague-Dawley rats were used in the experiment (purchased from Laboratory Animal Centre of Third Military Medical University). The rats were fed with full grain, had free access to forage and tap water, and were kept under controlled indoor temperature of (20±2) °C, with the light and dark cycle exchanging every 12 hours. Rats were housed in plastic cages (45 cm×32 cm×19 cm) with four rats per cage. The cages were supplied with sawdust as bedding and a metal grid on top. Animals' general health during the experiment was checked by measuring body weight at the beginning and the termination of the experiment.

Experimental design

After the adaptation period of one week, the rats were randomised into six groups of eight, as follows: the control group, 16 % DF, Mn, Mn+4 % DF, Mn+8 % DF, and Mn+16 % DF. Manganese (as $MnCl_2$) was given in a dose of 50 mg kg^{-1} body weight by gavage once a day. Soybean DF was mixed with rat forage in the following percentages: 4 %, 8 %, and 16 %. This dose was calculated using a simple formula: soybean DF weight was divided by total forage weight and multiplied with 100. The control and the 16 % DF group received physiological saline in the dose of 10 mL kg^{-1} body weight using the same procedure as for Mn. All animals were treated for one month.

The experimental protocol was reviewed and approved by the Ethics Review Committee for Laboratory Animal Research at the Affiliated Hospital of Zunyi Medical College.

Neurobehavioral tests

Morris water maze test was performed on all rats (22) after the one-month treatment. The water maze was a grey-circular tub filled with water at 25 °C. The diameter of the water maze was 120 cm and total depth 35 cm. Water was filled up to 15 cm from the brim. In the centre of the northeast quadrant of the water maze at the depth of 2 cm under the water surface, there was a black platform with a diameter of 12 cm. In order to hide the platform, water was stained with milk. The pool was always in the same position and the experiment carried out by the same people. In the locating navigation experiment, rats were placed at one of the four starting points (northeast, southeast, southwest, and northwest) facing the wall of the pool to enter the water, and the same rats started from the same points for the five consecutive test days**.** If the rats failed to get to the platform in 120 s (escape latency), they were guided to it, stayed there for 30 s, and their escape latency counted as 120 s. At the end of every trial, the rats were towelled, fan dried, and put back to their cages for a 15-minute rest, then they were re-tested and the tests were run twice a day. All results were recorded automatically by the computer. Here we report average time.

For the *step-down test* the rats were put onto a floor grid inside a rectangular box for 3 min to adapt to the environment and then shocked with 36 V alternating current. A normal reaction was to jumping onto a platform above the floor grid to escape the shock. After a while, the rats would return step back down the floor grid, and most repeated this action twice or even several times. The training lasted 5 min. After 24 h, the test was repeated and the latency of the first time to step down from the platform (step-down latency) and the number of errors within 3 min (number of errors) recorded for every rat. If a rat did not step down onto the grid platform within 3 min, the number of errors was counted as 0, and the step-down latency was counted as 180 s.

Manganese tissue analysis

Twenty-four-hour stool samples were collected using metabolic cages 24 h before the rats were decapitated under diethyl ether and dissected. Blood was collected after decapitation, and liver and cerebral cortex dissected for Mn determination.

One gram of liver, 0.2 g of cerebral cortex, and 0.2 g of wet stool were placed into a crucible and baked at 100 °C for 16 h. Blood (0.2 mL) was taken by suction and digested in a microwave after evaporation on an electric hot plate. Then the samples were dissolved with 10 mL of nitric acid for the final tests. Blood and cerebral cortex Mn level was determined by graphite furnace atomic absorption spectrometry

(AAS, Varian Inc., USA); and stool and liver Mn level by flame AAS. Mn excretion over 24 h was computed using the following equation:

Mn-excretion rate (%) = 24-h Mn in the stool / 24-h Mn by gavage administration × 100

Essential micronutrient tissue analysis

Blood, liver, and cerebral cortex Zn, Cu, and Fe concentrations were determined using an automatic biochemical analyser (AU2700, OLMPUS Inc., Japan).

Statistical analysis

The results are presented as mean ± standard deviation (SD). All data were analysed using the analysis of variance (ANOVA), and *post hoc* LSD tests were used for multiple comparisons. Dose-dependency was tested with factorial ANOVA using linear trend testing. All tests were two-tailed. Difference at the level of $P<0.05$ was considered statistically significant. We used the Statistical Package for Solution and Services software (version 18.0; SPSS Institute, California, USA).

RESULTS

At the beginning of the experiment, average rat weight per group was about 180 g. After one-month of treatment, their body weight increased about 60 g in average in all groups. The groups did not differ in total forage intake and body weight gain.

Escape latency

The overall ANOVA test showed a significant difference in escape latency between all groups ($F=36.6$, $P<0.001$). Multiple comparisons showed a significant increase in escape latency in the Mn group in respect to control for test days 2 through 5. The 16 % DF group did not differ from control. Over the same test days, the escape latency in Mn+DF-treated groups was significantly lower than in the Mn group and the lowering trend correlated with increasing DF percentage (Table 1, first five columns). It also dropped with test days 2 to 5.

Step-down latency and number of errors

Compared to control, step-down latency significantly dropped in the Mn group and the average number of errors significantly increased. No significant difference was found between control and the 16 % DF group. Latency also improved in the Mn+8 % DF and Mn+16 % DF groups compared to the Mn group and reached control values. Adding DF to the forage significantly decreased the number of errors and reached control value in all three Mn+DF groups (Table 1, last two columns).

Tissue Mn concentrations

Compared to control, blood, liver, and cerebral cortex Mn concentrations significantly increased in the Mn group, whereas blood, liver, cerebral cortex, and stool Mn was comparable between control and the 16 % DF group. The addition of DF, significantly lowered blood, liver, and cerebral cortex Mn concentrations in all three Mn+DF groups, but they remained higher than control, save for cerebral cortex in the Mn+16 % DF group. Mn excretion was significantly higher in all Mn+DF groups (Table 2) and showed a linear correlation with increasing DF percentage. A similar linear correlation was found for Mn concentrations, which dropped in the liver and

Table 1 *Effects of oral exposure to manganese (50 mg kg^{-1} of body weight in the form of $MnCl_2$ over one month) alone and in combination with increasing concentrations of dietary fibres (4 % DF, 8 % DF, 16 % DF) on neurobehavioral tests: escape latency, step-down latency, and number of errors in rats*

Group	Escape latency in Morris Maze Test(s)					Step-down latency(s)	Number of errors
	1st Day	2nd Day	3rd Day	4th Day	5th Day		
Control	98.6±8.57^{a}	38.8±5.82^{a}	27.2±2.93^{a}	19.3±4.76^{a}	5.75±2.36^{a}	152±6.07^{a}	1.13±0.640^{a}
16 % DF	100±15.1^{a}	37.7±7.45^{a}	23.2±8.02^{a}	15.2±3.87^{a}	9.00±3.43^{a}	150±7.36^{a}	1.00±0.534^{a}
Mn	95.4±11.6^{a}	74.7±10.7^{b}	46.1±9.22^{b}	50.4±17.5^{b}	31.4±4.02^{b}	133±12.7^{b}	2.17±0.753^{b}
Mn+4 % DF	94.5±11.9^{a}	48.5±9.60^{c}	35.2±6.70^{c}	31.6±4.74^{c}	33.7±5.47^{b}	135±6.65^{b}	1.88±0.641^{a}
Mn+8 % DF	97.8±11.6^{a}	41.1±6.28^{ac}	27.5±3.69^{ac}	23.0±3.46^{ac}	17.9±2.70^{c}	157±7.31^{a}	0.750±0.886^{a}
Mn+16 % DF	103±9.53^{a}	40.3±4.00$^{ac\#}$	27.0±4.56$^{ac\#}$	20.1±4.65$^{ac\#}$	20.9±4.72$^{c\#}$	162±12.3$^{a\#}$	0.875±0.641$^{a\#}$

Different superscript letters in each column denote significant difference ($P<0.05$) between respective groups.
$^{\#}$Significant linear trend ($P<0.05$) with higher DF levels.

cerebral cortex and rose in the stool as DF levels increased ($P<0.05$) (Table 2).

Micronutrients tissue concentrations

No difference in any of the observed micronutrient (Zn, Cu, and Fe) in blood, liver, or cerebral cortex concentration was found between the groups (Table 3).

DISCUSSION

Neurobehavioral changes induced by manganese

Beside factors related to the host organism, chemical forms may influence Mn absorption, distribution, and toxicity (23). Our choice of $MnCl_2$ was based on following reports. Reaney et al. (24) reported that Mn^{2+} and Mn^{3+} differed in toxic properties and Gunter et al. (25) reported Mn^{3+} could not be positively identified in the mitochondria of neuroteratocarcinoma (NT_2) cells, liver, brain, or heart. These findings suggest that Mn^{2+} is the toxic form for humans.

Although Mn is essential for normal brain development and function, it could be harmful to the immune, reproductive, and other systems and has a neurotoxic potential at high levels (1-3). Learning and memory are two most important facets of cognition and thus indispensable for the research of cerebral functions. Regarded as a standard procedure, Morris water maze test is extensively used to evaluate memory and spatial learning of rodents (22, 26). Step-down test is another common method to study learning and

Table 2 *Effects of oral exposure to manganese (50 mg kg^{-1} body weight in the form of $MnCl_2$ over one month) alone and in combination with increasing concentrations of dietary fibres (4 % DF, 8 % DF, 16 % DF) on blood, liver, cerebral cortex, and stool levels in rats*

Group	Mn levels				Mn-excretion rate* / %
	Blood / μg mL^{-1}	Liver / μg g^{-1}	Cerebral cortex / μg g^{-1}	Stool / mg g^{-1}	
Control	0.243±0.0357^{a}	1.78±0.333^{a}	1.37±0.219^{a}	0.330±0.148^{a}	—
16 % DF	0.245±0.0416^{a}	2.03±0.260^{a}	1.42±0.221^{a}	0.280±0.0894^{a}	—
Mn	0.483±0.0665^{b}	3.22±0.248^{b}	3.59±0.441^{b}	2.49±0.629^{b}	20.9±5.16^{a}
Mn+4 % DF	0.371±0.0789^{c}	2.70±0.347^{c}	2.57±0.247^{c}	2.91±0.726^{b}	26.2±7.59^{b}
Mn+8 % DF	0.375±0.0460^{c}	2.54±0.183^{c}	2.24±0.178^{c}	3.19±0.557^{c}	28.5±5.78^{b}
Mn+16 % DF	0.351±0.0548^{c}	2.73±0.192$^{c\#}$	1.62±0.132$^{ac\#}$	3.31±0.735$^{c\#}$	29.4±5.57^{b§}

Different superscript letters in each column denote significant difference ($P<0.05$) between respective groups.
**Mn-excretion rate / % = 24-h Mn in the stool / 24-h Mn by gavage administration × 100.*
\#Significant linear trend ($P<0.05$) with higher DF levels.
§ Borderline significant linear trend ($P=0.06$) with higher DF levels.

Table 3 *Effects of oral exposure to manganese (50 mg kg^{-1} body weight in the form of $MnCl_2$ over one month) alone and in combination with increasing doses of dietary fibres (4 % DF, 8 % DF, 16 % DF) on blood, liver, and cerebral cortex micronutrient levels in rats*

Group	Concentration in blood / μmol L^{-1}			Mass fraction in liver / μg g^{-1}			Mass fraction in cerebral cortex / μg g^{-1}		
	Zn	Cu	Fe	Zn	Cu	Fe	Zn	Cu	Fe
Control	10.7±1.32	20.1±4.97	51.2±4.51	57.3±6.85	1.83±0.358	130±21.1	42.5±3.06	0.652±0.0190	45.9±6.66
16 % DF	11.0±2.36	22.4±5.64	49.6±8.14	57.2±4.65	1.78±0.218	131±18.9	42.9±4.93	0.634±0.0279	44.8±6.82
Mn	9.47±2.38	20.3±2.95	43.5±7.61	52.5±3.94	1.69±0.221	116±18.5	39.4±6.08	0.613±0.0646	43.3±7.94
Mn+4 % DF	11.3±1.38	24.1±4.39	44.5±4.72	56.9±4.27	1.70±0.441	121±11.9	41.2±4.21	0.642±0.0301	43.2±3.91
Mn+8 % DF	10.7±0.77	22.8±2.62	54.1±14.4	54.6±4.14	1.78±0.410	123±18.6	43.9±5.07	0.654±0.0189	43.8±4.84
Mn+16 % DF	10.1±2.15	23.1±6.30	53.9±13.7	55.2±6.20	1.71±0.409	131±17.1	43.7±6.55	0.667±0.0706	44.2±6.99

No statistical difference was determined between the study groups.

memory. In this study, we evaluated both learning and memory and found that oral Mn exposure significantly increased the escape latency, as the exposed rats needed more time to find the platform beneath the water surface. Shorter latency and more errors in the step-down test also confirmed that exposure to Mn could affect learning and memory. Our findings corroborate the findings of earlier studies, which showed that exposure to Mn caused permanent aberrations in spontaneous behaviour, learning, and memory functions in mammals, including humans (1).

Protective effects of dietary fibres against Mn neurotoxicity

The primary objective of our study was to assess the protective effects of DF against Mn neurotoxicity. We found that DF antagonised Mn in rats co-exposed to 4 %, 8 %, and 16 % DF and 50 mg kg^{-1} body weight Mn, as their escape latency significantly shortened compared to rats exposed to Mn alone. However, only in groups that supplemented with 8 % DF and 16 % DF was escape latency similar to control. Moreover, rats supplemented with DF showed a significantly lower step-down latency and error proneness than rats exposed to Mn alone. These results suggest that DF can provide significant protection against Mn-induced neurotoxic effects, especially at 8 % and 16 % concentrations. These findings are consistent with the protective effects of DF against other heavy metals (Cd, Pb, Hg, Cu and Mg) (17-20).

However, our results were partly differ from those of Callegaro et al. (27), who reported that high-level DF supplements could reduce the absorption of calcium (Ca), phosphorus (P), Mg, and Cu, whereas Mn absorption increased in spite of high DF supplementation. The main reason for this difference may be that our DF was extracted from soybean and was metal free whereas in the study by Callegaro et al. it was a mixture with a high content of various minerals that affect the binding capacity of DF.

Our findings on the Mn excretion rate suggest that DF can facilitate Mn excretion and thus reduce its accumulation in tissues such as blood, liver, and cerebral cortex. The protective effect of DF on the neurotoxicity of Mn appears even more valuable if we take into account that it did not change micronutrient levels.

Manganism treatment is still in want of successful methods (1). Our study suggests that DF can alleviate manganism symptoms and offers a new approach to its treatment and prevention.

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Sažetak

ZAŠTITNO DJELOVANJE VLAKNASTOGA TKIVA U HRANI OD NEUROBIHEJVIORALNIH POREMEĆAJA UZROKOVANIH MANGANOM U ŠTAKORA

U ovome je ispitivanju testirana hipoteza da vlaknasto tkivo u hrani (DF - od engl. *dietary fibre*) štiti od neurotoksičnoga djelovanja mangana (Mn). Četrdeset i osam Sprague-Dawley štakora nasumce je raspoređeno u šest skupina: kontrolnu, skupinu koja je primala 16 %-tni udio vlaknastoga tkiva u hrani (DF 16 %), Mn (50 mg kg^{-1} tjelesne težine), Mn+DF 4 %, Mn+DF 8 % i Mn+DF 16 %. Nakon peroralne primjene Mn (u obliku $MnCl_2$) kroz gastričnu cjevčicu u trajanju od mjesec dana, utvrdili smo koncentracije Mn u krvi, jetri, moždanoj kori i stolici te napravili pretrage neurobihejvioralnih funkcija. Primjena Mn bila je povezana s povišenom koncentracijom Mn u krvi, jetri i moždanoj kori te s povećanim uklanjanjem stolicom. Poremećaji neurobihejvioralne funkcije obuhvaćali su produljeno vrijeme bijega te veći broj pogrešaka i skraćeno vrijeme silaska s platforme. Bez obzira na primijenjenu dozu, dodavanje vlaknastoga tkiva u hranu dovelo je do pada koncentracija Mn i njegova povećanog uklanjanja stolicom za 20 % do 35 %. Također su se popravili nalazi svih neurobihejvioralnih testova. Naši nalazi pokazuju da izlaganje Mn oralnim putom može dovesti do neurobihejvioralnih poremećaja u odraslih štakora, koji se mogu uspješno ublažiti istodobnim dodavanjem vlaknastoga tkiva u hranu.

KLJUČNE RIJEČI: *broj pogrešaka, neurobihejvioralna funkcija, oralna izloženost, vrijeme bijega, vrijeme silaska s platforme*

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Scientific paper

ALTERED CANONICAL HEDGEHOG-GLI SIGNALLING AXIS IN PESTICIDE-INDUCED BONE MARROW APLASIA MOUSE MODEL

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The mechanistic interplay between pesticide exposure and development of marrow aplasia is not yet well established but there are indices that chronic pesticide exposure in some instances causes marrow aplasia like haematopoietic degenerative condition in human beings. Canonical Hedgehog (Hh) signalling has multiple roles in a wide range of developmental processes, including haematopoiesis. The present study was designed to explore the status of four important components of the canonical Hedgehog signalling cascade, the Sonic Hedgehog (Shh), Ptch1, Smo, and Gli1, in a mouse model of chronic pesticide-induced bone marrow aplasia. We used 5 % aqueous mixture of pesticides (chlorpyriphos, prophenophos, cypermethrin, alpha-methrin, and hexaconazole) for inhalation and dermal exposure of 6 hours per day and 5 days a week up to 90 days. Murine bone marrow aplasia related to chronic pesticide treatment was confirmed primarily by haemogram, bone marrow cellularity, short term bone marrow explant culture for cellular kinetics, bone marrow smear, and flow cytometric $Lin^{-}Sca\text{-}1^{+}C\text{-}kit^{+}$ extracellular receptor expression pattern. Later, components of hedgehog signalling were analysed in the bone marrow of both control and pesticide-treated aplastic groups of animals. The results depicted pancytopenic feature of peripheral blood, developmental anomaly of neutrophils, depression of primitive stem and progenitor population along with Shh, Ptch1, Smo and Gli1 expression in aplasia group. This investigation suggests that pesticide-induced downregulation of two critically important proteins - Ptch1 and Gli1 - inside the haematopoietic stem and progenitor cell population impairs haematopoietic homeostasis and regeneration mechanism *in vivo* concurrent with bone marrow aplasia.

KEY WORDS: *bone marrow suppression, haematopoietic stem cell, patched, smoothened, sonic hedgehog*

A surge of numerous organophosphates, pyrethroids (analogs of naturally occurring phytopyrethrins), and thiazole fungicides replaced DDT after the ban in 1993. Depending on the physiological activity, synthetic pyrethroids are of two distinct types: Type I pyrethroids, which do not contain the α-cyano group, and Type II pyrethroids, which have the α-cyano group (1-3). Previous studies have affirmed the genotoxic and/or cytotoxic effects of Type II pyrethroids such as cyfluthrin, cypermethrin etc. (4-9). The carcinogenic potential of pyrethroids was also reported on the murine model (9). A few recent studies revealed that besides blocking neurotransmission, both pyrethroids and organophosphate pesticides can cause

immunosuppression and deregulation of many signalling molecules (10-15). Organophosphates (derivatives of phosphonic acid) and systemic azole fungicides have profound cytotoxic activities, too (16, 17). Despite a few epidemiological reports (18-20), we thus far have meagre understanding of the haematopoietic failure and the development of bone marrow aplasia by pesticide toxicity. Bone marrow aplasia represents a rare disorder of haematopoietic stem cell failure, often with severe life-threatening pathological consequences. More than half of the diagnosed cases of the aplastic anaemia are idiopathic, rest being acquired or inherited. Toxic chemicals, radiation, viral infections, and many other environmental factors are associated with frequent occurrence of acquired aplastic anaemia (18, 21-26). Acquired bone marrow aplasia may arise due to one of the three events: (i) intrinsic stem cell defect due to cytotoxic chemicals, (ii) disturbed stem cell microenvironment or (ii) deregulated stem cell differentiation process. Our previous studies have confirmed stem cell depletion in the pesticide exposed mouse model of marrow aplasia and in aplastic patients with history of chronic pesticide exposure (27-29).

The present study was planned to explore the probable mechanism of bone marrow aplasia development induced by chronic pesticide toxicity in mouse involving Hedgehog (Hh) signalling. Hedgehog-Gli signalling axis is an important cytosolic regulator that controls many fundamental processes from embryonic life to adulthood. Members of hedgehog family proteins are the key entities in growth patterning and morphogenesis of different regions within the body plan, from Drosophila to human beings. Intracellular Hedgehog signal transduction is executed through the binding of Hedgehog ligands to the transporter-like receptor, a tumour suppressor protein Patched 1 (Ptch1), which releases an inhibitory effect of its own on Smoothened (Smo), a seven pass transmembrane protein, and ultimately the signal goes down to the nucleus by Gli transcription factors thereby activating Hedgehog targeted genes (30-35). Besides different activities, Hedgehog-Gli signalling axis controls primitive and definitive haematopoiesis during embryogenesis and postnatal condition (36-39). In contrast, two contemporary articles reported that hedgehog signalling was dispensable for definitive haematopoiesis in conditionally deleted $Smo^{-/-}$ in mice (40-41). But, in support of Hh and definitive haematopoiesis, Trowbridge et al. (44) demonstrated that constitutively Hh activated $Ptch^{-/+}$ in mice showed increased cycling and expansion of haematopoietic stem cells (HSC) under homeostatic conditions. On the other hand, Dierks et al. (45) showed that $Smo^{-/-}$ in murine cells lost the colony forming ability at the second replating time, in contrast to the $Ptch^{-/+}$ in murine cells where Smo was activated, which showed both regeneration and enhanced engraftment ability of the bone marrow result.

The involvement of Hedgehog signalling in definitive haematopoiesis ranges from normal haematopoiesis to differentiation and malignancy and these have been supported by numerous investigators (46-51). Hedgehog activity specifies cell fate either through long-range or short-range individual signalling and has also been associated with proliferative responses of target cells (31). Expression of Hedgehog in human haematopoietic population and cells comprising haematopoietic microenvironment suggests that Hedgehog proteins play a functional role in blood cells. Blood cells and haematopoietic microenvironment transduce Hedgehog signalling which has downstream Gli involvement (47). Therefore, the studies on self-renewal and expansion of HSC from bone marrow in pesticide-induced aplastic animal model were taken into consideration as an innovative experimental strategy to reveal the mechanism that lies beneath the Hh-Gli signalling pathway. In corroboration of the aforesaid studies related to hedgehog signalling and definitive haematopoiesis, we are the first group to suggest that chronic long-term pesticide exposure downregulate Hh-Gli signalling axis, which accelerates bone marrow failure or aplasia in a mouse model.

MATERIALS AND METHODS

Animals

Ten to twelve-week-old Swiss albino mice of both sexes (20 males and 20 females) for pesticide exposure purpose and 10 animals (5 males and 5 females) for control weighing 20 g to 24 g were selected from an inbred colony maintained under controlled room temperature (22±2) °C in the animal house of the Calcutta School of Tropical Medicine (India). Both male and female animals were equally used in all following assays. The animals were fed a standard recommended diet and water *ad libitum*, under

standard conditions with a 12-hour light-dark period. Throughout the experiment, a maximum of six animals were housed in one cage containing sterile paddy husk as bedding. The procedures followed were in agreement with the approved guide for the care and use of laboratory animals, the Institutional Animal Ethical Committee (IAEC), and EU Directive 2010/63/EU.

Pesticide mixture preparation

In India, farmers and others use pesticides in a mixture and without any self-protection, so, we took a mixture of pesticides for the experimental purpose to mimic the normal exposure of individuals. We also observed and published the immunohaemotoxic effects of pesticide exposure where patients with aplastic anaemia, mainly farmers, used mixed pesticides (29). We used a 5 % aqueous mixture of DURSBAN (Chlorpyriphos 20 %; Dow Agrosciences, India), PROFEX SUPER (Prophenophos 40 % + Cypermethrin 4 %; Nagarjun Agrochemicals Ltd. India), STOP (alpha-Methrin 10 %; Biostadt, India), and Hexaconazole (Sigma-Aldrich, USA). The first three easily available commercial pesticide preparations were of organophosphate and pyrethroid groups and were manufactured by local Indian agrochemical companies as mentioned above. All aforementioned chemicals were dissolved in aromatic hydrocarbon solvent as per information provided by the manufacturer on each pesticide container. The last one was a systemic fungicide rarely available in the local market as HEXACON SUPER (Hexaconazole 5.43 %). Due to its (HEXACON SUPER) paucity, we used Hexaconazole from Sigma-Aldrich, USA, in pure form and maintained the same concentration as in HEXACON SUPER.

Pesticide exposure

Adult mice (10 to 12 weeks old) were divided into two groups. Experimental animal group (N=40) received inhalation and dermal exposure [by handheld glass atomiser (local made), atomising time was 5 min] to 10 mL of 5 % aqueous solution of aforesaid pesticides mixture for 6 hours per day and 5 days a week up to 90 days (LD_{50} of 10 % aqueous solution was found to be 20 days in our previous experiments). Control group (N=10) received inhalation and dermal exposure of aqueous saline solution without any trace of pesticide contamination over the same period of time (28). Altogether we took 24 exposed animals (8 animals per experiment) and 6 control animals (2 animals per experiment) for the following flow cytometric experiments which were repeated three times along with bone marrow cellularity assessment. The rest of the exposed animals (N=16) and control animals (N=4) were used for the bone marrow smear analysis and explant culture experiments. However, peripheral blood haemogram experiment was done for each and every animal as the investigation was related to the degeneration of haematopoietic machinery. Each haemogram experiment was performed with randomly selected five control animals and twenty pesticide-treated animals at a time.

Blood haemogram profile

We randomly selected experimentally pesticide-treated (N=20) and control (N=5) animals from respective cages at a time for blood haemogram profiling after completing 90-day exposure. Approximately 200 µL of blood was collected in heparinised vial by tail vein puncture from the animals. Total erythrocyte (RBC) count, total leukocyte (WBC) count, platelets, reticulocytes, differential WBC count, and haemoglobin estimation were performed manually as per standard laboratory protocol. Total RBC, WBC, and platelet count were performed by haemocytometer (Rohem, India) and binocular light microscope (Olympus *Ch20i*). Reticulocyte count was performed by Brilliant Cresyl Blue (Sigma) staining method and haemoglobin concentration was estimated by colorimetric (Systronics, India) cyanomethaemoglobin method. At the end, differential WBC count was done by Leishman staining (Himedia, India).

Bone marrow isolation and single cell preparation

The chronic pesticide-exposed experimental and control groups of mice were sacrificed to isolate the long bones (femur and tibia). Bone marrow was flushed out from the isolated bones by syringe (Dispovan 2 mL) containing RPMI-1640 (Sigma-Aldrich, USA) supplemented with 10 % FBS (Foetal Bovine Serum, Lonza, South American origin). Following bone marrow collection, a part of it was kept as undisturbed for explant culture and bone marrow smear analysis. Other part was subjected to single cell preparation for the assays such as total cellularity assessment, quantification of bone marrow-derived stem/progenitor cells, and status of hedgehog signalling in the bone marrow haematopoietic cells.

Bone marrow smears analysis

Bone marrows of the normal and exposed groups were Leishman stained (Himedia; India) and examined under light microscopy (1000x magnification; Olympus *Ch20i*) (52).

Bone marrow cellularity assessment

Femoral marrow cells were aseptically collected by flushing. Total cell counts of marrow single cell suspensions of both control and pesticide-exposed animals were then performed in Hemocytometer chamber (Rohem INDIA) by well known Erythrosin-B (Sigma-Aldrich, USA) dye exclusion method (53).

Bone marrow explants culture and cell release pattern study at (0, 24, 48, 72, 96) hours

Small portions of the isolated (described previously) marrow were cut into small pieces (0.2 mm^3) and subjected to culture in triplicate (for each of the normal and experimental groups) in 75 mm culture dish (Corning, USA) containing 3 mL of RPMI-1640 supplemented with 15 % FBS (Lonza, South American origin). The cultures were incubated at 37°C inside CO_2 incubator and a time-course [(0, 24, 48, 72, 96) h] cell release pattern was monitored and calculated by the aid of haemocytometer (Rohem; India).

Flow cytometry

Evaluation of murine bone marrow stem and progenitor population

To analyse haematopoietic stem cell population and signalling, $1x10^6$ pooled cells from each group were fixed with 1.5 % para-formaldehyde (PFA, Ranbaxy, India) for 30 min at 37 °C in dark. To identify the murine haematopoietic progenitors, a PerCP-Cy5.5™ conjugated Lineage (Lin) antibody cocktail (BD Pharmingen, USA) was used (CD3e, CD11b, CD45R/B220, TER-119, and Ly-6G and Ly-6C) in RBC depleted population for 20 min. This was followed by labelling with anti-mouse Sca-1-PE monoclonal antibody (BD-Bioscience, USA) and anti-CD117 antibody-FITC (BD-Bioscience, USA) in 1 % bovine serum albumin (Sigma), and dissolution in phosphate buffered saline (PBS) for another 30 min incubation. Furthermore, a single rapid wash was performed by PBS to remove unbound antibody from each sample. Samples were then analysed by BD-FACS Callibur (Becton Dickinson, USA) using CellQuest Pro software (v9.1 Becton-Dickinson, USA).

Flow cytometric analysis of Shh, Ptch1, Smo, and Gli1: components of canonical Hedgehog signalling pathway

Shh, Ptc1, and Gli1 proteins were stained by cell permeabilisation technique (27) in which $3x10^6$ bone marrow haematopoietic cells were first fixed at room temperature in 1.5 % PFA and pelleted. Fixed cells were permeabilised by resuspension with vortexing in 500 µL of chilled 90 % methanol (SRL, India.) per $1.5x10^6$ cells and incubated at 4 °C for 15 min to 20 min. Thereafter, cells were washed twice in FACS fluid (PBS containing 1 % BSA) and divided in five sorting tubes with $1.5x10^6$ cells per 100 µL of fresh FACS fluid. 2 µL anti-Shh antibody (Santa Cruz Biotechnology, USA), Ptc1antibody (H-267) (Santa Cruz Biotechnology, USA), and anti Gli1 antibody (H-300) (Santa Cruz Biotechnology, USA) were then added into respective sorting tubes and incubated for 30 min at 37 °C. This was followed by the addition of goat anti-rabbit secondary antibody conjugated with AlexaFluor-488 (Invitrogen, USA) to each primary antibody containing tubes and incubated further for 30 min at 37 °C.

One part of the non-permeabilised PFA-fixed bone marrow haematopoietic cells (1×10^6 cells) were subjected to 2 µL of anti-Smo antibody (N-19) (Santa Cruz Biotechnology, USA), which was directed against extracellular part of Smo receptor and incubated for another 30 min. This was followed by rabbit anti-goat secondary antibody conjugated with FITC (Santa Cruz Biotechnology, USA) staining. Then, all the samples were washed to remove excess fluorescence and resuspended to staining media. They were analysed by BD FACS Calibur (Becton-Dickinson, USA) using CellQuest Pro software (v9.1 Becton-Dickinson, USA).

Statistical analysis

All the values of flow cytometric studies, cell release kinetics, and haemogram were represented as mean ± SD (Standard Deviation). Statistical analysis was performed by paired Student's *t*-test and the level of statistical significance was $P<0.05$ and $P<0.0001$.

RESULTS

Peripheral blood pancytopenia

Comparative haemogram study in control and chronic pesticide-exposed group of mice revealed that

the pesticide-exposed animal group had moderate to severe pancytopenia with depressed haemoglobin level (~7 g dL^{-1}) and uniformly reduced corpuscular counts, of which reticulocyte count was significantly lower (~0.23 %) throughout the study compared to normal mice (0.89 % to 1.5 %). Significantly low absolute neutrophil count (498 μL^{-1}), appearance of abnormal neutrophils with their distorted nuclear lobulation pattern, and monocytopoenia (≤48 μL^{-1}) have further been recorded. The deserted situation of peripheral blood signified the commencement of bone marrow aplasia only inside these experimental animals as compared to the control group, which had normal total WBC (~6.0x10^3 μL^{-1}), RBC (8.5x10^6 μL^{-1}), and standard lymphocyte (~4320 μL^{-1}), neutrophil (~1420 μL^{-1}), and monocyte (~220 μL^{-1}) counts (Table 1).

Bone marrow smear study

Bone marrow smear of control group of mice revealed normal haematopoietic cellular distribution (Figure 1a). On the other hand, pesticide-exposed bone marrow cell pool started to be replaced by stromal fibres, large adipocytes, and empty spaces in contrast to the normal bone marrow (Figures 1b - 1d).

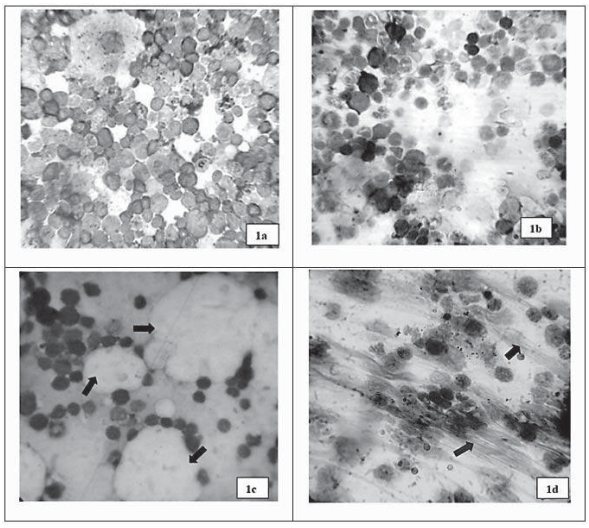

Figure 1 *Light microscopic study of bone marrow smear*
Figure 1a shows the smear of a control bone marrow comprised of numerous haematopoietic cells at different stages of developmental cascade. In the pesticide-treated aplastic bone marrow (Figures 1b-1d), most of the spaces in smear are occupied by large adipocytes or stromal fibres (arrow marked), which developed from pre-adipocytic fibroblasts and collagen, respectively, and very few haematopoietic cells are present there.

Table 1 *Comparative haemogram of control and pesticide-treated mice.*

Parameter	Control (N=10) Mean ±SD	Treated (N=40) Mean ±SD
Haemoglobin / g dL^{-1}	15.9±2.20	7.0±1.50 #
Reticulocyte / %	0.9±0.15	0.23±0.07 #
Total count		
Total RBC / x 10^6 μL^{-1}	8.5±0.70	3.2±0.60 #
Total WBC / x 10^3 μL^{-1}	6.0±1.20	3.6±0.30 #
Platelets / x 10^3 μL^{-1}	440±13.92	169±10.00 #
Differential WBC count		
PMNs / μL^{-1}	1402±2.00	498.8±3.12 #
Lymphocytes / μL^{-1}	4320±2.15	3045±3.60 #
Monocytes / μL^{-1}	220±1.30	48±1.50 #
Basophiles / μL^{-1}	58±0.01	10±0.05 #

 # Marked values are significantly different (P<0.001) from the values of the control animals within the same row.

Total bone marrow cellularity

Chronic pesticide exposure diminished (~2.5 fold) the total bone marrow cellularity of the pesticide-treated animals in comparison to the control group of animals. The difference in the bone marrow cellularity between control and pesticide-treated animals was statistically significant (P<0.05) (Figure 2).

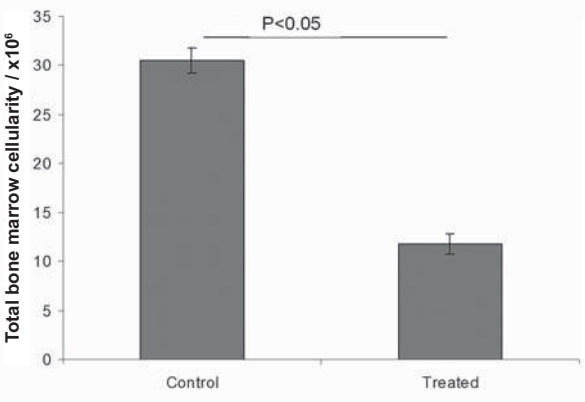

Figure 2 *Total bone marrow cellularity study*
Comparative whole bone marrow assessment of control and chronic pesticide-treated group of animals revealed a significant difference in its cellularity. Chronic pesticide exposure reduced the bone marrow cellular components approximately 2.5-fold in comparison to control group and observed difference was statistically verified (P<0.05).

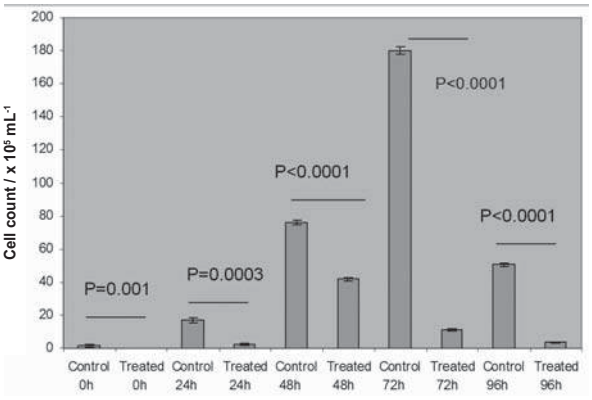

Figure 3 *Bone marrow cell release kinetics*

Bone marrow explant culture and cell release kinetics further revealed the hypocellularity of bone marrow after chronic pesticide exposure

Initially (0 h), normal bone marrow explant started to release cells and the number of cells gradually increased with time. The number of released cells was the highest at 72 h [(180±2.0) x10^5 cells mL^{-1}] in contrast to 24 h [(16.63±1.5) x10^5 cells mL^{-1}], 48 h [(76±1.3) x10^5 cells mL^{-1}], and 96 h [(50.6 ± 1.1) x10^5 cells mL^{-1}] (Figures 3 and 4). However, the scenario of aplastic bone marrow explant was not same (Figures 3 and 4). At the beginning (0 h) and after 24 h of culture, very few cells were released from pesticide-exposed bone marrow and it was almost negligible in comparison to normal cell release kinetics at 24 h (P=0.0003). But, the accelerated cell release was observed at 48 h [(41.76±1.32) x10^5 cells mL^{-1}] and was considered significant (P<0.0001). Cell release kinetics of aplastic bone marrow again decreased after 72 h [(11±0.5) x10^5 cells mL^{-1}] and 96 h [(3.5±0.2) x 10^5 cells mL^{-1}] (P<0.0001).

Hypocellularity was due to the shortage of stem/ progenitor cells in the bone marrow

The percentage of gated $Lin^-Sca\text{-}1^+C\text{-}kt^+$ (LSK) cells, (0.47±0.05) % [i.e. (0.01±0.002) % of whole bone marrow] in the bone marrow, showed a nearly sevenfold decrease in the pesticide exposed animals (P<0.001) compared to control animals, (3.42±0.7) % [i.e. (0.07±0.05) % of whole bone marrow] (Figure 5). It signifies that chronic pesticide exposures severely hampered the primitive haematopoietic stem cell compartment and its effects were manifested through hypocellular bone marrow and pancytopoenia in the treated group of animals.

Pesticide-mediated deregulation of canonical Hedgehog signalling: latent cause of bone marrow hypocellularity and degeneration

There was a severe depression in the expression of Ptch1 (Mean Fluorescence Intensity, MFI=320.4 ± 1.90), Smo (MFI=40.32±2.01), and Gli1 (MFI=365.89 ±3.45) proteins in the pesticide-treated bone marrow aplasia condition in comparison with the control bone marrow (MFI of Ptch1=400.0±3.2, MFI of Smo=75.85±1.65 and MFI of Gli1=550±3.54). Furthermore, bone marrow of control animals showed a good amount of autocrine Shh ligand expression (MFI=178.20±2.7) in contrast to the pesticide-treated bone marrow where Shh expression (MFI=46.35±3.70) was hampered. All the data were statistically significant

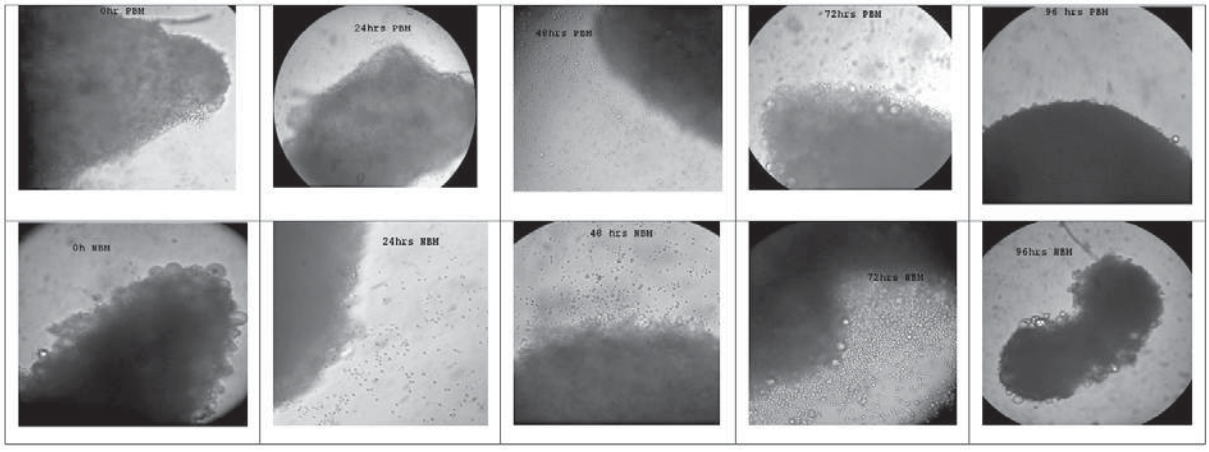

Figure 4 *Short term bone marrow explant culture*
A time-course experimental setup revealed the exact bone marrow microenvironmental status [control (NBM) vs. pesticide treated (PBM)] under the in vitro *short term culture condition where control marrow cell population moved rapidly towards the nutritionally enriched* in vitro *media to thrive and further repopulate in contrast to pesticide-treated marrow.*

Table 2 *Flow cytometrically measured Mean Fluorescence Intensity (MFI) values of Sonic hedgehog (Shh), Patched1 (Ptch1), Smothened (Smo), and Gli1 protein expression in control and experimentally pesticide-treated (aplastic bone marrow) haematopoietic cells.*

Marker	Mean Fluorescence Intensity	
	Treated (N=24)	Control (N=6)
Shh	178.2±2.7	46.35±3.70 #
Ptch1	400±3.2	320.4±1.90 #
Smo	75.85±1.65	40.32±2.01 #
Gli1	550±3.54	365.9±3.45 #

 # Marked values are significantly different (P<0.0001) from the values of the control animals within the same row.

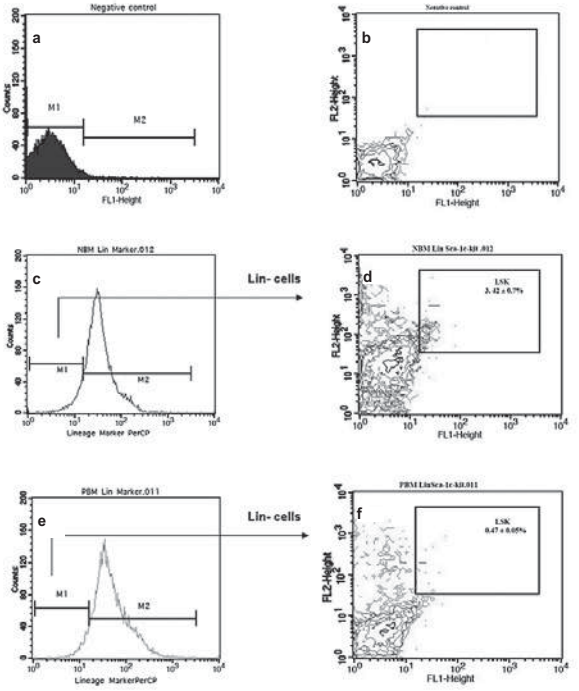

Figure 5 *Flow cytometric analysis of haematopoietic stem and progenitor (LSK) cell population in control and pesticide exposed animals*
Figures 5a to 5f represent a quantitative comparison of primitive haematopoietic stem and progenitor cell population in control (NBM; N=6) and pesticide-treated bone marrow (PBM) from a pooled preparation (N=8 for each experiment repeated three times).

(P<0.0001) (Table 2). Here, MFI of epitope-bound fluorophore-tagged antibodies was directly proportional to the concerned protein expression in the subjected cells measured by flow cytometry (Figures 6a-6d, Table 2).

DISCUSSION

Agrochemicals like pesticides are designed to target agricultural pests but incidentally these

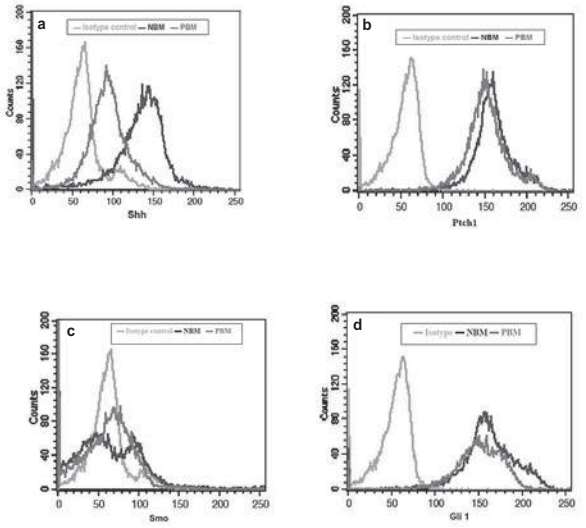

Figure 6 *Flow cytometric evaluations of Hedgehog signalling and its components*
Comparative flow cytometric expression study of Shh and Ptch-1 show a significant amount of downregulation in pesticide-treated bone marrow (PBM) haematopoietic cellular compartment in comparison to control bone marrow (NBM) haematopoietic compartment (Figures 6a and 6b). Smo, another transmembrane component and a positive regulator of Hedgehog signalling pathway also faced downregulation in aplastic condition in comparison with the control group (Figure 6c). Figure 6d shows further downregulation of Gli-1 expression in the aplastic bone marrow. Here, the flow cytometric expression estimation of Hh signalling components was performed on the basis of an analysis gate, which included mostly stem and progenitor population of scattergram. After gating MFI was determined by histogram analysis.

chemicals also harm a broad range of non-targeted organisms including humans. The widespread use of pesticides means we face a continuous risk of exposure to these compounds, which can have several adverse effects. In such cases previous research elucidated the mechanistic correlation of pesticide exposure and neurological disorders. Reports concerning the haematopoietic system, on the other hand, are scanty (4-7, 27-29). Here we considered adult definitive haematopoietic system i.e. bone marrow as our organ of interest to study the mechanism of long term pesticide exposure through inhalation and haematopoietic failure.

The present study revealed an overall diminution of peripheral haemogram parameters and it led us to propose an inference that chronic pesticide exposure hindered neutrophilopoiesis and distorted the morphology of nucleus along with leucopoenia. Low haemoglobin level and diminished total RBC were the ultimate results of impaired erythropoiesis and

generation of low number of reticulocytes, an immediate progenitor of mature RBC, which signified the presence of aplasia in pesticide exposed mice.

On the other hand, a declining state of haematopoietic cells was observed in aplastic marrow smear with scattered lymphocytes and numerous large adipocytes which developed from pre-adipocytic fibroblasts, a special feature of aplastic bone marrow (Figures 1b and 1c). Stromal collagenous fibres were also evident in experimental bone marrow. These large adipocytes and stromal fibres actually replaced the empty spaces of bone marrow produced by long-term pesticide treatment. Furthermore, chronic pesticide inhalation also reduced the total bone marrow cellular components in comparison to the control mice.

Time-bound and comparative bone marrow explant cultures of two aforesaid groups of animals represented a very authentic scenario regarding pesticide exposure and bone marrow cell release pattern under *in vitro* condition. Hypocellularity of pesticide-exposed bone marrow was further established by the present experimental effort. Unlike its normal counterpart, pesticide-exposed bone marrow explant released its cell pool very slowly and in low quantity over time. The low cell-releasing pattern was also an evidence of hypocellularity of bone marrow, which indicated the shortage of marrow haematopoietic cellular storage, low self-renewal, and proliferation activity. Bone marrow explant culture study also exhibited a phenomenon that the pesticide-exposed sample, following a 48-hour lagging period, starts releasing the haematopoietic cells which could not thrive under *in vitro* condition as compared to the normal counterpart. This finding reinforces the fact that chronic pesticide exposure hampers the cellular proliferation rhythm, which ultimately delays the cell release behaviour in comparison to normal bone marrow (Figure 3).

The reduced proliferation, as well as depletion of haematopoietic stem and progenitor cells, added an extra dimension to the reason behind marrow hypocellularity, impaired neutrophilopoiesis, erythropoiesis, and ultimately the occurrence of peripheral blood pancytopoenia. Besides, it also explained how progressive loss of primitive $Lin^{-}Sca\text{-}1^{+}C\text{-}kit^{+}$ (LSK) haematopoietic population in endosteal niche or bone marrow microenvironment of pesticide-exposed mice failed to replenish the pancytopoenia in the vascular niche, as a result of which we observed a significant diminution in peripheral blood elements.

The reason behind the shortage of self-renewing, proliferating haematopoietic stem cells in the bone marrow of pesticide-induced aplastic mice is mostly unexplained. We tried to study the event by our flow cytometric analysis (Figure 6) of the expression pattern of important protein components related to the canonical Hedgehog-Gli1 signalling axis. As we were already aware that hedgehog signalling had a profound effect on embryogenesis and adult tissue homeostasis, we considered the expression status of Shh, Ptch-1, Smo, and Gli1, a transcription factor of the zinc finger family, which activates the hedgehog targeted genes in the haematopoietic stem-progenitor rich compartment. In 2009, Hoffamann et al. (40) and Gao et al. (41) separately reported that the Hh pathway was dispensable for the adult HSC maintenance and differentiation process. However, their findings were not consistent with several studies by Trowbridge et al. (44), Dierks et al. (45), Zhao et al. (46), and Bharadwaj et al. (47). The limitations of their (Hoffman et al. and Gao et al.) studies (40, 41) were discussed in ′The Niche′-Nature Stem Cell Blogs (54) and in a spotlight review of ′Leukaemia′ journal by Aifantis et al. (43) and Merchant et al (42). Irrespective of their controversial studies, we observed sharp downregulation in Hh signalling cascade from the upstream regulator Ptch-1 to the downstream executor Gli-1 along with its ligands Shh in our pesticide-induced aplastic murine bone marrow haematopoietic compartment. It is known to us that Ptch-1 is a protooncogene which affects cell cycle as well as lineage commitment of haematopoietic stem cells (34). In our study, diminished expression of Ptch-1 in pesticide-induced aplastic bone marrow reflected low conversion of primitive LSK population to more mature progenitors and blood corpuscles in peripheral circulation.

Diminution of bone marrow LSK population and downregulation of Smo expression in the pesticide-exposed animals presented a parallel observation with the study of Zhao et al. who showed that $Smo^{-/-}$ and cyclopamine (Smo inhibitor) treatment in mice drastically reduced bone marrow LSK population in comparison to the control (46). Downregulation of Smo expression in our study might be the direct possible manifestation of pesticide exposure. We suspect that the Hexaconazole, a systemic fungicide of the ′azole′ family used in our experiment, possibly acted as an inhibitor for Smo downregulation in pesticide-induced bone marrow failure because it has been found that Itraconazole, another clinically well

known systemic fungicide of the azole family, showed Smo antagonism in a recent study on murine tumour (16). Smo failed to transduce the signal up to the final downstream executor Gli1, which was seen as a usual depressive status in pesticide-induced aplastic bone marrow in contrast to its normal counterpart (Figure 6).

Reduced Gli1 expression also affected the hedgehog pathway by downregulating the intracellular feedback loop of Ptch1 and Gli1. Under normal conditions, Gli1 protein helps to transcribe Gli1 by means of a positive feedback mechanism and also upregulates the signalling repressor Ptch1, a negative feedback that reins the Gli1 regulatory loop. It actually represents a signalling network composed of a positive transcriptional feedback loop embedded within a negative signalling feedback loop.

Our present experimental data revealed a steady dampening condition in the Gli1–Ptch1 feedback loop, in which downregulation of Gli-1 gene product automatically suppressed the Ptch-1 expression by direct action of pesticide-deregulated Smo repression. Henceforth, it is clear that chronic pesticide exposure also hampers the direct feedback loop between Gli-1 and Ptch-1 inside the bone marrow haematopoietic stem/progenitor cell population, in the context of Smo downregulation, which leads to a progressive degeneration of haematopoietic machinery. In these contexts, the altered internal regulatory loop between Ptch1-Smo-Gli1 of Hedgehog signalling crippled the self-renewal, proliferation, and differentiation capacity of haematopoietic stem cells and resulted in bone marrow suppression. However, future studies might be designed to further elucidate the role of Hedgehog signalling in the pathology of the pesticide-induced bone marrow toxicity by constitutive over-expression of the active components of the Hh signalling pathway in the stem cells. Only then could one determine whether this approach rescues the marrow failure observed in our established animal model.

CONCLUSION

At the end we conclude that the unresolved paradox of the development of intrinsic stem cell defect of acquired aplastic anaemia through chronic pesticide exposure lies in the deregulation of the canonical Hedgehog-Gli1 signalling cascade. Besides, commercially available pesticides are harmful not only to the nervous system of the non-target organisms but also to other developmentally important signalling cascades like Hedgehog signalling, which functions as “stem cell fate switch”. Finally, our experiment suggests the possibility of using primarily Gli-1 as the biomarker for those patients who are chronically exposed to pesticides in agricultural field and are either suffering from aplastic anaemia or having propensity towards the development of bone marrow aplasia.

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Sažetak

PROMIJENJENI KANONIJSKI SIGNALNI PUT HEDGEHOG-GLI KOD PESTICIDIMA INDUCIRANE APLAZIJE KOŠTANE SRŽI ISPITAN NA MIŠJEM MODELU

Mehaničko međudjelovanje izlaganja pesticidima i razvoja aplazije koštane srži još uvijek nije u potpunosti utvrđeno, ali postoje naznake da kronično izlaganje pesticidima u nekim slučajevima može uzrokovati aplaziju koštane srži poput hematopoetskoga degenerativnog stanja u ljudi. Kanonijski signalni put Hedgehog (Hh) ima višestruke uloge u mnogim razvojnim procesima, uključujući i hematopoezu. Ovo je ispitivanje imalo za cilj istražiti status četiri glavne sastavnice kanonijskoga signalnoga puta Hedgehog, Sonic Hedgehog (Shh), Ptch1, Smo i Gli1, na mišjem modelu pesticidima inducirane aplazije koštane srži. Koristili smo 5 % vodenu mješavinu pesticida (klorpirifos, profenofos, cipermetrin, alfa-metrin i heksakonazol) kojoj smo miševe izložili udisanjem i preko kože tijekom 6 sati dnevno i 5 dana tjedno do najviše 90 dana. Kronično izlaganje pesticidima vezano uz aplaziju koštane srži bilo je primarno potvrđeno krvnom slikom, celularnošću koštane srži, kratkotrajnom kulturom eksplantata koštane srži radi stanične kinetike, razmazom koštane srži i ekspresijskim obrascem protočne citometrije izvanstaničnog receptora Lin-Sca-1^{+}C-kit^{+}. Potom su analizirane sastavnice signalnog puta hedgehog u koštanoj srži kontrolnih jedinki i aplastičnih životinja koje su tretirane pesticidima. Rezultati su pokazali pancitopeniju periferne krvi, razvoju anomaliju neutrofila, depresiju primitivnih matičnih stanica i prastanica uz Shh, Ptch1, Smo i Gli1 ekspresiju u skupini koja je imala aplaziju. Ovo istraživanje navodi na zaključak da pesticidi uzrokuju sniženje dvaju kritičnih proteina - Ptch1 i Gli1 - unutar hematopoetskih matičnih stanica i prastanica uzrokujući time hematopoetsku homeostazu i poremećaje regeneracijskog mehanizma *in vivo* zajedno s aplazijom koštane srži.

KLJUČNE RIJEČI: *hematopoetske matične stanice, "patched" protein, "smoothened" protein, "sonic hedgehog" protein, supresija koštane srži*

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Scientific Paper

PREDICTION OF MUTAGENICITY, CARCINOGENICITY, DEVELOPMENTAL TOXICITY, AND SKIN SENSITISATION WITH CAESAR PROGRAM FOR A SET OF CONAZOLES

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This article presents models to predict mutagenicity, carcinogenicity, developmental toxicity, and skin sensitisation for a set of 27 conazoles. The predictions were performed with the program package CAESAR, which is available on the Internet. The CAESAR programs were developed to support the European Community Regulation on chemicals and their safe use (REACH) and follow the OECD principles for (Q)SAR models used for regulatory purposes. The programs provide a number of information, including a binary classification of a compound as toxic or non-toxic and information on similar compounds from the model's training sets (similarity sets). In this study we analysed conazole sets using principal component analysis (PCA). The predictions were compared to the currently valid classification of these substances in the European Union (EU) or to the classification proposed at expert meetings of the Pesticide Risk Assessment and Peer Review (PRAPeR) group. The predicted classification for mutagenicity was in good agreement with regulatory classification, the predictions for carcinogenicity and developmental toxicity showed some discrepancy in particular cases, while the predictions for skin sensitisation showed even greater discrepancy.

KEY WORDS: *European Chemical Regulation – REACH, Principal Component Analysis, QSAR modelling*

Registration of chemicals requires proper risk assessment. It consists of four steps: hazard identification, hazard assessment, exposure assessment, and risk characterisation. Hazard assessment involves determination of toxicological properties of a compound. They are usually determined experimentally applying *in vivo* or *in vitro* measurements, which are expensive, time-consuming, and sometimes involve ethical concerns. Computer-assisted (*in silico*) methods, including Quantitative Structure-Activity/ Property Relationship (QSAR/QSPR) modelling, provide alternative methods for the evaluation of toxicological endpoints. The use of these methods is supported by the European Community Regulation on chemicals and their safe use to protect human health and environment (REACH - Registration, Evaluation, Authorisation and Restriction of Chemical Substances) (1). Beside QSAR/QSPR, REACH acknowledges other *in silico* methods for read-across and grouping. The REACH Guidance on Information Requirements and Chemical Safety Assessment recommends that all these methods be used in hierarchical (tiered) order (2). Furthermore, the European Chemical Agency (3), a body which implements REACH, advises the industry to use *in silico* methods (4). An important message is that in their guidelines for (Q)SAR models

REACH, US Environmental Protection Agency (US EPA) and the Organisation for Economic Co-operation and Development (OECD) promote alternative methods to evaluate chemical hazards, both to save animals and to increase information robustness (5, 6). QSAR/QSPR modelling rests on the assumption that compounds with "similar molecular structures show similar properties" (7). QSAR models take several steps (8, 9). The first is to determine the data set that represents the knowledge base for the model. The data set must contain molecular structures and their specific properties. It is the quality of these data that essentially determines the quality of the model. Structural data should be selected carefully while the property data should be obtained under the same laboratory protocols.

The second step is to determine molecular structures, where different approaches are possible. A molecule can be represented with two-dimensional or three-dimensional structures, which is defined by the positions of all atoms constituting a molecule. In the QSAR/QSPR model the structures are represented with descriptors. Nowadays, a variety of computer software is available to calculate hundreds of descriptors at a time. A common method to select the most relevant descriptors is the genetic algorithm.

The third step involves selection of the modelling method. The most common is multi-dimensional linear regression (MLR), which is very fast, and a result is given in the form of a multi-linear equation. More advanced methods are the principal component analysis (PCA), partial least square, Reach regression, artificial neural networks of different architectures, and learning algorithms. In a standard QSAR model the property is expressed as a continuous variable, as for example dose of activity. Alternatively, the property can be given as affiliation to a particular class of activity. For classification, a variety of methods is available such as linear discriminate analysis, support vector machine, or artificial neural networks of specific architectures.

The last step is the testing and the validation of models. The questions are: how to test a model and how to express the quality of a model. Today, a basic concept is accepted that a model should be tested with an independent test set. An independent test set means a set that was never used in a model-developing procedure. Before modelling starts, a test set is excluded from the compiled data set. Usually, test sets are selected at random from an entire model's pool of data sets. When the model is presented in its final form it is tested with this test set. The quality of a model is usually expressed as the correlation coefficient between predicted and measured values. When a model is used for classification its performance is usually expressed as a ratio between correctly and falsely classified objects. In a binary classification, in which response is either positive or negative, model responses can be true positive (TP), true negative (TN), false positive (FP), or false negative (FN). The models are evaluated for their precision (P), specificity (SP), and sensitivity (SE) using the following formulas: P=(TP+TN)/(TP+TN+FP+FN); SE=TP/(TP+FN); and SP=TN/(TN+FP).

To ensure the transparency of the QSAR/QSPR models used in regulatory procedures, the OECD adopted five principles for their evaluation (10): *Principle 1: defined endpoint*; *Principle 2: unambiguous algorithm; Principle 3: definite applicability domain; Principle 4: measure of goodness-of-fit, robustness and predictivity*; and *Principle 5: mechanistic interpretation, if possible.*

CAESAR programs were developed to support the use of *in silico* methods in REACH. They provide complex information about the examined structure and the models. In addition to prediction of activity, these include information about applicability that describes how well the examined structure fits in the training set. Our aim was to analyse these results for the entire set of conazoles. The analysis may show some common features in the set which may support the classification pattern of conazoles. The information on applicability should support this classification. Some details are described in Methods. The data set for conazoles is described in the next section.

Data set

The predictions were performed for a set of 27 conazoles. Conazoles form a group of chemicals which are used in agriculture, in human and veterinary anti-mycotic therapies, and as non-steroidal anti-oestrogens in the treatment of oestrogen-responsive breast tumours in postmenopausal women. As active substances in plant protection, conazoles marketed in the EU must be tested for mutagenicity, carcinogenicity, developmental toxicity, and skin sensitisation. Conazoles are mostly biologically active substances with known mechanisms of activities. For example, the anti-mycotic activity of conazole fungicides is based on the competitive and reversible inhibition of two cytochrome P450 enzymes, sterol 14α-demethylase and aromatase. These two enzymes are also present

in mammals, and their inhibition can affect mammalian steroidogenesis (endocrine disruption) (11). Some conazoles like flusilazole inhibit aromatase with an IC_{50} in the range of cytostatic drugs. Bitertanol, triadimenol, tebuconazole, and propiconazole are weak inhibitors of aromatase (12). Table 1 presents the set of 27 conazoles and their CAESAR classification. Some of the substances are fungicides [bitertanol, bromuconazole, cyproconazole, difenoconazole, diniconazole (-M), epoxiconazole, fenbuconazole, floquiconazole, flusilazole, hexaconazole, ipconazole, metconazole, myclobutanil, penconazole, propiconazole, prothioconazole, tebuconazole, tetraconazole, triadimenol, triticonazole], some are herbicides (amitrole, cafenstrole, epronaz, flupoxam), and some are of unknown biological activity. Metabolite triazole acetic acid, which is sometimes found in plants treated with conazole pesticides, was also added to the list. Data on this set have been reported in references (11-15).

METHODS

CAESAR models

CAESAR (8, 16) is a project funded by the European Commission dedicated to developing *in silico* models for the prediction of five endpoints relevant for REACH. These models were developed according to the OECD principles for (Q)SAR models (10) on high quality data sets following the OECD or US EPA standards. To develop the models, different programs for the calculation of molecular descriptors and descriptor selection were used. These models are publicly available over the Internet and with regard to the input, only the SMILES code of molecular structure is required. For bioconcentration, prediction is expressed as a real number and for other four endpoints prediction takes the form of a binary classification. Beside classification, CAESAR provides additional information such a comment, if the descriptors are out of range, and about the similarity set. The similarity set consists of six compounds from the training set, which are the most similar to the evaluated one. Similarity is expressed with the similarity index.

Mutagenicity

Details of the CAESAR mutagenicity model are given in reference (17). The model is built on a large data set of 4204 compounds with their Ames test results, which were extracted from the original set reported by Kazius et al. (18). For all structures the descriptor pool was calculated using MDL software. BestFirst algorithm from the Waikato Environment for Knowledge Analysis software (WEKA) was applied to select the 27 relevant descriptors. Modelling combines support to vector algorithm and a rule-based system checking for structural alerts. In classification, mutagenicity is expressed as "non-mutagen", "mutagen", or "suspected mutagen".

Carcinogenicity

Details of the CAESAR carcinogenicity model are given in reference (19). The model was built on a set of 805 non-congeneric compounds extracted from the Carcinogenic Potency Database (CPDBAS). The Hybrid Selection Algorithm developed by BioChemics Consulting SAS (BCX), France was applied to selected eight descriptors from a set of 254 MDL descriptors. Furthermore, a cross correlation matrix, multi-colinearity, and Fisher ratio were applied to 12 descriptors taken from a set of 835 DRAGON descriptors. For classification we used Counter Propagation Artificial Neural Networks. A compound in the training set was classified as non-carcinogenic when mice and rats tested negative, and vice versa, it was classified as carcinogenic when at least one test was positive. The prediction is expressed as "negative" or "positive" together with the class index, which indicates how reliable the prediction is.

Developmental toxicity

Details of the CAESAR program for developmental toxicity are given in reference (20). The classification model is based on the random forest algorithm. It is built on the Arena data set, which includes 292 compounds (21). In the CAESAR program, the compounds are binarily classified as developmental non-toxicants, if they belong to the FDA categories A or B, or as developmental toxicants, if they belong to the FDA categories C, D, or X. Descriptors were calculated with DRAGON, T.E.S.T., and MDL programs. Thirteen molecular descriptors were selected using the WEKA software. The testing strategy and test results are given in reference (20). In the prediction, a compound is predicted as "developmental toxicant" or "developmental non-toxicant".

Skin sensitisation

Details of the CAESAR model for skin sensitisation are given in reference (22). The data set consists of 209 compounds selected from the Gerberick data set of 211 compounds (23). From a pool of 502 descriptors, which were calculated with DRAGON, the hybrid selection algorithm singled out seven descriptors. The hybrid selection algorithm combines the genetic algorithm with regression technique. The model itself was based on an adaptive fuzzy partition algorithm (24). The prediction is expressed as "active" (for sensitisers) or "inactive" (for non-sensitisers), accompanied by a class index.

Analysis of data

The predicted classifications for the four endpoints are presented in Table 1. In the further step we analysed the similarity sets, which are given for each prediction. For each endpoint we constructed a representation space for molecules, which combines all similarity sets. In other words, a molecule is represented by a multidimensional binary vector, where each vector component indicates a compound of the similarity set (data available from authors upon request). First, we analysed how many times individual compounds of the training set appeared in the similarity sets. The question was whether a single compound (or several compounds) predominated in the predictions for all conazoles. In the next step, we considered the binary representation vectors as descriptors and implemented principal component analysis (PCA) to explore similarities between compounds. We wanted to see if individual principal axes were predominately composed of active or inactive compounds. We also wanted to see if the principal axes separated the conazoles set in two or more clusters.

The aim of the study was also to predict the toxicological class for the five substances in the conazole set for which no classification at the EU level had been proposed. The predictions for other compounds obtained with the CAESAR models were compared to the EU classification (15) or to the one proposed at Pesticide Risk Assessment and Peer Review (PRAPeR) group meetings (14) where no EU classification was available.

RESULTS

Mutagenicity

The predictions are shown in Table 1. Testing showed that predictions for 22 of 27 compounds were true negative, one compound was wrongly predicted as suspected positive, and for four compounds no experimental data were available. Two compounds, climbazole[1] and omoconazole[2], appeared in ten similarity sets and econazole[3] in eight. All three compounds were verified as (tested) non-mutagenic.

The loadings for the PCA were as follows: the first and the second axes combined mofezolac[4], climbazole[1], omoconazole[2], and econazole[3]. The third axis added indolebutyric acid[5], and haloperidol[6]. All the compounds constituting the first three axes tested non-mutagenic.

The score plots for the first and second axis are shown in Figure 1a. Four clusters are evident; the first consists of 1,2,4-triazole, amitrole, and 1,2,4-triazol acetic acid, the second of epronaz and prothioconazole, the third of ipconazole, myclobutanil, penconazole, cafenstrole, hexaconazole, tetraconazole, metconazole, tebuconazole, triadimenol, and cyproconazole, and the fourth of bitertanol, flusilazole, fenbuconazole, flupoxam, epoxiconazole, and difenoconazole. As almost all compounds were predicted as non-mutagenic, the clusters show only the relationship regarding the similarity sets. Only cafenstrole and epronaz were predicted as mutagenic and epoxiconazole as a suspected mutagen. Epronaz, for which no data on classification is available, was predicted as mutagenic. This prediction was supported by similarity with six compounds in the same set, which all tested mutagenic. On the other hand, the predictions for cafenstrole and epoxiconazole were not supported by similarity sets. The similarity set for cafenstrole was composed of six compounds tested non-mutagenic and for epoxiconazole of four compounds tested non-mutagenic and two mutagenic. The false positive prediction for epoxiconazole was probably due to the presence of epoxide and aziridine groups in the molecule, which were recognised as mutagenic by the program. Diniconazole-M was predicted as non-mutagenic with an equal number of compounds tested mutagenic and non-mutagenic in the similarity set, just like the set for diniconazole. Ipconazole was predicted as non-mutagenic, as all six compounds in its similarity set tested non-mutagenic. The calculated

[1] 1-(4-chlorophenoxy)-1-imidazol-1-yl-3,3-dimethylbuxan-2-one
[2] 1-[1-[2-(4-chlorophenoxy)ethoxy]-1-(2,4-dichlorophenyl)prop-1-en-2-yl]imidazole
[3] 1-[2-[(4-chlorophenyl)methoxy]-2-(2,4-dichlorophenyl)ethyl]imidazole
[4] 2-[3,4-bis(4-methoxyphenyl)-1,2-oxazol-5-yl]acetic acid
[5] 4-(1H-indol-3-yl)butanoic acid
[6] 4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one

Table 1 *CAESAR predictions and* comparison *with EC regulation for the set of 27 conazoles.*

No	Substance	CAS	Mutagenicity		Carcinogenicity		Developmental toxicity		Skin sensitisation	
			CAESAR	Regulation or PRAPeR proposal (14, 15)	CAESAR	Regulation or PRAPeR proposal (14, 15)	CAESAR	Regulation or PRAPeR proposal (14, 15)	CAESAR	Regulation or PRAPeR proposal (14, 15)
1	1H-1,2,4-triazole[1]	288-88-0	N	N	P	N	N (descriptors out of range)	P	P	N
2	amitrole[2]	61-82-5	N	N	P	N	P	P	P	N
3	bitertanol[3]	55179-31-2	N	N	N	N	N	P	P	N
4	bromuconazole[4]	116255-48-2	N	N	P	N	P	P	P	N
5	cafenstrole[5]	125306-83-4	P	no data	P	no data	P	no data	P	no data
6	cyproconazole[6]	94361-06-5	N	N	N	N	P	P	P	N
7	difenoconazole[7]	119446-68-3	N	N	P	N	N	N	P	N
8	diniconazole[8]	76714-88-0	N	N	N	N	P	N	P	N
9	Diniconazole M[9]	83657-18-5	N	no data	N	no data	P	no data	P	no data
10	epoxiconazole[10]	133855-98-8 or 106325-08-0	SP	N	P	P	N	P	P	N
11	epronaz[11]	59026-08-3	P	no data	P	no data	P	no data	P	no data
12	fenbuconazole[12]	114369-43-6	N	N	N	N	P	N	P	N
13	fluquinconazole[13]	136426-54-5	N	N	P	N	P	N	P	N
14	flupoxam[14]	119126-15-7	N	N	N	N	P	N	N	N
15	flusilazole[15]	85509-19-9	N	N	P	P	N	P	P	N
16	hexaconazole[16]	79983-71-4	N	N	P	N	P	N	P	P
17	ipconazole[17]	125225-28-7	N	no data	P	no data	P	no data	P	no data
18	metconazole[18]	125116-23-6	N	N	P	N	P	P	P	N
19	myclobutanil[19]	88671-89-0	N	N	N	N	P	P	P	N
20	penconazole[20]	66246-88-6	N	N	N	N	P	P	P	N
21	propiconazole[21]	60207-90-1	N	N	P	N	P	N	P	P
22	prothioconazole[22]	178928-70-6	N	N	N	N	P	P	P	P
23	tebuconazole[23]	107534-96-3	N	N	N	N	P	P	P	N
24	tetraconazole[24]	112281-77-3	N	N	P	N	P	N	N	N
25	triadimenol[25]	55219-65-3	N	N	N	N	P	P	P	N
26	1,2,4-triazol-1-acetic acid	28711-29-7	N	N	N	no data	P	no data	P	no data
27	triticonazole[26]	131983-72-7	N	N	P	N	P	N	P	N

N = non-toxic; P = toxic; SP = suspected toxic

[1] 1H-1,2,4-triazole
[2] 1H-1,2,4-triazol-5-amine
[3] 3,3-dimethyl-1-(4-phenylphenoxy)-1-(1,2,4-triazol-1-yl)butan-2-ol
[4] 1-[[4-bromo-2-(2,4-dichlorophenyl)oxolan-2-yl]methyl]-1,2,4-triazole
[5] N,N-diethyl-3-(2,4,6-trimethylphenyl)sulfonyl-1,2,4-triazole-1-carboxamide
[6] 2-(4-chlorophenyl)-3-cyclopropyl-1-(1,2,4-triazol-1-yl)butan-2-ol
[7] 1-[[2-[2-chloro-4-(4-chlorophenoxy)phenyl]-4-methyl-1,3-dioxolan-2-yl]methyl]-1,2,4-triazole
[8] 1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)pent-1-en-3-ol
[9] (E,3R)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)pent-1-en-3-ol
[10] 1-[[3-(2-chlorophenyl)-2-(4-fluorophenyl)oxiran-2-yl]methyl]-1,2,4-triazole
[11] N-ethyl-N-propyl-3-propylsulfonyl-1,2,4-triazole-1-carboxamide
[12] 4-(4-chlorophenyl)-2-phenyl-2-(1,2,4-triazol-1-ylmethyl)butanenitrile
[13] 3-(2,4-dichlorophenyl)-6-fluoro-2-(1,2,4-triazol-1-yl)quinazolin-4-one
[14] 1-[4-chloro-3-(2,2,3,3,3-pentafluoropropoxymethyl)phenyl]-5-phenyl-1,2,4-triazole-3-carboxamide
[15] bis(4-fluorophenyl)-methyl-(1,2,4-triazol-1-ylmethyl)silane
[16] 2-(2,4-dichlorophenyl)-1-(1,2,4-triazol-1-yl)hexan-2-ol
[17] 2-[(4-chlorophenyl)methyl]-5-propan-2-yl-1-(1,2,4-triazol-1-ylmethyl)cyclopentan-1-ol
[18] 5-[(4-chlorophenyl)methyl]-2,2-dimethyl-1-(1,2,4-triazol-1-ylmethyl)cyclopentan-1-ol
[19] 2-(4-chlorophenyl)-2-(1,2,4-triazol-1-ylmethyl)hexanenitrile
[20] 1-[2-(2,4-dichlorophenyl)pentyl]-1,2,4-triazole
[21] 1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1,2,4-triazole
[22] 2-[2-(1-chlorocyclopropyl)-3-(2-chlorophenyl)-2-hydroxypropyl]-1H-1,2,4-triazole-3-thione
[23] 1-(4-chlorophenyl)-4,4-dimethyl-3-(1,2,4-triazol-1-ylmethyl)pentan-3-ol
[24] 1-[2-(2,4-dichlorophenyl)-3-(1,1,2,2-tetrafluoroethoxy)propyl]-1,2,4-triazole
[25] 1-(4-chlorophenoxy)-3,3-dimethyl-1-(1,2,4-triazol-1-yl)butan-2-ol
[26] (5E)-5-[(4-chlorophenyl)methylidene]-2,2-dimethyl-1-(1,2,4-triazol-1-ylmethyl)cyclopentan-1-ol

precision (P) of the model for the analysed set of conazoles was 95.6 %, specificity (SP) 95.6 %, while sensitivity (SE) could not be calculated because the set did not contain mutagenic compounds.

Carcinogenicity

Table 1 shows that of the 27 conazoles, 15 were predicted as positive (carcinogenic) and 12 as negative (non-carcinogenic). The loadings show the following: the first axis is a combination of pirinixic acid (tested positive), fluconazole (tested positive), and nefiracetam[7] (tested negative). The second axis is a combination of bemitradine (tested positive) loxtidine[8] (tested positive), and pyrazapon[9] (tested negative), and the third axis is a combination of entramin[10] (tested positive), formic acid 2-(4-methyl-2-thiazolyl)hydrazide[11] (tested positive), and ionidamine[12] (tested negative).

The score plot shows five clusters (Figure 1b). Three clusters are dominated by compounds predicted as positive and two clusters by compounds predicted as negative. The clusters of compounds predominately predicted as positive are: 1,2,4-triazole (false positive), amitrole (false positive) and 1,2,4-triazol acetic acid (predicted as negative with no available data), cluster of fluquinconazole (false positive), flupoxam (true negative) and flusilazole (false positive) and cluster of ipconazole (positive with no data), propiconazole (false positive), cyproconazole (true negative), metconazole (false positive), triticonazole (false positive). The first cluster of (mostly) negative compounds consists of hexaconazole (false positive), myclobutanil (true negative), penconazole (true negative), tebuconazole (true negative), and the second one of cafenstrole (predicted as positive with no available data), diniconazole-M (predicted as negative with no available data), diniconazole (true negative), tetraconazole (false positive), and triadimenol (true negative). The model correctly predicted epoxiconazole and flusilazole which are classified as carcinogenic in the Regulation (EC) No. 1272/2008 (15). Three out of five substances, which are not listed in the Regulation (EC) No. 1272/2008 (15) or were not evaluated by PRAPeR group (14) (see Table 1), were predicted as carcinogenic (cafenstrole, epronaz, ipconazole) and two as non-carcinogenic (diniconazole-M, 1,2,4-triazol acetic acid). Looking at the similarity sets for still unclassified compounds, we can see that a set for cafenstrole, diniconazole-M, and epronaz consists of three compounds tested and predicted as carcinogenic and three as non-carcinogenic. For ipconazole the set consists of two tested and predicted carcinogenic compounds, one correctly predicted non carcinogenic compound, and three carcinogenic substances predicted as non-carcinogenic. The similarity set for 1,2,4-triazol acetic acid is composed of three predicted and tested positive compounds and three compounds predicted negative, but positive. The calculated precision (P) of the model was 54.5 %, specificity (SP) 50 %, and sensitivity (SE) 100 %. Low specificity indicates high number of false positive predictions. On the other hand, sensitivity is very high. This is because only two compounds from the data set (Table 1) were predicted and confirmed as positive (true positive) and no compound was predicted negative and tested positive (false negatives).

Developmental toxicity

Five conazoles were predicted as developmental non-toxicants and 22 as developmental toxicants. 1,2,4-triazole, a known developmental toxicant, was predicted as non-toxicant with the remark to be "out of descriptors" range. This remark means that the numerical values of one of more descriptors are out of the interval, which is defined by compounds included in the training set. Such a prediction should be evaluated with caution.

Looking at the similarity sets of conazoles in the first cluster, we identified three compounds that appear in all sets: acetazolamide (tested toxicant), allantoin[13] (tested non-toxicant), and nitrofurazone[14] (tested non-toxicant).

Loadings are the following: the largest part of the first axis includes tolmetin (tested toxicant), chlorpheniramine (tested non-toxicant), and acemetacin[15] (tested non-toxicant); the second axis includes haloperidol[16] (tested toxicant), triprolidine (tested toxicant), and Spectrum_000171[17] (tested toxicant); the third axis includes acetazolamide[18]

[7] N-(2,6-dimethylphenyl)-2-(2-oxopyrrolidin-1-yl)acetamide
[8] [1-methyl-5-[3-[3-(piperidin-1-ylmethyl)phenoxy]propylamino]-1,2,4-triazol-3-yl]methanol
[9] 1-ethyl-3-methyl-8-phenyl-4,6-dihydropyrazolo[4,3-e][1,4]diazepin-5-one
[10] 5-nitro-1,3-thiazol-2-amine
[11] N-[(4-methyl-1,3-thiazol-2-yl)amino]formamide
[12] 1-[(2,4-dichlorophenyl)methyl]indazole-3-carboxylic acid
[13] (2,5-dioxoimidazolidin-4-yl)urea
[14] [(5-nitrofuran-2-yl)methylideneamino]urea
[15] 2-[2-[1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl]acetyl]oxyacetic acid
[16] 4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one
[17] 2-[1-(4-methylphenyl)-3-pyrrolidin-1-ylprop-1-enyl]pyridine
[18] N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)acetamide

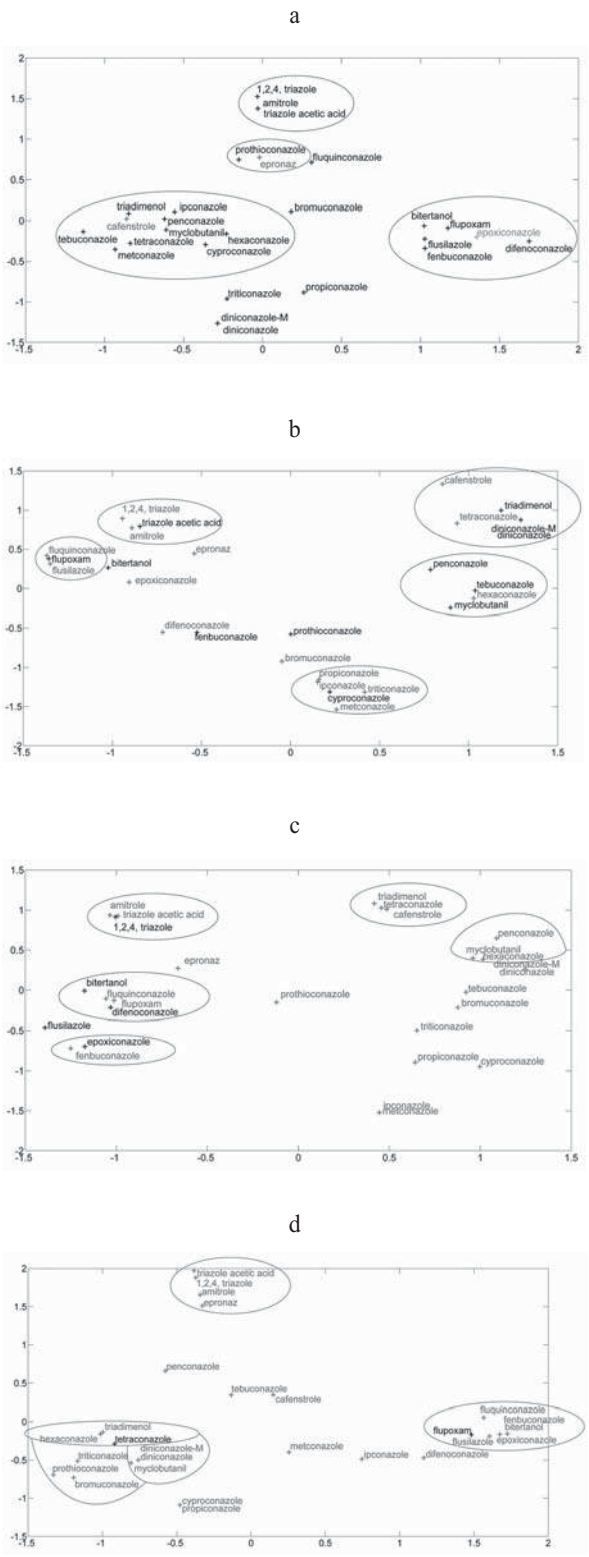

Figure 1 *Figures show score plots for mutagenicity (a), carcinogenicity (b), developmental toxicity (c), and skin sensitisation (d). The x and y axes represent the first and the second principal axes, respectively. In all plots, bold indicates toxic, regular non-toxic, and italic suspected toxic compounds. Clusters are indicated with circles.*

(tested toxicant), allantoin (tested non-toxicant), acemetacin (tested non-toxicant), and nitrofurazone (tested non-toxicant).

In the score plot (Figure 1c) five clusters are evident: the first consists of 1,2,4-triazole (false negative), amitrole (true positive), and 1,2,4-triazol acetic acid (predicted positive with no available data), the second cluster consists of bitertanol (false negative), difenconazole (true negative), fluquinconazole (false positive), and flupoxam (false positive), the third cluster of epoxiconazole (false negative) and fenbuconazole (false positive), the fourth of hexaconazole (false positive) and myclobutanil (true positive), and the fifth cluster of cafenstrole (predicted as positive with available no data), tetraconazole (false positive), and triadimenol (true positive). Amitrole was correctly predicted as a developmental toxicant (true positive). Its similarity set was composed of four correctly predicted developmental non-toxicants and two developmental toxicants. On the other hand, 1,2,4-triazole was wrongly predicted as a developmental non-toxicant (false negative). Cafenstrole, which is in the score plot situated close to both compounds, is predicted as toxicant. Due to different predictions for both neighbours no final conclusion on its toxicity can be made.

Triadimenol and tetraconazole were predicted as developmental toxicants. The predictions were supported by similarity sets, which contain compounds predicted and tested as developmental toxicants.

Ipconazole, diniconazole-M, and epronaz, for which no data are available, were predicted as toxicants. The similarity set for ipconazole is composed of three developmental toxicants and three developmental non-toxicants. Most of these compounds were also found in the similarity set of metconazole, which was correctly predicted as toxicant (true positive). The similarity sets for diniconazole and diniconazole-M, which include five correctly predicted developmental toxicants and two correctly predicted developmental non-toxicants are identical. This is not a surprise taking into consideration that diniconazole-M is the R isomer of diniconazole. Epronaz was predicted as developmental toxicant with the similarity set composed of four correctly predicted toxicants and two correctly predicted non-toxicants. However, because of dissimilarity between epronaz and the rest of the training set, it is difficult to predict its developmental toxicity.

The calculated precision (P) of the model for a set of conazoles was 45.5 %, specificity (SP) 11.1 %, and sensitivity (SE) 69.2 %.

Skin sensitisation

All compounds with the exception of flupoxam and tetraconazole were predicted as active, which means that only these two compounds were not expected to sensitise skin.

The predictions and the analysis of similarity sets show that most conazoles are on the active side of representation space. Only five conazoles were correctly predicted, three of them active and two inactive. Even the right prediction for flupoxam and tetraconazole as inactive was not supported by the similarity sets, where most compounds were predicted and confirmed as active. The correct prediction for prothioconazole (active) should be considered with a caution, because the proposed classification as a skin sensitiser (14) was based on the presence of an impurity in the technical material and not because of the properties of the substance itself. Table 1 shows that in most cases the predictions are in conflict with the classification in the EU Regulation (14), where most of the compounds are classified as inactive. Five conazoles for which no classification is available, were predicted as active. The prediction was supported by the similarity set of six compounds, where active (sensitising) compounds prevailed. Due to the general discrepancy between predictions and measured data, these predictions are less reliable (16). The calculated precision (P) of the model for the set of conazoles was 22.7 %, specificity (SP) 10.5 %, and sensitivity (SE) 100 %.

CONCLUSIONS

This article reports classification of 27 conazoles on four different endpoints. The similarity analysis was performed on four different representation spaces, which were constructed from similarity sets obtained from CAESAR programs. The representation spaces are parts of training sets, i.e., the sets used for developing models and are different for four endpoints. When using the QSAR/QSPR models, the analysis of applicability domain is crucial. An example is the prediction of developmental toxicity for 1,2,4-triazole, which is a known developmental toxicant. The CAESAR prediction is non-toxic with the remark "out of descriptor range". The model for the four endpoints shows clearly different properties. In some cases the predicted and regulatory classification are in accordance, in other cases not. The reason for discrepancies may be that the models were built on limited data sets using structural descriptors. On the other hand, regulatory classification is an extensive procedure, which includes different steps and takes into consideration different facts. Our aim is to promote *in silico* models to become a part of this procedure. For now, they can be used as a valuable method for setting priorities among chemicals for further testing, but not as standalone methods for classification.

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Izvleček

NAPOVEDI MUTAGENOSTI, KARCENOGENOSTI, RAZVOJNE TOKSIČNOSTI IN KOŽNE OBČUTLJIVOSTI ZA NIZ KONAZOLOV

V članku predstavimo modele za napoved mutagenosti, karcenogenosti, razvojne toksičnosti in kožne občutljivosti za niz 27 konazolov. Uporabili smo programski paket CAESAR, ki je dostopen preko interneta. Programi so bili razviti v podporo Evropske Regulacije za varno uporabo kemikaljij REACH in ob upoštevanju OECD principov za validacijo (Q)SAR modelov, ki se uporabljajo za regulatorne namene. Pri napovedi dobimo različne informacije; binarno klasifikacijo kot toksičen ali ne-toksičen in informacijo o šestih najbolj podobnih spojinah iz testnega niza (podobnostni niz). Ti nizi so bili analizirani z metodo glavnih osi (PCA). Napovedi smo primerjali z trenutno veljavnimi klasifikacijami spojin, ki so bile na Evropski komisiji že sprejete, ali pa so predlagane na srečanjih ekspertov (Pesticide Risk Assessment and Peer Review (PRAPeR) group). Napovedane klasifikacije se dobro ujemajo z sprejeto klasifikacijo za mutagenost. Za karcenogenost in razvojno toksičnost se napovedi v nekaterih primerih ujemajo, v nekaterih ne. Pri kožni občutljivosti smo našli več diskrepanc. Za pet spojin, za katere ni eksperimentalnih podatkov smo diskutirali napovedi.

KLJUČNE BESEDE: *Evropska Kemijska Regulativa – REACH, metoda glavnih osi, QSAR modeliranje*

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Scientific Paper

COMPARISON OF BUFFERS FOR EXTRACTION OF MITE ALLERGEN DER P 1 FROM DUST

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Der p 1 is the main allergen of house dust mite *Dermatophagoides pteronyssinus*, which has routinely been detected in residential dust. However, the procedure for extracting Der p 1 from reservoir dust has not been well defined. The aim of this study was to compare Der p 1 mass fractions in dust extracts prepared using the following extraction buffers: phosphate (pH 7.4), borate (pH 8.0), and ammonium bicarbonate (pH 8.0), all with 0.05 % Tween 20. Twenty-eight dust samples were divided into three aliquots and each portion was extracted with one of the three buffers at room temperature. Der p 1 mass fractions were measured in a total of 84 dust extracts using the enzyme immunoassay (range: 0.1 μg g^{-1} to 7.53 μg g^{-1}). Statistical methods including intraclass correlation showed a high agreement between Der p 1 mass fractions irrespective of the extracting medium. Our results suggest that all three buffers are suitable for the extraction of mite allergens and routine Der p 1 analysis in dust.

KEY WORDS: Dermatophagoides pteronyssinus, *ELISA, extraction buffers, indoor allergens, intraclass correlation, settled dust*

House dust mites are the source of 21 allergens identified so far. The major allergens of the common dust mite (*Dermatophagoides pteronyssinus*) are Der p 1 and Der p 2 (1-3). Exposure to these allergens is associated with allergic symptoms and asthma in sensitised people (1-3). Reservoir dust samples have been used as a proxy for Der p 1 exposure in residential (1, 4) and occupational (5) settings. In order to compare results from different studies investigating allergen exposure and related health effects, both the collection techniques and laboratory protocols (analysis, extraction, and storage) should be comparable. Enzyme-linked immunosorbent assay (ELISA) is the standard method for quantification of common indoor allergens in reservoir dust (6). However, there is no standard protocol for the extraction of allergens from dust (7). Several solutions have regularly been used for extraction of Der p 1 in laboratories worldwide including phosphate, borate, and ammonium bicarbonate buffers (Table 1). Some laboratories add the non-ionic surfactant Tween 20 to the extraction medium and some do not. Little information is available about the extraction efficiency of buffers on Der p 1 measurements. Siebers et al. (8) found that the type of buffer affected measurement of Der p 1 levels. In this short report, Der p 1 concentrations (high exposure level) in a borate extract were much higher than in phosphate and ammonium bicarbonate extracts. However, other operating conditions (such as time and temperature) appeared to be very important for Der p 1 extraction and Der p 1 measurement with ELISA. Extraction at a lower temperature (4 °C) resulted in lower Der p 1 level, irrespective of the buffer type. This lack of standardised operating conditions, certainly contributes to great inter-laboratory differences in Der p 1 measurements (7, 9).

The aim of our study was to establish correlations between Der p 1 mass fractions in dust samples extracted with three common buffers, namely phosphate, borate, and ammonium bicarbonate, all containing 0.05 % Tween 20, at room temperature.

MATERIALS AND METHODS

Dust collection

Twenty-eight dust samples were collected from 18 urban households in Zagreb, Croatia between 2007 and 2009. Samples were taken by vacuuming a carpeted area of the living rooms using a standard vacuum cleaner adapter and cellulose filter (Heska AG, Freiburg, Switzerland) as described earlier (10).

Dust extraction and analysis

Three buffers were used for Der p 1 extraction from settled dust: phosphate (PBS; pH 7.4), borate (BBS; pH 8.0; Titrisol, Merck, Germany), and 0.125 mol L^{-1} ammonium bicarbonate (ABS; pH 8.0; Kemika, Zagreb). The final concentrations of the PBS components were 137 mmol L^{-1} for NaCl, 10 mmol L^{-1} for $Na_2HPO_4x2H_2O$, 2 mmol L^{-1} for KH_2PO_4, and 2.7 mmol L^{-1} for KCl. According to the manufacturer (Merck, Germany), the borate buffer consisted of 0.11 mol L^{-1} H_3B0_3, 0.044 mol L^{-1} HCl, and 0.056 mol L^{-1} NaOH. All buffers contained 0.05 %

Table 1 *Summary of reported buffers and operating conditions for extraction of Der p 1 and other indoor allergens from dust*

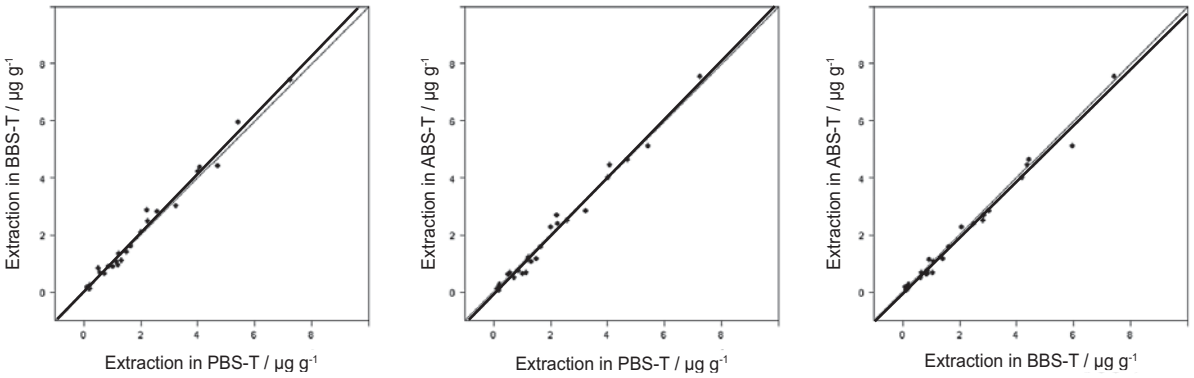
Extraction buffer	Tween 20	Reference
Phosphate (PBS) pH 7.4		
PBS[a]	/	23, 24
PBS (30 min, RT)	0.05	20, 25
PBS (1 h, 30 °C)	0.05	26
PBS (2 h, RT)	0.05	27, 28, this work
PBS-1 % BSA (2h, RT)	0.05	29
PBS-1 % BSA (overnight, RT)	0.5	30
PBS-0.2 % BSA (overnight, 4 °C)	0.2	31
Borate (BBS) pH 8.0		
BBS[a]	/	13, 32-35
BBS (2 h, RT)	0.05	this work
BBS-5 % BSA[a]	/	36
BBS-5 % BSA (overnight, 4 °C)	/	37
BBS-aprotinin (2h, 4 °C)	0.1	38
Ammonium bicarbonate (ABS) pH 8.0		
ABS (2h, RT)	/	39
ABS (2h, RT)	0.05	21, this work

RT - room temperature
BSA - bovine serum albumin
[a]- extraction conditions not available

Table 2 *Der p 1 findings (μg g^{-1}) by extraction medium at two exposure levels*

Der p 1	Median	Mean±SD	Range	n
low level				
PBS-T	0.648	0.721±0.53	0.12 to 1.655	18
BBS-T	0.753	0.696±0.497	0.105 to 1.62	18
ABS-T	0.645	0.616±0.462	0.060 to 1.565	18
moderate level				
PBS-T	3.620	3.774±1.681	2.015 to 7.255	10
BBS-T	3.605	3.958±1.685	2.075 to 7.42	10
ABS-T	3.422	3.838±1.667	2.270 to 7.525	10

PBS-T - phosphate buffer-Tween
BBS-T - borate buffer-Tween
ABS-T - ammonium bicarbonate buffer-Tween



— *identity line*
— *linear regression line*

Figure 1 *Linear regression analysis for different pairs of extraction solutions*

Tween 20 (T) (Merck, Germany). Before extraction, each sample was manually sieved through a 300 µm sieve, mixed until homogenous, and weighed. Fine dust samples were divided into three 100-mg aliquots, and 2 mL of extraction solution was added to each aliquot. Extractions were done at room temperature with constant shaking on a Vortex mixer (Ika Vortex, Germany) for 2 h. After 10 min of centrifugation at 1,000x*g*, supernatants were stored in plastic tubes at -20 °C until analysis for Der p 1 content. A total of 84 dust extracts were analysed for Der p 1 content.

The mass fractions of Der p 1 were determined with capture ELISA, using a commercial kit (Indoor Biotechnologies Ltd, Cardiff, UK) as described in our earlier article (10). The kit contained monoclonal antibody 5H8 (mouse anti-Derp 1, IgG2A) (lot number 30034) as capture antibody, biotinylated monoclonal antibody 4C1 (mouse IgG1; lot number 30068) as secondary antibody, and Der p 1 standard (2500 ng mL^{-1}). All antibodies (except capture mAb), standards, dust extracts, and positive and negative controls were diluted in PBS-T containing 1 % bovine serum albumin (BSA; PBS-T-BSA; Sigma, USA). Dust extracts were diluted three or six times depending on the Der p 1 level. Aliquots of extracts were placed into a 96-well microtitre plate (Maxi Sorp, Nunc, Denmarkt) following the manufacturer's instructions. After all reagent incubations, optical densities were read at 450 nm using a microtitre plate ELISA reader (IASON, Vienna, Austria). The limit of detection was 0.1 µg g^{-1}. The intra-assay coefficient of variation (CV) and inter-assay CV for Der p 1 ELISA were 6.9 % and 13.1 %, respectively (10).

Statistical analysis

All data were analysed using free statistical software R, version 2.13.2. Descriptive statistics was used to illustrate the distribution of Der p 1 in PBS-T, BBS-T, and ABS-T extracts. According to allergen mass fraction, Der p 1 values were grouped in low (from 0.1 µg g^{-1} to 2.0 µg g^{-1}) and moderate (from 2.01 µg g^{-1} to 7. 53 µg g^{-1}) levels. Der p 1 measurements were compared for each pair of buffers and, in a separate procedure, for all three buffers. For each pair of buffers, Der p 1 measurements were compared using correlation coefficients (Pearson's and Spearman's), and linear regression coefficients (slope and intercept, with 95 % confidence intervals, 95 % CI) in two mass ranges. The agreement between Der p 1 values for each pair of buffers was estimated using the intraclass correlation coefficient (ICC) (11, 12). This coefficient was also used to determine the agreement between Der p 1 values in all three buffers, since other coefficients allow only paired measurements.

Intraclass correlation coefficients were calculated from two-way random effects model with both buffers and dust batches as covariates (11) using R software. Intraclass coefficient represents the proportion of data variation that can be explained by between-class variability (variability in Der p 1 values between batches). All coefficients were calculated with their 95 % confidence intervals. The level of significance was set at 0.05.

Table 3 *Regression analysis (slopes, intercept with 95 % confidence interval) and correlation coefficients (Pearson's, Spearman's and intraclass) for Der p 1 mass fractions in PBS-T, PBS-T, and ABS-T extracts*

Extraction comparison	Der p 1 range / μg g^{-1}	Linear regression		Correlation		
		Slope (95 % CI)	Intercept (95 % CI)	Pearson (95 % CI)	Spearman	ICC (95 % CI)
PBS-T *vs*. BBS-T	0.1 to 2.0	0.906 (0.779, 1.032)	0.043 (-0.07, 0.155)	0.967 (0.912, 0.988)	0.931	0.966 (0.912, 0.987)
	2.01 to 7.53	0.988 (0.849, 1.126)	0.23 (-0.338, 0.798)	0.985 (0.938, 0.997)	0.964	0.981 (0.909, 0.995)
	overall	1.037 (0.991, 1.084)	-0.018 (-0.137, 0.101)	0.994 (0.986, 0.997)	0.980	0.993 (0.985, 0.997)
PBS-T *vs*. ABS-T	0.1 to 2.0	0.833 (0.695, 0.971)	0.015 (-0.107, 0.138)	0.954 (0.879, 0.983)	0.946	0.927 (0.753, 0.975)
	2.01 to 7.53	0.977 (0.84, 1.115)	0.15 (-0.413, 0.714)	0.985 (0.937, 0.997)	0.964	0.986 (0.948, 0.996)
	overall	1.022 (0.973, 1.071)	-0.084 (-0.209, 0.041)	0.993 (0.985, 0.997)	0.984	0.993 (0.984, 0.997)
BBS-T *vs*. ABS-T	0.1 to 2.0	0.9 (0.772, 1.027)	-0.011 (-0.118, 0.097)	0.966 (0.91, 0.988)	0.933	0.952 (0.836, 0.984)
	2.01 to 7.53	0.972 (0.823, 1.121)	-0.01 (-0.646, 0.625)	0.983 (0.927, 0.996)	1	0.982 (0.933, 0.995)
	overall	0.98 (0.937, 1.023)	-0.057 (-0.171, 0.057)	0.994 (0.987, 0.997)	0.982	0.993 (0.983, 0.997)

PBS-T - phosphate buffer-Tween
BBS-T - borate buffer-Tween,
ABS-T - ammonium bicarbonate buffer-Tween,
ICC - intraclass correlation coefficient
All correlation coefficients and regression slopes significantly differed from zero ($p<0.001$)

RESULTS

Table 2 shows medians, means, and ranges of Der p 1 mass fractions in dust extracts. Table 3 shows the results of the statistical analysis. Respective Pearson's correlation coefficients between Der p 1 mass fractions in PBS-T *vs.* BBS-T, PBS-T *vs.* ABS-T, and BBS-T *vs.* ABS-T extracts were 0.967, 0.954, and 0.966 for low exposure range (0.1 μg g^{-1} to 2 μg g^{-1}) and 0.985, 0.985, and 0.983 for moderate exposure range (2.01 μg g^{-1} to 7.53 μg g^{-1}). Spearman's correlation coefficients between Der p 1 mass fractions in the extraction buffers and exposure levels ranged from 0.931 to 1, while intraclass correlation coefficients ranged from 0.927 to 0.993, showing a statistically significant and high agreement in both level ranges. The lowest agreement was observed for Der p 1 measurements in PBS-T and ABS-T extracts (ICC and Pearson's coefficient of 0.927 and 0.954, respectively) for low exposure range. In the moderate allergen level group, ICC ranged from 0.981 to 0.986 between all three extraction buffers (without significant differences between coefficients), suggesting very good agreements between Der p 1 measurement. Table 3 also shows linear regression coefficients (slope, intercept, and 95 % confidence intervals) for each pair of extraction buffers. Figure 1 shows a very good agreement between Der p 1 measurements between pairs of buffers obtained with linear regression. Each plot shows both the identity line and regression line. The identity line was not included in the 95 % confidence interval for linear regression parameters only in the case of Der p 1 measurements in PBS-T and ABS-T extracts that contained low allergen level.

The agreement between all three extraction buffers estimated with ICC was 0.949 (95 % CI: 0.881, 0.98) for low exposure range, 0.983 (95 % CI: 0.952, 0.995) for moderate exposure range, and 0.993 (95 % CI: 0.986, 0.996) overall.

DISCUSSION

A number of studies have measured Der p 1 in settled dust worldwide in order to assess exposure risk, especially in children and adults with asthma (4, 13). Monitoring household allergens may play an important role in asthma control (14), but it needs standardised and harmonised protocols for indoor allergen sampling and measurement. Our results show a high correlation and agreement between Der p 1 measurements in PBS-T, BBS-T, and ABS-T at either low or moderate allergen levels. Furthermore, overall ICCs for Der p 1 measurements are high (0.993) for all three buffers. ICC values were slightly lower for the low exposure range than for the moderate because the latter range is wider and involves greater between-class variability and, consequently, a higher intraclass coefficient. Regression analysis showed the slope very close to 1 and small y-axis intercept for each pair of extraction data (Table 3, Figure 1). This reflects high homogeneity of Der p 1 values and excellent agreement between measurements for each pair of extraction buffers (PBS-T, BBS-T, and ABS-T) and may help in standardising the extraction procedure.

Similarly, Martin et al. (15) found no buffer effect on the extraction of Fel d 1 (cat allergen) from dust. Pate et al. (7) also observed that the extraction step was not a significant source of variability. In contrast, Siebers et al. (8) found that borate buffer was superior to PBS and ABS. However, they did not use Tween 20 in that study and their results are not fully comparable with ours. Several investigators reported that adding Tween 20 (as a dispersing and solubilising agent) to the extraction media improved endotoxin detection in dust extracts (16-18). Furthermore, adding Tween 20 to pyrogen-free water has been recommended for endotoxin analysis by the European Committee for Standardization (CEN) (19). However, the influence of Tween 20 on allergen extraction efficiency has not been investigated or its use universally accepted. Therefore, further research should investigate the effect of Tween 20 on extraction efficiency of indoor allergens from dust.

However, variety between laboratories can be great in other operating conditions such as temperature and time of extraction (7). At a lower extraction temperature (4 °C), Sieber et al. (8) reported lower Der p 1 level irrespective of the buffer type. Furthermore, dust sampling and storage may also affect laboratory performance (20, 21). According to Fahlbusch et al. (21), storing dust at -20 °C for up to 10 months had no effect on mite allergen levels but Fel d 1 concentration significantly dropped with storage time. However, the freeze-thaw effects on Der p 1 concentrations in dust extracts or dust samples have not yet been investigated.

In 2005, Pate et al. (7) reported the results of the first quality control of common indoor allergen measurements (mite, cockroach, and pets) in residential dust. They found a strong inter-laboratory variability in the levels of all indoor allergens, which pointed to poor standardisation of some steps in allergen measurements. Harmonising protocols for indoor allergen measurement can make results more comparable and lower inter-laboratory variability. Recently, Filep et al. (22) have developed a single standard for eight common indoor allergens. Adding a such control sample with declared allergen levels can improve performance and reduce variability between laboratories.

In this study the efficiency of buffers on Der p 1 extraction from dust samples was compared using correlation coefficients (intraclass, Pearson's and Spearman's) and linear regression coefficients. Generally, intraclass correlation is a better indicator of agreement between different measurements than Pearson's and Spearman's correlation which may yield misleadingly higher values of agreement in case one extraction solution is constantly giving higher values than the other. Similarly, the linear regression model can produce regression line equal to the identity line even when data points are far from the estimated line. Another drawback of the regression model is the assumption that data points for at least one solution are free of measurement error, which is unrealistic in our case. However, we decided to include linear regression and Pearson's and Spearman's correlation coefficients as an addition to intraclass correlation to make possible a comparison with future studies.

CONCLUSION

Our results have shown excellent agreement between Der p 1 measurements regardless of the extraction buffers (PBS-T, BBS-T, and ABS-T) or exposure level. Therefore, all three buffers plus 0.05 % Tween 20 have proved equally efficient in the extraction of Der p 1 from residential dust at room temperature. In order to harmonise extracting procedures, further studies should include extraction from samples with high Der p 1 levels. In addition,

further studies are necessary to find out if the results reported in this study can be generalised for other allergens in reservoir dust samples.

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Sažetak

USPOREDBA PUFERA ZA EKSTRAKCIJU ALERGENA GRINJE Der p 1 IZ PRAŠINE

Der p 1 glavni je alergen grinje *Dermatophagoides pteronyssinus* koji se rutinski određuje u kućnoj prašini. Postupak ekstrakcije Der p 1 iz prašine nije dobro definiran. Cilj je ovoga rada ispitati korelaciju i slaganje između Der p 1 masenih udjela u ekstraktima prašine koji su sadržavali fosfatni (pH 7,4), boratni (pH 8,0) ili amonij-hidrogenkarbonatni (pH 8,0) pufer s dodatkom 0,05 % Tween 20. Dvadeset i osam uzoraka prašine podijeljeno je u tri skupine za ekstrakciju s jednim od tri pufera na sobnoj temperaturi. Maseni udio Der p 1 određen je u ukupno 84 ekstrakta enzim-imunokemijskom metodom (raspon: 0,1 μg g^{-1} do 7,53 μg g^{-1}). Statističke metode, uključujući i "intraclass" korelaciju, pokazale su visoku korelaciju i slaganje između masenih udjela Der p 1 u svim ekstraktima. Rezultati pokazuju da su sva tri pufera prikladna za ekstrakciju alergena grinje i rutinsko određivanje Der p 1 u prašini.

KLJUČNE RIJEČI: *alergeni unutarnjih prostora,* Dermatophagoides pteronyssinus*, ekstrakcijski puferi, ELISA, "intraclass" korelacija, sedimentirana prašina*

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Scientific paper

SULPHUR CYCLING BETWEEN TERRESTRIAL AGROECOSYSTEM AND ATMOSPHERE*

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Central gas station of the natural gas borehole system Podravina is located near the village Molve. It delivers more than a quarter of total energy used in Croatia to its consumers. Over the years, adapting technology to increasingly demanding and rigorous standards in environmental protection has become paramount. Yet, despite all the industry has undertaken to address the risk of harmful substances entering the food chain, a multidisciplinary research team of independent scientists monitors the content of specific substances in all components of the ecosystem.

This paper presents measurements of total sulphur contents in soil surface [(0 to 3) cm] and subsurface [(3 to 8) cm] layers (study period: autumn 2006 - spring 2010) and in plants (study period: spring 2000 - spring 2010), and the concentration of gaseous sulphur compounds in the air. Concentrations of hydrogen sulphide (H_2S) and mercaptans (RSH) were measured from the summer of 2002 until the autumn of 2010, while concentrations of sulphur dioxide (SO_2) were measured from the spring of 2008 until the autumn of 2010. The paper also shows total annual atmospheric sulphur ($S\text{-}SO_4$) deposition at Bilogora measuring station (study period: 2001 - 2010). Average monthly concentrations of H_2S in air varied between 0.2 μg m^{-3} and 2.0 μg m^{-3}, RSH between 0.1 μg m^{-3} and 24.5 μg m^{-3}, and SO_2 between 0.4 μg m^{-3} and 2.8 μg m^{-3} depending on the location and the season of sampling.

Mean values of total sulphur in soil and in *Plantago lanceolata* plant ranged between 610 mg kg^{-1} and 1,599 mg kg^{-1} and between 3,614 mg kg^{-1} and 4,342 mg kg^{-1}, respectively, depending on the soil type, location, and sampling depth. Average values of total sulphur mass ratio for all examined single soil samples (n=80) were 1,080 mg kg^{-1} for both studied layers, and 4,108 mg kg^{-1} for all analysed plant samples (n=85). Average total annual atmospheric sulphur deposition at Bilogora measuring station was 6.3 kg of $S\text{-}SO_4$ per hectar.

KEY WORDS: *air, deposition, H_2S, mercaptans,* Plantago lanceolata*, SO_2, soil, total sulphur*

The northern part of the Pannonian plain in Croatia is a traditional agricultural region [Western Pannonian region according to Bašić et al. (1)] rich in natural gas. The coexistence of agriculture and energy is clearly essential to stakeholders. Central gas station (CGS) of the natural gas borehole system Podravina is located near the village Molve. Natural gas is not a pure product. When gas is extracted from a field under the supercritical conditions (high pressure and temperature) it may contain harmful and corrosive components such as CO_2, H_2S, RHS, Hg^0 etc. In CGS,

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natural gas is collected, treated, purified, and distributed to consumers. For the past 21 years, the scientists within a multidisciplinary research team have monitored its influence on air, water, soil, plants, forests, animals (wild and domestic), as well as humans. Since 1991, the Institute for Medical Research and Occupational Health, Zagreb (Environmental Hygiene Unit) has been in charge of the environment and air quality monitoring on carefully selected representative locations in CGS, while the soil and plant quality monitoring has been the task of the Faculty of Agriculture, University of Zagreb (Department of General Agronomy).

In the past, all pollutants from different industrial sources were emitted in the atmosphere. As landowners became more aware of the ecological and health problems, they insisted on some improvements. As a result, some technological processes were modified, such as the installation of two mercury (Hg) absorbers using active carbon impregnated with sulphur, LO-CAT unit (H_2S oxidation in elementary S), and RTO unit (oxidation of reduced sulphur compounds to SO_2). Despite all the measures that were taken to protect the environment and local public health, it was still necessary to establish a quality multidisciplinary monitoring programme since only independently collected scientific data could provide relevant information for conclusions and possible solutions for the environment protection.

Sulphur is a biogeoelement in agroecosystem and a macronutrient for plants, as well as a constituent of certain essential amino acids, vitamins, enzymes, and aromatic oils. Sulphur deficient plants tend to have low sugar but high nitrate content in their sap and leaf chlorosis, which can cause lower yield and lower plant quality (2, 3). But high concentrations of S in soil or air can be toxic to plants, animals, and humans (4-6). The three major natural sources of sulphur that can be available for plant uptake are (1) organic matter; (2) soil minerals; and (3) sulphur gases in the atmosphere (7).

According to Deloch (8), natural total sulphur content range is different in grasses (corn 1,700 mg kg^{-1} of dry matter (DM), barley 1,800 mg kg^{-1} of DM), legumes (bean 2,400 mg kg^{-1} of DM, soybean 3,200 mg kg^{-1} of DM) and cruciferae (rape 10,000 mg kg^{-1} of DM, radicchio 17,000 of DM). Some authors have studied the influence of atmospheric sulphur (SO_2 and H_2S) from different anthropogenic sources, mostly power plants, on soil and the uptake in some plant species. Reimann et al. (9) measured total sulphur concentrations in some plants from different catchments in northern Europe and one location near the nickel smelter and refinery. They found the median S concentration of 965 mg kg^{-1} in moss; 990 mg kg^{-1} in conifers; 1,490 mg kg^{-1} in shrubs; and 1,900 mg kg^{-1} in deciduous trees with significantly higher concentrations observed on a polluted location. In a field study conducted in the vicinity of two thermal power plants in India, Agrawal et al. (10) noted a high correlation between the emission from power plants and elemental concentrations of sulphur in the leaves of evergreen and deciduous plants (significantly higher concentrations on polluted than on unpolluted sites). Fidalgo-Hijano et al. (11) obtained the following results in a study conducted in the city of Madrid on different plants as bioindicators of sulphur emission: a higher accumulation of sulphur was found in vegetable species located near highways and dense traffic roads and near the areas with high population density. The minimal accumulation of SO_2 was registered in winter and spring seasons (from January to April) due to the end of vegetative growth, while maximal values were recorded during the summer season (from June to September), due to stomatal opening. Regarding sulphur in agroecosystem, our team has already studied sulphur behaviour in central Croatia through field experiments on an unpolluted site and has published data related to sulphur wheat grain uptake depending on the fertilisation (12) and $S\text{-}SO_4$ losses from soil with drainage water (13).

The goal of this paper is to study sulphur cycling between terrestrial agroecosystem and atmosphere and to present the results of measurements of total sulphur content in soils, total sulphur content in plants, concentration of gaseous sulphur compounds (H_2S, RSH, SO_2) in air, and annual atmospheric sulphur ($S\text{-}SO_4$) deposition measured during the monitoring that was carried out over several years in the vicinity of CGS Molve.

MATERIAL AND METHODS

Samples of air, soil, and plants were collected at four locations in the vicinity of boreholes Molve 9 (M9), Molve 10 (M10), Molve 11 (M11), and Molve 12 (M12). Geographic locations of sampling sites in the area of CGS Molve were described and summarised in our previous study (14) regarding arsenic in soil and air measured in the surroundings of CGS Molve.

Air sampling and analysis

Measurements of H_2S, RSH, and SO_2 concentrations in air were done twice a year, in a 30-day period during the warmer season (summer time) and a 30-day period during the colder season (winter or early spring or late autumn). Samples of H_2S and RSH were collected during 24 hours by forcing the air to the filter paper Whatman No. 41 impregnated with mercury(II) chloride with the addition of urea as an antioxidant (H_2S) and mercury(II) acetate with the addition of acetic acid (RSH). Analysis of H_2S was carried out spectrophotometrically by molybdenum blue method (15, 16). Mercaptans were also determined spectrophotometrically after adding *N,N*-dimethyl-p-phenylenediamine hydrochloride and Reissner reagens (17). Samples of SO_2 were collected by forcing air through the absorption solution of hydrogen peroxide (18) and the resulting concentration of sulfate ions was determined by ion chromatography (19). The analysis was performed on Dionex DX 120 chromatograph equipped with suppressed conductivity detection, Dionex AS14 analytical column, and AG14 guard column. The eluent was 3.5 mmol L^{-1} Na_2CO_3/ 1 mmol L^{-1} $NaHCO_3$ solution.

This paper presents average monthly mass concentrations of gaseous sulphur compounds (H_2S, RSH, and SO_2) in air during the study period of summer 2002 - autumn 2010 (H_2S and RSH) and during the study period of autumn 2008 - autumn 2010 (SO_2). Generally, the measured concentrations were very low, and sometimes even below the limit of detection of the used method.

Soil and plant sampling and analysis

Soil and plant samples were collected twice a year, in spring and autumn, on four locations in the vicinity of the above mentioned boreholes. Surface [(3 to 8) cm] and subsurface soil samples [(3 to 8) cm] were taken with a Holland probe and a knife following the Protocols of soil sampling (20). Plant samples were also collected with a knife. Ribwort Plantain (*Plantago lanceolata* L.) plant (leaves and stems) was gathered as an indicator. In the borehole M9 area, soil and plant samples were collected for two different types of soil (locations M9 and M9 hill). All examined locations included five soil types, dominant in respect of their texture and moisture regime: according to Croatian classification (21) they are classified as: M9 and M12: Eugley, M9 hill: Distric Regosols, M10: Stagnic Luvisols and M11: Gleysols or According to the World Reference Base (WRB) for soil resources (22): M9: Gleysol vertic dystric and M9 hill: Regosol acric, M12: Gleysol vertic, M10: Stagnosol luvic and M11: Gleysols clayic. According to their texture, two of them belong to heavy clay soils (M9 and M12), two to silt loam soil (M10 and M11), and one to light sandy soils (M9 hill).

Soil samples were air-dried, ground, sieved (< 2 mm) and homogenised according to the ISO Protocol (23). Plant samples were first dried in the oven at 105 °C until a constant mass was reached, and were then ground and homogenised.

Analyses of *Plantago laceolata* samples and soil samples on total sulphur content were done by elementary analyser Vario Macro CHNS, Elementar, Deutschland, 2006 following the ISO Protocol (24).

Accuracy and precision were controlled by Reference Material (RM: ISE and IPE, Wepal) and by repeating the same sample measurements, respectively. Accuracy and precision limits were determined within the standards and were acceptable.

This paper presents measurements of total sulphur mass ratio in soil, in surface and subsurface layers (study period: autumn 2006 - spring 2010), as well as in plants (study period: spring 2000 - spring 2010).

The paper also shows total annual atmospheric sulphur ($S\text{-}SO_4$) deposition at Bilogora measuring station (study period: 2001 - 2010) (25).

RESULTS AND DISCUSSION

Sulphur components in air

Monthly average mass concentrations of H_2S for each measuring period measured in CGS surroundings are presented in Figure 1. Monthly average concentrations of H_2S in air varied between 0.2 μg m^{-3} (spring 2006) and 2.0 μg m^{-3} (summer 2002), and the maximal daily value (6.6 μg m^{-3}) was measured during the summer of 2009. According to Croatian legislation, in particular the Regulation on Limit Values of Pollutants in Air (26), the limit values (LVs) for H_2S in air are 2 μg m^{-3} (for an average time of one year) and 5 μg m^{-3} (for a 24-hour collecting period, but LVs may not be exceeded more than seven times during a calendar year). The results cannot be compared with the LV (for one year) because we performed measurements only 60 days per year which is only 17 % of all data; however, they could be

indicative. During the examination period, the measured values (average daily concentrations) exceeded LV (5 μg m^{-3}) three times in 2002, and once in 2004, 2009, and 2010.

Monthly average mass concentrations of RSH for each examination period measured in CGS surroundings are presented in Figure 2. These varied between 0.1 μg m^{-3} (winter 2006) and 24.5 μg m^{-3} (summer 2005), and the maximal daily value measured was 89.2 μg m^{-3}. According to national legislation (26), LVs for RSH in air are 1 μg m^{-3} (for an average time of one year) and 3 μg m^{-3} (for a 24-hour collecting period, but LVs may not be exceeded more than seven times during a calendar year). As we can see in Figure 2, in the period from 2002 until 2006, mass concentrations of RSH were frequently higher than 3 μg m^{-3} while in the period from 2007 until 2010, the limit value of 3 μg m^{-3} was not exceeded. In the period from 2002 until 2006, there were a number of days with daily concentrations of RSH higher than 3 μg m^{-3}. There were months when only one day per month exceeded the LV (at M9, M10, and M12 in summer 2002 and at M12 in summer 2005), but there were also months where all 30 daily measured values were higher than 3 μg m^{-3} (at M11 in summer 2004 and at M10 in summer 2005). In 2007, a system for burning waste gases was built in CGS Molve (RTO unit - oxidation of reduced sulphur components to SO_2), which is a probable reason for such a decrease in the RSH content in air.

Monthly average mass concentrations of SO_2 for each measuring period observed in air in CGS surroundings are presented in Figure 3. These were very low and ranged between 0.4 μg m^{-3} (at M11, in summer 2005) and 2.8 μg m^{-3} (at M10, in summer 2010) depending on the location and the season of sampling, while the maximal daily value (9.3 μg m^{-3}) was measured during the autumn of 2009. According

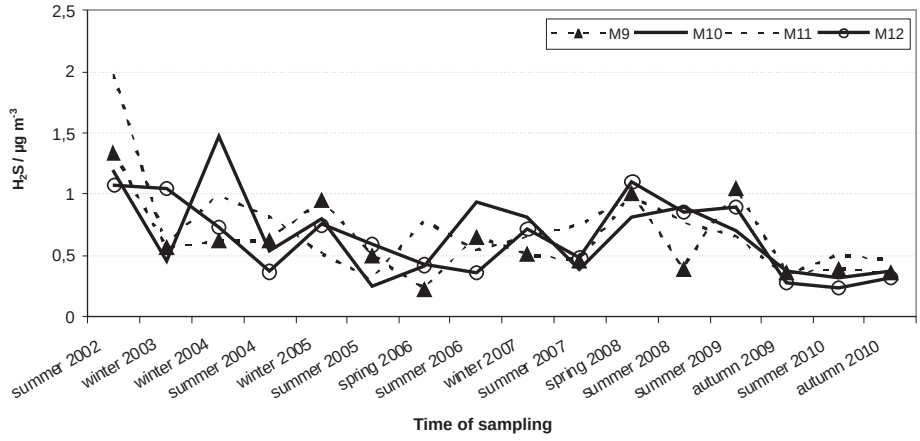

Figure 1 *Average monthly concentration of H_2S measured in air in central gas station (CGS) surroundings*

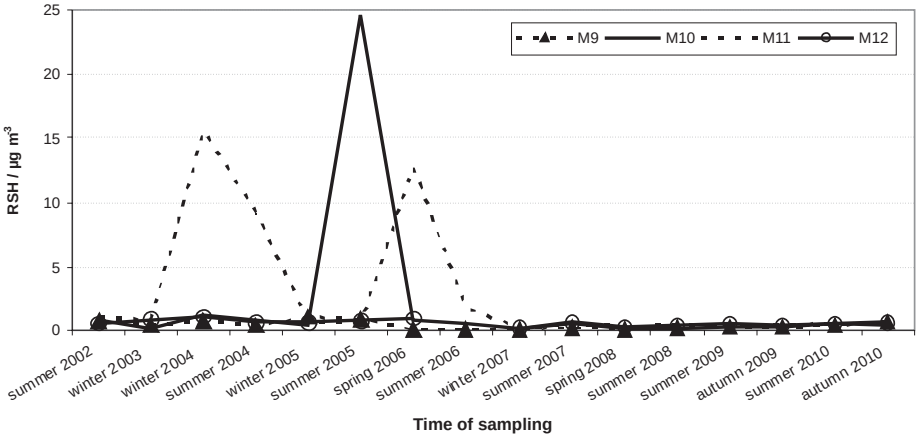

Figure 2 *Average monthly concentration of RSH measured in air in central gas station CGS surroundings*

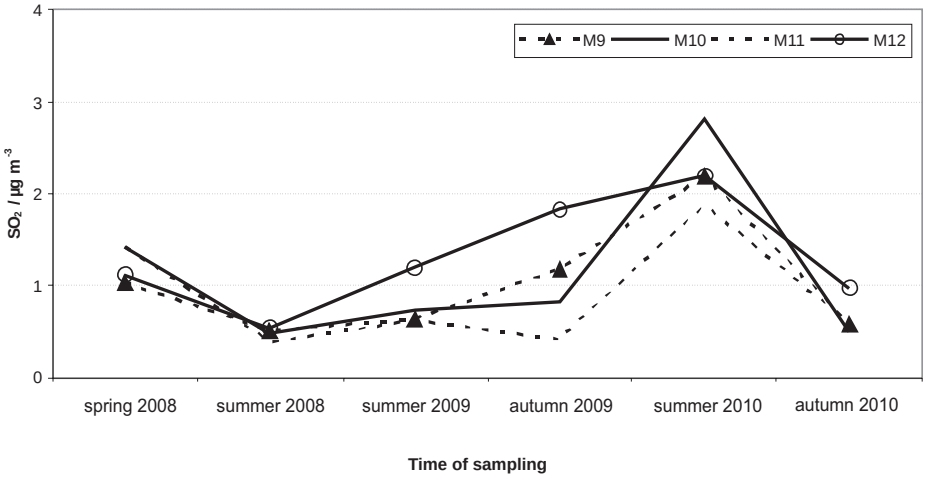

Figure 3 *Average monthly concentration of SO_2 measured in air in central gas station (CGS) surroundings*

to national legislation (26), LVs for SO_2 in air are 50 µg m^{-3} (for an average time of one year) and 125 µg m^{-3} (for a 24-hour collecting period, but LVs may not be exceeded more than seven times during a calendar year). During the study period, the LV (125 µg m^{-3}) was not exceeded. Considering the fact that the investigated area is relatively unpopulated, it is relevant to mention that national legislation (26) also prescribes LVs for the concentration of pollutants (SO_2 and NO_x) in air for the protection of ecosystem and vegetation. For SO_2, LV in air is 20 µg m^{-3} (for an average time of one calendar year and winter period), which is still high above the observed values in our study.

Sulphur in soil

Total sulphur mass ratio measured in surface [(0 to 8) cm] and subsurface [(3 to 8) cm] soil layers for each measuring period in CGS surroundings is shown in Figures 4 and 5, respectively.

According to Isaac and Kerber (27), normal concentration range of total S in soils is 500 mg kg^{-1} to 4,000 mg kg^{-1}. Total S in mineral soils may range from <20 mg kg^{-1} in sandy soils to >600 mg kg^{-1} in heavy texture soils. Organic soils may contain as much as 5,000 mg kg^{-1}. Moist soils, however, contain sulphur between 100 mg kg^{-1} and 500 mg kg^{-1} (28).

In a study conducted in the USA on Alberta soil samples (n=18), total sulphur content in different soil

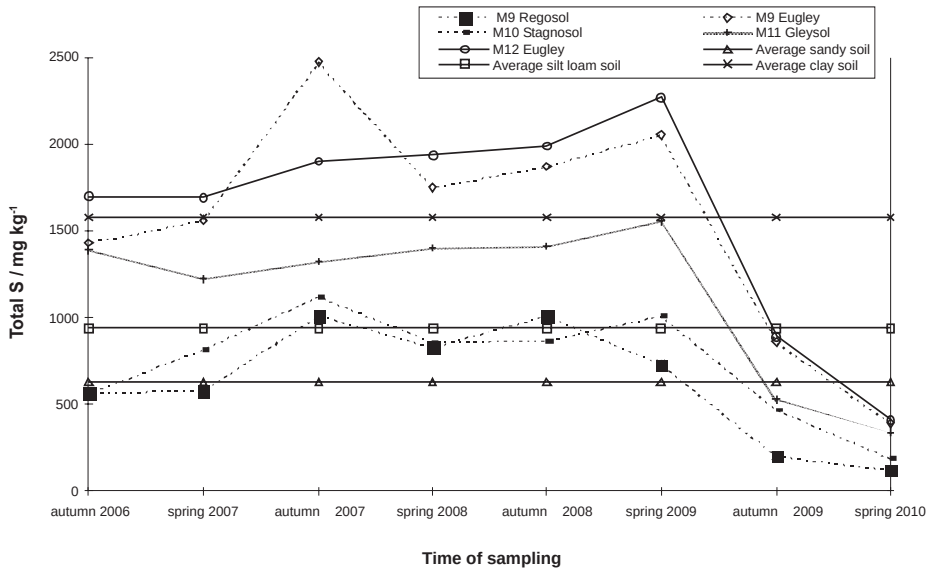

Figure 4 *Total sulphur mass ratio measured in surface soil layer [(0 to 3) cm] in central gas station (CGS) surroundings*

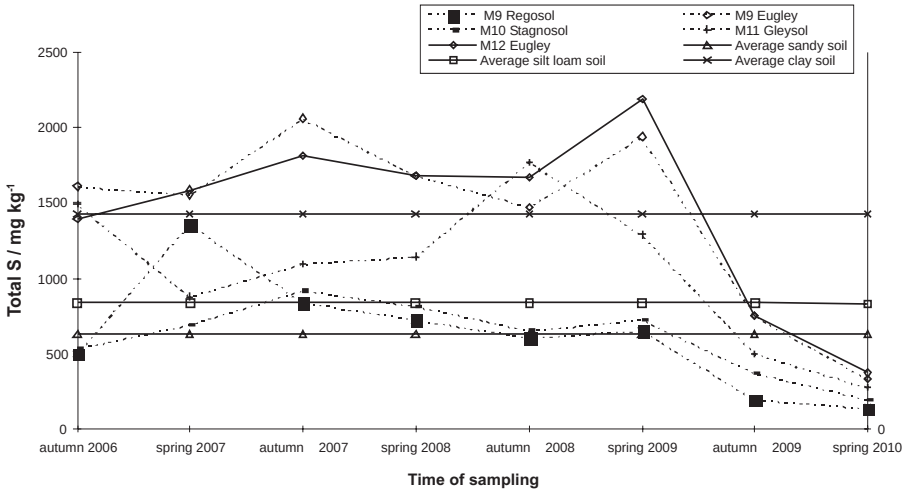

Figure 5 *Total sulphur mass ratio measured in subsurface soil layer [(0 to 3) cm] in central gas station (CGS) surroundings*

types in surface horizon were: up to 343 mg kg^{-1} in humic eluviated gleysol (lacustrine) in (0 to 10) cm; up to 869 mg kg^{-1} in orthic humic gleysol (lacustrine) in (0 to 12) cm; and up to 1,040 mg kg^{-1} in humic eluviated gleysol (till) in (0 to 3) cm (29).

In our case, mean values of total sulphur in soil ranged between 610 mg kg^{-1} observed on Stagnosol (M10) in subsurface horizon and 1,599 mg kg^{-1} measured on Eugley (M12) in surface horizon. For all examined periods, from autumn 2006 until spring 2010, single values of total sulphur in soil ranged between minimal 120 mg kg^{-1} measured on Regosol (M9 hill) in surface horizon in spring 2010 and maximal 2,470 mg kg^{-1} measured on Eugley (M 9) also in surface horizon in autumn 2007. Average values measured for sandy (n=8), silt loam (n=16), and clay (n=16) soils were in surface soil layer 631 mg kg^{-1}, 941 mg kg^{-1}, and 1,573 mg kg^{-1} and in subsurface soil layer 625 mg kg^{-1}, 832 mg kg^{-1}, and 1,426 mg kg^{-1}, respectively. Furthermore, the average values of all examined single samples (n=40) in surface and subsurface soil layers, were 1,132 mg kg^{-1} and 1,028 mg kg^{-1}, respectively. We observed a slightly lower mass ratio of total sulphur in deeper horizons (Figures 4 and 5) and an increasing trend of total S with a decrease in soil particle size (sandy < silt loam < clay).

Sulphur in plants

Total sulphur mass ratio recorded in Ribwort Plantain for each measuring period in CGS surroundings is shown in Figure 6.

According to Isaac and Johnson (30), normal concentration ranges of total S in plant tissue are between 500 mg kg^{-1} and 20,000 mg kg^{-1}.

Mean values of total sulphur in *Plantago lanceolata* ranged between 3,614 mg kg^{-1} measured in the plant grown on Stagnosol (M10) and 4,342 mg kg^{-1} measured in the plant grown on Eugley (M12).

Single values for all examined periods, from spring 2000 until spring 2010, for total sulphur in the plant were between minimal 1,910 mg kg^{-1} measured in the plant grown on Gleysol (M11) in spring 2000 and maximal 8,040 mg kg^{-1} measured in the plant grown on the same location and the same soil type on Gleysol (M 11) but in autumn 2008.

Average values observed in *Plantago lanceolata* grown on sandy (n=17), silt loam (n=34), and clay (n=34) soils where 4,184 mg kg^{-1}, 3,967 mg kg^{-1}, 4,212 mg kg^{-1}, respectively. Furthermore, the average values of total sulphur mass ratio for all examined single plant samples (n=85) were 4,108 mg kg^{-1} (Figure 6). As we already mentioned in the Introduction, according to Deloch (8), natural total sulphur content varies in different plants, depending on the plant species and cultivar; for example, in grasses like barley its content can be 1,800 mg kg^{-1} of DM, in legumes like soybean 3,200 mg kg^{-1} of DM and in cruciferae like rape as much as 10,000 mg kg^{-1} of DM (Figure 6). We observed an increasing trend in sulphur mass ratio in *Plantago lanceolata* over the years (from spring 2000 until spring 2010). The correlation was positive and ranged from medium to strong depending on the soil type, but we cannot connect this increase only and exclusively with the CGS Molve.

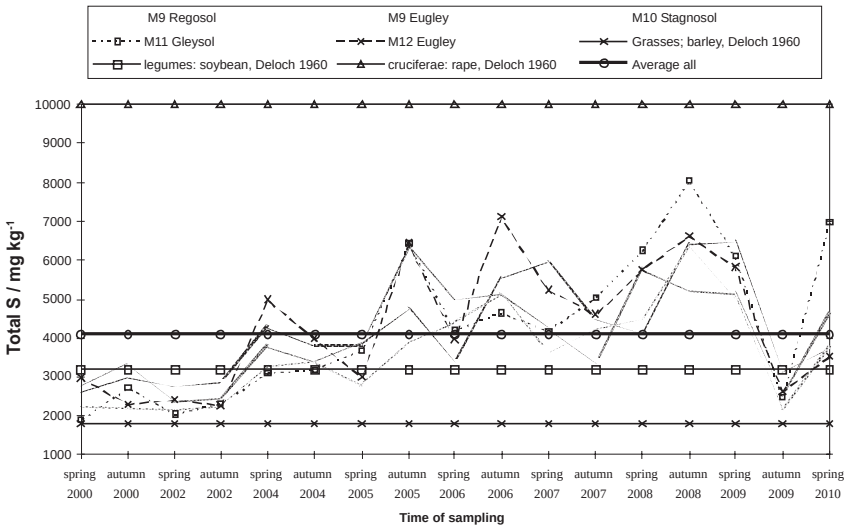

Figure 6 *Total sulphur mass ratio measured in Ribwort Plantain (*Plantago lanceolata*) in central gas station (CGS) surroundings*

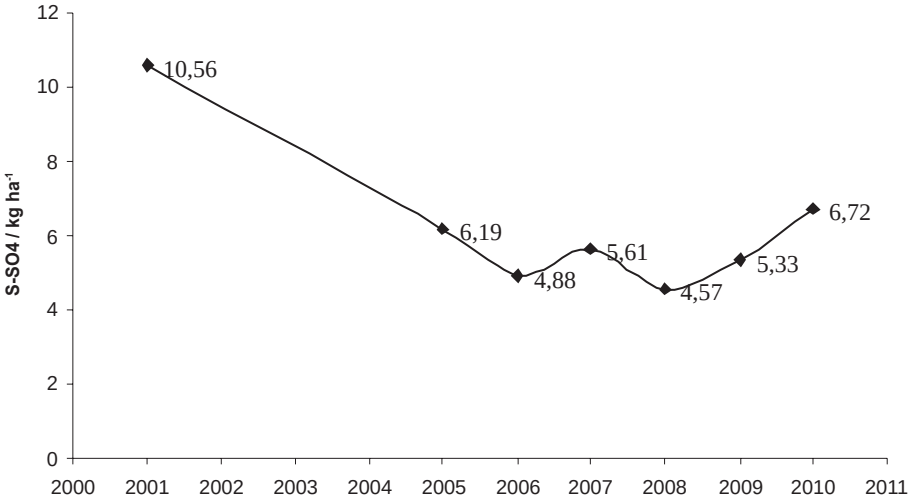

Figure 7 *Annual atmospheric total sulphur depositions at Bilogora measuring station; data from Central Bureau of Statistics RH, Statistical Yearbooks of Republic of Croatia (2001-2011), Meteorological and Hydrological Service*

Sulphur deposition and sulphur plant requirements

Annual atmospheric total sulphur deposition at Bilogora measuring station for the period 2001 through 2010 is presented in Figure 7 (25). This value ranged from 4.57 kg ha^{-1} (2008) up to 10.56 kg ha^{-1} (2001), the average deposition of S-SO_4 being 6.3 kg ha^{-1}. Nowadays in eastern North America, annual S deposition is more commonly below (8 to 10) kg ha^{-1}, due to the aplication of clean technology which is more energy efficient than the clasical industrial technology. The latter is still used in industrial China and India, where coal and oil burning cause as much as (50 to 75) kg ha^{-1} of sulphur fall level per year, while also in these countries, but in areas with no industry, deposition of S is generally as small as (1 to 5) kg ha^{-1} (7). The total S requirement in different crops depends on plant material production and crop species. Crops with high production of organic material, protein rich crops or cruciferae have a higher demand for S, for example 50 kg ha^{-1} for sugarbeet or cabbage. For this reason, they respond most sensitively to an inadequate S supply. Other crops have relatively low requirements for S, about 3 kg ha^{-1} for barley or wheat grain, for instance (5).

According to our previous studies and regarding sulphur in agroecosystem, we observed that the sulphur uptake in winter wheat grain increased on drained Stagnosol in central Croatia from 6.1 kg ha^{-1}

up to 16.9 kg ha^{-1} (12) and S-SO4 losses from soil with drainage water increased from 10.6 kg ha^{-1} to 15.2 kg ha^{-1} (13) depending on different nitrogen fertilisation doses that were applied.

Considering that sulphur is one of 17 plant nutrients (31) that are essential to the growth and development of all plants, it plays a key role in plant health. Losses (plant removal and leaching) and gains (precipitation) of sulphur in agroecosystem are usually in deficit, thus sulphur deficiencies can only be and usually are corrected by the application of an S fertiliser.

CONCLUSION

We have not observed elevated levels of sulphur compounds or its potential or mutual association with industrial activity for total sulphur in soil and total sulphur in the examined plant material Ribwort Plantain (*Plantago lanceolata* L.), but we have observed an increasing trend in sulphur mass ratio in *Plantago lanceolata* during the study period (from the spring of 2000 until the spring of 2010).

The concentrations of SO_2 and H_2S in air were generally low for the whole examined period.

Concentrations of RSH in air were relatively high by 2006 and often exceeded the limit daily value (LV=3 μg m^{-3}), but then ensued a period in which measured values were below the limit daily value. The most likely reason for this kind of RSH behaviour and abundance in air was the installation of the equipment for the incineration of waste gases (RTO units).

We definitely recommend further monitoring.

Acknowledgement

This paper was done within the framework of a multidisciplinary project entitled "Ecosystem Monitoring in Environment of Central Gas Station Molve". The authors would especially like to thank the National Institute of Public Health (Koprivničko-križevačka County) as a coordinator of this demanding project and the Oil and Gas Company (INA) for a complete and active cooperation. Of course, many thanks go to all those who participated in this project, for their hard work and professionalism.

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Sažetak

KRUŽENJE SUMPORA IZMEĐU TERESTRIČKOG AGROEKOSUSTAVA I ATMOSFERE

Centralna plinska stanica plinskobušotinskog sustava Podravina nalazi se u Molvama, a potrošačima energije isporučuje više od četvrtine ukupne energije koja se troši u Hrvatskoj. Prilagodba tehnologije sve zahtjevnijim i strožim standardima zaštite okoliša tijekom godina bila je neupitna, no bez obzira na sve učinjeno od strane industrije, a s obzirom na rizik ulaska štetnih tvari u hranidbeni lanac, u okviru multidisciplinarnog istraživačkog tima nezavisni stručnjaci motre sadržaj potencijalno štetnih tvari i prate utjecaje na sve sastavnice ekosustava.

U ovom radu prikazane su vrijednosti ukupnog sumpora izmjerenog u tlu (u periodu od jeseni 2006. do proljeća 2010.) i u biljci (u periodu od proljeća 2000. do proljeća 2010.) te koncentracije plinovitih sumporovih spojeva u zraku. Koncentracije sumporovodika (H_2S) i merkaptana (RSH) mjerene su u razdoblju ljeto 2002-jesen 2010, dok su koncentracije sumporova(IV) oksida (SO_2) određivane u razdoblju proljeće 2008-jesen 2010. Prikazane su i godišnje vrijednosti ukupne atmosferske depozicije sumpora ($S\text{-}SO_4$) izmjerene na mjernoj stanici Bilogora (za period od 2001. do 2010.).

Srednje mjesečne koncentracije H_2S u zraku kretale su se između 0,2 μg m^{-3} i 2,0 μg m^{-3}, merkaptana između 0,1 μg m^{-3} i 24,5 μg m^{-3} te SO_2 između 0,4 μg m^{-3} i 2,8 μg m^{-3}, ovisno o lokaciji i sezoni uzorkovanja. Srednje vrijednosti ukupnog sumpora u tlu i u trpucu kretale su se redom od 610 mg kg^{-1} do 1 599 mg kg^{-1} te od 3 614 mg kg^{-1} do 4 342 mg kg^{-1}, ovisno o tipu tla, lokaciji i dubini uzorkovanja, dok su prosječne vrijednosti masenog udjela ukupnog sumpora, za cijeli period istraživanja, za tlo, iznosile 1 080 mg kg^{-1} (n = 80) za obje ispitivane dubine te 4 108 mg kg^{-1} za sve ispitivane uzorke trpuca (n = 85).

Prosječno godišnje ukupno atmosfersko taloženje sumpora na mjernoj stanici Bilogora iznosilo je 6,3 kg ha^{-1} $S\text{-}SO_4$.

KLJUČNE RIJEČI: *depozicija, H_2S, merkaptani,* Plantago lanceolata*, SO_2, tlo, ukupni sumpor, zrak*

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Scientific Paper

ASSOCIATIONS BETWEEN WEATHER CONDITIONS AND RAGWEED POLLEN VARIATIONS IN SZEGED, HUNGARY

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This study analyses potential associations between day-to-day variations in common ragweed pollen counts in the southern Hungarian district of Szeged and meteorological variables using adapted factor analysis. The database includes ten years (1997-2006) worth of data on daily common ragweed pollen ratios (value on the given day per value on the day before) and daily differences (value on the given day minus value on the day before) in eight meteorological variables (mean temperature, minimum temperature, maximum temperature, temperature range, irradiance, relative humidity, wind speed, and rainfall) over the ragweed pollen season. This method is new, as it has only been applied in the economics. In factor analysis it is advisable to combine all the weights of the factors and the resultant variable into one factor indicating the rank of importance of the given explanatory variables in influencing the resultant variable, while the remaining factors are uncorrelated with the resultant variable. The procedure shows that wind speed, rainfall, and temperature range are the most important, while minimum temperature and irradiance are the least important meteorological variables influencing daily pollen ratios. We found a tendency to stronger associations between the meteorological variables and the pollen variable when the pollen ratio was 1 or below. This is due to the fact that data corresponding to the pollen ratio over 1 come mainly from the pre-peak pollen season, while data corresponding to less than 1 are characteristic of the post-peak pollen season (late summer to early autumn).

KEY WORDS: *allergenic pollen,* Ambrosia artemisiifolia*, meteorological parameters, plant physiology, pollen transport, respiratory disease*

Air pollution, as a major and ever increasing environmental hazard, is associated with increased cost of health insurance (1). The prevalence of allergic respiratory diseases has also increased extensively over the last three decades (2). Historical records have demonstrated that the prevalence of allergic rhinitis and allergic asthma have significantly increased over the past two centuries (3). Although the reasons for this increase are not entirely clear, epidemiological data suggest that particular pollutants produced by burning fossil fuels may have played a substantial role in increasing this prevalence (3).

Pollen allergy has become a widespread disease by the end of the 20th century. Around 20 % of the general population in Europe suffers from this immune system disorder (4). Hungary is exposed to one of the most severe air pollutions in Europe (5); in addition, airborne pollen levels here are also high. The Carpathian basin, which includes Hungary (Figure 1), is considered the most polluted region with airborne

ragweed (*Ambrosia artemisiifolia*) pollen in Europe (6-10). Ragweed in Hungary discharges the most pollen of all taxa (11, 12). In Szeged in the southern Hungary, the ratio of its pollen release compared to the total pollen release in the late summer is around 60 % to 71 % (13). The highest counts on peak days in Szeged are about one order of magnitude higher than those over other cities in Europe (14). The prevalence of sensitivity to ragweed in Szeged is 83.7 % (15). Ragweed-related allergy and asthma have become the dominant health issue in Hungary over the past few decades (16, 17). Recently, 20 % of the total population suffers from allergic diseases and for one-third of these patients may also suffer from asthma (18). In 60 % to 90 % of patients pollen allergy is ragweed-related (19). The number of patients with registered allergic diseases has doubled over the last 40 years, and the number of patients with allergic asthma has quadrupled in the southern Hungary by the late 1990s (12).

Knowledge of the association between daily ragweed pollen concentrations and daily meteorological parameters may have a great practical importance. Applying simple statistical analysis, several studies have detected significant positive correlations of daily ragweed pollen counts with daily maximum temperature (20), daily mean temperature (6, 7, 21-23), daily mean wind speed (23), and daily maximum wind speed (22) and significant negative correlations with relative humidity (21-23) and rainfall (7, 23, 24). Furthermore, Ziska et al. (25) established that the association between higher temperatures and higher ragweed pollen counts was stronger in urban than in rural locations. Wind direction analysis has shown that either long-range transport or local sources could play an important role in ragweed pollen concentrations (20, 23).

However, meteorological elements affect pollen concentration not by means of their individual values but through their interrelationships (26). This is why it could be useful to study the association between daily ragweed pollen concentrations with daily values of meteorological parameters as a whole. Only a few papers have reported results of this kind of approach, using multivariate statistical analysis. They generally define the most homogeneous groups as objective classes of meteorological parameters (27-30) using factor and cluster analyses to associate them with a given pollen variable.

The aim of this study was to determine possible reasons for day-to-day variations in ragweed pollen counts for Szeged in view of meteorological parameters. For this purpose, we used factor analysis with special transformation of daily meteorological and ragweed pollen data to establish the strength of associations between meteorological (explanatory) variables and ragweed pollen ratio (resultant) variable. Factor analysis with special transformation is a procedure that has not yet been applied for studying this kind of relationships.

MATERIALS AND METHODS

Szeged (46.25°N; 20.10°E) is the largest city in the southeast of Hungary (Figure 1). The area is located in the Great Hungarian Plain with an elevation of 79 m above mean sea level. The city area covers about 46 km^2. The city is the centre of the Szeged region with 203,000 inhabitants. According to the Köppen system, the climate in Szeged is of the Ca type (warm temperate climate) with relatively mild and short winters and hot summers (31). Airborne ragweed pollen content was measured using a 7-day recording "Hirst-type" volumetric trap (32). The air sampler was located about 20 m above the ground (Figure 1, lower panel).

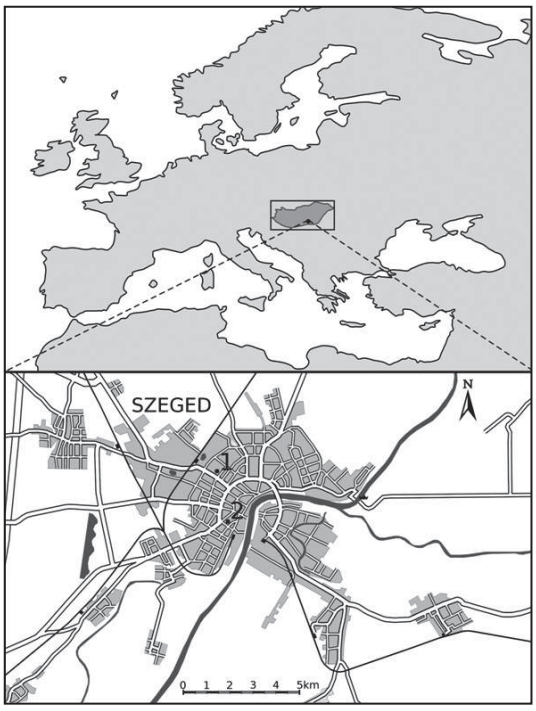

Figure 1 *Location of Hungary in Europe (upper panel) and the map of Szeged with the locations of 1 - the meteorological monitoring station and 2 - aerobiological station (lower panel)*

Our database includes ten years (1997 to 2006) of daily ratios of ragweed pollen counts (A - a value on the given day per value on the day before) and daily differences (a value on the given day minus the value on the day before) of eight meteorological variables (mean temperature, minimum temperature, maximum temperature, temperature range, irradiance, relative humidity, wind speed, and rainfall) over the ragweed pollen season (15 July to 16 October) (Figure 2).

The *Ambrosia* genus has only one species, *Ambrosia artemisiifolia* (common ragweed) in the Szeged region. It is present both in the city and in the countryside. Ragweed is particularly common in the west of the town. The prevailing northwest wind can easily transport pollen into the city. Since in the sandy region northwest of Szeged, stubble stripping is not necessary for ground-clearance due to the mechanical properties of sandy soils, ragweed can spread unchecked. Furthermore, due to newly-built motorways around Szeged several farmlands have remained uncultivated, which also favours the spread of ragweed.

For the start and the end of the season we used the first and the last date on which at least one pollen grain per cubic metre of air was recorded for at least five consecutive days (33). Evidently, the pollen season varies from year to year. For our analysis we used the absolute longest observed pollen season over the ten-year period even if the remaining years had either remarkably late start or early end.

For each day of the analysis, daily differences in meteorological variables were paired with the daily ratios of ragweed pollen counts (A). Three data sets were subjected to an analysis: (1) the total data set, (2) those daily differences in meteorological variables for which $A \leq 1$, and (3) those for which $A > 1$. Days falling in respective data sets were classified into four categories: (a) rainy day preceded by a rainy day; (b) rainy day preceded by a dry day; (c) dry day preceded by a rainy day; and (d) dry day preceded by a dry day.

Factor analysis with special transformation

Factor analysis identifies linear relationships between subsets of variables, and this helps to reduce the dimensionality of the initial database without substantial loss of information. We started with a dataset consisting of eight variables (seven meteorological parameters as explanatory variables and daily ratios of ragweed pollen counts as the resultant variable) in order to reduce the original number of variables. The newly combined variables (called factors) can explain the behaviour of meteorological and ragweed pollen variables. The optimum number of retained factors can be determined using different statistical criteria (34). The most common and widely accepted criterion is setting the lowest percentage (80 %) of total variance in the original variables (35). After factor analysis, we transformed the retained factors to see to what degree the explanatory variables affected the resultant variable and to rank their influence (36). In factor analysis of standardised variables the received factor loadings are the correlation coefficients between the

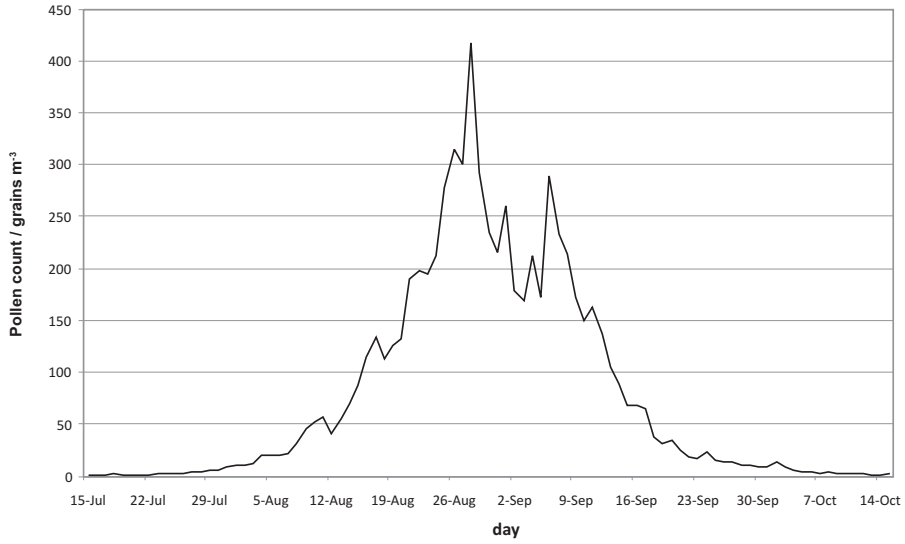

Figure 2 *Mean daily ragweed pollen counts over the pollen season (15 July to 16 October) in Szeged between 1997 and 2006*

Table 1 *Special transformation. The effect of the daily differences in meteorological (explanatory) variables[1] on the daily ragweed pollen ratios (A)[2] (resultant variables) and the ranking of the explanatory variables. Significant factor loadings are indicated with figures in underlined italics ($x_{0.10}$), bold ($x_{0.05}$), and underlined bold ($x_{0.01}$).*

[1]Daily differences in meteorological variables	[2]Daily ratios of ragweed pollen counts (A)							
	a		b		c		d	
	thresholds of significance							
	0.139		0.140		0.139		0.073	
	0.165		0.166		0.166		0.087	
	0.217		0.218		0.218		0.115	
	weight	rank	weight	rank	weight	rank	weight	rank
Ragweed	0.869	–	0.895	–	0.999	–	0.988	–
T_{mean}	**0.250**	3	0.118	3	0.083	2	-0.023	6
T_{min}	-0.029	8	0.063	6	0.117	1	0.059	3
T_{max}	**0.263**	2	0.074	4	0.049	4	-0.007	7
DT	**0.199**	4	-0.009	8	-0.082	3	-0.058	4
I	0.108	6	-0.069	5	0.029	6	-0.071	2
RH	0.092	7	0.056	7	0.046	5	**-0.166**	1
V	*-0.165*	5	**-0.291**	2	0.002	7	-0.034	5
R	0.548	1	**-0.359**	1	–	–	–	–

[1]: value on the given day minus value on the day before;
[2]: value on the given day divided by value on the day before;
a: rainy day preceded by a rainy day;
b: rainy day preceded by a dry day;
c: dry day preceded by a rainy day;
d: dry day preceded by a dry day;
T_{mean} = daily mean temperature; T_{min} = daily minimum temperature,
T_{max} = daily maximum temperature, DT = daily temperature range;
I = irradiance, RH = relative humidity; V = wind speed; R = rainfall;

Table 2 *Special transformation. The effect of the daily differences in meteorological (explanatory) variables[1] on the daily ratios of ragweed pollen ratios (A)[2], A≤1 (resultant variables) and the ranking of the explanatory variables. Significant factor loadings are indicated with figures in underlined italics ($x_{0.10}$), bold ($x_{0.05}$), and underlined bold ($x_{0.01}$).*

[1]Daily differences in meteorological variables	[2]Daily ratios of ragweed pollen counts (A), A ≤ 1							
	a		b		c		d	
	thresholds of significance							
	0.185		0.197		0.191		0.106	
	0.220		0.233		0.225		0.126	
	0.286		0.304		0.294		0.167	
	weight	rank	weight	rank	weight	rank	weight	rank
Ragweed	-0.790	–	-0.813	–	0.656	–	-0.929	–
T_{mean}	*0.193*	4	0.087	5	0.181	5	**0.260**	1
T_{min}	-0.166	6	0.165	2	**-0.316**	4	-0.023	7
T_{max}	0.170	5	0.027	8	**0.397**	3	**0.234**	2
ΔT	**0.249**	2	-0.129	4	**0.553**	2	**0.151**	4
I	0.000	8	-0.156	3	0.160	6	**-0.144**	5
RH	-0.072	7	-0.064	6	-0.084	7	**0.176**	3
V	**-0.243**	3	**-0.657**	1	**-0.631**	1	*-0.119*	6
R	**-0.725**	1	-0.058	7	–	–	–	–

[1]: value on the given day minus value on the day before;
[2]: value on the given day divided by value on the day before;
a: rainy day, preceded by a rainy day;
b: rainy day, preceded by a dry day;
c: dry day, preceded by a rainy day;
d: dry day, preceded by a dry day;
T_{mean} = daily mean temperature; T_{min} = daily minimum temperature,
T_{max} = daily maximum temperature, DT = daily temperature range;
I = irradiance, RH = relative humidity; V = wind speed; R = rainfall;

original variables and the factors. Consequently, if the resultant variable strongly correlates with a factor (if a factor has high factor loading at the place of the resultant variable), and an influencing variable highly correlates with the factor, then the influencing variable also highly correlates with the resultant variable. Therefore, it is advisable to combine all the weights of the factors into one factor. This can done by rotating the factors. As a result of the rotation, factor loadings will be very close to either 0 or 1. In this way we can more easily determine to which group of variables an individual factors belongs to. There are different rotation techniques. We use varimax rotation that produces uncorrelated factors after factor rotation. It is useful to rotate the factors so that only one factor has a great load on the resultant variable and the remaining factors are uncorrelated with the resultant variable (35). This procedure is called special transformation.

RESULTS AND DISCUSSION

Factor analysis with special transformation

In the total set, factor analysis yielded four factors for category (a), five for category (b), four for category (c), and four for category (d) (Table 1). In the data set with A≤1, it yielded four factors in category (a), five in category (b), three in category (c), and four in category (d) (Table 2). In the data set A>1, four factors were retained for each category (Table 3). In order to rank the importance of the explanatory (meteorological) variables for the resultant variable (daily ratios of ragweed pollen counts), loadings of the retained factors were projected onto Factor 1 (the one that has the greatest load on the resultant variable) for all twelve factor analyses with the special transformation (Tables 1-3) (36).

We analysed only the relationships between the meteorological and pollen variables that were significant at 10 %, 5 %, and 1 % probability levels. In the total data set (Table 1), rainfall (R), maximum temperature (T_{max}), mean temperature (T_{mean}), and temperature range (ΔT) were the most important variables for category (a) with a proportional correlation with daily ratios of ragweed pollen counts. At the same time, wind speed (V) showed a weak inverse correlation with the resultant variable. For category (b), rainfall (R) and wind speed (V) were the only relevant meteorological parameters, both influencing inversely the resultant variable. For category (c), there were no significant explanatory variables, while for category (d) only relative humidity (RH) showed a significant inverse correlation with daily pollen ratios.

In the data set A≤1 (Table 2), rainfall (R) and wind speed (V) showed proportional, while temperature range (ΔT) and mean temperature (T_{mean}) showed inverse correlation with the resultant variable for category (a). Wind speed (V) was the only significant parameter for category (b), with a positive correlation with daily pollen ratios. For category (c) significant inverse correlation was found for wind speed (V) and minimum temperature (T_{mean}), while temperature range (ΔT) and maximum temperature (T_{max}) showed a positive correlation with the resultant variable. For category (d), mean temperature (T_{mean}), maximum temperature (T_{max}), relative humidity (RH), and temperature range (ΔT) showed significant inverse correlation, while irradiance (I) and wind speed (V) positive correlation with the resultant variable.

In the data set A>1 (Table 3), the resultant variable positively correlated with rainfall (R) for category (a), while for category (b) it inversely correlated with rainfall (R) and wind speed (V) as the most important variables. For category (c), temperature range (ΔT) showed negative, while minimum temperature (T_{mean}) showed positive correlation with the resultant variable. For category (d), irradiance (I) and relative humidity (RH) were the most important variables, influencing inversely daily pollen ratios.

Variable ranking

Rainfall (R) is one of the first two most important meteorological parameters for all three data sets, except for category (b) of the A≤1 dataset (Table 2). For category (a) rainfall positively correlates with daily pollen ratios in all three data sets (Table 1-3). Rainfall generally stimulates pollen production, but this is not an immediate effect. Increased rainfall results in a higher biomass that leads to higher pollen concentration. For category (b) in the total data set (Table 1) and in the data set A>1 (Table 3) rainfall is in inverse correlation with daily pollen ratios. This association can be explained by the immediate wash-out effect; air pollen content drops sharply after rainfall (23, 37, 38). Another reason may be that rainfall is accompanied by a temperature drop, which slows down plant metabolism (39) (Table 1; Table 3). However, this association is not direct. Pollen release depends on the dehiscence of the flowers, which in

turn is linked to meteorological conditions, relative humidity in particular. Experimental studies on excised ragweed flowers and whole plants confirmed that the opening of flowers can be controlled by regulating temperature and relative humidity. Lower temperature slows down or inhibits extension of anthers while higher humidity delays or stops the opening of pollen sacs (40). The role of rainfall in daily pollen counts is not clear. Fornaciari et al. (41) and Galán et al. (42) find it complex because intense rain can lower pollen count. Fornaciari et al. (41) obtained the best correlation by comparing pollen concentrations of *Urticaceae* and meteorological parameters on dry days. Ragweed pollen count negatively correlated with rainfall in several studies (7, 23, 24, 37, 43), but Bartkova-Scevkova (21) found no statistically significant association.

The importance of mean temperature (T_{mean}) in our study varied between data sets and categories (Tables 1-3). For category (a) in the total data set (Table 1) it was in positive, while for categories (a) and (d) in the data set A≤1 (Table 2) it was in inverse correlation with daily pollen ratios. If humidity is favourable, an increase in mean temperature (T_{mean}), if it is not too far from its optimum value, can accelerate vegetative and hence generative functions, which can increase airborne pollen concentrations [Table 1, category (a)] (21, 23, 45). If humidity is low, an excessive increase in mean temperature (T_{mean}) can inhibit pollination, as the plant seeks to preserve water to maintain its vegetative life functions in contrast to the generative functions (39). This is why mean temperature (T_{mean}) showed inverse correlation with daily ratios of ragweed pollen counts for categories (a) and (d) (Table 2).

Minimum temperature (T_{min}) was relevant for category (c), inverse in the data set A≤1 (Table 2) and positive in the data set A>1 (Table 3). The reason for the inverse relationship is that if preceding day is rainy, rainfall can lower temperature early in the morning. On the other hand, the reason for the positive correlation may be that low minimum temperature can inhibit ragweed pollen release as it slows down life functions (Table 3).

Maximum temperature (T_{max}) significantly correlated with daily pollen ratios for category (a) in the total data set (Table 1). However, it was in proportional and inverse correlation with the resultant variable for categories (c) and (d) in the data sets A≤1, respectively (Table 2). The proportional relationship may be explained as follows: anthers dehisce and release pollen when anther sacs walls dehydrate (44), which is facilitated by higher maximum temperatures. This association may not be valid on a dry day preceded by a dry day [category (d) in the data set with A≤1; Table 2]. In the summer, extremely high maximum temperatures may limit pollen release as water deficiency can prompt the plant to preserve the remaining water and stop releasing pollen.

Temperature range (DT) is in a significant positive correlation with the daily ratios of ragweed pollen counts for category (a) in the total data set (Table 1) and for category (c) in the data set A≤1 (Table 2). In the same data set, this variable showed an inverse correlation for categories (a) and (d) (Table 2). The same is true for category (c) in the data set A>1 (Table 3). The reason for inverse correlation is that very low temperatures slow down plant metabolism and pollen release, while extremely high temperatures force the plant to preserve water, which also lowers pollen release. Accordingly, the greater the temperature range (DT), the lower the pollen count.

Irradiance (I) showed positive correlation with daily ragweed pollen ratios for category (d) in the data set A≤1 (Table 2) and negative correlation in the data set A>1 (Table 3). The positive correlation is due to the fact that irradiance favours vegetative processes important for releasing pollen. Inverse correlation may be owed to extremely high irradiance (I) that increases mean temperature (T_{mean}), forcing the plant to limit its functions on preserving water (39).

Relative humidity (RH) was inversely associated with daily pollen ratios for category (c) in all three data sets (Tables 1-3). In general, pollen shedding is associated with shrinkage and rupture of anther walls at low relative humidity (44). Hence, relative humidity is inversely associated with pollen release (21, 45). Furthermore, humid air makes pollen grains stick together, which in turn contributes to the inverse association (23).

Wind speed (V) showed inverse correlation with daily ragweed pollen ratios for categories (a) and (b) in the total data set (Table 1), for all categories in the data set A>1 (Table 3), and for category (c) in the data set A≤1 (Table 2). Correlation was positive for categories (a), (b), and (d) in the data set A≤1 (Table 2). When analysing the role of wind speed one should take into account its physical properties (39), plant physiology (39), and the range of pollen transport (46). Wind can hinder sticking of pollen grains (46), and higher speeds increase evapotranspiration, forcing the plant to preserve water at the expense of pollination.

Long-range pollen transport may also have a substantial effect on local pollen counts (46). Gioulekas et al. (45), Kasprzyk (23), and Hernández-Ceballos et al. (38) reported positive association between wind speed and daily pollen ratios. However, if mean temperature (T_{max}) favours ragweed pollen release, then wind may take away locally released pollen and bring a smaller amount of pollen from far away to the local environment. Extremely high mean temperature (T_{max}) may significantly reduce available water and limit pollen release. In this case, wind may bring a greater amount of pollen from far away than the amount of locally released pollen.

CONCLUSIONS

Our factor analysis with special transformation has singled out wind speed (V), rainfall (R), and temperature range (ΔT) as the most important parameters for daily ratios of ragweed pollen counts. In contrast, minimum temperature (T_{min}) and irradiance (I) were the least important meteorological variables influencing the resultant variable. After dividing the total data set in two groups, we established stronger associations between meteorological variables and the pollen ratio in the data set A≤1 (Table 2) than A>1 (Table 3). This is due to the fact that A>1 data mainly correspond to the pre-peak pollen season, while A≤1 data mainly correspond to the post-peak pollen season. Weather conditions during early autumn are not optimal for ragweed pollen release. Additionally, although particular meteorological parameters might favour pollen release, the association might be inverse if the pollen season is nearing its end, and plants are flowering less. This is may be the main reason for the differences between the two datasets.

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Table 3 *Special transformation. The effect of the daily differences in meteorological variables[1] on the daily ratios of ragweed pollen ratios (A)[2], A>1 (resultant variables) and the ranking of the explanatory variables. Significant factor loadings are indicated with figures in underlined italics ($x_{0.10}$), bold ($x_{0.05}$), and underlined bold ($x_{0.01}$).*

[1]Daily differences in meteorological variables	[2]Daily ratios of ragweed pollen counts (A), A > 1							
	a		b		c		d	
	thresholds of significance							
	0.233		0.218		0.224		0.105	
	0.276		0.258		0.265		0.125	
	0.358		0.335		0.344		0.164	
	weight	rank	weight	rank	weight	rank	weight	rank
Ragweed	-0.985	–	0.794	–	-0.931	–	0.991	–
T_{mean}	0.014	7	0.176	3	0.102	5	0.096	3
T_{min}	0.031	6	0.107	5	**-0.391**	2	0.071	4
T_{max}	0.067	5	0.138	4	0.153	4	0.066	5
DT	0.010	8	-0.010	7	**0.454**	1	-0.042	6
I	0.083	4	-0.081	6	0.007	7	**-0.127**	1
RH	-0.165	3	0.002	8	0.061	6	*-0.117*	2
V	0.185	2	**-0.623**	1	0.215	3	-0.023	7
R	**-0.294**	1	*-0.254*	2	–	–	–	–

[1]: value on the given day minus value on the day before;
[2]: value on the given day divided by value on the day before;
a: rainy day, preceded by a rainy day;
b: rainy day, preceded by a dry day;
c: dry day, preceded by a rainy day;
d: dry day, preceded by a dry day;
T_{mean} = daily mean temperature; T_{min} = daily minimum temperature,
T_{max} = daily maximum temperature, DT = daily temperature range;
I = irradiance, RH = relative humidity; V = wind speed; R = rainfall

4.2.1/B-09/1/KONV-2010-0005 and TAMOP-4.2.2/B-10/1-2010-0012).

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Sažetak

POVEZANOST IZMEĐU VREMENSKIH UVJETA I VARIJACIJA U PELUDU AMBROZIJE U OKOLICI GRADA SZEGEDA, MAĐARSKA

Primjenom prilagođene metode faktorske analize istražili smo povezanost između dnevnih varijacija u koncentracijama peludnih zrnaca ambrozije u zraku i meteoroloških varijabli u okolici grada Szegeda u južnoj Mađarskoj. Koristili smo bazu podataka prikupljenih tijekom desetogodišnjeg razdoblja (1997.-2006.) za dnevne omjere u koncentracijama peludnih zrnaca ambrozije (vrijednost određena na promatrani dan podijeljena s vrijednošću određenom prethodnog dana) i dnevne razlike u koncentracijama peludnih zrnaca ambrozije (vrijednost određena na promatrani dan od koje je oduzeta vrijednost određena prethodnog dana) te podatke o osam meteoroloških varijabli (prosječna temperatura, minimalna temperatura, maksimalna temperatura, raspon temperatura, sijanje sunca, relativna vlažnost, brzina vjetra, padaline) tijekom sezona cvatnje ambrozije. Prikazali smo novu metodu za ovo područje istraživanja, jer je ona do sada bila primjenjivana samo u ekonomiji. U faktorskoj analizi poželjno je sve faktorske težine i izlaznu varijablu kombinirati u jedan faktor koji označava razinu važnosti pojedinih nezavisnih varijabli u utjecaju na izlaznu varijablu, dok preostali faktori nisu u korelaciji s izlaznom varijablom. Primjenom spomenutoga statističkog modela utvrdili smo da su najvažnije meteorološke varijable brzina vjetra, padaline i raspon temperatura, dok su minimalna temperatura i sijanje sunca imali najmanje utjecaja na dnevne omjere u koncentracijama peludnih zrnaca ambrozije u promatranu razdoblju. Uočili smo sklonost prema jačoj povezanosti između meteoroloških varijabli i varijabli peluda u slučajevima kad je omjer u koncentracijama peludnih zrnaca iznosio 1,00 ili niže. To tumačimo činjenicom da su podaci koji se odnose na omjer u koncentracijama peludnih zrnaca veći od 1,00 uglavnom dobiveni u razdoblju koje prethodi vrhuncu sezone cvatnje ambrozije, dok su podaci koji se odnose na omjer u koncentracijama peludnih zrnaca manji od 1,00 karakteristični za razdoblje koje slijedi nakon vrhunca sezone cvatnje ambrozije (kasno ljeto – rana jesen).

KLJUČNE RIJEČI: *alergeni pelud, ambrozija, bolesti dišnog sustava, fiziologija bilja, meteorološki parametri, prijenos peludnih zrnaca*

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Scientific paper

CADMIUM AND ZINC INDUCED SIMILAR CHANGES IN PROTEIN AND GLYCOPROTEIN PATTERNS IN TOBACCO (*NICOTIANA TABACUM* L.) SEEDLINGS AND PLANTS

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The effects of 10 μmol L^{-1} and 15 μmol L^{-1} cadmium (Cd), a nonessential toxic element and 25 μmol L^{-1} and 50 μmol L^{-1} zinc (Zn), an essential micronutrient, on proteins and glycoproteins of *Nicotiana tabacum* L. seedlings and plants were investigated after exposure to each metal alone or to their combinations. Changes in only few polypeptides related to heavy metal treatments were observed in tobacco seedlings and leaves of adult plants, while the greatest change in total soluble protein pattern was observed in plant roots. Differences between control and treated tobacco tissues were more pronounced in the glycoprotein pattern, which was analysed by application of different lectins. The majority of the detected glycoproteins in leaves and roots of adult plants can be considered as a result of enhanced glycosylation due to heavy metal stress. The difference in glycoproteins between Cd and Zn application on tobacco seedlings and adult plants could not be determined since enhanced glycosylation was noticed after treatment with either metal alone or in combination. Therefore, it can be concluded that both metals induced N- and O-glycosylation as a result of changed environmental conditions.

KEY WORDS: *heavy metals, lectins, protein pattern*

Agricultural plants face a variety of abiotic and biotic stresses, which are major causes limiting crop production. Among abiotic stressors, heavy metal contamination represents a global environmental problem endangering humans, animals, and plants. Cadmium (Cd) is very toxic and without any metabolic significance (1). It is especially dangerous due to its long biological half-life. When released into the environment, Cd becomes accessible to plants, where its toxic effects involve a decrease in chlorophyll and carotenoid content, as well as changes in photosynthetic activity (2), growth inhibition and root damage (1), reduction of water and nutrient uptake (3), lipid peroxidation (4) and protein degradation (1, 5). Toxicological properties of Cd originate from its chemical similarity to zinc (Zn), an essential micronutrient of great chemical and metabolic significance in plant systems (6). Zn plays a fundamental role in several cellular functions such as protein metabolism, gene expression, chromatin structure, photosynthetic carbon metabolism, and indole acetic acid metabolism (7) and is involved in the catalytic function of many enzymes and structural stability of various cell proteins (8). Cd frequently accompanies Zn minerals in the environment (9), which is why they have often been investigated together for their mutual effect on different plants. Up to this moment, mostly antagonistic interaction was suggested; it has been

found that Zn can lessen physiological damage caused by Cd (10), suppress Cd uptake (6), and decrease Cd-induced oxidative stress (11).

It has been documented that exposure to heavy metals can cause changes in the expression of cellular plant proteins (12, 13). Some heavy metals, such as manganese (Mn) and Zn, stimulate protein synthesis (14), while other metabolites, such as Cd, interfere with this process (15). Sobkowiak and Deckert (16) reported that Cd induced changes in the protein pattern in soybean cell suspension culture. Moreover, dramatic changes in the protein pattern of barley seedlings exposed to Cd were revealed by SDS-PAGE (17), while a proteomic approach has been adopted for the analysis of protein profile alternations during the germination of rice following exposure to Cd (18).

Posttranslational modifications such as glycosylation are important for altering the properties and functions of proteins. The oligosaccharide chain can be either N- or O-linked. N-glycosylation occurs in the endoplasmic reticulum (ER) and the primary oligosaccharide chain is further processed when it exits the ER and passes through the Golgi apparatus (GA). The latter cell compartment is also a site of protein O-glycosylation. Glycosylation can change basic biological functions of a protein including immunogenicity, specific activity, and the ligand-receptor interaction. Only few studies have been conducted on plants to reveal whether environmental conditions act upon protein glycosylation. The analysis of N-linked glycans of soluble endogenous glycoproteins from the leaves of tobacco demonstrated that developmental processes and different growth conditions could influence glycosylation (19, 20). It was found that N-glycosylation of extracellular (21-23) as well as cellular proteins (22, 24-27) was affected by *in vitro* culture conditions. However, modest information about the abiotic stress related to glycoprotein patterns is available so far. It has been revealed that the mutation in *STT3* gene, which encodes important subunits of the enzyme oligosaccharyltransferase involved in protein N-glycosylation, increases the sensibility to salt and osmotic stress (28). Moreover, Kang et al. (29) found that salt tolerance of *Arabidopsis thaliana* requires of N-glycosylated proteins to mature in the GA. In the basal parts of rice leaf sheaths, cold stress altered the glycosylation profile of calreticulin, a key protein that regulates the quality control of other proteins (30). Carpena et al. (31) suggested that glycoprotein accumulation might be considered as a useful indicator of Cd-induced stress in white lupin nodules. In a recent publication Zhang et al. (32) suggest that protein N-glycoslyation might have a key role in plant development and abiotic stress response. The role of O-glycosylation in plant cells grown in a stressful environment is even less studied than N-glycosylation. The report by Johnson et al. (33) indicates that arabinogalactans, a group of plant specific O-glycoslyated proteins, are likely to be important during plant development and in response to abiotic stress.

Tobacco, *Nicotiana tabacum* L., became a model plant in many studies due to its relative tolerance to environmental stress and wide distribution (34). All of the first achievements in plant genetic engineering are mostly based on the work with tobacco. This plant has been employed in studies on the production of useful recombinant proteins and antibodies, which have an application in medicine and industry (35). Moreover, tobacco has been found to be an extremely versatile system for all aspects of abiotic stress research, including the studies of physiological mechanisms, which lead to adverse environmental conditions (36, 37).

The aim of the present work was to investigate the effects of Cd and Zn, applied separately or in combinations, on protein and glycoprotein patterns of soluble cellular proteins in seedlings and adult plants of tobacco.

MATERIALS AND METHODS

Chemicals and instrumentation

$CdCl_2$, $ZnCl_2$, $AgNO_3$, glycine, sucrose, ascorbic acid, methanol, HCl and NaCl were obtained from Kemika (Zagreb, Croatia), while polyvinylpyrrolidone (PVP), bovine serum albumin (BSA), Tris, Phytagel, Tween® 20, EDTA, Con A, 4-chloro-1-naphthol and peroxidase were purchased from Sigma (Steinheim, Germany). Distilled and deionized water from Milli-Q water systems (Millipore, Bedford, MA, USA) was used to prepare the nutrient media and extraction buffers. UV-VIS spectrophotometer ATI/Unicam UV4-100, (Cambridge, UK) was used to determine the protein concentration in extracts. Pure nitrocellulose membrane – 0.45 mm as well as electrophoresis system for protein separation and electroblotting (Mini-PROTEAN 3 Cell and Mini Trans-Blot Cell)

were purchased from Bio-Rad (Bio-Rad Laboratories GmbH München, Germany). Protein Molecular Weight Marker was obtained from Fermentas (St. Leon-Rot, Germany). Ponceau-S stain and digoxigenin-labeled lectins GNA, DSA, PNA MAA, and SNA (their descriptions are given in Table 1) (DIG Glycan Differentiation Kit) were obtained from Roche Applied Science (Mannheim, Germany). Gels and membranes were scanned using HP Scanjet 2400 scanner (Hewlett-Packard Company, USA).

Plant material and heavy metal treatments

Seeds of *Nicotiana tabacum* L. cv Burley were surface sterilised with 50 % NaOCl, washed with distilled H_2O several times and subsequently germinated in sterilised nutrient medium. The medium was prepared according to Murashige and Skoog (38) with addition of the 500 mg L^{-1} MES [2-(N-morpholino)ethanesulfonic acid], 1.5 g L^{-1} sucrose, and 2.2 g L^{-1} Phytagel (pH 5.6) (39) at 24 °C with 16 h : 8 h light/dark cycle and light intensity of 90 µE m^{-2} s^{-1}. In these conditions seedlings were grown for 90 days until adult plants were obtained.

For heavy metal treatment of seedlings, tobacco seeds were germinated for 30 days on solid MS nutrient medium with the addition of Cd, Zn or their combinations. Cd treatments in concentrations of 10 µmol L^{-1} and 15 µmol L^{-1} were prepared by adding a stock solution of $CdCl_2$ to the nutrient medium. Zn was added as $ZnCl_2$ to the nutrient medium in the amounts suitable to achieve concentrations of 25 µmol L^{-1} and 50 µmol L^{-1}. Tobacco seedlings were also exposed to the combinations of metals (10 µmol L^{-1} or 15 µmol L^{-1} Cd with 25 µmol L^{-1} and 50 µmol L^{-1} Zn). For protein extraction, whole tobacco seedlings were used.

For exposure to heavy metals, adult plants obtained on the solid MS medium were transferred to the liquid medium of the same composition but with the addition of either metal alone (10 µmol L^{-1} or 15 µmol L^{-1} Cd and 25 µmol L^{-1} or 50 µmol L^{-1} Zn) or their combinations (10 µmol L^{-1} or 15 µmol L^{-1} Cd with 25 µmol L^{-1} and 50 µmol L^{-1} Zn) and then treated for 7 days. For protein and glycoprotein pattern analyses, leaves and roots were analysed separately.

Protein extracts, SDS-PAGE and electroblotting

Total soluble proteins from tobacco seedlings were extracted by grinding 0.4 g of fresh tissues in 1.5 mL of 0.1 mol L^{-1} Tris/HCl buffer, pH 8.0 at 4 °C containing 17.1 % sucrose, 0.1 % ascorbic acid, 0.1 % cystein (40), and (10 to 15) mg of PVP. For the extraction of total soluble proteins from leaves and roots of adult plants, 0.4 g of fresh tissue was homogenized in 1.0 mL cold (0 to 4) °C extraction buffer (pH 7.5) containing 30 mmol L^{-1} Tris/HCl, 5 mmol L^{-1} EDTA, 20 mmol L^{-1} NaCl, and (10 to 15) mg of PVP. Homogenates were centrifuged at 20,000 x*g* and 4 °C for 15 min. Supernatants were centrifuged again at 20,000 x*g* and 4 °C for 60 min. The supernatant was collected and protein content was determined according to Bradford (41) using BSA as a standard. Obtained supernatants were used for the analysis of total soluble proteins and glycoproteins.

Total soluble proteins were analysed by SDS-PAGE in 8 % to 18 % T (2.67 % C) gradient gels, with the buffer system of Laemmli (42). For the SDS-PAGE, the same amount of protein (10 µg) per sample was loaded. Proteins migrated through stacking and separating gels at 100 V and 200 V, respectively. Protein bands were visualised by silver staining (43) and gels were scanned as an 8 bit grey scale Tiff-images.

Table 1 *Major N- and O-linked carbohydrate binding specificities of different plant lectins applied.*

Lectin	Taxonomic lectin source	Specificity toward carbohydrate moieties	Type of glycans
Con A	*Canavalia ensiformis*	*α*-D-Glu *α*-D-Man	core N-glycan structure (44)
GNA	*Galanthus nivalis*	*α*-D-Man	high-mannose N-glycans (44)
PNA	*Arachis hypogaea*	*β*-Gal-(1,3)-GalNAc	O-glycosidically linked carbohydrates (45)
DSA	*Datura stramonium*	Gal-(1,4)-GlcNAc	complex and hybrid N-glycans (46)
MAA	*Maackia amurensis*	NeuAc-α(2,3)-Gal	sialic acid in complex N- and O-glycans (47)
SNA	*Sambucus nigra*	NeuAc- α (2,6)-Gal	sialic acid in complex N- and O-glycans (47)

For lectin assays, proteins were separated by SDS-PAGE in 12 % T (2.67 % C) polyacrylamide gels (42). The same amount of protein (15 µg) per sample was loaded. Proteins migrated through stacking and separating gels at 100 V and 200 V, respectively. Subsequently, proteins were electroblotted to the nitrocellulose membrane in a mini trans-blot cell at 60 V for 60 min. The transfer buffer was 20 mmol L^{-1} Tris-HCl, 150 mmol L^{-1} glycine and 10 % methanol. The membrane was stained with Ponceau-S stain to confirm the complete transfer of proteins. The stain was washed off with distilled water. The unoccupied sites of the membrane were blocked by incubating the membrane with 0.1 % Tween® 20 in TBS buffer, pH 7.5 at 4 °C overnight. Glycoproteins with D-manose in their glycan component were detected on nitrocellulose membrane by the reaction with Con A. Bands were visualised by peroxidase reaction using 4-chloro-1-naphthol as a substrate (44). The glycan part of proteins was further characterised according to binding of digoxigenin-labelled lectins GNA (45), DSA (46), PNA (46), MAA (47), and SNA (47) (Table1). The staining procedure was performed following the manufacturer's instructions. Membranes were scanned as an 8 bit grey scale Tiff-images.

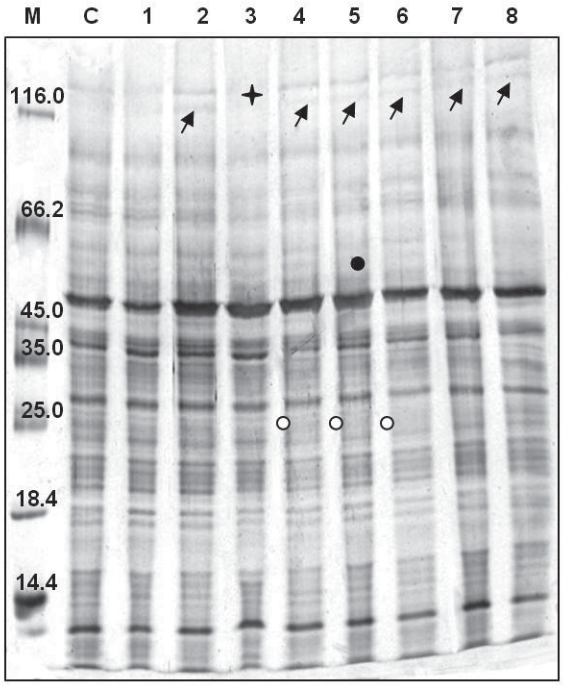

Figure 1 *Total soluble cell proteins of tobacco seedlings separated by SDS-PAGE in gradient 8 % to 18 % gel and silver stained. M – protein molecular weight marker, C – control; (1 to 10) µmol L^{-1} Cd; (2 to 15) µmol L^{-1} Cd; (3 to 25) µmol L^{-1} Zn; (4 to 50) µmol L^{-1} Zn; (5 to 10) µmol L^{-1} Cd and 25 µmol L^{-1} Zn; (6 to 10) µmol L^{-1} Cd and 50 µmol L^{-1} Zn; (7 to 15) µmol L^{-1} Cd and 25 µmol L^{-1} Zn; (8 to 15) µmol L^{-1} Cd and 50 µmol L^{-1} Zn.*

RESULTS

Total soluble cell proteins

We observed changes in only few polypeptides related to heavy metal treatments in tobacco seedlings (Figure 1): protein band of 116 kDa (arrows) was present in all samples, although with weaker intensity in the control and treatments with lower concentration of both metals, 10 µmol L^{-1} Cd and 25 µmol L^{-1} Zn. Moreover, seedlings exposed separately to 10 µmol L^{-1} Cd and 25 µmol L^{-1} Zn exhibited weak expression of 118 kDa protein (star) in comparison with other treatments, while the 56 kDa (black circle) was missing after the combined treatments with 10 µmol L^{-1} Cd and 25 µmol L^{-1} Zn as well as with 15 µmol L^{-1} Cd and 50 µmol L^{-1} Zn. 25 kDa protein (white circles) was missing after the treatment with 50 µmol L^{-1} Zn as well as with combinations of 10 µmol L^{-1} Cd and both concentrations of Zn.

In tobacco leaves, a protein of 120 kDa (arrows) was expressed after exposure of adult plants to 10 µmol L^{-1} Cd, both Zn concentrations, and combined treatments with both Cd concentrations and 50 µmol L^{-1} Zn (Figure 2A). The 60 kDa polypeptide (star) was specific for combined treatments of 15 µmol L^{-1} Cd with 25 and 50 µmol L^{-1} Zn. In comparison to control, all leaves from treated plants exhibited a stronger expression of 45 kDa, 50 kDa and 55 kDa proteins (black circles).

The greatest change in total soluble protein pattern was observed in roots of adult plants (Figure 2B). The 20 kDa and 25 kDa proteins had a stronger expression in control in comparison with treated roots (stars). After the exposure to 50 µmol L^{-1} Zn, protein bands of 50 kDa, 70 kDa and 116 kDa (black circles) were observed in roots of adult plants with the strongest staining intensity. The 45 kDa proteins (black squares) were expressed after the treatment with 15 µmol L^{-1} Cd, 25 µmol L^{-1} Zn as well as in combined treatment with 15 µmol L^{-1} Cd and 50 µmol L^{-1} Zn, although they were present as very faint bands in combined treatments with 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn as well as 15 µmol L^{-1} Cd and 25 µmol L^{-1} Zn. Roots treated with 15 µmol L^{-1} Cd in combination with both

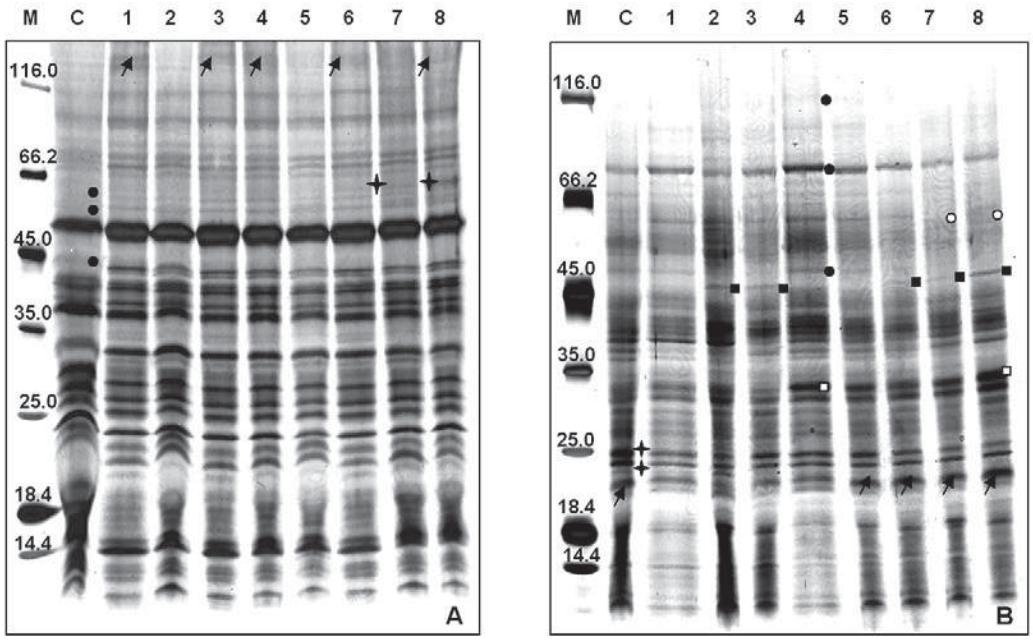

Figure 2 *Total soluble cell proteins of A) leaves and B) roots of adult tobacco plants separated by SDS-PAGE in gradient 8 % to 18 % gel and silver stained. For lane labels see Figure 1.*

Zn concentrations were devoid of the 60 kDa protein (white circles). Protein band of 30 kDa (white squares) had a stronger expression after the treatment with 50 µmol L^{-1} Zn alone and in combination with 15 µmol L^{-1} Cd in comparison with other treatments, while the 20 kDa polypeptide (arrows) was more pronounced in control roots and those exposed to combinations of metals compared to each metal alone.

Glycoproteins

In control and in treated seedlings, the 45 kDa glycoprotein (black frame) was detected, although in the treatment with 15 µmol L^{-1} Cd and 50 µmol L^{-1} Zn was present as a very faint band (Figure 3A). The 50 kDa glycoprotein (grey frame) was revealed in all samples, except in combined treatment with 15 µmol L^{-1} Cd and 50 µmol L^{-1} Zn. Control leaves and those treated with 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn alone and with combination of 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn, as well as 15 µmol L^{-1} Cd with 25 µmol L^{-1} Zn were characterised by the 35 kDa band (dashed frame). GNA revealed glycoproteins of 40 kDa (white circle) and 43 kDa (black circle) of the strongest staining intensity (Figure 3B), which were present in control and all treatments, except in combined treatment with 15 µmol L^{-1} Cd and 50 µmol L^{-1} Zn. Glycoproteins of 46 kDa (grey arrow) and 50 kDa (black arrow) were detected as very faint bands and were more pronounced in treatments with either metal alone than in combined treatments. The 20 kDa glycoprotein (star) was detected in control seedlings as well as in those treated with 10 µmol L^{-1} Cd, 50 µmol L^{-1} Zn, 15 µmol L^{-1} Cd and 25 µmol L^{-1} Zn, as well as 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn. Exposure to 50 µmol L^{-1} Zn resulted in the appearance of 15 kDa, 16 kDa and 17 kDa bands (asterisks), which were also detected in treatments with 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn as well as with 15 µmol L^{-1} Cd and 25 µmol L^{-1} Zn, but with weaker expression. In control and in treated seedlings, PNA revealed the 43 kDa glycoprotein (black arrow), whose expression was the strongest after treatment with 50 µmol L^{-1} Zn (Figure 3C). Combined treatment with 15 µmol L^{-1} Cd and 50 µmol L^{-1} Zn was devoid of the 38 kDa band (grey arrow), whose expression was the strongest in the treatment with 50 µmol L^{-1} Cd. Control seedlings and those exposed to 50 µmol L^{-1} Zn and combinations of 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn as well as 15 µmol L^{-1} Cd and 25 µmol L^{-1} Zn, were characterised by the presence of the 18 kDa glycoprotein (black circle). In the above mentioned treatments 17 kDa band was detected (star), while the exposure to 50 µmol L^{-1} Zn and to a combination of 15 µmol L^{-1} Cd and 25 µmol L^{-1} Zn resulted in the appearance of the 16 kDa glycoprotein (asterisk). DSA revealed only

two glycoproteins (Figure 3D). The 43 kDa one (black arrow) was missing from the treatments with 15 μmol L^{-1} Cd, 25 μmol L^{-1} Zn as well as from combined treatment with 15 μmol L^{-1} Cd and 50 μmol L^{-1} Zn. Exposure to 50 μmol L^{-1} Zn alone and to combinations of 10 μmol L^{-1} Cd and 50 μmol L^{-1} Zn as well as 15 μmol L^{-1} Cd and 25 μmol L^{-1} Zn resulted in the appearance of the 38 kDa glycoprotein (grey arrow). Nine glycosylated proteins were detected in seedlings with MAA (Figure 3E). The 43 kDa (black arrow) and 45 kDa (grey arrow) glycoproteins were present in control and all treatments as very strong bands, while the 66 kDa (black circle) and 68 kDa (white circle) ones were visible as proteins with weak staining intensity. Seedlings exposed to 10 μmol L^{-1} Cd and 50 μmol L^{-1} Zn as well as 15 μmol L^{-1} Cd and 25 μmol L^{-1} Zn were characterised by the presence of the 55 kDa glycoprotein (star), which was also detected as a very faint band in control seedlings and in those treated with single 10 μmol L^{-1} Cd and 50 μmol L^{-1} Zn. The 22 kDa band (asterisk) was present in treatments with 50 μmol L^{-1} Zn as well as with 15 μmol L^{-1} Cd and 25 μmol L^{-1} Zn, while the 20 kDa one (white star) was detected in control and in treatments with 10 μmol L^{-1} Cd, 50 μmol L^{-1} Zn, 10 μmol L^{-1} Cd and 50 μmol L^{-1} Zn as well as 15 μmol L^{-1} Cd and 25 μmol L^{-1} Zn. Combined treatment with 15 μmol L^{-1} Cd and 25 μmol L^{-1} Zn revealed the 16 kDa (white square) and 17 kDa (black square) glycoproteins. SNA revealed the 35 kDa glycoprotein (black arrow) in control and all treatments except for the combined treatment with 15 μmol L^{-1} Cd and 50 μmol L^{-1} Zn (Figure 3F). Glycosylated protein of 32 kDa (grey arrow) was missing from the

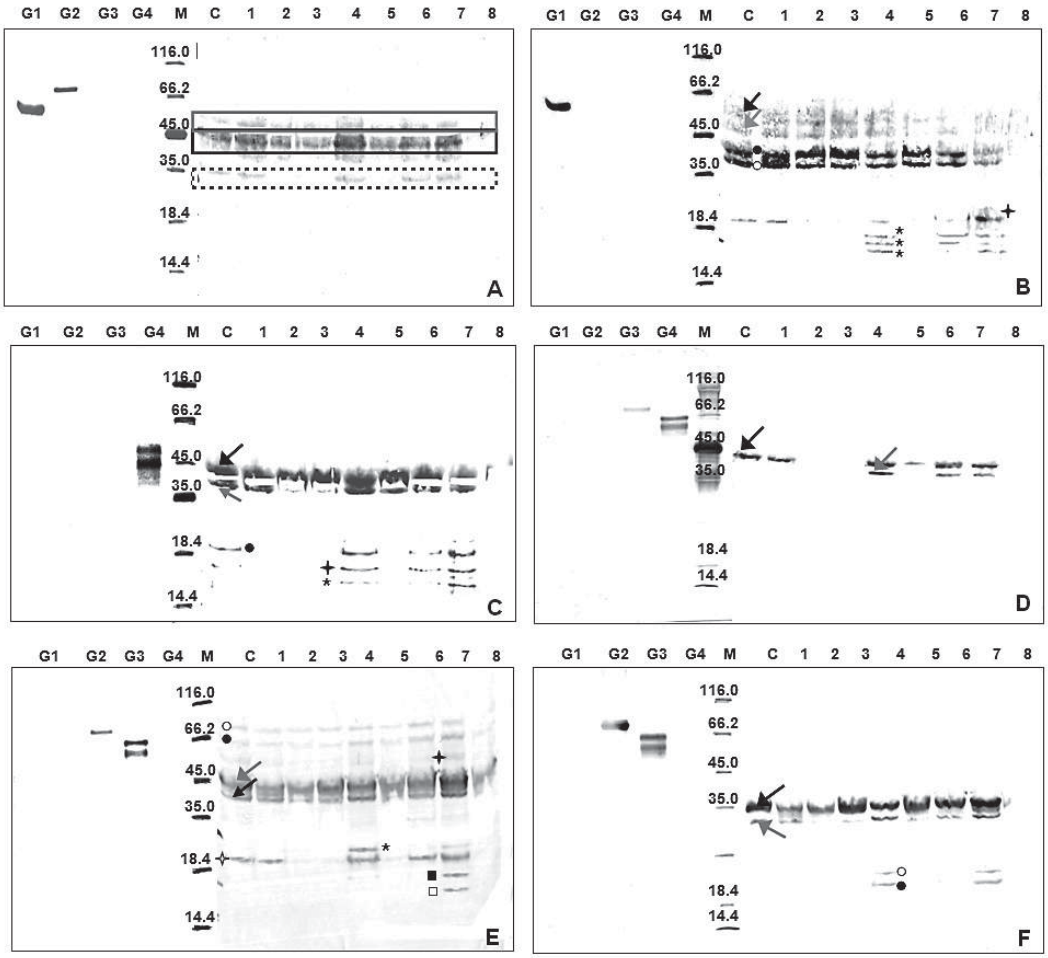

Figure 3 *Glycoprotein pattern of tobacco seedlings detected by lectins A) Con A, B) GNA, C) PNA, D) DSA, E) MAA, and F) SNA. G1 – control glycoprotein carboxypeptidase Y (positive control for Con A and GNA), G2 – control glycoprotein transferrin (positive control for MAA and SNA), G3 – fetuin (positive control for DSA, MAA and SNA), G4 – asialofetuin (positive control for DSA and PNA). For lane labels see Figure 1*

treatment with 15 µmol L^{-1} Cd as well as with 15 µmol L^{-1} Cd and 50 µmol L^{-1} Zn. Very faint bands of 22 kDa and 23 kDa (black and white circle) were present only after exposure to 50 µmol L^{-1} Zn and combination of 15 µmol L^{-1} Cd and 25 µmol L^{-1} Zn.

Three glycosylated proteins were detected by Con A (Figure 4A), according to which all leaves of tobacco adult plants were characterised by the presence of 60 kDa glycoprotein (grey frame), which was the most pronounced one. This glycoprotein band showed a stronger expression in all treatments in comparison to control. Control leaves and those treated with 15 µmol L^{-1} Cd were devoid of the 43 kDa (dashed frame) and 68 kDa glycoprotein (black frame) of weaker staining intensities. The pattern of glycosylated proteins detected with GNA (Figure 4B) was very similar to that obtained with Con A; 43 kDa (grey arrow) and 60 kDa glycoproteins (black arrow) were present in leaves of all treated plants, while they were missing in control plants. Moreover, GNA revealed two additional bands of very weak expression; 15 kDa glycoprotein (black circle) was present in the treatment with 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn, while the 20 kDa (white circle) one was detected after exposure to 25 µmol L^{-1} Zn and combined treatment with 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn. PNA revealed the 40 kDa protein (black arrow) of very strong expression in all treated samples except in control leaves (Figure 4C), while very faint band of 68 kDa (grey arrow) was detected in leaves of plants exposed to single 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn. Exposure to 10 µmol L^{-1} Cd as well as to both concentrations of Zn alone resulted in very low expression of 35 kDa glycoprotein (black circle). The 27 kDa band (white circle) was missing from the control and treatments with 15 µmol L^{-1} Cd alone and

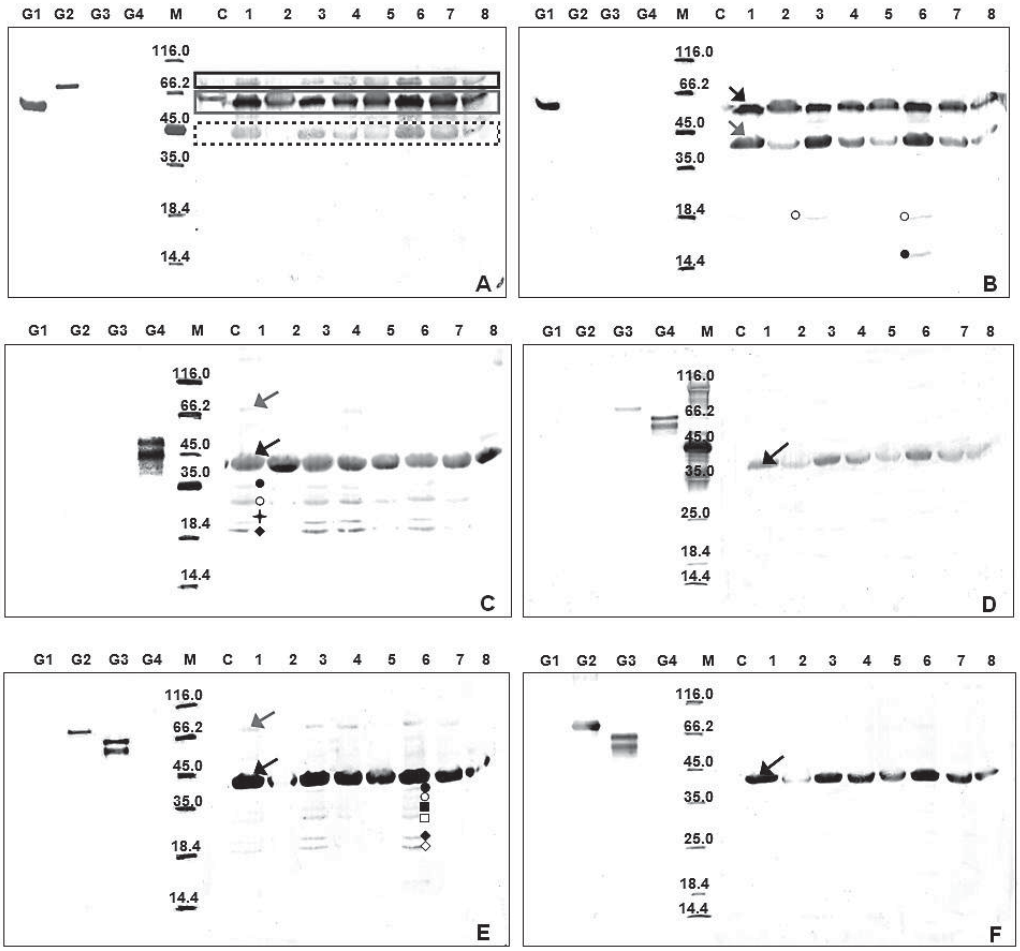

Figure 4 *Glycoprotein pattern of leaves of adult tobacco plants detected by lectins A) Con A, B) GNA, C) PNA, D) DSA, E) MAA, and F) SNA. G1 – control glycoprotein carboxypeptidase Y (positive control for Con A and GNA), G2 – control glycoprotein transferrin (positive control for MAA and SNA), G3 – fetuin (positive control for DSA, MAA and SNA), G4 – asialofetuin (positive control for DSA and PNA). For lane labels see Figure 1*

combination of 15µmol L^{-1} Cd and 50 µmol L^{-1} Zn, while the 20 kDa (diamond) and 22 kDa (star) ones were observed in treatments with single 10 µmol L^{-1} Cd, 25 µmol L^{-1}, and 50 µmol L^{-1} Zn as well as in combined treatments of 10 µmol L^{-1} Cd with 25 µmol L^{-1}, and 50 µmol L^{-1} Zn. Leaves from adult plants exposed to all investigated treatments revealed with DSA only one glycoprotein of 40 kDa (black arrow), whose expression was the strongest in the treatments with single 10 µmol L^{-1} Cd and 25 µmol L^{-1} Zn as well as in combined treatment with 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn (Figure 4D). Control leaves were devoid of any glycoproteins after the MAA was applied (Figure 4E). In total, eight bands were detected with this lectin in leaves of treated plants, among which the 43 kDa one (black arrow) was present in all the treatments with the strongest staining intensity. The 70 kDa protein (grey arrow) of the weaker expression was detected in all treatments except in the 15 µmol L^{-1} Cd treatment and in combined treatments with 10 µmol L^{-1} Cd and 25 µmol L^{-1} Zn as well as 15 µmol L^{-1} Cd and 50 µmol L^{-1} Zn. Very faint glycoproteins of 20 kDa (white diamond), 22 kDa (black diamond), 32 kDa (white square), and 36 kDa (black square) were detected after exposure to single 10 µmol L^{-1} Cd and 25 µmol L^{-1} Zn as well as to combined treatment with 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn, while the 40 kDa one (black circle) was present only in treatment with 25 µmol L^{-1} Zn alone as well as with combination of 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn. Combined treatment of 10 µmol L^{-1} Cd and 50 µmol L^{-1} Zn was characterised by the presence of 38 kDa glycosylated protein (white circle). After incubation with SNA,

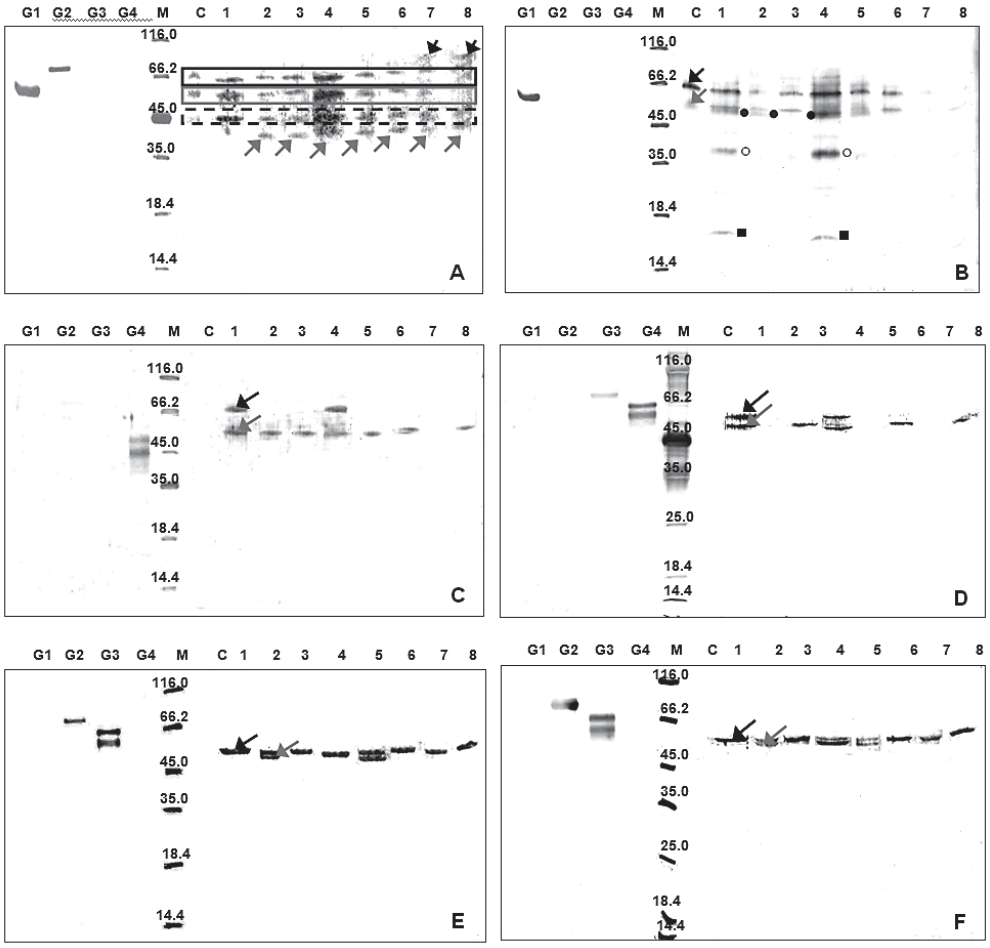

Figure 5 *Glycoprotein pattern of roots of adult tobacco plants detected by lectins A) Con A, B) GNA, C) PNA, D) DSA, E) MAA, and F) SNA. G1 – control glycoprotein carboxypeptidase Y (positive control for Con A and GNA), G2 – control glycoprotein transferrin (positive control for MAA and SNA), G3 – fetuin (positive control for DSA, MAA and SNA), G4 – asialofetuin (positive control for DSA and PNA). For lane labels see Figure 1*

leaves from the treated plants revealed only one glycoprotein of 43 kDa (black arrow), the expression of which was much weaker after exposure to 15 μmol L^{-1} Cd in comparison with other treatments (Figure 4F).

Roots of adult plants revealed in total five glycoprotein bands with Con A (Figure 5A). The 45 kDa (dashed frame), 60 kDa (grey frame), and 66 kDa (black frame) glycoproteins were detected in control and all the treatments, although their expression was the strongest after exposure to 50 μmol L^{-1} Zn. Combined treatments with 15 μmol L^{-1} Cd and 25 μmol L^{-1} Zn as well as 15 μmol L^{-1} Cd and 50 μmol L^{-1} Zn were characterised by the presence of 70 kDa band (black arrow). The 43 kDa glycoprotein (grey arrows) was detected in roots of adult plants exposed to single 15 μmol L^{-1} Cd and to Zn at both concentrations as well as to all combined treatments, with the strongest staining intensity after exposure of single 50 μmol L^{-1} Zn. Five glycoprotein bands were detected in roots of adult plants with GNA (Figure 5B). The 50 kDa (grey arrow) and 60 kDa (black arrow) bands were present in control and all treatments except in the combined exposure to 15 μmol L^{-1} Cd with 25 μmol L^{-1} or 50 μmol L^{-1} Zn. The treatments with single Cd at both concentrations as well as with 50 μmol L^{-1} Zn were characterised by the presence of the 48 kDa band (black circles). The 16 kDa (black squares) and 40 kDa (white circles) glycoproteins appeared in roots after exposure to single 10 μmol L^{-1} Cd and 50 μmol L^{-1} Zn. Only roots of adult plants exposed to metal treatments revealed bands with PNA; the 66 kDa band (black arrow) was detected in treatments with single 10 μmol L^{-1} Cd and 50 μmol L^{-1} Zn, while the 50 kDa one (grey arrow) was present in all treatments, except for the combination of 15 μmol L^{-1} Cd and 25 μmol L^{-1} Zn (Figure 5C). DSA also reacted with root proteins of adult plants (Figure 5D). The 60 kDa glycoprotein (black arrow) was detected after exposure to single 10 μmol L^{-1} Cd and 50 μmol L^{-1} Zn, with stronger staining intensity in Cd treatment. The 10 μmol L^{-1} Cd treatment gave strong expression of 50 kDa band (grey arrow), while the same glycoprotein was also detected in treatments with single Zn at both concentrations and in combined treatments with 10 μmol L^{-1} Cd and 50 μmol L^{-1} Zn as well as 15 μmol L^{-1} Cd and 50 μmol L^{-1} Zn. MAA (Figure 5E) and SNA (Figure 5F) lectins revealed two glycoprotein bands in roots of treated plants. All treatments were characterized by the presence of the 55 kDa band (black arrow) with both lectins. The 53 kDa band (grey arrow) appeared in treatments with 15 μmol L^{-1} Cd and combinations of 10 μmol L^{-1} Cd and 25 μmol L^{-1} Zn with MAA, while with SNA the same band also appeared in the treatment with single 50 μmol L^{-1} Zn.

DISCUSSION

Proteins, as direct gene products, reflect characteristic gene expression. Numerous researches revealing the full genome sequence of model plants, such as Arabidopsis and rice, have been published recently (48-50). However, gene sequence does not give complete information about gene function, developmental and regulatory biology or biochemical kinetics (51). Proteins are macromolecules directly responsible for most biological processes in a living cell, while protein function is directly influenced by posttranslational modifications, which cannot be followed by genome studies. Therefore, it is necessary to conduct proteomic studies which elucidate protein presence and role under certain environmental conditions (52). It is well known that heavy metal stress can activate a range of potential cellular mechanisms in plants, some of which being the mobilization of specific molecules such as phytochelatins and stress proteins that play a very significant role in Cd detoxification and tolerance in plants (53, 54). In a study performed on *Datura innoxia*, a Cd-induced expression of numerous proteins in a molecular mass range from 10 kDa to 70 kDa was reported (55), while Cd-binding proteins have been isolated from Cd-exposed pea plants (56, 57). In our study, changes in protein expression were observed in tobacco seedlings as well as in leaves and roots of adult plants exposed to both cadmium and zinc. An enhanced expression of 116 kDa protein was noticed in seedlings grown on media supplemented with both Cd and/or Zn, except on the medium containing lower Cd and Zn concentration. Moreover, in leaves of adult plants, a 120 kDa protein appeared after treatment with 10 μmol L^{-1} Cd, both concentrations of Zn, as well as in combined treatments of 10 μmol L^{-1} and 15 μmol L^{-1} Cd with 50 μmol L^{-1} Zn. In general, no specific effect of cadmium on protein profiles could be observed based on the SDS-PAGE results obtained in this study. Namely, both metals induced similar changes in protein patterns in tobacco seedlings and plants. In a study of Cd and Zn effect on leaves of *Arabis paniculata*, most identified

proteins suggested that Zn and Cd share similar pathway to certain extent (58). However, our investigation was restricted to cellular proteins which can be easily extracted due to their high solubility in aqueous buffers at neutral pH. Several publications report that heavy metals affect the rigidity of the cell wall and the cell membrane as well as the adsorption of certain secreted proteins to the cell wall (59-61). Therefore, in search of a more reliable molecular marker of heavy metal stress, some additional analyses should be performed. First of all, in order to identify the complete set of cadmium- and/or zinc-induced proteins the SDS-PAGE analyses of expression patterns should be extended to membrane and extracellular proteins. Moreover, 2D-PAGE electrophoresis should be applied, since this technique has a higher resolving power for separating complex mixtures of heterogenous polypeptides than SDS-PAGE. Finally, employing 2D-PAGE in combination with mass spectrometry analyses will allow some of the heavy metal stress related proteins to be identified and their localization, modifications, interactions, activities, and ultimately their function to be determined.

Jomová and Morovič (13) found enhanced protein signals in the pattern of cellular stress protein expression in root tips of *Lupinus luteus* after exposure to Cd and suggested that increased proteosynthetic activity indicated the possible participation of these proteins in the cell defence reactions. It has been documented that heavy metals, as other abiotic stressors, can induce formation of reactive oxygen species (ROS) (62). The excess ROS produced under abiotic stress leads to oxidative damages of lipids, proteins, and nucleic acids (63). When exposed to such unfavourable environmental conditions, plants activate an arsenal of defence mechanisms, both passive and active. The active defence responses require *de novo* synthesis of proteins, some of these being antioxidative enzymes such as the superoxide dismutase, ascorbate peroxidase, gluthatione reductase, and class III plant peroxidases (64). Since elevated activities of these particular enzymes as well as an increased number of their isoforms were found in tobacco tissues exposed to Cd and/or Zn (Šikić et al. unpublished results), it is possible that at least some of the detected proteins belong to this group of defenders of plant cells. A study conducted with Cd on whole rice seeds, including the endosperm during germination, showed that similar proteins increased in response to Cd as with Cu, so it appears that these proteins are involved in a general metal or stress response (18).

Except at the proteome level, abiotic stress can induce changes in posttranslational modifications, among which glycosylation is the most frequent one. Many proteins destined for secretion or expression at the surface of plant cells are glycoproteins. N- as well as O-glycosylation are essential protein modifications required for many different aspects of their structure and function, including their targeting to the appropriate destinations, their stability, solubility and antigenicity, as well as their capacity to be recognized by receptors. In several studies it was reported that protein glycosylation could have an important role in plant response to different abiotic stresses (28, 29, 33). However, the influence of heavy metals on protein glycosylation has been poorly studied so far and only one extensive investigation could be found (31). In our study, characterization of glycosylated proteins was performed by lectin-based glycan profiling, the essence of which is to extract core information (like N-glycosylated or O-glycosylated, high-mannose type or complex type, core-fucosylated or not, fully or partially sialylated and other information) about glycan structures by means of lectin affinity technology (65). The use of lectins in glycan profiling provides considerable advantages, such as discrimination between the isomers on the basis of biological rather than physicochemical principles (66). Lectin-based glycan profiling has already been successfully applied in several plant glycoprotein studies (23, 26, 27). In the present research, differences between control and treated tobacco tissues were more pronounced in glycoprotein compared to soluble protein pattern, which suggests that the oligosaccharide structures could be important determinants for heavy metal stress.

Analysis of N-glycosylated proteins from tobacco seedlings, leaves, and roots revealed common glycoproteins, which implies an enhanced protein glycosylation in all tobacco tissues after exposure to heavy metal stress. Namely, numerous papers indicate that N-glycans can protect protein from proteolytic degradation and are responsible for thermal stability, solubility, and biological activity of the protein (32, 67). Komatsu et al. (30) reported that stress induced by a low temperature changed reactivity of 12 out of 22 glycoproteins detected with Con A in rice. Moreover, in tobacco roots, additional glycoproteins of 43 kDa and 70 kDa were detected with Con A, which is in agreement with the fact that plant roots

are the main site of heavy metals uptake and thus serve as the first barrier in defence mechanism. Interestingly, when GNA lectin was applied, all treated tobacco tissues revealed the bands, which according to their molecular weight (40 kDa in seedlings as well as 43 kDa in leaves and roots) corresponded to signals detected with horseradish peroxidase antibody (Šikić et al. unpublished results). Since most of the plant peroxidases are glycoproteins, this result suggests enhanced expression and glycosylation of this enzyme which has a very important role in plant defence against ROS (64). However, to be completely certain about this, more elaborated techniques for characterising protein and glycan part of glycoproteins further by their isoelectric point and molecular weight as well as by mass spectrometry should be applied. This would give significant information about the expressed glycoslyated proteins that are apparently subdued to changes in glycosylation during the exposure to heavy metal treatments. Lectin GNA revealed N-glycosylated proteins of high-mannose type of lower molecular weight (up to 28 kDa) in seedlings, leaves and roots of tobacco exposed to heavy metals, which proves enhanced protein glycosylation that occurs in ER. Zhang et al. (32) reported that N-glycosylation has a key role in response to abiotic stress and its absence leads to accumulation of damaged proteins in ER.

Lectin DSA, which mostly binds to Gal-(1,4)-GlcNAc, detected glycoproteins with complex and/or hybrid N-glycans in tobacco seedlings and adult plants. In treated leaves, only the glycoprotein of 40 kDa was observed, while glycoslyated proteins of 50 kDa and 60 kDa were detected in root tissue after exposure to certain treatments. This result confirms enhanced modification of N-glycan component, which occurs in GA, where complex and hybrid N-glycans are formed (68, 69). Correct N-glycosylation is important for normal growth and morphology of plant cells (70). Moreover, recent investigations, conducted on the model plant *Arabidopsis thaliana*, suggest that plant cell capability to form complex N-glycan is very important in response to abiotic stress (28, 32). Kang et al. (29) reported that *A. thaliana* resistance to salt stress requires maturation of N-glycosylated proteins in GA. Moreover, von Schaewen et al. (71) reported that *A. thaliana* mutants defective in complex N-glycans show enhanced salt sensitivity, establishing that complex N-glycans protect plants from salt and osmotic stress.

Information about plant O-glycans are scarce. In plants, O-glycosylation has been described mainly for the hydroxyl groups of Hyp, Ser, and Thr residues. Plant and mammalian O-glycans are usually considered structurally different. The main O-glycosylated proteins in plants, extensins and arabinogalactan proteins (AGPs), belong to a large group of glycoproteins known as Hyp-rich glycoproteins (HRGPs). HRGPs are involved in many aspects of plant growth and development (72), and many effects of O-linked glycosylation on the biological activity of these proteins have been described (73). In our study, PNA, which specifically recognizes the *β*-Gal-(1,3)-GalNAc sequence present in O-glycans, revealed several glycoproteins that also reacted with GNA. This result suggests that these proteins might possess both N- and O-glycosylation sites, although PNA and GNA may alternatively recognize different glycoproteins of similar molecular size, which are not resolved in SDS-PAGE (26). In seedlings treated with 50 μmol L^{-1} Zn and combined treatments 10 μmol L^{-1} Cd and 50 μmol L^{-1} Zn as well as 15 μmol L^{-1} Cd and 25 μmol L^{-1} Zn PNA detected glycoproteins of 16 kDa and 17 kDa, which were not present in control tissues. In tobacco leaves and roots no band was detected in control tissues with PNA, while the majority of treatments responded with at least one band. Interestingly, the greatest number of PNA-reacting proteins was found after exposure to 10 μmol L^{-1} Cd as well as to 50 μmol L^{-1} Zn in tobacco leaves. These findings indicate that heavy metal stress can also induce O-glycosylation, even though it is difficult to say which type of plant O-glycoproteins these bands belong to. To get this answer, some more powerful techniques such as HPAEC-PAD and mass spectrometry should be applied.

MAA and SNA detected sialic acids in glycoproteins of tobacco tissues. Sialylated glycoconjugates have already been found in suspension-cultured cells of *Arabidopsis thaliana* (47), in cellular and extracellular proteins of sugar beet tissue lines (23) as well as in soluble cellular glycoproteins of *Mammillaria gracilis* (24), which suggests that a genetic and enzymatic basis for sialylation exits in plants. In our study, the 43 kDa glycoprotein from all tobacco seedlings reacted with MAA, but also with GNA and PNA. These findings indicate that the 43 kDa is a multiglycosylated protein which has sialic acid (NeuAc) α(2,3)-linked to galactose (Gal). This suggests that this could be a stress-related protein. Balen et al. (27) reported enhanced sialylation of proteins in plant tissues

obtained in *in vitro* culture, which indicates that sialylation could be induced by stressful environmental conditions. Arillo et al. (74) pointed out that the sialic acid content can be used as an index for environmental stress. Moreover, it was found that increased ROS generation in human (75, 76) and animal cells (77) can be correlated with the overproduction of sialoglycoproteins.

Considering all the glycoprotein results, the greater number of glycosylated proteins was detected in tobacco seedlings compared to both leaves and roots of adult plants. In this study, control seedlings reacted with all the applied lectins, while the majority of detected bands were common for all samples. This result is in accordance with the findings that glycosylation has an important role during plant growth and development (19, 20, 22-25). On the other hand, in leaves and roots of adult plants, only Con A among the applied lectins reacted with control samples. Therefore, the majority of detected glycoproteins in these tobacco tissues can be considered as a result of enhanced glycosylation due to heavy metal stress. Furthermore, the difference in glycosylation pattern between Cd and Zn application on tobacco seedlings and adult plants could not be determined since enhanced glycosylation was noticed after treatment with either metal alone or in combination. Therefore, it can be concluded that both metals induced N- and O-glycosylation as a result of changed environmental conditions, although more sophisticated analytical techniques should be employed to acquire more information.

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Sažetak

KADMIJ I CINK INDUCIRAJU SLIČNE PROMJENE U UZORKU PROTEINA I GLIKOPROTEINA U KLIJANCIMA I ODRASLIM BILJKAMA DUHANA (*NICOTIANA TABACUM* L.)

Ispitivali smo učinke 10 μmol L^{-1} i 15 μmol L^{-1} kadmija (Cd), neesencijalnoga toksičnog elementa i 25 μmol L^{-1} i 50 μmol L^{-1} cinka (Zn), esencijalnog mikronutrijenta, na proteine i glikoproteine u klijancima i odraslim biljkama *Nicotiana tabacum* L. nakon izlaganja svakomu metalu posebno ili njihovim kombinacijama. Promjene kod nekoliko polipeptida koje su uočene u klijancima i listovima odraslih biljaka nisu bile brojne, dok su one najvažnije zabilježene u uzorku ukupnih topljivih proteina u korijenu biljke. Razlike između kontrole i tretiranog tkiva duhana bile su izraženije kod glikoproteina koji su analizirani primjenom različitih lektina. Većina glikoproteina uočenih u listovima i korijenu odraslih biljaka može se smatrati rezultatom povećane glikozilacije zbog stresa koji uzrokuju teški metali. Nije bilo moguće utvrditi razliku u glikoproteinima između tretiranja klijanaca i odraslih biljaka duhana kadmijem i cinkom jer je povećana glikozilacija utvrđena i nakon tretmana svakim metalom posebno i nakon tretmana njihovim kombinacijama. Stoga se može zaključiti da su, kao rezultat promijenjenih uvjeta u okolišu, oba metala potaknula N- i O-glikozilaciju.

KLJUČNE RIJEČI: *glikozilacija, lektini, teški metali, uzorak proteina*

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Scientific Paper

INERTISATION OF GALVANIC SLUDGE WITH CALCIUM OXIDE, ACTIVATED CARBON, AND PHOSPHORIC ACID

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In this study we compared three methods for the treatment of electroplating sludge highly loaded with zinc and iron: (1) calcium oxide-based solidification/stabilisation; (2) conversion into inert material by adsorption of organic and inorganic pollutants onto activated carbon; and (3) conversion of mobile waste components into insoluble phosphates. All three methods proved highly efficient in the conversion of hazardous waste into inert material. Under optimum treatment conditions zinc concentration in the leachate of solidified waste was reduced by 99.7 % compared to untreated sludge. Zinc retention efficiency in the waste treated with activated carbon and phosphoric acid was 99.9 % and 98.7 %, respectively. The advantages of electroplating sludge treatment with activated carbon over the other two methods are high sorption capacity, insignificant pH and volume changes of the sludge, and simple use.

KEY WORDS: *heavy metals, immobilization, leaching, solidification*

Because of the high content of toxic heavy metals electroplating sludge is considered hazardous waste (1). On the other hand, this toxic waste could be reused as raw material for the extraction of zinc, chromium, and other valuable components to save natural resources. Various alkaline or acid based hydrometallurgical and electrochemical processes have been used for this purpose (1-6), but their major disadvantage is that they yield large amounts of sludge that needs to be neutralised (rendered inert) before landfilling. If metal extraction is not economically justified, such material has to be converted to non-hazardous waste by solidification/stabilisation and can be reused in cement industry (7-11). Although there are more than a hundred electroplating facilities in Croatia, most of them zinc plating, there either recovery or inertisation of electroplating sludge have been regulated (12). As this sludge contains high concentrations of zinc in the leachate, and the pH value is usually below 3, it poses a considerable threat to the environment and people, which has been confirmed by toxicological tests on various bio-systems for the concentration of zinc that are even lower than 2 mg L^{-1} (13-19).

Oreščanin et al. (20) developed the first method to treat electroplating sludge in Croatia that is based on solidification/stabilisation of sludge with CaO in order to convert hazardous waste into inert material. Our preliminary results have confirmed that the method is highly efficient for the specific purpose. In this study we wanted to analyse two new methods for sludge inertisation based on the application of powdered activated carbon and phosphoric acid and to compare them with the CaO method in terms of efficiency in

binding heavy metals, sludge production, simplicity of implementation, and cost.

MATERIALS AND METHODS

Sampling and sample handling

For inertisation experiments we took samples of electroplating sludge from Tvik electroplating facility (Knin, Croatia). Sampling and sample handling has already been described in detail in our previous article (20).

Solidification/stabilization procedure

The electroplating sludge was solidified/stabilised with calcium oxide (Lika lime factory, Ličko Lešće, Croatia) as described in our previous work (20) and after air-drying subjected to the leaching test according to DIN38414-S4 procedure.

Sludge inertisation using activated carbon

A composite sample of sludge was mixed with 1 %, 2 %, 3 %, 4 %, 5 %, and 6 % of powdered activated carbon (*p.a.* Kemika, Zagreb) in relation to the dry weight of the sludge. The mixture (5.9 g) was homogenised for 10 min, air dried at room temperature (22 °C), and subjected to the DIN38414-S4 leaching test.

Sludge inertisation using phosphoric acid

A composite sample of sludge was mixed with 1 %, 2 %, 3 %, 4 %, and 5 % of phosphoric acid (Kemika, Zagreb) in relation to the dry weight of the sludge. The mixture (5.9 g) was homogenised for 10 min, air dried at room temperature (22 °C), and subjected to the DIN38414-S4 leaching test.

Analysis of parameters in solid and liquid samples

Dry matter and organic matter content were measured overnight by loss on ignition at 105 °C and 375 °C, respectively. Elemental concentrations in solid samples were determined using the energy dispersive X-ray fluorescence method (EDXRF) (21). DIN 38414-S4 leachates of original and inertised samples were prepared as described in our earlier studies (20, 22). Instrumental settings as well as quality control have been described in detail in our earlier research (21, 22).

RESULTS AND DISCUSSION

Basic physico-chemical characteristic of the electroplating sludge

Table 1 shows that the prevailing elements of toxicological significance in untreated electroplating sludge were iron, zinc, chromium, nickel, and lead. Sludge also contained a significant amount of organic matter (9.4 % to 29.4 %). High chelating potential of these organic constituents was the most probable reason for low leaching of chromium, lead, and copper from the sludge. Lead concentration in the DIN38414-S4 leachate was below the detection limit (0.001 mg L^{-1}) in all tested samples while mean chromium and copper values were 0.096 mg L^{-1} and 0.053 mg L^{-1} of leachate, respectively (Table 2). These findings have

Table 1 *Element content in bulk samples of untreated electroplating sludge*

Measured parameter	Statistical parameter					
	Mean	**SD**	**RSD**	**Median**	**Minimum**	**Maximum**
Ca / mg kg^{-1}	73000	39900	0.55	65000	13000	152000
Ti / mg kg^{-1}	451.7	53.61	0.12	463	365	557
Cr / mg kg^{-1}	207.2	197.42	0.95	131	21	759
Mn / mg kg^{-1}	439.4	110.23	0.25	440.5	207	598
Fe / mg kg^{-1}	136000	47900	0.35	151600	38400	191400
Ni / mg kg^{-1}	278.3	67.99	0.24	273	185	418
Cu / mg kg^{-1}	133.3	72	0.54	122.5	16	317
Zn / mg kg^{-1}	33400	12600	0.38	31800	10900	58200
Pb / mg kg^{-1}	109.9	62.25	0.57	114.5	11	235
Dry Matter / mg kg^{-1}	253000	83200	0.33	243000	125000	455000
LOI 375 ˚C / mg kg^{-1}	197000	52900	0.27	183000	94000	294000

SD-standard deviation; RSD-relative standard deviation

confirmed our previous research (22-24) showing high susceptibility of these three elements to organic lygands. The DIN38414-S4 leachate composition shows that this sludge was not suitable for landfilling with inert waste since mean zinc and nickel were 10 and 1.5 times higher than the upper permissible limit, and the maximum zinc and nickel values were 27 and 2.5 times higher than the upper permissible limit, respectively (25).

Zinc concentration in the leachate of untreated sludge ranged from 0.3 mg L^{-1} to up to 107 mg L^{-1} with the mean value of 40.3 mg L^{-1}. In our earlier study (13), we found that zinc concentrations higher than 25 mg L^{-1} were cytotoxic to TA98 and TA100 *Salmonella typhimurium* strains while 100 mg L^{-1} of zinc significantly increased the frequency of micronucleated cells and reduced mitotic activity of human peripheral blood lymphocytes. In another study (14) the survival rates for HeLa and HEp2 human cells were only 2.3 % and 0.3 %, respectively, after treatment with electroplating wastewater having zinc concentration of 562 mg L^{-1}, while 99.5 % diluted sample significantly increased all comet assay parameters compared to negative control.

Durgo et al. reported (15) less than 15 % survival of the TA98 and TA100 *Salmonella typhimurium* strains after the treatment with electroplating wastewater having zinc concentration of 505 mg L^{-1} (15) while Horvat et al. (17) found that 51 mg L^{-1} and 126 mg L^{-1} of zinc have a significant toxic effect on *Lemna minor*.

In another study (19), zinc concentration of 86 mg L^{-1} significantly increased all comet assay parameters after exposure of peripheral blood lymphocytes to the leachate of electric arc furnace dust. All these findings suggest that untreated electroplating sludge could endanger human health and plant bio-systems if released directly into the environment (leaching by precipitation).

Sludge inertisation with CaO

Figure 1a shows to which extent calcium oxide treatment lowered heavy metal concentrations in the

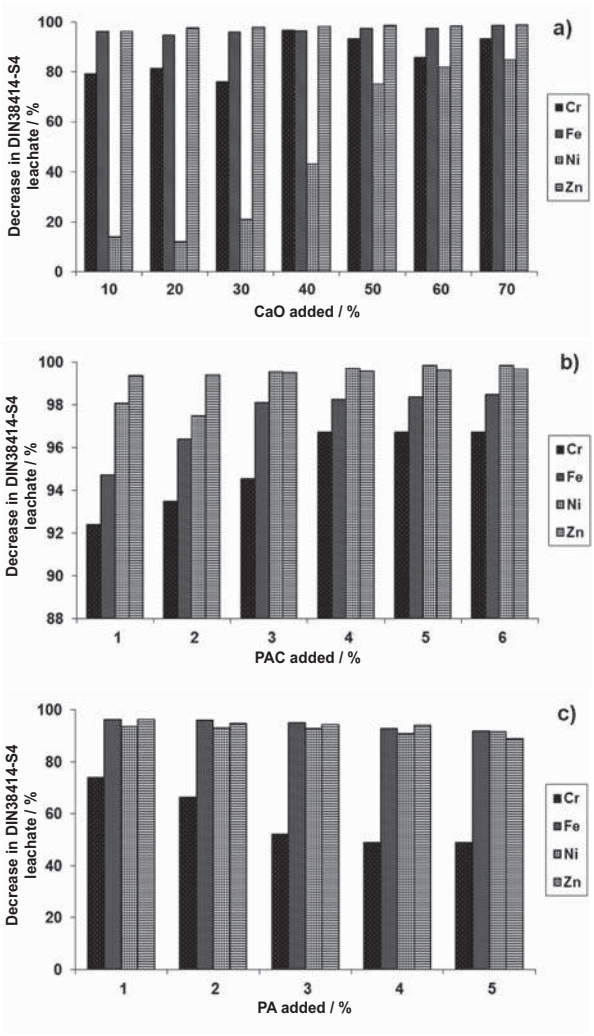

Figure 1 *Decrease in heavy metal concentrations in the DIN38414-S4 leachate of treated waste compared to untreated electroplating sludge for different dosages of: a) calcium oxide; b) powdered activated carbon (PAC); c) phosphoric acid (PA)*

Table 2 *Heavy metal concentrations in the leachate DIN38414-S4 of untreated electroplating sludge and maximum allowed concentrations*

Statistical parameter	Measured parameter					
	Cr / mg L^{-1}	Fe / mg L^{-1}	Ni / mg L^{-1}	Cu / mg L^{-1}	Zn /mg L^{-1}	pH
Mean	0.096	15.725	0.634	0.053	40.320	4.34
SD	0.047	9.970	0.306	0.194	27.997	0.80
RSD	0.486	0.634	0.483	3.691	0.694	0.19
Median	0.090	14.307	0.634	0.003	34.746	4.06
Minimum	0.006	0.345	0.082	0.001	0.349	3.42
Maximum	0.183	34.085	1.052	0.829	107.475	5.90
MAV (25)	0.5	-	0.4	2	4	-

SD-standard deviation; RSD-relative standard deviation

DIN38414-S4 leachate of electroplating sludge compared to untreated sludge. Treatment with the lowest dosage of CaO (10 %) already lowered all elemental concentrations below the lowest limit for the landfilling (25) and these concentrations dropped linearly with increasing CaO concentration. The exception is nickel, which dropped below the acceptable limit only at 40 % CaO.

Although, lime-based solidification is a method of choice for solidification of municipal solid waste (26-28), the use of this method for solidification of waste oil, oily wastes, and other waste by-products with high organic content has scarcely been investigated and only a few studies investigated its use in the solidification of inorganic sludges. Silva et al. (3) showed high efficiency of a two-phase sludge solidification method that included clay and lime in the first step and Portland cement, sand, and water in the second. Other authors (29) found that hydrated lime and black rice husk ash in the presence of either Na_2SiO_3 or Na_2CO_3 activators significantly reduced heavy metals leaching, especially of zinc, which was immobilised in the form of calcium zincate and calcium zinc silicate.

Extremely low leaching of arsenic was observed after solidification of inorganic sludge with cement, fly ash, and $Ca(OH)_2$ in the weight ratio 3:1:0.5:0.5 (30) confirming excellent binding capacity of this mixture.

Hydrated lime was also used for the immobilisation of heavy metals in highly contaminated soil having a similar chemical composition as electroplating sludge. At $Ca(OH)_2$ and soil ratio of 0.0375 the concentration of heavy metals Cd, Co, Ni, Pb and Zn in the leachate were decreased below minimum detection limit (31).

The advantage of CaO-based solidification is that solidification and chemical stabilisation of waste sludge occur simultaneously due to exothermic hydration of calcium oxide. The porosity, hydrophobicity, fire resistance, and good thermal and acoustic properties of the obtained solid matter make it suitable for use in sectors such as civil engineering. Moreover, CaO is readily available and less expensive than the other two additives. Solidification is less expensive in terms of equipment and trained operators and can be used easily in large companies and small family businesses alike.

The major disadvantage is that for CaO to be effective with heavy metals retention it has to be added in large quantities (at least 40 %), which generates more waste than the other two methods.

Sludge inertisation with powdered activated carbon

The regulatory requirements for landfilling of inert waste (25) were met for all elements with only 1 % of PAC (Table 4). Zinc concentration in the leachate dropped 159 times compared to untreated sludge. Cr, Fe, Ni and Zn concentration dropped 92.4 %, 94.7 %, 98.1 %, and 99.4 %, respectively (Figure 1b). Heavy metal concentrations dropped linearly with further addition of PAC.

Although, activated carbon, either in granular or powdered form, is commonly used for the adsorption of organic (32-34) and inorganic matter (35-37) in wastewaters, we have not found a single article on immobilization of heavy metals with this adsorbent in industrial sludges.

PAC turned out to be more effective in reducing heavy metal leaching than the other two methods (Figure 2) due to high adsorption capacity. PAC only

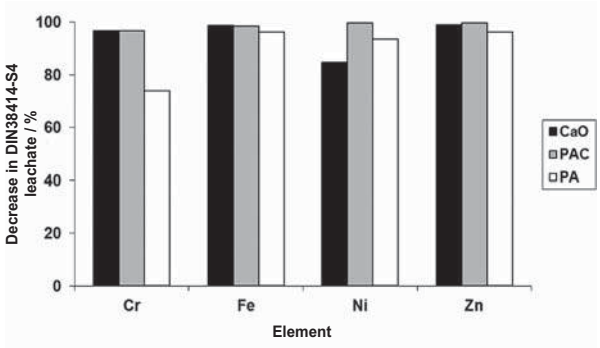

Figure 2 *Comparison of the best performance results for three treatment method of electroplating sludge. PAC-powdered activated carbon; PA-phosphoric acid*

slightly changes pH and the volume of the treated sludge in relation to untreated sludge. Treatment process is simple, does not require expensive equipment and trained personnel, and is easily applied by large companies and small family businesses alike. The adsorbent does not require special precautions for handling and storage. The major disadvantages over CaO are that PAC is less available and more expensive and that treated sludge needs additional drying.

Sludge inertisation with phosphoric acid

The addition of 1 % of phosphoric acid showed the best results in the retention of all elements in waste sludge. Cr, Fe, Ni, and Zn concentration dropped in the leachate by 73.9 %, 96.2 %, 93.7 %, and 96.3 %, respectively (Figure 1c) and all elements met regulatory requirements for landfilling of inert waste

Table 3 *Elemental concentration in DIN38414-S4 leachate of galvanic sludge before/after treatment with different dosages of CaO and upper permissible limit for inert waste (MAV)*

Element	Concentration before treatment / mg L^{-1}	MAV (25)	Concentration after CaO added / mg L^{-1}						
			10 %	20 %	30 %	40 %	50 %	60 %	70 %
Cr	0.092	0.5	0.019	0.017	0.022	0.003	0.006	0.013	0.006
Fe	13.113		0.477	0.673	0.504	0.439	0.333	0.317	0.175
Ni	0.681	0.4	0.586	0.599	0.537	0.389	0.169	0.122	0.103
Zn	37.900	4	1.380	0.787	0.781	0.679	0.488	0.532	0.348

Table 4 *Elemental concentration in DIN38414-S4 leachate of galvanic sludge before/after treatment with different dosages of powdered activated carbon (PAC) and upper permissible limit for inert waste (MAV)*

Element	Concentration before treatment / mg L^{-1}	MAV (25)	Concentrationa after PAC added / mg L^{-1}					
			1 %	2 %	3 %	4 %	5 %	6 %
Cr	0.092	0.5	0.007	0.006	0.005	0.003	0.003	0.003
Fe	13.113		0.691	0.473	0.247	0.229	0.213	0.198
Ni	0.681	0.4	0.013	0.017	0.003	0.002	0.001	0.001
Zn	37.900	4	0.239	0.222	0.174	0.153	0.139	0.127

Table 5 *Elemental concentration in DIN38414-S4 leachate of galvanic sludge before/after treatment with different dosages of phosphoric acid (PA) and upper permissible limit for inert waste (MAV)*

Element	Concentration before treatment / mg L-1	MAV (25)	Concentration after PA added / mg L^{-1}				
			1 %	2 %	3 %	4 %	5 %
Cr	0.092	0.5	0.024	0.031	0.044	0.047	0.047
Fe	13.113		0.495	0.528	0.653	0.941	1.059
Ni	0.681	0.4	0.043	0.047	0.049	0.061	0.057
Zn	37.900	4	1.393	1.964	2.101	2.201	4.201

(Table 5). Further addition of phosphoric acid caused linear increase in leachate heavy metals concentrations probably trough the formation of soluble complexes.

Over the last few years a new effective method has been introduced for waste inertisation with phosphoric acid. This method has been developed by Solvay for inertisation of sediments in ports and shipyards with high heavy metal and organic matter loads and has been patented under the name of Novosol®. It can transform highly mobile heavy metals into hardly soluble metal phosphates and destroy organic matter with heat (38, 39). Yang and Mosby (40) used phosphoric acid for successful *in situ* immobilisation of lead in contaminated soil while Zupančič et al. (41) used the same method to immobilise nickel and zinc in sewage sludge and reduced significantly leaching of either metal.

High retention rate of heavy metals (Table 5) and other components in treated sludge, slight changes in pH, and a small amount needed are the major advantages of the phosphoric acid-based method. Its major disadvantage is that it involves special handling and storage, higher operational costs, lower efficiency in heavy metal binding, treated sludge needs additional drying.

CONCLUSION

All three treatment methods can efficiently convert hazardous waste into inert material and reduce its adverse effects on the environment and human health (13-19). Inertization with powdered activated carbon showed best performance (Figure 2). Its other advantages are insignificant changes in pH and volume of treated sludge, small amount needed, and simplicity of the application. On the other hand, CaO-based solidification is the least expensive method and the obtained solidificate is suitable for reuse.

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Sažetak

INERTIZACIJA GALVANSKOG MULJA S POMOĆU KALCIJEVA OKSIDA, AKTIVNOG UGLJENA I FOSFORNE KISELINE

U radu su prikazani i uspoređeni rezultati triju metoda obrade galvanskog mulja visoko opterećenog cinkom i željezom: (1) solidifikacija/stabilizacija otpada primjenom kalcijeva oksida; (2) inertizacija otpada adsorpcijom organskog i anorganskog opterećenja na aktivni ugljen; (3) prevođenje mobilnih komponenata otpada u teško topljive fosfate. Sve tri metode pokazale su se efikasnima u prevođenju opasnog otpada u inertno stanje. Kod optimalnih uvjeta koncentracija cinka u eluatu solidificiranog otpada snizila se za 99,7 % u odnosu prema neobrađenom otpadu. Efikasnost retencije cinka u otpadu inertiziranom aktivnim ugljenom iznosila je 99,9 %, a fosfornom kiselinom 98,7 %. Prednost inertizacije aktivnim ugljenom u odnosu prema ostalim dvjema metodama očituje se visokim sorpcijskim kapacitetom, neznatnim promjenama pH-vrijednosti i volumena tretiranog otpada, kao i jednostavnošću primjene.

KLJUČNE RIJEČI: *CaO, izluživanje, praškasti aktivni ugljen, teški metali*

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Scientific Paper

COPD AND OCCUPATION: A RETROSPECTIVE COHORT STUDY OF INDUSTRIAL WORKERS*

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The aim of this paper was to ascertain chronic obstructive pulmonary disease (COPD) prevalence among industrial workers in the Russian Federation and determine relative contribution of smoking and occupational factors to COPD.

We recruited 1,375 workers aged 30 or over. Six hundred and twenty-four of them were occupationally exposed to vapours, gases, dust, and fumes (VGDF). Physical examination and baseline spirometry were performed for all the participants of the study. Those with airflow limitation of $FEV_1/FVC<0.70$ were considered having COPD and those with presence of cough and sputum production for at least three months in each of two consecutive years were considered having chronic bronchitis (CB), with no overlapping between these 2 groups. Data on occupational history and VGDF levels in the working area were collected from all participants. In total, 105 cases of COPD and 170 cases of CB were diagnosed in the cohort of examined workers. Occupational exposure to VGDF was twice as often present among COPD patients than among both patients with CB and the control group of healthy workers ($p<0.05$). More than 40 % of COPD patients were occupationally exposed to VGDF above the value of 3.0 of the occupational exposure limit (OEL) and more than 20 % to 6.0 OEL and higher. Overall odds ratio for COPD development due to occupational VGDF exposure was 5.9 (95 % CI=3.6 to 9.8, p=0.0001). Both smoking and VGDF seem to be important for the development of COPD. Analysis of the combined effect of tobacco smoking and occupational noxious particles and gases on COPD development has shown the following order of risk factors based on the strength of their influence: VGDF levels, smoking index, age, and heating microclimate. There is a statistically significant level of relationship and "dose-effect" dependence between occupational exposures to VGDF and the development of COPD. The effect of VGDF composition on the probability of COPD development was not found in the study. Results of this study were used to substantiate the inclusion of COPD into the National List of Occupational Diseases of the Russian Federation.

KEY WORDS: *chronic obstructive pulmonary disease, occupational exposure, risk assessment, silica dust, smoking*

Chronic obstructive pulmonary disease (COPD) is an increasing cause of chronic morbidity and mortality around the world (1). This disease, which has already affected 44 million people in Europe and is deemed the 4th leading cause of death worldwide, is likely to become the 3rd such cause by 2030 according to the predictions of the World Health Organization (3). Thus, the prognosis of CJ Murray

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and AD Lopez about the global burden of COPD, made in 1996 (2), has come true.

The major cause of COPD is smoking. However, tobacco smoking is not the only cause of COPD. According to the last updates of the Global Initiative for Chronic Obstructive Lung Disease (GOLD), occupational exposure is one of the two most important risk factors for COPD (4).

It is well known that tobacco smoke and occupational exposures exert a synergistic effect and increase each other′s influence (5). However, relative impacts of each of these factors are poorly understood. Therefore differentiation between the two risk factors′ individual effects may be important for planning strategies for the prevention and treatment of COPD.

Strong evidence implicates occupational exposures as one of the causes of COPD (6). A significant part of the literature accumulated over the past two decades demonstrated the relationship between vapour, gases, dust, and fumes (VGDF) and the development of COPD (7-13). However its importance remains underappreciated, especially in the Russian Federation. This applies particularly to the combined effect of occupational exposure and smoking. Since COPD develops predominantly during the working age, a comprehensive analysis of this joint effect seems to be important.

The aims of the present study were to ascertain the prevalence of COPD among industrial workers in the Russian Federation, to establish the relative contributions of smoking and occupational factors, and to investigate the accuracy of the following hypotheses: (1) COPD can be caused by VGDF only, irrespective of smoking; (2) there is a ″dose - effect″ dependence between VGDF and COPD development; and (3) the influence of smoking and VGDF on COPD development is similar.

Results of this study were used to corroborate the inclusion of COPD into the National List of Occupational Diseases of the Russian Federation.

METHODS

Study design

The retrospective cohort study was chosen due to its capability to study the outcomes after the exposure; the ability to yield true incidence rates, values of relative risks, and other measures of association.

Study population

One thousand three hundred and seventy-five workers, 879 men and 496 women, aged 30 to 60 years, were selected randomly for this study during periodical medical examinations of industrial workers, residents of three largest cities of the Republic of Tatarstan between June 2005 and December 2008 (Figure 1). The data were collected from workers of five enterprises - two foundry plants, one aircraft plant, and two oil extracting enterprises. Eligible participants were those who met the following inclusion criteria: (1) voluntary informed consent, (2) working for an industrial enterprise for at least five years. Exclusion criteria were: (1) refusal to participate in the study, (2) any other respiratory disease except COPD or chronic bronchitis (CB). Six hundred and twenty-four of included subjects were occupationally exposed to VGDF: 327 of these were exposed to silica dust, 244 to nonfibrogenic dusts, and 53 to nonfibrogenic dusts with vapours of irritants and sensitizers.

After medical examination, 22 workers were excluded from the study because four of them had been diagnosed with pneumoconiosis, and 18 with asthma. We compared three groups of workers: patients with COPD (N=105), those with CB (N=170), and a reference group of healthy workers with no signs of COPD/CB (N=1,100) (Figure 1).

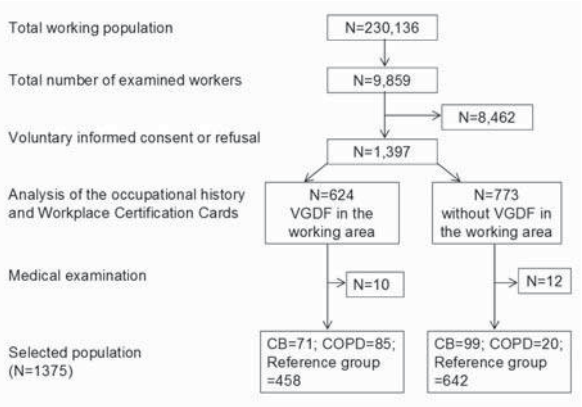

Figure 1 *Selection of the study population*
A hierarchical population sample was selected as follows: all subjects exposed to occupational hazards in the Republic of Tatarstan (n=230,136); a total number of workers, aged 30 to 60 years, examined at the University Clinic of Occupational Medicine during 2005-2008 (n=9,859), workers who agreed to take part in the study (n=1,397), subjects who had vapour, gases, dust and fumes (VGDF) in their working area (n=624), i.e. 10 excluded people (4 with pneumoconiosis, 6 with asthma), 71 with chronic bronchitis (CB), and 85 with chronic obstructive pulmonary disease (COPD); subjects without any VGDF in their working area (n=773), i.e. 12 were excluded due to asthma, 99 with CB, and 20 with COPD. The total number of selected population was 1,375 people.

Definition of exposures

Data on the smoking status and occupational exposures were collected from all study participants.

Subjects who had smoked a minimum of 100 cigarettes since they had started smoking were regarded as current smokers. Subjects who had smoked a minimum of 100 cigarettes in their lifetime but did not smoke at the time of the study were regarded as former smokers. Subjects who had never smoked or smoked less than 100 cigarettes were regarded as never smokers (14).

Workers who were regarded as both current and former smokers were included in the calculation of the smoking index. The average time elapsed since the former smokers quit smoking was 3.1 years.

Occupational exposure to VGDF was categorised into three groups: (1) silica dust, i.e. dust containing 10 or more percent of silica; (2) nonfibrogenic dusts; and (3) nonfibrogenic dust simultaneously with vapours of irritants and sensitizers. Occupational exposure limits (OEL) for each kind of VGDF were established by the Russian Federation regulations.

Definition of outcomes

We used common definitions for COPD/CB, used in the last GOLD revision (15). Therefore, we considered COPD as "a common preventable and treatable disease, characterised by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases". We confirmed the presence of persistent airflow limitation using spirometry. Subjects with forced expiratory volume in 1 second and forced vital capacity ratio (FEV_1/FVC) value of less than 0.7 were regarded as COPD patients. The stages of COPD were also determined according to GOLD criteria.

To evaluate chronic bronchitis, we used the GOLD (2011) diagnosis criteria: the presence of cough and sputum production for at least three months in each of two consecutive years (15).

Therefore, there was no overlapping between COPD and CB groups.

Questionnaires

We used the modified European Society for Coal and Steel (ECSC) questionnaire (16) for the pre-assessment of physical health status. We determined the degree of tobacco dependence using the modified Fagerstrom test (17). Both instruments were translated into Russian.

Spirometric measurements

Physical examination and baseline spirometry were performed for all the participants of the study. We used a portable computerised spirometer (Spirolab III, MIR, Italy). All spirometric measurements were performed by only one of the authors (MNN), respecting the American Thoracic Society (ATS)/European Respiratory Society (ERS) guidelines (18). We calculated the FEV_1/FVC and the percentage of the predicted values for FVC (FVC % predicted) and FEV_1 (FEV_1 % predicted) using published reference values for Europeans (19).

Study participants with value of FEV1/FVC of less than 0.7 were examined with post-bronchodilator test, which was performed according to the ATS / ERS guidelines (18) 15 minutes after the administration of 400 micrograms of salbutamol. The increase in FEV_1 by more than 15 % (or 200 mL) from baseline was regarded as reversible obstruction.

Occupational exposure assessment

We evaluated occupational exposures in two ways. First, we assessed the workers′ lifetime occupational exposures to VGDF directly by self-reports during the periodical medical examination. Subjects who answered affirmatively to at least one of the following two questions i.e.: "Have you ever worked for five years or more in any dusty job?" and/or "Have you ever been exposed (for five years or more) to gas or chemical fumes in your work?" were regarded as exposed to VGDF.

Second, we collected the data on current occupation, occupational history, and occupational exposure for all the study participants (N=1,375): the employers provided us with all these information from the workplace certification cards, established by the Russian Federation regulations.

We combined different types of VGDF into three groups, as was described above, due to a large variety of occupational hazards in the foundry plants, where 15 to 36 various chemical substances could simultaneously be present in the working area. Occupational exposure to VGDF in the aircraft and oil extracting enterprises, i.e. nonfibrogenic dusts or organic solvent vapours in values of less than OEL, were present at a much smaller number of workplaces.

We took into account the influence of occupational exposures other than VGDF. We considered heating microclimate and excessive physical activity as two

additional risk factors for occupational respiratory diseases because of their effects on the breathing rate. The information on the presence of overheating and excessive physical activity in the workplace were also drawn from workplace certification cards.

Occupational Exposure Limit values of VGDF were accepted according to the National hygienic standards.

Data Analysis

Analyses were conducted using the R statistical system (version 2.11.1) software (20). Prevalence of COPD and CB was estimated for the entire cohort by age, gender, education level, smoking status, and occupational exposures. The difference between variables was evaluated by Student′s t-test for continuous data and chi-square test for categorical data. The main epidemiological criteria traditionally used as measures of association, i.e. odds ratio (OR), attributable risk (AR), and population attributable risk (PAR) were also calculated. The relationship between the influence of smoking and occupational factors for COPD development was analysed by Cochran-Mantel-Haenszel test (CMH-test) of data stratification, with smoking being an additional affecting factor.

The logistic model of regression analysis was used for the same raw data to evaluate the combined effect of occupational exposures and smoking. Generalised additive model (GAM) (21) with binomial family was used to model the COPD probability; the model can include interactions between independent variables. The nonlinear dependence on smoking index was modeled using the smoothing term (22). "MGCV" package for R was used to fit the model.

To model the response with ordered values, Ordered Logistic Regression (23) was used. Model was fitted using the MASS R package to estimate

Table 1 *General characteristics of the studied cohort*

	COPD (n=105)	CB (n=170)	Reference group (n=1100)
Age / year, mean ± SD	51.7±8.4 †	49.5±8,1	47.5±9.9
Women, n (%)	3 (2.9) ‡	46 (26.7) †	447 (40.6)
Education level, n (%):			
Secondary	99 (94.3)*	152 (89.4)	930 (84.5)
Higher	6 (5.7) †	18 (10.6)*	170 (15.5)
Level of income, n (%):			
Low	16 (15.2) †	13 (7.6)	60 (5.5)
Middle	83 (79.0)	103 (60.6)*	910 (82.7)
High	6 (5.7)*	54 (31.8)*	130 (11.8)
Smoking status, n (%):			
Nonsmokers	20 (19.1) †	43 (25.3) †	713 (64.8)
Smokers	75 (71.4) †	113 (66.5) †	292 (26.5)
Former smokers	10 (9.5)	14 (8.2)	95 (8.6)
Smoking index / pack-years, n (%):			
Less than 20	55 (33.3) †	60 (35.3) †	231 (21.0)
20 or more	50 (47.6) †	67 (39.4) *	156 (14.2)
Occupational exposure to VGDF, n (%)	85 (80.9) †	71 (41.8)	458 (41.6)
VGDF levels, n (%):			
Low (<OEL to 3.0 OEL)	18 (17.1) †	36 (21.2)	325 (29.5)
Medium (3.1 OEL to 6 OEL)	46 (43.8) †	35 (20.6) †	106 (9.6)
High (>6.0 OEL)	21 (20) †	0	27 (2.5)

The difference between variables was evaluated by Student's t-test for continuous data and chi-square test for categorical data.
Marked differences ($p<0.01$, † - $p<0.05$, ‡ - $p<0.001$) between COPD and/or CB patients and control group.*
COPD – chronic obstructive pulmonary disease
CB – chronic bronchitis
VGOF – vapour, gases, dust and fumes
OEL – occupational exposure limit

confidence intervals. We built seven predictive models using the same initial data. As a dependent variable, relation to one of the following three groups of workers was taken into account: "healthy", "CB", and "COPD". The variables with discrete values were presented as the factors with fixed rates. Contributions of 14 factors in the model was estimated, i.e. sex, age, education, and income levels, VGDF levels in the working area, presence of other occupational factors, i.e. physical exertion, overheating, vibration, and noise. Besides, period of work, smoking status, smoking history, smoking index value, and the degree of nicotine dependence were estimated too. Contribution of each factor was evaluated separately. Significance of the factors was determined against the baseline, which was assumed to be the most important factor value in an importance order. The contribution of the baseline was considered as the zero value of the model to be defined.

The predictive capacity of models was evaluated by cross-validation and Receiver Operating Characteristic (ROC)-analysis. The repetitive cross-validation (bootstrap) was used: data was split into teaching (80 % of data) and validation (20 % of data) subsets, model was fitted, and the predictions on the validation subset were compared with the true values. This procedure was repeated 100 times to estimate the histograms and confidence intervals; these results were used to select the best predictive model. ROC was estimated and plotted using the ROCR package (24).

RESULTS

In total, 105 cases of COPD and 170 cases of chronic bronchitis (CB) have been diagnosed in the cohort of workers examined (Table 1). Thus, prevalence of COPD was 7.5 % for the entire cohort and 7.9 % for people older than 40; prevalence of CB was 12.1 % for the entire cohort and 13.4 % for the people over 40. Distribution of COPD patients across stages according to the GOLD criteria was as follows: stage I - 69 people (65.7 %); stage II - 29 people (27.6 %), stage III - 7 people (6.7 %). Proportion of smokers was significantly lower in healthy workers than among COPD and CB patients ($p<0.05$). The highest proportion of smokers was found among patients with COPD. Occupational exposure to VGDF occurred twice more often among COPD patients than among both the CB patients and the control group of healthy workers ($p<0.05$). More than 40 % of COPD patients were occupationally exposed to VGDF on the level of 3.0 OEL and more. The 6.0 OEL and higher levels were found in more than 20 % of COPD patients.

A strong link between exposure to VGDF at the workplace and COPD development was found (Table 2). Thus, the overall odds ratio for COPD development due to occupational VGDF exposure was 5.9 (95 % CI=3.6 to 9.8; $p=0.0001$).

The values of odds ratios for different types of occupational noxious particles and gases among COPD patients were highest for silica dust (OR=6.2; 95 % CI=3.6 to 10.7; $p<0.0001$). The same refers to other indicators of risk assessment, e.g. PAR % values for COPD patients. The difference between PAR % values for silica dust and other kinds of dust were not statistically significant (56.7 % and 49.8 %, $p>0.05$). The lowest value of PAR % for COPD patients was found for occupational exposure to dust with vapours of irritants and sensitizers (18.3 %). The overall value of PAR % obtained in our study was 65.3 %. This is more than three times higher than the value demonstrated in ATS statements (2003, 2010) (15, 16) dedicated to occupational burden of lung diseases.

Another kind of dependence was found for CB patients. The overall odds ratio for CB development was statistically non-significant (OR=1.0; 95 % CI=0.7 to 1.4; $p=0.4$). However, the statistically significant value of odds ratio for CB development was obtained for VGDF levels above 3.0 OEL. The highest value of odds ratio was reached for CB patients who had occupational exposure to dust with vapours of irritants and sensitizers (OR=2.0; 95 % CI=1.0 to 4.1; $p=0.07$) but not for silica dust.

Occupational risk assessment calculated separately for nonsmoking and smoking workers showed that the odds ratios for occupational COPD were significantly higher for nonsmokers. For the smoking workers, smoking was a major risk factor for COPD development (Table 3). Thus, the odds ratio for occupational COPD for non-smokers was almost seven times higher than that of smokers and their attributable risk for occupational COPD was more than 95 % compared to 65 % for smokers. Total risk of COPD development for smoking workers due to the influence of both major risk factors for COPD, i.e. smoking and VGDF, was also calculated. As Table 3 shows, the overall value was about four times higher than the risk for non-smoking workers. It is rather interesting that the risk

of COPD from occupational VGDF exposures established for non-smoking workers was comparable with that obtained for smokers who were not in contact with VGDF in the working area.

As regards the second hypothesis, there is a quite clear and statistically significant ″dose - effect″ dependence between the level of occupational exposures and COPD development (Table 2). The risk assessment for occupational COPD revealed a regular and statistically significant risk increase when working conditions deteriorated.

Cochran-Mantel-Haenszel test of data stratification with smoking as an additional affecting factor showed the relationship between the influence of smoking and occupational factors. Higher values of odds ratios of COPD development (see above) were obtained for non-smoking workers (Table 4). The difference caused by the smoking status was statistically significant for workers with occupational exposure to silica dust and both to dust and irritants (p=0.032 and 0.012, respectively). No significant difference between smokers and nonsmokers was found for

Table 2 *The risk of COPD/CB development from occupational exposures to VGDF, depending on their type and levels*

	OR (95 % CI)	p-level	AR / %	PAR/ %
COPD				
Overall	5.9 (3.6 to 9.8)	0.0001	80.7	65.3
Low levels of VGDF *	1.7 (0.9 to 3.4)	0.07	42.4	20.1
Medium levels of VGDF †	13.9 (7.9 to 24.5)	<0.0001	90.0	62.7
High levels of VGDF ‡	24.9 (12.1 to 51.5)	<0.0001	93.1	47.7
Silica dust, i.e. dust containing 10 % or >10 % silica	6.2 (3.6 to 10.7)	<0.0001	81.3	56.7
Nonfibrogenic dusts	5.7 (3.2 to 10.2)	<0.0001	80.0	49.8
Nonfibrogenic dust simultaneously with vapours of irritants and sensitizers	5.5 (2.1 to 14.6)	0.002	79.3	18.3
CB				
Contact with VGDF, including:	1.0 (0.7 to 1.4)	0.4	0.45	0.19
Low levels of VGDF *	0.7 (0.5 to 1.1)	0.1	<0	<0
Medium levels of VGDF †	2.1 (1.4 to 3.3)	0.0005	45.4	11.4
High levels of VGDF ‡	No data			
Silica dust, i.e. dust containing 10 % or >10 % silica	1.0 (0.7 to 1.5)	0.8	25	0.34
Nonfibrogenic dusts	0.8 (0.5 to 1.3)	0.4	<0	<0
Nonfibrogenic dust simultaneously with vapours of irritants and sensitizers	2.0 (1.0 to 4.1)	0.07	48.3	11.6

*Levels of VGDF in the working area: *<OEL to 3.0 OEL; † from 3.1 OEL to 6 OEL, ‡ >6.0 OEL.*
COPD – chronic obstructive pulmonary disease
CB – chronic bronchitis
VGDF – vapour, gases, dust and fumes
OEL – occupational exposure limit

Table 3 *The risk values of chronic obstructive pulmonary disease (COPD) development among dusty trade workers and smokers not having any occupational exposures*

	OR (95 % CI)	p-level	AR / %	PAR / %
Nonsmoking workers in dusty trades	22.2 (4.9 to 100.5)	<0.0001	95.1	81.5
Smoking workers in dusty trades (risk values due to occupational exposures only)	3.4 (1.8 to 6.5)	0.0001	65.3	42.5
Smoking workers in dusty trades (overall risk from occupational and non-occupational exposures)	82.7 (19.9 to 342.3)	<0.0001	98.3	95.2
Smokers not having any VGDF in the working area (risk value from smoking)	34.7 (7.9 to 151.5)	<0.0001	96.7	87.0

OR – odds ratio
CI – confidence internal
AR – attributable risk
PAR – population attributable risk
VGDF – vapour, gases, dust and fumes

the workers who were occupationally exposed to other kinds of dust different from silica.

The logistic regression model investigated the combined effect of smoking and occupational exposures on COPD development using the same raw data. As shown in Table 5, the following factors specified in order of their significance were important for COPD: VGDF composition (χ^2=64.4; p<0.0001), VGDF level (χ^2=63.3; p<0.0001), smoking index (χ^2=43.2; p<0.0001), age (χ^2=5.7; p=0.01), and heating microclimate in the workplace (χ^2=5.2; p=0.02). The contribution of all factors to the model was linear except for the smoking index.

Visual analysis of the curve of the influence of smoking index in the model of occupational COPD showed that its nonlinear dependence could be described by two linear curves: the effect of smoking index on the development of COPD in the model increased almost linearly from 0 to about 20 pack-years, and then it remained practically unchanged (Figure 2). For this reason, a new variable was introduced in the model with values of smoking index

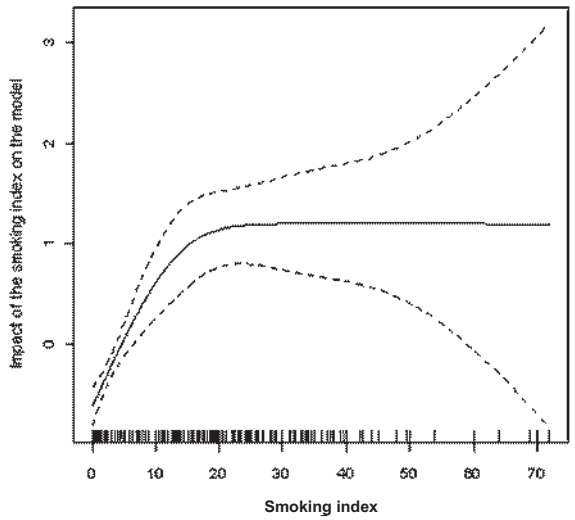

Figure 2 *Effect of smoking index on the probability of chronic obstructive pulmonary disease (COPD) development*

limited to the level of 20 pack-years. Assuming linear contribution of the smoking index in the model, the VGDF composition becomes insignificant for COPD development because of the counterbalancing effect of the smoking index and VGDF levels. In addition, the statistically significant interaction between two main predictors of occupational COPD (smoking index and VGDF levels) was found.

Analysis of the values of linear predictors in this model showed that the effect of smoking on COPD development in the cohort of workers studied ranged from 5 % to 40 % depending on other risk factors. The contribution of VGDF to COPD likelihood increased monotonously with the worsening of working conditions, with no changing in the likelihood of the COPD development, and was found statistically absolutely significant (p <0.0001). The VGDF impact was within 3 % to 5.2 % at low VGDF levels (<3.0 OEL), 33 % at middle levels (from 3.0 OEL to 6.0 OEL), and reached 44 % at high levels (>6.0 OEL).

Therefore, the degrees of smoking and VGDF influence on COPD development are probably rather similar. This suggestion was verified using seven predictive models with the same initial data. Ten from 14 studied occupational and non-occupational factors proved to be statistically significant in the models, i.e. age, gender, VGDF levels, type of VGDF, levels of irritants, physical stress, heating microclimate, smoking status, smoking index, and period of smoking. VGDF levels and the smoking index demonstrated maximal influence on CB/COPD probability. Age, gender, and levels of irritants exerted minimal but statistically significant influence.

Overall, average results of cross-validation showed high levels of reliability of predictiveness of the models on new data. The best results of cross-validation and ROC-analysis were obtained for two models, i.e. three-level generalised linear model (GLM) “(0) healthy workers & CB, (1) COPD”, and two-level GLM-model “(0) healthy workers, (1) COPD” (Figures 3, 4).

DISCUSSION

Prevalence of COPD and CB obtained in this study correspond to average data in the Russian Federation and internationally (4).

High values for odds ratios of COPD development obtained in our study indicate a high level of the occupationally determined risk for COPD. This allows discussing possible inclusion of the disease in the National List of Occupational Diseases. However, a well-known statement “Chronic bronchitis is the biggest single cause of sickness absence” (25) also explains the impressive difference between COPD and CB odds ratio values shown in this study. A substantial difference between OR, AR, and PAR % values for COPD and CB call for the possible update of the classification of occupational airways diseases.

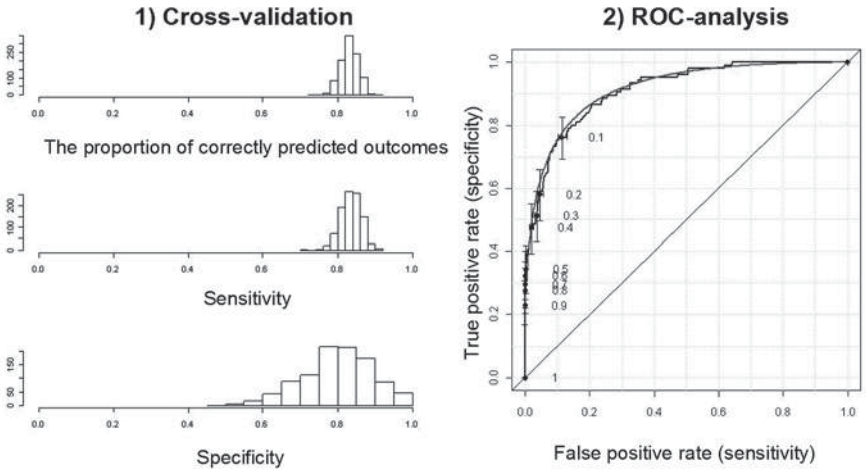

Figure 3 *Results of verification for the model "0 – healthy workers and chronic bronchitis (CB) patients; 1 – chronic obstructive pulmonary disease (COPD) patients"*
ROC-analysis = Receiver Operating Characteristic Analysis

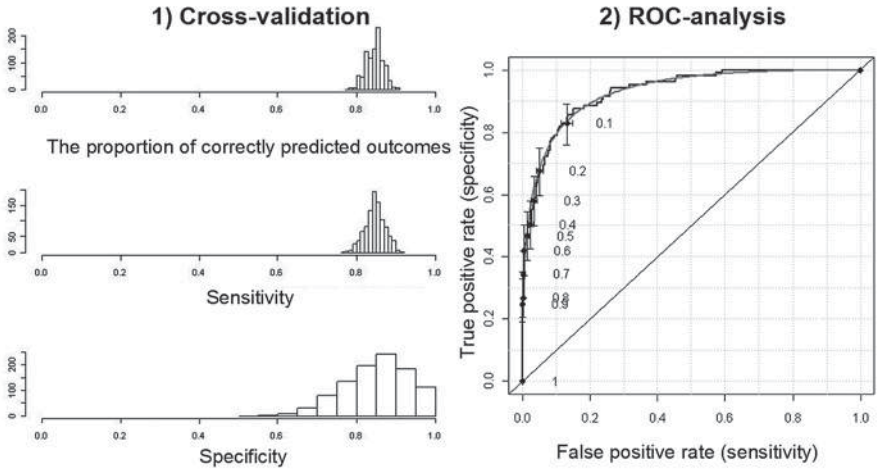

Figure 4 *Results of verification for the model "0 - healthy workers; 1- COPD patients"*
ROC-analysis = Receiver Operating Characteristic Analysis

"Dose - effect" dependence between VGDF levels and COPD development (see Table 2), is another argument in favour of the possibility of COPD being developed due to VGDF only.

The odds ratio and other risk assessment criteria of COPD development for the silica dust exposed workers obtained here confirm again the importance of this risk factor (26). Insignificant difference between PAR % values for silica dust and other kinds of dust indicates the importance of all kinds of occupational noxious particles for COPD development. Further research is required for the comparative impact of occupational factors to be evaluated.

As Table 2 shows, the values of PAR % for workers exposed to low VGDF levels (<3.0 OEL) are very close to the results obtained in other studies of occupational COPD published earlier (27-29). However, our study showed that the values of risk assessment were much higher for the workers exposed to higher VGDF levels. This might result from much poorer working conditions at the foundry plants in the Russian Federation, and relatively high VGDF levels in the working area of dusty trade workers included in the cohort studied. Therefore, the effect of VGDF should be evaluated according to the levels in the working area. This may be useful for future strategies of occupational risk management.

We have also found that non-smoking dusty trade workers run the risk of developing occupational COPD six times more than smoking dusty trade workers (see Table 3). This is similar to the results described earlier by Becklake (30) in her classic epidemiological study of the prevalence of occupational COPD.

Table 4 *The relationship between smoking and occupational factors in the development of chronic obstructive pulmonary disease (COPD)*

	OR (95 % CI)		CMH-test for homogeneity, χ^2	p-level
	Smoking workers	Nonsmoking workers		
Silica dust, i.e. dust containing 10 % or >10 % silica	3.5 (1.8 to 6.8)	20.6 (4.5 to 190.7)	4.61	0.033
Nonfibrogenic dusts	3.7 (1.9 to 7.4)	9.76 (1.4 to 109.4)	1.08	0.300
Nonfibrogenic dust simultaneously with vapours of irritants and sensitizers	1.75 (0.4 to 5.8)	32.1 (1.0 to 7.0)	6.28	0.013

CMH-test = Cochran-Mantel-Haenszel test

Table 5 *The significance of risk factors to the development of chronic obstructive pulmonary disease (COPD) in a model of multiple logistic regression*

	With initial values of smoking index		With transformed values of smoking index	
	χ^2	p	χ^2	p
Type of VGDF	64.5	<0.0001	0.9	0.6288
Level of VGDF	63.3	<0.0001	70.8	<0.0001
Smoking index	43.2	<0.0001	43.5	<0.0001
Age	5.7	<0.0200	5.9	<0.0140
Heating microclimate	5.2	<0.0230	5.3	<0.0220

VGDF – vapour, gases, dust and fumes

Data stratification performed by Cochran-Mantel-Haenszel test in our study showed again that non-smoking workers have higher probability of developing the disease (see Table 4) with the isolated impact of occupational risk factors for COPD. The difference due to the smoking status in the cohort studied was statistically significant for both silica dust and for dusts and irritants present simultaneously in the working area (p=0.032 and 0.012, respectively). For other types of dust, except quartz, no significant difference was found between smokers and nonsmokers. This points to a more important role of silica dust and irritants in the development of COPD.

It should be pointed out that smoking workers are influenced by both major risk factors for COPD. For non-smoking dusty trade workers, the risk of COPD is caused only by VGDF. Therefore, we evaluated the overall risk for COPD development from occupational and non-occupational exposures and the result was that the overall risk for workers influenced by both major risk factors for COPD is about four times higher than that caused by VGDF only. The studies performed by Meer et al. (31) and Blanc et al. (32) showed similar results. This makes us think about the necessity of planning preventive strategies and developing smoking cessation programs for dusty trade workers.

Similar results were obtained in our study for COPD risk due to occupational VGDF exposures for non-smoking dusty trade workers and for smokers who did not have contact with VGDF in the working area. This confirms again the well-known thesis of the two main risk factors for COPD, i.e., smoking and noxious particles and gases, whose influence seems comparable.

Analysis of the combined effect of tobacco smoking and occupational noxious particles and gases for COPD development showed the following order of risk factors based on the strength of their influence: VGDF levels, smoking index, age, and heating microclimate. The values of impact of VGDF and smoking for COPD development are similar in the regression model used.

The results of cross-validation and ROC-analysis revealed the highest level of sensitivity and specificity for two models made. The two-level GLM-model "(0) healthy workers; (1) COPD" showed the best result of cross-validation. However, the realisation of the method assuming the artificial exclusion of CB patients from the database does not allow the model for prediction to be applied.

Nevertheless, high analytical capacity of this model provides the application of its predictors as the assessment criteria for medical examination of workers. The three-level GLM-model "(0) healthy workers & CB; (1) COPD" showed the second best results of cross-validation. All of the results of cross-validation and ROC-analysis fully support the conclusion made.

CONCLUSIONS

The differentiation between the various effects of inhaled noxious particles and gases seems to be complex. But this task is particularly challenging for the Russian Federation because of a high number of smokers in the country. Therefore, a comprehensive assessment of contribution of the two leading risk factors for COPD is of special interest for occupational medicine. Future investigations of occupational COPD seem to be important for developing prevention strategies.

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Sažetak

KRONIČNA OPSTRUKTIVNA PLUĆNA BOLEST I RAD: RETROSPEKTIVA ISTRAŽIVANJA PROVEDENOG NA KOHORTI INDUSTRIJSKIH RADNIKA

Cilj ovog rada bio je potvrditi prevalenciju kronične opstruktivne plućne bolesti (engl. *chronic obstructive pulmonary disease*, COPD) među industrijskim radnicima u Ruskoj Federaciji i utvrditi relativni doprinos pušenja i profesionalnih čimbenika razvoju COPD-a.
Odabrali smo 1.375 radnika u dobi od 30 godina i starijih. Šeststo dvadeset i četiri radnika bila su izložena parama, plinovima, prašini i dimovima (engl. *vapours, gases, dust, and fumes* – VGDF) na radu. Svi su radnici bili podvrgnuti fizičkom pregledu i temeljnoj spirometriji. Za radnike koji su imali smanjen protok zraka (FEV_1/FVC<0,70) smatralo se da imaju COPD, a za one koji su imali kašalj i pojačan sputum barem 3 mjeseca tijekom dvije uzastopne godine smatralo se da boluju od kroničnog bronhitisa (CB). Između ove dvije skupine nije bilo preklapanja. Od svih su sudionika prikupljeni podaci o radnoj anamnezi i razinama izloženosti VGDF-u na radu. Profesionalna izloženost VGDF-u bila je dvostruko češće prisutna kod radnika s COPD-om nego kod radnika s CB-om i kod kontrolne skupine zdravih radnika (p<0,05). Više od 40 % bolesnika s COPD-om bilo je profesionalno izloženo VGDF-u iznad 3,0 OEL (engl. *occupational exposure limit* – granica profesionalne izloženosti), a više od 20 % granici od 6,0 OEL i više. Ukupni omjer izgleda (engl. *odds ratio* – OR) za razvoj KOPB-a zbog izloženosti VGDF-u bio je 5,9 (95 %-tni CI=3,6 do 9,8; p=0,0001). Čini se da su i pušenje i VGDF važni za razvoj COPD-a. Analiza sjedinjenog učinka pušenja i profesionalne izloženosti štetnim česticama i plinovima na razvoj COPD-a pokazala je ovakav redoslijed čimbenika rizika prema jačini njihova utjecaja: razine VGDF-a, indeks pušenja, dob i mikroklima grijanja. Postoji statistički značajna veza i ovisnost „doza-učinak" između profesionalne izloženosti VGDF-u i KOPB-a. Utjecaj sastava VGDF-a na vjerojatnost razvoja COPD-a nije utvrđen u istraživanju. Rezultati ovog rada potvrdili su potrebu uvrštavanja COPD-a u Nacionalni popis profesionalnih bolesti Ruske Federacije.

KLJUČNE RIJEČI: *COPD, filtarska prašina, procjena rizika, profesionalna izloženost, pušenje*

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Scientific Paper

CHRONIC EFFECTS OF ENVIRONMENTAL BIOMASS SMOKE ON LUNG HISTOPATHOLOGY IN TURKISH NON-SMOKING WOMEN: A CASE SERIES

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Biomass is widely used for fuel in developing countries. Particles and gases of biomass burning may cause changes in the lung. In this prospective study we investigated histopathological changes in the lungs of 42 non-smoking women [mean age (59±10) years] caused by biomass smoke. We valuated exposure to biomass smoke, case histories, and the findings of physical examination, radiology, bronchoscopy, and lung histopathology. Mean exposure to biomass smoke was (28±9) hour-year (1 hour-year equals 365 hours of exposure per year with average exposure of 1 hour a day). The radiological findings were mass (42 %), reticulonodular opacities (31 %), mediastinal lymphadenopathy (26 %), pleuro-parenchymal fibrotic banding (19 %), widening of the pulmonary artery (14 %), ground glass (11 %), mosaic perfusion (9 %), consolidation (9 %), segmental or subsegmental atelectasis (7 %), and bronchiectasis (7 %). The patients were diagnosed with lung cancer (35 %), interstitial lung disease (31 %), sarcoidosis (9 %), tuberculosis (9 %), chronic obstructive pulmonary disease (4 %), chronic bronchitis (9 %), and metastasis (4 %). Bronchoscopy showed pilies, oedema, erythema, bronchus narrowing, endobronchial tumour, mucosal irregularity, increased vascularisation, blue-black anthracotic plaques, mucosal oedema, and purulent secretion. Transbronchial biopsies revealed neutrophil and lymphocyte leucocytes in the perivascular, peribronchiolar, and interalveolar septa, slightly enlarged connective tissue, thickening of the basal membrane, thickening of interalveolar septa, intimal and medial thickening of the vascular wall and vascular lumen narrowing, anthracosis between the cells and in the bronchiole epithelium. These findings confirm that biomass smoke has important toxic effects on the lung parenchyma, interstitium, and pulmonary vessels that may result in malignancies.

KEY WORDS: *biomass smoke exposure, bronchoscopy, histopathological changes, lung diseases*

Heat releasing sources composed of biological material are called biomass'. Burning biomass and poor indoor air exchange may cause chronic lung diseases, interstitial lung disease, and cancer. The use of biomass in Turkey is common due to social, economic, and cultural reasons.

The most important aetiological factor in biomass-related disease is the pollutant being released (1-3). In suburban areas of Turkey women who make fire, bake, and cook with biomass primarily composed of cowpat are exposed to its smoke. that may contain harmful pollutants such as aldehydes, phenols, and

toluene (4, 5). Incomplete burning of animal waste also releases CO, NO_2, and SO_2 (5). Exposure in this population, who are poor and have little access to healthcare services, is often associated with chronic lung damage and respiratory infections.

Our earlier experimental study (6) showed histopathological changes in the trachea and the lungs of rats exposed to biomass smoke for one hour a day for three, six, and nine months. Histopathological findings included lymphocyte, eosinophil, and macrophage infiltrations in the interstitium, small papillary structures in the airway mucosa, thickening of the intimae and media of the pulmonary vessels, narrowing of pulmonary vessel lumens, and papillary adenomatous proliferation of the mucosa (6).

The aim of this study was to determine if similar changes occurred in human lungs after biomass smoke exposure. The number of studies which include bronchoscopy in people exposed to biomass smoke is very small and we hoped to fill in this gap by analysing histopathological findings in transbronchial tissue samples from women who had bronchoscopy performed and who were exposed to biomass smoke.

MATERIALS AND METHODS

This prospective study was approved by bioethics committees of the Yuzuncu Yil University Faculty of Medicine, and observed the Declaration of Helsinki. It was carried out between January 2007 and September 2009 in 42 women who had pathological findings on lung roentgenograms. The women were exposed to biomass smoke for various hour-years (1 hour-year equals 365 hours of exposure per year with average exposure of 1 hour a day). Biomass exposure in the absence of other pollutant exposure is rare in men, and we excluded male patients from the study. Women who smoked tobacco and were exposed to biomass smoke were also excluded from the study. All of the patients lived in the countryside, which excludes an influence of metropolitan atmospheric air pollution to a reasonable extent.

Cigarette smoking and biomass exposure were determined by interview. The length of exposure to biomass smoke was expressed in years and the density of exposure in hours per day. Total exposure is therefore expressed in hour-years.

Posterior-anterior lung roentgenograms and findings of computed tomography (CT) scans were analysed to confirm that the patients met our inclusion criteria.

Bronchoscopy was performed in all of the patients included in the study according to our diagnostic algorithm. All patients signed an informed consent form before the procedure. They were also tested for platelet count, prothrombin time, and partial thromboplastin time. The patients underwent bronchoscopy after a four-hour fast and remained in the same operation room with resuscitation equipment ready throughout the procedure. Those experiencing a bronchospasm received a bronchodilator. For local anaesthesia they received an 8 mg kg^{-1} dose of 2 % lidocaine solution in spray. The nasal passageway was preferred for the use of fiberoptic bronchoscope (Olympus, Center Valley, PA, USA). Patients were monitored by pulse oximetry. Macroscopic finding were recorded. Transbronchial biopsy was collected from sub-lobe basal segments of patients with interstitial changes and common lesions, and from the lobes with limited lesions. Three hours after the transbronchial biopsy, a posterior-anterior lung roentgenogram was taken to exclude pneumothorax. Forceps biopsy was performed in patients with endobronchial lesions, and transbronchial needle aspiration biopsy in patients with mediastinal lymphadenopathy. Bacteriological and cytopathological analyses were performed on the bronchial lavage and biopsy samples. Anthracotic changes, inflammatory infiltration, thickening at alveolar septum, fibrosis, and malignancies were assessed by an experienced pathologist. Photos of histopathological samples were taken microscopically.

Continuous variables were expressed as mean ±standard deviation, while categorical variables were expressed as number and percentage. To determine the difference between the diagnostic groups in terms of continuous variables, we used the Mann-Whitney *U* test. To determine the relationships between the diagnostic groups and other categorical variables, we used the chi-square test and calculated the odds ratio. Statistical significance was set at $P<0.05$. Data were processed using the SPSS (version 16) statistical software.

RESULTS

The age, biomass exposure, bronchoscopy findings, and diagnosis for individual patients are shown in Table 1. Average biomass exposure years and total

Table 1 *Age of the patients, duration of exposure to biomass smoke, bronchoscopy findings, and diagnosis*

Age / years	Biomass exposure / hour-years	Years of exposure	Bronchoscopy findings	Diagnosis
36	8.5	11	Normal	Chronic bronchitis
59	25.7	35	Narrowing in the orifice of apicoposterior segment of the left upper lobe	Chronic bronchitis
51	25.7	29	Anthracotic plaque in the orifice of right upper lobe	Chronic bronchitis
69	38.5	45	Hyperaemia and swelling in the lingular segment of the left upper lobe	Chronic bronchitis
63	21.4	30	Normal	Chronic bronchitis+ILD
60	34.2	35	Normal	Chronic bronchitis+ILD
70	51.4	43	Anthracotic plaque in the orifice of right upper lobe, and pale mucosa	Chronic bronchitis +ILD
59	34.2	33	Normal	COPD+ILD
61	34.2	36	Normal	COPD+ILD
51	14.2	30	Mass in the lingular segment of the left upper lobe	Oesophageal cancer
43	25.7	15	Normal	ILD
69	25.7	41	Mucosal erythema and oedema	ILD
54	25.7	32	Normal	ILD
65	38.5	40	Widespread anthracosis and swelling in mucosa	ILD
77	42.8	50	Normal	ILD
69	42.8	37	Widespread anthracotic plaques	ILD
63	17.1	31	Indirect tumour findings in left bronchial system	ILD+PE
64	17.1	29	Narrowing in the orifice of right middle lob	Lung cancer
65	17.1	32	Anthracosis and narrowing in the orifice of anterior segment of right upper lobe	Lung cancer
57	17.1	28	A mass in right middle lobe	Lung cancer
58	17.1	35	A mass in right main bronchus	Lung cancer
46	21.4	20	A mass in left main bronchus	Lung cancer
57	25.7	32	A mass in right main bronchus	Lung cancer
71	25.7	50	A mass in the intermediary bronchus	Lung cancer
58	30.0	32	A mass in the intermediary bronchus	Lung cancer
43	30.0	18	A mass in the intermediary bronchus	Lung cancer
55	34.2	31	Narrowing in the orifice of right upper lobe	Lung cancer
66	34.2	43	A mass in left main bronchus	Lung cancer
65	38.5	40	Paralysis of left vocal cord and mass in left main broncus	Lung cancer
74	42.8	51	Submucosal swelling and narrowing in the orifice of right upper lobe	Lung cancer
76	42.8	60	Total obstruction in right lower lobe bronchus	Lung cancer
68	42.8	41	Narrowing in the orifice of left upper lobe	Lung cancer
68	38.5	39	Normal	Pancreatic cancer
34	1.1	14	Normal	Pneumonia
50	21.4	29	Subcarinal enlargement	Sarcoidosis
53	25.7	31	Subcarinal enlargement	Sarcoidosis
54	25.7	33	Subcarinal enlargement	Sarcoidosis
56	34.2	29	Subcarinal enlargement	Sarcoidosis
45	25.7	17	Erythema in the orifice of right lower lobe	Tuberculosis
59	25.7	40	Normal	Tuberculosis
55	30.0	35	Narrowing in the orifice of posterior and anterior segments of right upper lobe	Tuberculosis
67	34.2	42	Anthracosis and obstruction in the orifice of superior segment of right lower lobe	Tuberculosis

COPD = chronic obstructive pulmonary disease; ILD = interstitial lung disease; PE = pulmonary embolism

exposure were (13±5) years and (28.6±9.1) hour-years, respectively. The utilised biomass fuel is called *tezek*, and is produced by dehumidifying animal manure. The percent (mean ± SD) of the predicted FEV_1 and FVC were (90±7) % [(2.6±0.48) L s^{-1}] and (87.8±7.1) % [(2.9±0.44) L s^{-1}], respectively, and the FEV_1/FVC was (85±6) %. Seven percent of the patients had an FEV_1/FVC below 70 %.

Radiological findings were as follows: mass (18 patients, 42.9 %); reticulonodular opacity (13 patients, 31 %); mediastinal lymphadenopathy (11 patients, 26.2 %); pleuroparenchymal fibrotic stripes (8 patients, 19 %); dilatation of the pulmonary artery (6 patients, 14.3 %); ground glass opacities (5 patients, 11.9 %); consolidation (4 patients, 9.5 %); mosaic perfusion (4 patients, 9.5 %); segmenter or sub-segmenter atelectasis (3 patients, 7.1 %); and bronchiectasis (3 patients, 7.1 %).

Bleeding was seen in only one patient as a bronchoscopy complication and was controlled by lavage with a solution including adrenalin. Pneumothorax was not observed in any of the patients. The main findings of bronchoscopy were indirect tumour findings such as oedema, erythema, and narrowing of the bronchus, indicating acute and chronic airway inflammation. Findings of chronic bronchitis included swollen mucosa, purulent sputum, and dark blue or black anthracotic plates, generally in the upper lung lobes, and especially in the bifurcation regions. Direct tumour findings included endobronchial neoplasm, mucous instability, and increased blood vessels.

We also performed bronchoscopic transbronchial biopsy (16 patients, 38 %); mucous biopsy (16 patients, 38 %); transcarinal needle aspiration biopsy (4 patients, 9.5 %); and bronchial lavage (6 patients, 14 %). Most of the patients had lung cancer and interstitial lung disease. Lung disease in these patients was diagnosed after more than 27 hours per year of biomass smoke exposure. However, one patient with 11 hours per year of biomass smoke exposure had a normal biopsy finding. The incision was performed to find the cause of haemoptysis.

There was a highly negative correlation between interstitial lung disease and lung cancer (P=0.001). That biomass smoke exposure may cause different diseases in different people may partially be explained by a person's genetic predisposition to a disease.

Table 2 shows the histopathological findings in the patients with interstitial lung disease (ILD). Biopsy tissue of four patients was lost during examination.

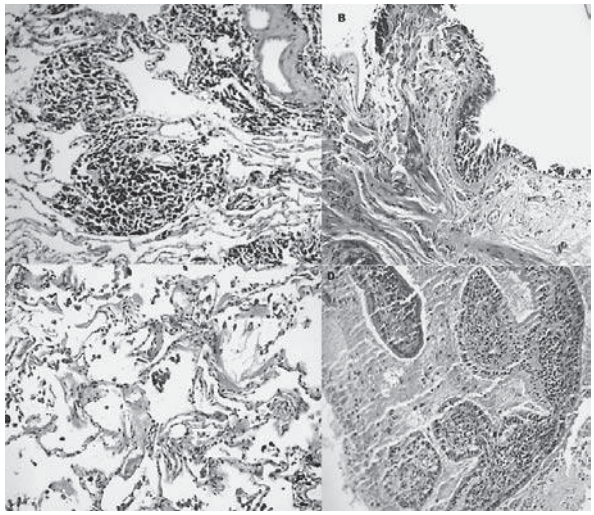

Figure 1 *Histology of representative tissue samples obtained by bronchoscopy. The following tissue samples are shown with H&E staining at 100x magnification except where noted otherwise: (A) Anthracosis in the lung parenchyma with vascular wall thickening; (B) Basal membrane thickening, eosinophilic enhancement in bronchial mucosa (200x); (C) Inflammatory focal thickening of alveolar septipara and lung parenchyma; (D) Dominated by lymphocytes, few neutrophils and plasma cells showing signs of chronic inflammation of the bronchial mucosa*

Transbronchial biopsy taken from 16 of 21 patients who were thought to have ILD. Bronchial lavage was performed on five patients. Cytological analysis showed no malignant cells in these patients. Neutrophils were the dominant cell type for many patients. In one patient, lymphocytes accounted for 70 % of the cells. Tissue samples stained with haematoxylin and eosin (H&E) were examined under a light microscope (Olympus BX50F–3, Olympus Optical Co., Japan) at either 100x or 200x magnification. Figure 1 shows the typical tissue samples obtained by bronchoscopy.

In the interalveolar septum, we observed perivascular and peribronchial neutrophils, lymphocytes, and alveolar macrophage infiltration. In some cases this cell infiltration let to the thickening of the interalveolar septa. In some patients, white blood cells infiltrated the spaces around vessels, the intima and the media of the vessels thickened, and their lumens narrowed. Black coal dust in the form of granular debris or little piles were scattered between the cells. Similar piles were found in the bronchioles on the apical surface of the epithelium and in the intracytoplasmic space. In one patient, nodular fibrotic thickening was observed in some places. Bilateral and many nodular lesions, more obvious in the sub-lobes, were detected by lung tomography in one patient, who

was thought to have ILD, but the bronchoscopic lavage histopathology did not support this diagnosis. However, a bulk was detected at the head of the pancreas in an abdomen tomography during examination of the patient, and the lung lesions were interpreted as metastasis.

Computer tomography detected a mass indicative of lung cancer in 14 patients, who then underwent bronchoscopy to confirm or rule out this preliminary diagnosis. The following histopathological types of lung cancer were detected: small cell in six patients, squamous cell in two patients, adenocarcinoma in two patients, and bronchoalveolar carcinoma in one patient. In two patients, the malignant epithelial tumour could not be determined by the cell type, and the tumour in one patient was evaluated as benign.

Chronic inflammation in a patient who complained of difficulty swallowing was followed up by endoscopy. Transbronchial biopsy revealed esophageal squamous carcinoma.

One of the four patients with mediastinal lymphadenopathy had right hilar lymphadenopathy and one had a consolidation at the right-mid lobe. The smear obtained by transcarinal thin needle aspiration biopsy did not show malignant cells. Patients diagnosed with sarcoidosis were introduced steroid treatment. All patients with lymphadenopathy showed a degree of regression due to treatment.

Tuberculosis was diagnosed in four patients. Mediastinal lymphadenopathy was established in one; a consolidation in the sub-lobe superior segment of the right lung in another; a consolidation in the sub-lobe of the right lung together with mediastinal lymphadenopathy in one patient; and a consolidation with fusion fields in the upper lobe of the right lung in one patient. Histopathological analysis showed a negative finding in the first patient, an active chronic inflammation in the second, chronic inflammation in the third, and anthracosis in the fourth. Tuberculosis culture of bronchial lavage tested positive and all four patients were started antituberculosis treatment.

DISCUSSION

Exposure to biomass in buildings without proper ventilation increases the risk of acute airway infections in children and COPD, ILD, and lung cancer in the adults (2 ,3 ,7 ,8). In Turkey, biomass smoke exposure is an important risk factor for lung diseases in non-smoking women, especially in rural areas. However, tobacco smoke and biomass smoke-related lung diseases have similar characteristics.

Biomass smoke contains carbon monoxide, nitric acid, sulphur oxides, formaldehyde, polycyclic organic substances, carcinogens like benzopyrene, and particles smaller than 10 micrometers (9). Most of these components are mutagenic *in vitro* (9). Smoke is an irritant toxic to cilia and contains coagulant agents which affect the respiratory system defence and increase the risk of chronic lung infection (10, 11). Domestically-acquired particulate lung disease (DAPLD) is a term used to identify biomass-related lung diseases (12). DAPLD manifests itself in a broad range of disorders from chronic bronchitis and COPD to advanced interstitial lung disease and malignancy.

Studies around the world have established a relationship between lung disease and exposure to biomass smoke. Pandey et al. (13) have shown that COPD prevalence rises with age and is greater Nepalese men than women (13). Akhtar et al. (9) identified chronic bronchitis in 100 of 1426 Pakistani women burning biomass and established a strong relationship between bronchitis and biomass use compared to a control group. Demirtaş et al. (2) found that women who exposed to biomass smoke were significantly more prone to COPD than unexposed women. Özbay et al. (1) detected a severe obstruction and an increase in the lung volume in pulmonary function tests of biomass-exposed women. They observed radiologically diffuse emphysema, thickening at interlobular septa, focal emphysematosa fields, and an increase in bronchovascular arborisation. They showed that biomass smoke increased obstructive and restrictive effects on the pulmonary function and structure. Our results seem to confirm the above studies, especially in reference to COPD. Sandaval et al. (14) have suggested that dense anthracotic staining is more characteristic of women exposed to biomass than of heavy tobacco smokers. Chung et al. (15) found anthracotic staining in people exposed to biomass smoke, in heavy smokers, miners, and city population. Bruce et al. (16) found that tuberculosis prevalence was higher in homes using biomass than in homes using cleaner combustibles. Similarly, Perez Podilla et al. (17) support the finding that heating with biomass at home contributes to tuberculosis. Shprykov et al. (18) found that tobacco smoke had a great effect on the structure of *Mycobacterium tuberculosis*. These structural changes suggest that smoke supports bacterial growth. In our study, in the four pulmonary tuberculosis diagnosed cases, dark anthracotic

pigmentation and chronic inflammation were detected that may have contributed to bacterial growth.

Biomass smoke contains numerous chemicals that are known or suspected human carcinogens. Mutations in human cancer are the most frequent in the p53 gene (19). Mutation in the p53 gene is found in 50 % of non-small cell lung cancers and in 90 % of small cell lung cancers (20).

Cigarette smoke contains almost 60 carcinogenic components including benz(a)pyrene (BaP) and nicotine-derived nitrosamine ketone (NNK), which induce p53 gene mutations (21). Wood smoke contains BaP, just like cigarette smoke (16). Delgado et al. (3) detected mutation in the p53 gene in lung cancer cases related to wood smoke and in cancers caused by cigarette smoke. They proposed that, just like cigarette smoke, wood smoke can play a role in lung cancer. Malatz et al. (22) found higher frequency of genetic polymorphism in glutathione S-transferase enzymes in non-smoking lung cancer patients who were exposed to wood smoke for more than 20 years. In the regions of China in which lung cancer is common in humans, Liang et al. (23) investigated exposure to coal and wood smoke in mice and rats and found that wood smoke exposure involved a higher risk of cancer than control, but a lower risk compared to coal smoke exposed animals. Consistent with all these studies, our study has shown that biomass smoke exposure is an important risk factor for human lung cancer

According to Gold et al. (24), cooking may produce high levels of BaP that equal a packs of cigarettes a day. In another study (16), BaP levels detected over three hours of cooking a day equalled two packs of cigarettes a day. These analogies help to explain how biomass smoke exposure can contribute to the growth of malignancy. Behera et al. (25) showed that adenocarcinoma was the dominant histopathological type of lung cancer in non-smoking women exposed to wood smoke. Similarly in Delgado et al. (3), the main histopathological type was adenosarcoma in patients exposed to wood smoke (3). Hoffman et al. (26) showed that nicotine-derived nitrosamine ketone (NNK), contributed to adenocarcinoma and BaP to squamous cell carcinoma in lab animals. It is thought that wood smoke, like cigarette smoke, contains BaP, but it may also contain NNK and other carcinogens. In our patients, small cell carcinoma was the primary type of lung cancer, possibly due to a specific composition of the biomass.

Kajdaz et al. (27) found a significant correlation between wood smoke exposure and sarcoidosis. In another study (28) wood burning and organic dust exposure had a significantly higher correlation with sarcoidosis. In our study, four patients were diagnosed with sarcoidosis.

Chronic biomass smoke exposure changes the defense mechanisms of the lung mucociliary system and decreases the antibacterial capacity of macrophage (15, 29). It may cause bronchial narrowing or blockage due to anthracosis or mucoid secretion piles at distal lung fields, and development of pneumonia at segments or lobes. Repeating infections increase bronchiectasis. Consistent with our study, Kara et al. (30) concluded that biomass smoke caused many diseases, from chronic bronchitis to interstitial lung disease.

Like in some of our patients, a transbronchial biopsy conducted in a patient exposed to wood smoke in India showed the existence of coal macules (31). The main pathological changes in the transbronchial tissue samples of patients exposed to inorganic dust were similar to other interstitial lung diseases (32-34). The samples showed focal thickening of the alveolar septum and a mix of inflammatory infiltration, fibrosis and basal membrane thickening. Restrepo et al. (35) found similar pathological fibrotis changes in animals and patients exposed to wood smoke for a long time. Ramage et al. (36) identified a similar relationship between interstitial lung disease and wood smoke exposure.

Table 2 *Histopathological features of patients (N=11) with interstitial lung disease*

Histopathology Feature	**Patients with feature n (%)**
Anthracotic pigment	7 (63)
Basal membrane thickening	6 (54)
Alveolar septal thickening	2 (18)
Goblet cell metaplasia	2 (18)
Fibrosis	1 (9)
Squamous metaplasia	0
Chronic inflammation	11 (100)

Our study has showed that exposure to biomass can cause histopathological lung lesions that range from the upper airway inflammation to interstitial fibrosis and malignancy. These histopathological changes provide evidence of specific diseases attributable to biomass smoke exposure in non-smoking women. One of the limitations of our study is that we have not established whether the exposed women were also exposed to secondary tobacco smoke.

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Sažetak

KRONIČNI UČINCI AMBIJENTALNOGA DIMA IZ BIOMASE NA HISTOPATOLOGIJU PLUĆA U LJUDI: OPIS NIZA SLUČAJEVA

Biomasa se u mnogim zemljama u razvoju rabi za gorivo. Čestice i plinovi izgorene biomase mogu dovesti do plućnih promjena. Ovdje smo istražili histološke promjene u plućima ljudi uzrokovane biomasom. Ovo je prospektivno ispitivanje obuhvatilo 42 nepušačice izložene dimu iz biomase u kojih je ocijenjena izloženost dimu, uzeta povijest bolesti, napravljen fizikalni pregled te analizirani radiološki, bronhoskopski i histopatološki nalazi. Srednja dob ispitanica bila je (59±10) godina. Srednja izloženost dimu iz biomase iznosila je (28±9) sati na godinu. Radiološki nalazi upozorili su na tumorsku tvorbu (u njih 42 %), retikulonodularna zasjenjenja (31 %), limfadenopatiju medijastinuma (26 %), fibrozne promjene pleure i plućnog parenhima (19 %), proširenje plućne arterije (14 %), sliku smrvljenog stakla (11 %), sliku mozaične perfuzije (9 %), konsolidacije (9 %), segmentnu i subsegmentnu atelektazu (7 %) te bronhijektazije (7 %). U njih 35 % dijagnosticiran je rak pluća, u 31 % bolesti plućnog intersticija, u 9 % sarkoidoza, u 9 % tuberkuloza, u 4 % KOPB, u 9 % kronični bronhitis te u 4 % metastaze. Bronhoskopija je pokazala pilije, edem, eritem, sužavanje bronhija, endobronhijalni tumor, neurednu sluznicu, izrazitiju vaskularizaciju, plavo-crne antrakotične plakove, otjecanje sluznice te purulentnu sekreciju. Transbronhijalnom biopsijom pronađeni su neutrofili i limfociti u perivaskularnom, peribronhiolarnom tkivu i interalveolarnim septumima, zadebljanje vezivnoga tkiva, zadebljanje bazalne membrane, zadebljanje interalveolarnih septuma, zadebljanja stijenke žila intimalno i medijalno te suženje njihova lumena, crna antrakoza između stanica te u epitelu bronhiola. Sve to upozorava na značajne promjene uzrokovane biomasom, koje obuhvaćaju zloćudne formacije uslijed toksičnoga djelovanja na plućni parenhim, intersticij i žile.

KLJUČNE RIJEČI: *izloženost dimu biomase, plućne bolesti*

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Short communication

CHROMOSOMAL INSTABILITY IN PERIPHERAL BLOOD LYMPHOCYTES OF PATIENTS WITH REPRODUCTIVE FAILURE ASSESSED BY MICRONUCLEUS ASSAY

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We investigated chromosomal instability in peripheral blood lymphocytes (PBL) of patients with reproductive failure in respect to age, smoking habits, gender, miscarriages, and semen parameters. The study involved 36 individual cases of reproductive failure (18 men and 18 women) attended at the Clinical Centre of Kragujevac, Serbia, and 30 healthy subjects (15 men and 15 women). Micronuclei (MN) frequency was estimated in PBL using the cytokinesis-block micronucleus (CBMN) assay. The baseline MN frequencies were significantly higher ($p=0.031$; $p<0.001$) in male [(9.22 ± 4.70) MN per 1000 BN cells] and female patients [(13.50 ± 2.5) MN per 1000 BN cells] than in male and female healthy controls [(6.27 ± 2.66) MN per 1000 BN cells; (6.80 ± 2.98) MN per 1000 BN cells]. The mean baseline MN frequency did not significantly differ between miscarriage groups and between patients with and without normal values of semen parameters. The correlations between poor sperm concentration ($<20x10^6$ mL^{-1}), rapid progressive motility (<25 %), normal morphology (<30 %), and MN frequencies were negative, but not statistically significant. We found that only gender significantly influenced the MN rates in analysed patients. There were no significant differences between age groups and between smokers and non-smokers in patients and control samples. We conclude that the increase in baseline MN frequency in PBL of patients with reproductive failure corresponds to the increase in chromosomal damage, which occurs as a result of complex events that cause reproductive disorders.

KEY WORDS: *age, gender, infertility, micronuclei, semen quality, smoking*

Infertility is defined as the inability to conceive after twelve months of regular unprotected sexual intercourse. It may be related to a variety of genetic (1, 2), as well as nongenetic factors (3-5).

Previous studies confirmed higher frequency of chromosomal abnormalities in the sperm of men with abnormal semen parameters (6, 7). Chromosomal abnormalities were also found in peripheral blood lymphocytes of infertile men (8, 9). Two comet assay studies (10, 11) reported a significantly higher level of DNA strand breaks in the sperm of infertile men compared to the controls. Duzcan et al. (12) suggested a greater incidence of sex aneuploidy in somatic cells of oligozoospermic men.

Female infertility is also associated with genomic instability. High incidence of genomic instability in lymphocytes of women with polycystic

ovary syndrome was revealed in the studies of Yesilada et al. (13) and Moran et al. (14). Moreover, couples with a history of spontaneous abortions and idiopathic infertility tend to have an increased micronuclei frequency in lymphocytes (15).

Micronuclei (MN) are defined as small, round nuclei clearly separated from the main cell nucleus which forms from acentric chromosome fragments or whole chromosome(s) during cell division. The frequency of MN as an index of chromosomal damage and genome instability is widely used for evaluating the genotoxic impact of demographic, habitual (16), occupational, and environmental factors (17, 18). Moreover, Bonassi et al. (19) consider that MN frequency may serve for predicting individual cancer risk.

Assuming that MN persistence in cells reflects chromosomal instability, the objective of this study was to evaluate spontaneous chromosomal damages in peripheral blood lymphocytes (PBL) of patients with reproductive failure and healthy controls by using cytokinesis-block micronucleus assay (CBMN) in respect of the factors that may affect MN frequency (i.e. age, smoking habits, gender, miscarriages, and semen parameters).

MATERIALS AND METHODS

Study populations

This study has been approved by the Ethics Committee of the Clinic of Kragujevac (No 01-4886). It included 36 individual cases of reproductive failure (18 men and 18 women) refereed to the Clinical Centre of Kragujevac, Serbia. All participants were informed about the aim of the study and completed a standardised questionnaire that included standard demographic, medical, lifestyle, and occupational questions.

The mean age of male patients was (35.22±6.68) years (range from 27 to 48 years). All of them underwent sperm analysis and three standard sperm parameters (sperm concentration, rapid progressive motility, and morphology) were determined. Numbers of spermatozoa per unit volume refers to the sperm concentration. Actively moving spermatozoa, linearly or in large circles, were scored for determining the progressive motility, while spermatozoa without malformations (head, neck, and tail defects) were scored for estimating the proportion of spermatozoa with normal morphology. Sperm concentration ≥20 $x10^6$ mL^{-1}, spermatozoa with rapid progressive motility ≥25 %, and normal sperm morphology ≥30 % were referred to as normal values. The samples of five patients revealed azoospermia, four were diagnosed with abnormalities only in one parameter, two in two parameters, and three in all three major parameters. Four men from couples with idiopathic infertility were assigned to the infertile group despite normal sperm parameter values.

All male patients kept standard dietary habits. Nine of them were smokers.

Mean age of female patients was (31.67±3.50) years (range from 24 to 37 years). Eleven of them were smokers and only one patient kept diet. Seven women had a history of miscarriages (ranged from 1 to 2).

The control sample included 15 age-matched healthy fertile male donors aging from 27 to 50 years [mean age (38.27±7.76) years], and 15 age-matched healthy fertile female donors, aging from 24 to 36 years [mean age (32.4±2.99) years] without any recent exposure to known mutagenic agents. Fifteen of them (7 men and 8 women) were smokers.

Cytokinesis – block micronucleus test (CBMN)

We applied the CBMN test, as proposed by Fenech (20), for the analysis of MN frequency in peripheral blood lymphocytes. Heparinised whole blood was cultivated in the complete medium (PBMax Karyotyping, Invitrogen, USA) for a total of 72 h at 37 ºC. Cytochalasin B (Sigma-Aldrich, USA), at a final concentration of 4 μg mL^{-1}, was added to cultures after 44 h of incubation. Using the standard procedure, cells were harvested to prepare microscopic slides that were then stained in the 2 % Giemsa solution (Alfapanon, Novi Sad, Serbia). The MN frequencies were determined by analysing 1000 BN cells per person following the standard criteria for scoring MN in BN cells as described by Fenech (21).

Statistics

Data were presented as mean ± standard deviation (S.D.). The differences between baseline MN frequencies in lymphocytes in male patients and healthy men, healthy women and healthy men, among male samples, and among healthy female samples

were compared using the Student′s t-test. The differences between MN frequencies of female patients and female controls, male and female patients, and among female patients were determined using Mann-Whitney U test. The relationship between age, smoking status, and gender and MN frequency in both patients and control samples was determined by multiple linear regression analysis. The relationship between poor semen parameters and MN frequency was determined by Pearson′s correlation. Level of significance was $p<0.05$.

RESULTS

Results of the CBMN assay in PBL of patients with reproductive failure and PBL of healthy controls are shown in Tables 1-5.

We found significantly higher baseline MN frequencies ($p<0.001$) in female patients [(13.50±2.50) MN per 1000 BN cells] in comparison with healthy female controls [(6.80±2.98) MN per 1000 BN cells]. The mean MN frequency in male patients was significantly higher ($p = 0.031$) than in control healthy men [(9.22±4.70) MN per 1000 BN cells vs. (6.27±2.66) MN per 1000 BN cells]. In all samples gender affected MN rates, but only in patients was the sample statistically significant ($p=0.011$).

In all analysed samples, BN cells with 1 MN were most frequently present. Cells with 2 MN were less common, while BN cells with 3 MN were found only in the patient sample (Table 3).

The mean MN frequency did not differ between smokers and non-smokers both in male patients and healthy men. Age had also no effect on the MN frequency in both analysed samples of men - patients and healthy controls.

Male patients were grouped according to their values for each analysed parameter. Statistical analyses for each analysed parameter (sperm concentration, rapid progressive motility, morphology) did not show any significant difference between the groups of

Table 1 *General characteristics, micronuclei frequency and MN distribution in male patients with reproductive failure*

No	Age / year	Smoking status (+/-)	Occupation	Semen parameters			MN per1000 BN cells	Distribution of MN		
				Sperm concentration / $x10^6$ mL^{-1}	Rapid progressive motility / %	Normal morphology / %		1 MN	2 MN	3 MN
1.	32	-	worker	5.50	37	22	3	3	0	0
2.	44	+	lawyer	0	0	0	13	11	1	0
3.	30	+	policeman	0	0	0	9	9	0	0
4.	35	-	engineer	51.37	38	26	9	7	1	0
5.	48	+	car mechanic	1.10	9	19	6	6	0	0
6.	48	-	instructor	58.9	34	35	17	14	0	1
7.	41	-	seller	0	0	0	4	2	1	0
8.	29	+	farmer	89.50	39	45	9	9	0	0
9.	29	+	doctor	5.90	7	28	6	6	0	0
10.	28	-	seller	0	0	0	16	13	0	1
11.	40	+	worker	27.6	30	48	12	10	1	0
12.	31	+	driver/ technician	8.58	28	32	1	1	0	0
13.	38	-	economist	50.25	21	45	7	7	0	0
14.	36	+	locksmith	6.10	35	22	14	12	1	0
15.	36	-	doctor	0	0	0	14	8	3	0
16.	32	-	engineer	141.25	25	41	9	9	0	0
17	27	+	driver	43.00	5	53	13	9	2	0
18.	30	-	seller	13.875	18	24	4	4	0	0
Mean ± S.D.	35.22 ± 6.68			27.94 ± 38.75	18.11 ± 15.41	24.44 ± 18.31	9.22 ± 4.70	7.78 ± 3.67	0.56 ± 0.86	0.11 ± 0.32

MN - micronuclei, BN - binucleated

Table 2 *General characteristics, micronuclei frequency and MN distribution in female patients with reproductive failure*

No	Age / year	Smoking status (+/-)	Dietary status (+/-)	Occupation	Miscarriages	MN per 1000 BN cells	Distribution of MN		
							1 MN	2 MN	3 MN
1.	31	-	-	engineer	0	21	19	1	0
2.	35	+	-	technician	0	17	12	1	1
3.	36	+	-	technician	1	12	12	0	0
4.	32	+	-	cook	0	13	13	0	0
5.	34	-	-	nurse	0	15	13	1	0
6.	33	-	-	doctor	2	16	16	0	0
7.	31	+	-	technician	2	12	12	0	0
8.	37	-	-	nurse	0	14	12	1	0
9.	29	+	-	locksmith	0	11	9	1	0
10.	25	+	-	worker	0	12	10	1	0
11.	30	+	-	worker	0	13	13	0	0
12.	28	-	-	seller	2	14	12	1	0
13.	33	+	-	cleaner	0	13	11	1	0
14.	33	-	+	seller	1	12	12	0	0
15.	32	+	-	technician	1	11	11	0	0
16.	24	+	-	worker	0	13	11	1	0
17.	35	+	-	housewife	1	13	13	0	0
18.	32	-	-	seller	0	11	11	0	0
Mean ±S.D.	31.67± 3.50					13.50±2.50	12.33± 2.22	0.5± 0.51	0.05± 0.23

MN - micronuclei, BN - binucleated

Table 3 *Micronuclei frequency and MN distribution in analysed patients and healthy controls*

	No.	Age / years Mean±S.D. (range)	No. of analysed cells	MN per 1000 BN cells Mean±S.D. (range)	No. of BN cell with MN / %	Distribution of MN (%)		
						1 MN No. (%)	2 MN No. (%)	3 MN No. (%)
Controls	30							
Male	15	38.27±7.76 (27 to 50)	15 000	6.27±2.66 (2 to 10)	92 (0.61)	90 (0.60)	2 (0.01)	0 (0.0)
Female	15	32.4±2.99 (24 to 36)	15 000	6.80±2.98 (1 to 11)[a]	102 (0.68)	102 (0.68)	0 (0.0)	0 (0.0)
Patients	36							
Male	18	35.22±6.68 (27 to 48)	18 000	9.22±4.70 (1 to 17)[b]	152 (0.84)	140 (0.78)	10 (0.05)	2 (0.01)
Female	18	31.67±3.50 (24 to 37)	18 000	13.50±2.5 (11 to 21)[c,d]	232 (1.29)	222 (1.23)	9 (0.05)	1 (0.01)

[a] *no statistically significant difference in MN frequencies between healthy women and healthy men, p=0.609 (Student's t- test)*
[b] *statistically significant difference in MN frequencies between male patients and male controls, p=0.031 (Student's t- test)*
[c] *statistically significant difference in MN frequencies between female patients and female controls, p<0.001 (Mann-Whitney U test)*
[d] *statistically significant difference in MN frequencies between female patients and male patients, p=0.011 (Mann-Whitney U test)*
MN - micronuclei, BN – binucleated

Table 4 *Micronuclei frequencies in male patients and healthy male controls with regard to demographic, lifestyle and medical characteristics*

Groups (number)	MN per 1000 BN cells Mean±SD (range)	p value
Patients [a]		
Smoking status		
Smoker (9)	9.22±4.29 (1 to 14)	1.00
Non-smoker (9)	9.22±5.33 (3 to 17)	
Age / years		
27 to 35 (10)	7.90±4.56 (1 to 16)	0.190
36 to 48 (8)	10.87±4.61 (4 to 17)	
Semen parameters		
Sperm concentration / $x10^6$ mL^{-1}		
<20 (11)	8.18±5.25 (1 to 16)	0.250
≥20 (7)	10. 86±3.39 (7 to 17)	
Rapid progressive motile spermatozoa (grade a)		
<25 % (10)	9.20±4.44 (4 - 16)	0.983
≥25 % (8)	9.25±5.31 (1 - 17)	
Normal morphology		
<30 % (11)	8.91±4.68 (3 to 16)	0.734
≥30 % (7)	9.71±5.06 (1 to 17)	
Healthy controls [a]		
Smoking status		
Smoker (7)	6.00±2.31 (3 to 9)	0.731
Non-smoker (8)	6.50±3.07 (2 to 10)	
Age / years		
27 to 39 (9)	6.55±3.13 (2 to 10)	0.624
40 to 50 (6)	5.83±1.94 (3 to 8)	

[a] *MN frequencies between groups were compared by Student's t-test*
MN - micronuclei, BN - binucleated

patients with normal values of semen parameters and those who had lower values (Table 4).

The mean MN frequency did not differ between non-smokers and smokers in both female patients and female healthy controls. In both analysed samples of women, patients and controls, age had no effect on the MN values. The MN frequency between female patients with and without miscarriage history was not significantly different (Table 5).

Multiple linear regression analyses showed that among the analysed confounding factors (i.e., age, smoking habits, and gender), only gender had a significant effect on the MN values in the analysed sample of patients (p=0.001). Neither of the analysed factors affected MN frequency in the analysed healthy control sample.

The result of Pearson′s correlation analyses showed a negative correlation between poor values of sperm concentration (<$20x10^6$ mL^{-1}), rapid progressive motility (<25 %), normal morphology (<30 %) and MN frequencies but without statistical significance (r=-0.524, p=0.098; r=-0.545, p=0.103; r=-0.463, p=0.152).

DISCUSSION

Chromosomal instability can be defined as an increased rate of numerical and structural chromosomal changes during cell division. Different human health problems are associated with chromosomal instability in lymphocytes (22–24). Today, cytogenetics provides numerous different biomarkers for evaluating chromosomal instability and scoring of MN in lymphocytes is one that draws a lot of interest.

Our results show that mean MN frequencies in both male and female patients increased in comparison with mean MN frequencies of healthy male and female controls. The approximately 1.5-

Table 5 *Micronuclei frequencies in female patients and healthy female controls with regard to demographic, lifestyle and medical characteristics*

Groups (number)	MN per 1000 BN cells Mean±SD (range)	p value
Patients [a]		
Smoking status		
Smoker (11)	12.73±1.62 (11 to 17)	0.117
Non-smoker (7)	14.71±3.25 (11 to 21)	
Age / years		
24 to 31 (7)	13.71±3.35 (11 to 21)	0.854
32 to 37 (11)	13.36±1.96 (11 to 17)	
History of miscarriages		
Yes (7)	12.86±1.68 (11 to 16)	0.433
No (11)	13.91±2.91 (11 to 21)	
Healthy controls [b]		
Smoking status		
Smoker (8)	6.25±3.24 (1 to 11)	0.465
Non-smoker (7)	7.43±2.76 (4 to 11)	
Age / years		
24 to 32 (6)	6.83±2.99 (3 to 11)	0.973
33 to 36 (9)	6.78±3.15 (1 to 11)	

MN – micronuclei, BN - binucleated
[a] MN frequencies between groups were compared by Mann-Whitney U test
[b] MN frequencies between groups were compared by Student's t- test

fold MN increase in male patients and more than a two-fold MN increase in female patients indicated greater chromosomal damages in PBL of patients with reproductive failure. Recently, the association between MN frequency in PBL and impaired reproductive history has been reviewed by Fenech (25).

Similar, De Palma et al. (26) demonstrated an increase in sex chromosome aneuploidy rate in peripheral leukocytes of patients with impaired spermatogenesis and suggested that they had generalised defective cell division mechanism. Higher MN frequency in analysed patients with reproductive failure may be explained by abnormality in the mechanism that controls division.

Increased MN frequencies in patients may also be the consequences of oxidative stress (OS), which has been considered a contributing factor to infertility. The events that lead to MN formation in the cells may be induced by OS (19).

In our study, we obtained a great inter-individual variation in MN frequencies. Age, smoking, gender, dietary habits, and individual susceptibility to environmental or occupational mutagens are factors that account for the MN frequency variation. According to Fenech (27), age is an important demographic contributing factor. The same age effect on MN was reported in the studies of Ishikawa et al. (16) and Mahrous (28). In our study, age had no effect on MN frequency in both healthy subjects′ and patients′ samples. Trkova et al. (15) explained the lack of significant correlation between age and MN frequency by age homogeneity of tested samples. Besides this explanation, the lack of effect of age on MN frequency in analysed samples might be due to a small range of years. However, these results have to be confirmed in larger samples primarily designed to evaluate the effect of age.

Gender, as one of the contributing factors, was analysed in many studies. In most of them, MN frequencies have been reported to be higher in women than in men (27, 29); generally by 1.2 to 1.6 times (27). This appearance could be explained by over-prevalence of X chromosome in female MN (30). Although in our study, women had higher MN frequency in both healthy control and patient samples, a significant difference in MN frequencies was evident only in the patient sample. Women had approximately 1.5-fold higher MN frequency than analysed male patients.

In addition to age and gender, smoking habits may modulate MN frequency. Bonassi et al. (31) reported a significant effect of smoking on MN frequency only in the heavy-smoker group. In the present study, we did not find a statistically significant difference in MN frequency between smokers and non-smokers. No association between smoking habit and MN frequency was also observed in the study of Hessel et al. (32) and Costa et al. (33). It is possible that exposure to genotoxic substances from cigarette smoke induces serious impairments of cell division or cell death. However, negative results of CBMN assay in smokers might be explained by the "escape" of highly damaged cells from scoring. Contrary to these, micronucleated cells with one MN and less genome damages have a better chance of surviving (34). Thus, Bonassi et al. (31) explain that if damaged cells do not divide, they will not form binucleated cells, so these will not be scored for MN. Furthermore, adaptive response is often mentioned as a possible reason for lower MN frequency in the PBL of light - medium smokers (31, 35).

With regard to miscarriage history, in the analysed patient sample, women with a history of miscarriages had the same level of chromosomal damages in PBL as women with no conception in reproductive history. According to the study of Milošević-Djordjević et al. (36) and an earlier study of Grujičić et al. (37), women with threaded miscarriages had an increased MN frequency in lymphocytes and this could be the explanation for MN similarity between analysed women.

In our study, male patients with poor semen parameters did not have a significantly lower MN frequency in PBL compared to patients with normal values of semen parameters. As in smokers, lower MN frequencies in these patients might be explained by greater genome damages in their lymphocytes that escaped from scoring.

Although we did not find a statistically significant association between the levels of chromosomal instability (presented in the form of MN) and poor semen quality, negative correlation between these two parameters points to a tendency of higher chromosomal damages in PBL of infertile men.

CONCLUSION

From the present study it is evident that an increase in the baseline MN frequency in PBL of untreated men and women with reproductive failure corresponds to the increase in chromosomal damage, which occurs as a result of complex events that cause reproductive disorders.

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Sažetak

PROCJENA KROMOSOMSKE NESTABILNOSTI U LIMFOCITIMA PERIFERNE KRVI BOLESNIKA S POREMEĆAJIMA REPRODUKTIVNOG SUSTAVA POMOĆU MIKRONUKLEUS-TESTA

Ispitivali smo kromosomsku nestabilnost u limfocitima periferne krvi (engl. *peripheral blood lymphocytes* - PBL) bolesnika s poremećajima reproduktivnog sustava u odnosu na parametre dobi, navike pušenja, spola, spontanih pobačaja i kvalitete sjemena. Ispitivanje je uključivalo 36 pojedinačnih ispitanika s poremećajima reproduktivnog sustava (18 muškaraca i 18 žena) u Kliničkom centru u Kragujevcu, Srbiji, te 30 zdravih ispitanika (15 muškaraca i 15 žena). Učestalost pojave mikronukleusa (MN) utvrđena je u PBL-ima primjenom mikronukleus-testa s tehnikom blokirane citokineze (engl. *cytokinesis-block micronucleus* - CBMN). Učestalosti MN bile su značajno povišene ($p=0,031$; $p<0,001$) kod muških [(9,22±4,70) MN na 1000 BN stanica] i ženskih bolesnika [(13,50±2,5) MN na 1000 BN stanica] u odnosu na osnovne vrijednosti utvrđene u muških i ženskih zdravih kontrolnih ispitanika [(6,27±2,66) MN na 1000 BN stanica; (6,80±2,98) MN na 1000 BN stanica]. Prosječna se učestalost MN nije značajno razlikovala među skupinama kod kojih je došlo do spontanih pobačaja te među skupinama koje su imale normalne vrijednosti parametara kvalitete sjemena i onih koje nisu imale takve vrijednosti. Korelacije između niske koncentracije spermija ($<20x10^6$ mL^{-1}), smanjene pokretljivosti spermija (<25 %), normalne morfologije (<30 %) i učestalosti MN bile su negativne, ali ne i statistički značajne. Utvrdili smo da je samo spol značajno utjecao na pojavnost MN u svih ispitanih bolesnika. Nije bilo značajnih razlika između dobnih skupina, kao ni između pušača i nepušača kod bolesnika i kontrolnih ispitanika. Zaključujemo da pojavnost MN u limfocitima bolesnika s poremećajima reproduktivnog sustava prati porast razine kromosomskih oštećenja koja nastaju kao posljedica složenih događaja koji uzrokuju poremećaje reproduktivnog sustava.

KLJUČNE RIJEČI*: dob, kvaliteta sjemena, spol, mikronukleusi, neplodnost, pušenje*

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Case Report

MONOCROTOPHOS POISONING THROUGH CONTAMINATED MILLET FLOUR

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Several episodes of mass poisoning by organophosphates (OPs) have been reported from the developing countries. The diagnosis of OP-poisoning is mainly based on the characteristic clinical features and history of exposure to a known OP compound. Estimation of serum and red blood cell (RBC) cholinesterase activities are helpful in confirming the diagnosis. However, there is controversy regarding a definite relationship between serum cholinesterase activity and the severity of clinical manifestations and prognosis. This report describes an episode of mass monocrotophos poisoning that occurred due to accidental ingestion of monocrotophos-contaminated millet (so called *bavta*) flour involving eight severely poisoned persons. Clinical presentation included severe abdominal pain, diarrhoea, vomiting, pupil narrowing, and difficulty breathing. On hospital admission, plasma cholinesterase (PChE) and especially RBC acetylcholinesterase (AChE) activities correlated well with clinical symptoms presented by the patients. This case study highlights the need for clinicians to be aware of OP-pesticide poisoning from food sources and the need to look for depressed PChE and AChE activities that may point to OP exposure, so that OP-poisoning can be identified immediately and patients can receive specific treatment, rather than general treatment for food poisoning.

KEY WORDS: *abdominal pain, acetylcholinesterase, acute OP poisoning, blood cholinesterase, diarrhoea, difficulty breathing, organophosphate pesticides, plasma cholinesterase, pupil narrowing, vomiting*

Organophosphate (OP) pesticides continue to be the most common type of pesticides involved in acute poisoning in countries like India and Sri Lanka (1, 2). A large number of them, including monocrotophos, are registered for use in India. Despite structural differences, the mechanism by which they elicit their toxicity is identical and is associated with the inhibition of the nervous tissue acetyl cholinesterase. Morbidity and mortality from OP pesticides remains especially high in rural settings where facilities for intensive care are either absent or very limited. The World Health Organization (WHO) has estimated that each year more than 200,000 people in the world die from pesticide poisoning (3). Most of the poisonings occur in Asia and at least 50 % are OP-related (1, 3). Episodes of mass poisoning due to OP pesticides have been reported from developing countries like Pakistan and India (4, 5); the first from India was reported in 1958 and occurred following the consumption of parathion-contaminated wheat, killing more than 100 people (6).

In the past five years, several episodes of mass poisoning by different pesticides have been reported to the Poison Information Centre (PIC) of the National Institute of Occupational Health (NIOH) in Ahmedabad; most notably endosulfan (7), phorate and ethion (8) poisonings. It has been observed that OP poisoning from contaminated food ingestion is all too often treated empirically for food poisoning instead of specific treatment. In this paper, we outline the

medical history of patients involved in OP poisoning with details of the investigations conducted to ascertain the involvement of monocrotophos.

CASE HISTORY

This episode of mass poisoning occurred at Deva village about 60 km from Ahmedabad following a meal containing breads made from contaminated *bavta* (a variety of millet) flour. Estimated average consumption of the flour (based on number of breads eaten and estimated amount of flour required for preparing a bread) was 107 g in children and 200 g in adults. The affected people were initially admitted to the civil hospital in Nadiad and then to the civil hospital in Ahmedabad. The site of the incident and the hospitals were visited and the information was collected from the relatives, neighbours and the medical personnel who treated the victims. In addition, routine tests were run at the civil hospital in Ahmedabad, including haematology and urine analysis, random blood sugar, liver and renal function tests, arterial blood gases, chest X-rays, ECG, blood pressure (by sphygmomanometry), and respiratory and heart rate. Plasma cholinesterase (PChE) and red blood cell (RBC) acetylcholinesterase (AChE) activities were measured at the PIC. For the study, ethical clearance was obtained from the Ahmedabad-civil hospital. A written consent in local language was obtained from the patients, and they were informed of the importance of the study.

For cholinesterase estimations, two to three millilitres of blood was collected from the antecubital vein into vacutainer tubes containing EDTA, and blood samples taken to the PIC laboratory within half an hour. PChE and AChE activities were determined by spectrophotometry using a modified Ellman's method with acetyl thiocholine as substrate (9). To 1.5 mL phosphate buffer (pH 7.2, 52 mmol L^{-1}) containing 0.26 mmol L^{-1} 5,5'-dithiobis-(2-nitrobenzoic acid), 10 µL of sample was added and the reaction was started by adding 50 µL of 156 mmol L^{-1} acetyl thiocholine. The rate of change in optical density was recorded at 405 nm on RA-50 Chemistry Analyzer (Technicon) at 37 °C. Blood sugar, serum creatinine, blood urea, and serum bilirubin were analysed using standard commercial kits (Ranbaxy Diagnostics, India).

For identification of the OP pesticide, a sample of *bavta* flour was collected and stored at -20 °C in an airtight glass bottle protected from light. Information on the approximate amount of flour (bread) consumed by the individuals was also collected to estimate the amount of the pesticide ingested. The flour sample was analyzed for identification and quantification of the organophosphate pesticide using gas chromatography with a nitrogen phosphorus detector (GC-NPD, TRACE GC Ultra System, Thermo Finnigan, USA) (10). We added 50 g of flour from the sample to a 100 mL mixture of acetone and water (65:35), and shook for 3 min using a vertical blender with a homogeniser at high speed. The extract was filtered through a Buchner funnel using moderate vacuum. From the obtained filtrates, we transferred an aliquot of 40 mL, representing 20 g of the sample, to a separating funnel and partitioned liquid using a mixture of *n*-hexane and methylene chloride (1:1). The extract thus collected was evaporated to about 1 mL under a vacuum rotary evaporator. The evaporation was repeated thrice in the presence of *n*-hexane to remove the traces of methylene chloride. The final volume of the extract was made with *n*-hexane/acetone (9:1) mixture for the GC-NPD analysis. Monocrotophos (AccuStandard) was used as standard. Acetyl thiocholine and 5,5'-dithiobis-(2-nitrobenzoic acid) were from Sigma-Aldrich, USA. All other chemicals used were of analytical grade.

CASE REPORT

Patients

On 6 October 2006 around noon eight persons (five adults and three children) from two families ate a meal of *bavta* bread and a vegetable. Within two to three hours, all started complaining of severe abdominal pain, difficulty breathing, diarrhoea, and vomiting. After hospitalisation, all manifested narrowed pupils and some had frothing at the mouth. Three (all children) lost consciousness within three hours of the incident. On the same day, seven of the affected people were taken to the civil hospital in Nadiad, where they were treated for food poisoning and received intravenous (*i.v.*) fluids and antibiotics, while one adult who had mild effects got primary treatment at a nearby private hospital. Two of the children regained consciousness after five to six hours of treatment, while one child did not regain consciousness and developed respiratory distress. He was intubated and

given bag tube ventilation. The three children and the four adults were transferred to the civil hospital in Ahmedabad on the second and the third day, respectively. The PIC, NIOH was contacted for blood analysis, and it confirmed involvement of an organophosphate pesticide by establishing lower blood cholinesterase activity levels. All patients were treated for OP poisoning with atropine (*i.v.*) and 2-pralidoxime (*i.v.*); children received 9 mg to 12 mg and adults 36 mg to 50 mg of atropine over four to five days; 2-pralidoxime was administered in the dose of 2 g to 4 g to children and 10 g to 16 g to adults over three days. The child who had respiratory distress died at 5.00 am on the third day, 53 hours after poisoning.

Analysis

The data on the clinical status, outcome, and blood cholinesterase activities of seven patients are shown in Table 1. All had significantly lower activities of PChE and AChE compared to reference intervals of 2900 U L^{-1} to 5800 U L^{-1} and 1700 U L^{-1} to 2300 U L^{-1}, respectively (8). The children (Patient 1, 2, and 3) were drowsy at the time hospitalisation and had very low activities of AChE, ranging from 0 U L^{-1} to 61 U L^{-1}. One child (patient 1), who had narrowed, pinpoint pupils and the lowest PChE and AChE activities, died while the rest survived. Patients 4, 5, 6, and 7 showed comparatively higher activities of PChE and AChE. They were conscious at the time of

Table 1 *Patient data at the time of hospital admission*

Parameter	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Sex	Male	Male	Male	Male	Female	Female	Female
Age / years	7	5	8	48	45	30	26
Body weight / kg	17	15	22	42	50	47	62
Level of conscioness	Drowsy	Drowsy	Drowsy	Conscious	Conscious	Conscious	Conscious
*PChE / UL^{-1}	81	126	401	874	945	675	520
**AChE / UL^{-1}	0	61	0	640	493	652	468
AChE / PChE	0	0.48	0	0.73	0.52	0.97	0.90
Routine investigations ***	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Pupils	Constricted, pinpoint	Semi-constricted, not pinpoint	Constricted, not pinpoint	Semi-constricted, not pinpoint	Semi-constricted, not pinpoint	Semi-constricted, not pinpoint	Semi-constricted, not pinpoint
Other symptoms	Tachycardia, stupor, no fever	Fever	Fever	No fever	Bradycardia, no fever	No fever	No fever
Blood pressure syst/diast / mm Hg	110/75	100/70	108/74	114/70	110/70	110/70	116/78
Heart rate / bpm	148	90	90	64	55	80	98
Respiratory rate / bpm	36	32	52	20	22	22	24

** Normal range (2900 to 5800) U L^{-1}*
*** Normal range (1700 to 2300) U L^{-1}*
**** Includes routine urine analysis, arterial blood gases, chest X-ray, and ECG*
PChE – plasma cholinesterase
AChE - acetylcholinesterase

Table 2 *Patient data - other routine haematological and biochemical findings*

Parameter	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Blood haemoglobin / g dL^{-1}	11.6	11.1	11.7	11.0	11.5	10.9	11.9
Total WBC count	12,100	19,200	10,800	10,200	9,100	10,600	9,100
Differential WBC count (N/L/M/E/B)	55/38/04/03/00	50/46/02/02/00	56/40/02/02/00	59/35/03/02/01	53/31/04/02/00	51/33/04/02/00	60/34/04/01/01
SpO_2 / %	96	98	98	-	-	-	-
Random blood sugar * / mg dL^{-1}	128	118	110	132	110	115	120
Blood urea ** / mg dL^{-1}	26.1	22.2	24.4	30.1	28.0	24.2	22.0
Serum creatinine *** / mg dL^{-1}	0.62	0.44	0.72	0.87	0.84	0.72	0.83
Serum total bilirubin **** / mg dL^{-1}	0.67	0.70	0.82	1.22	1.10	0.78	0.52
Complications developed	Respiratory failure	No	No	No	No	No	No
Outcome	Died after 53 h	Complete recovery	Complete recovery	Complete recovery	Complete recovery	Complete recovery	Complete recovery

* *Normal range <130 mg dL^{-1}*
** *Normal range (15 to 40) mg dL^{-1}*
*** *Normal range (0.5 to 1.40) mg dL^{-1}*
**** *Normal range (0.50 to 1.10) mg dL^{-1}*

hospitalisation and did not develop any complications. The ratios of AChE to PChE activity varied from 0 to 0.97 (Table 1) and did not correlate with the clinical picture at the time of admission or with patient outcome. Cholinesterase activities, especially AChE, correlated well with the clinical picture of the patients on the day of admission. The activities of blood urea, serum creatinine and total bilirubin were within normal range (Table 2). The analysis of the *bavta* flour revealed very high amounts of monocrotophos (938 mg kg^{-1}). Estimated ingestion of the pesticide by the subjects ranged from 75 mg to 225 mg. The child who died after respiratory distress was estimated to have ingested about 113 mg of the pesticide.

DISCUSSION

Monocrotophos is a highly toxic OP pesticide belonging to the WHO class 1b. Approximately 6000 tons of this pesticide are used annually in India. It is a small (molar mass: 223.2 g mol^{-1}), water-soluble molecule that can be absorbed rapidly by the oral route. The time course of the described events leading to severe toxicity within a few hours points toward quick absorption of this pesticide. Quick absorption of and delayed treatment seem to be the main culprits for very high morbidity observed in this episode.

Symptoms and signs of OP poisoning vary with the age of the affected person. Young children present with altered levels of consciousness, rather than with the classic DUMBELS (diarrhoea/diaphoresis, urination, miosis, bradycardia/bronchospasm, emesis, lacrimation, salivation) signs, which are common in adults. After retrospective examination of 36 children aged two to eight years, Lifshitz et al. (11) observed a decreased level of consciousness including coma, stupor and hypotonicity upon exposure to organophosphates or carbamates in Israel. Similarly, all the three children (aged five to eight years) in our report were drowsy at the time of hospital admission. The child who presented with narrowed pupils and the lowest PChE and AChE activities died after the development of respiratory distress. It is unclear whether this was due to a larger exposure or greater sensitivity. Four adults showed comparatively higher

activities of PChE and AChE and were all conscious at the time of admission and did not develop any complications. This suggests that cholinesterase activities, especially AChE activities, correlate well with the clinical picture of the patient, which is in line with our earlier observations about acute ethion (OP pesticide) poisoning (8). Even though we know that the poisoning was due to ingestion of contaminated food, we did not establish the exact reason, but it was most probably unintentional.

Pesticides are known to affect lipid, protein, and carbohydrate metabolism. OPs increase Ach, which contributes to the secretion of insulin and glucagon by activating protein kinase C and by increasing the efficiency of free cytosolic calcium on exocytosis of insulin granules. Oral administration of 1/10 LD_{50} of monocrotophos caused reversible hyperglycaemia in rats, peaking 2 h following administration, accompanied by significant inhibition of acetylcholinesterase (AChE) activity. At 4 h following administration, glucose was back to normal (12). Similarly, feeding broiler chicks with poultry mash containing 2 mg kg^{-1} of monocrotophos for eight weeks significantly decreased blood glucose concentration and serum AChE activity compared to control, without significant changes in blood urea nitrogen, total red blood cell (RBC) count, packed cell volume, haemoglobin, eosinophil and monocyte count (13). We, however, found no changes in blood glucose levels after acute monocrotophos poisoning. This may be due to the reversible nature of hyperglycaemia, as has already been observed in experimental rats (12).

Another study on rats (14) showed a significant decrease in haemoglobin concentrations, total RBC and white blood cell (WBC) counts and in haematocrit after chronic sublethal oral exposure (150 mg kg^{-1} body weight per day) to dimethoate, another OP insecticide. The levels of blood glucose, cholesterol, urea, and total bilirubin soared, but the activities of acid phosphatase and cholinesterase significantly dropped. We, however, found no changes in blood urea, serum creatinine and total bilirubin concentrations in the poisoning episode reported here.

The oral lethal dose of monocrotophos to humans has been estimated to (5 to 50) mg kg^{-1} of body weight (15), while the WHO reports that ingestion of 120 mg of monocrotophos can be fatal to humans (16). The consumption of the pesticide by the child who died was estimated to 113 mg or about 6.7 mg kg^{-1} of body weight. Quite expectedly, this points to far greater susceptibility of children than of adults.

Organophosphate poisoning continues to be a serious problem in developing countries that calls for urgent revision of marketing policies, availability, safe use, and treatment facilities. This report highlights the problem of accidental pesticide poisoning in developing countries resulting in high morbidity and mortality, as facilities for immediate treatment of acutely poisoned persons are seldom at hand. It also highlights the need for clinicians to be aware of OP-pesticide poisoning from food sources and of the need to check cholinesterase activities to establish OP exposure rather than introduce food poisoning treatment alone.

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Sažetak

TROVANJE MONOKROTOFOSOM IZ ONEČIŠĆENOGA BRAŠNA OD PROSA

Zemlje u razvoju nerijetko se susreću sa slučajevima masovnih trovanja organofosfatima (OP). Dijagnoza trovanja organofosfatima uglavnom se zasniva na karakterističnim kliničkim simptomima koji se pojavljuju nakon izloženosti nekome od poznatih organofosfornih spojeva. U potvrđivanju dijagnoze važnu ulogu ima mjerenje aktivnosti enzima kolinesteraza u serumu i crvenim krvnim stanicama. Međutim, povezanost između aktivnosti kolinesteraze u serumu i ozbiljnosti kliničkih manifestacija i prognoze još uvijek je predmet mnogih dvojba. Ovo istraživanje donosi prikaz slučaja masovnog trovanja monokrotofosom koje je nastupilo uslijed akcidentalne konzumacije prosenog brašna (tzv. *bavta*) onečišćenog monokrotofosom. U osmero otrovanih osoba uočeni su sljedeći klinički simptomi: snažni bolovi u trbuhu, proljev, povraćanje, sužavanje zjenica i poteškoće u disanju. Razine kolinesteraza: kolinesteraze u plazmi (PChE) te posebice razine acetilkolinesteraze u crvenim krvnim stanicama (AChE), izmjerene na dan prijema u bolnicu, visoko su korelirale s kliničkim simptomima zamijećenim u bolesnika. Ovaj prikaz slučaja naglašava potrebu za boljim informiranjem liječnika o brzom prepoznavanju simptoma trovanja hranom onečišćenom OP-pesticidima i potrebom provjere jesu li u otrovanih osoba prisutne snižene razine PChE i AChE koje mogu upućivati na izloženost organofosfatima. Sve to moglo bi značajno pridonijeti ranom postavljanju dijagnoze trovanja organofosfatima, čime bi bolesnici na vrijeme mogli primiti specifičnu, a ne općenitu terapiju kao u slučajevima „običnog“ trovanja hranom.

KLJUČNE RIJEČI: *acetilkolinesteraza, akutno trovanje organofosfatima, bolovi u trbuhu, dijareja, kolinesteraza u plazmi, pesticidi, poteškoće u disanju, povraćanje, sužavanje zjenica*

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Professional Paper

IZGUBLJENO VRIJEME U OTKRIVANJU I POČETKU LIJEČENJA TUBERKULOZE: ŠTO TREBA NAPRAVITI?

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Nastavni zavod za javno zdravstvo Splitsko-dalmatinske županije, Split[1], Klinika za plućne bolesti Jordanovac, Zagreb[2], Medicinski fakultet Sveučilišta u Splitu[3], Klinika za plućne bolesti Kliničkog bolničkog centra Split[4], Split, Hrvatska

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Ovim istraživanjem željelo se odrediti veličinu i analizirati čimbenike koji utječu na kašnjenje u otkrivanju i započinjanju liječenja tuberkuloze. Oboljeli od plućne tuberkuloze detaljno su intervjuirani o rizičnom načinu života, različitim demografskim, socioekonomskim i zdravstvenim obilježjima te je izračunano vrijeme od početka simptoma do početka liječenja.

Medijan i 75. percentil navedenog vremena iznosili su 68 i 120 dana. Unutar mjesec dana od početka simptoma liječiti se počelo 16,7 % bolesnika, u drugome mjesecu 23,8 %, 23,3 % u trećem mjesecu, 12,9 % u četvrtome mjesecu, dok je 23,3 % ispitanika liječenje započelo više od četiri mjeseca nakon početka simptoma. Uporaba droga bila je povezana s vremenom dužim od medijana, što se smatralo dugim kašnjenjem (p=0,021) u otkrivanju i liječenju tuberkuloze. Najniži stupanj obrazovanja (p=0,021), minimalan (p=0,039) te minimalni do prosječni mjesečni obiteljski prihod (p=0,020), pušenje (p=0,050) i komorbiditet (p=0,048) pokazali su se značajnima kad je promatran 75. percentil izmjerenog vremena, što se smatralo ekstremnim kašnjenjem. U multiplome modelu uporaba droga ostala je značajno povezana s dugim kašnjenjem, a najniži stupanj obrazovanja (p=0,033), sadašnje (p=0,017) i bivše (p=0,045) pušačke navike s ekstremnim kašnjenjem.

U uvjetima smanjivanja incidencije tuberkuloze kašnjenje u otkrivanju i liječenju tuberkuloze može se smanjiti zdravstvenim prosvjećivanjem opće populacije ne samo o tuberkulozi nego i općenito o zdravlju te stavovima i navikama glede prevencije i ranog liječenja. Istodobno se mora povećati i znanje o tuberkulozi te dijagnostičke sposobnosti zdravstvenih radnika.

KLJUČNE RIJEČI: *komorbiditet, obrazovanje, pijenje alkohola, prevencija, pušenje, zdravstvena skrb, zloporaba droga*

Premda je tuberkuloza bolest stara koliko i čovječanstvo, svijet je još daleko od njezina iskorjenjivanja. Štoviše, broj oboljelih od tuberkuloze u svijetu raste, uz popratne probleme poput infekcije virusom humane imunodeficijencije (HIV) te multirezistentnih i prošireno rezistentnih oblika bolesti. Svjetska zdravstvena organizacija (SZO) izvijestila je da je u 2010. godini 8,8 milijuna ljudi oboljelo, a 1,4 milijuna umrlo od tuberkuloze, dok je trećina svjetske populacije (2 milijarde ljudi) inficirana bacilom tuberkuloze (1).

Strategija Stop TB koju je osmislila i promovira Svjetska zdravstvena organizacija (SZO) poziva na intenzivne aktivnosti usmjerene borbi protiv tuberkuloze s različitih polazišnih točaka. Šesta komponenta strategije posebno je usmjerena omogućivanju i podupiranju različitih istraživanja. Dizajniranje i provođenje istraživanja bitnih na nacionalnoj razini može pomoći u prepoznavanju problema, pronalaženju rješenja i provođenju prikladnih mjera. Osnovni princip nadzora nad tuberkulozom jest smanjivanje prijenosa infekcije ranim otkrivanjem i ranim liječenjem zaraznih tuberkuloznih bolesnika (2).

Najvažniji izvor infekcije u populaciji jest neotkriveni bolesnik koji ima zarazni oblik tuberkuloze. Procijenjeno je da takav bolesnik može inficirati do 15 osoba na godinu i više od 20 osoba tijekom prirodnog razvoja bolesti do smrti (3). Zakasnjelo otkrivanje i liječenje oboljelih dovodi do težih oblika bolesti i većeg mortaliteta na razini pojedinca. Osim toga, ono je odgovorno za duže vrijeme zaraznosti na razini populacije (4).

Međutim nema globalno dogovorene dužine razdoblja između pojave simptoma bolesti i početka liječenja koja se smatra prihvatljivom. Prijelomna točka dužine kašnjenja u istraživanjima koja su se fokusirala na ovaj problem obično je definirana na dva načina: konsenzusom skupine stručnjaka o tome što smatraju prihvatljivim kašnjenjem (manje od 30, 60 ili xy dana) ili se rabio medijan dobivenih podataka (5). Većina takvih istraživanja provedena je u zemljama visoke ili niske incidencije tuberkuloze (6, 7). S druge strane, radovi s tom problematikom nisu brojni u zemljama srednje incidencije gdje je potrebno intenzivno djelovati kako bi se tuberkuloza spustila na ljestvici vodećih nacionalnih javnozdravstvenih prioriteta.

Stopa incidencije tuberkuloze u Hrvatskoj iznosi 19 na 100 000 stanovnika te je u polaganom, ali kontinuiranom padu (8). Kako više nije prioritetan javnozdravstveni problem, svijest o tuberkulozi i među zdravstvenim radnicima i među općom populacijom može se smanjiti te dovesti do kašnjenja u otkrivanju i početku liječenja tuberkuloze (9).

Cilj je ovog rada odrediti dužinu razdoblja između početka simptoma i početka terapije te analizirati čimbenike koji na to utječu. Rezultati istraživanja mogli bi se upotrijebiti kao koristan vodič u planiranju aktivnosti kojima je cilj unaprijediti postojeći sustav nadzora nad tuberkulozom.

METODE

Ustroj istraživanja

U ovome presječnom istraživanju sudjelovalo je sedam slučajno odabranih hrvatskih županija (Grad Zagreb, Istarska, Krapinsko-zagorska, Osječko-baranjska, Splitsko-dalmatinska, Zadarska i Zagrebačka županija), čime je obuhvaćeno gotovo 50 % svih slučajeva tuberkuloze u Hrvatskoj tijekom prethodne godine (10) i više od 50 % ukupnog stanovništva Hrvatske. Skupina iskusnih epidemiologa koji sudjeluju u nadzoru nad tuberkulozom na županijskim razinama nakon dva jednodnevna treninga za potrebe izvođenja istraživanja započela je s anketiranjem tuberkuloznih bolesnika. Intervjui su provedeni u bolnicama ili domovima bolesnika sa strukturiranim upitnikom te je pregledana njihova medicinska dokumentacija odmah nakon što je tuberkuloza dijagnosticirana ili unutar mjesec dana. Upitnik je sadržavao podatke o rizičnom načinu života te različitim demografskim, socioekonomskim i zdravstvenim obilježjima. Iz medicinske dokumentacije zabilježeni su potrebni podaci (primjerice simptomi, dijagnostički proces, laboratorijski i radiološki nalazi i dr.). Podaci o nalazima rendgenskog snimanja pluća zabilježeni su iz nalaza radiologa, a ne rendgenskih slika. Ako se trajanje simptoma razlikovalo između podataka iz medicinske dokumentacije i odgovora ispitanika, zabilježen je datum kraćeg trajanja simptoma.

Istraživanje je odobrilo Etičko povjerenstvo Nastavnog zavoda za javno zdravstvo Splitsko-dalmatinske županije, a svi ispitanici dali su informirani pristanak.

Kriterij uključenja i isključenja ispitanika

U istraživanje su uključeni svi bolesnici s plućnom tuberkulozom, s pozitivnim kulturama na *Mycobacterium tuberculosis* u izabranim županijama, u dobi od 15 i više godina, a oboljeli su između travnja i prosinca 2006. godine.

Bolesnici koji su otkriveni tijekom obrade kontakata drugog slučaja tuberkuloze, koji su otkriveni iz nekih drugih razloga (primjerice prijeoperativna obrada i sl.) i oni koji su već imali tuberkulozu isključeni su iz istraživanja.

Definicije

Kašnjenjem se smatrao broj dana od početka simptoma do početka liječenja tuberkuloze. Premda

datum dijagnoze rijetko kada nije bio i dan na koji je započeta terapija, izabrali smo datum početka terapije kao posljednji promatrani dan. U nedostatku standardnih definicija dugo je kašnjenje definirano kao razdoblje koje prelazi medijan kašnjenja u promatranoj populaciji, dok je za ekstremno kašnjenje kao prijelomna točka uzet 75. percentil.

Prema pušačkomu statusu bolesnici su podijeljeni u nepušače (oni koji nisu nikad pušili), bivše pušače (nekoć su pušili, ali više ne puše) i trenutačne pušače. Prema konzumaciji alkohola u posljednjih 12 mjeseci bolesnici su svrstani u ove skupine: nisu konzumirali, prije su konzumirali (oni koji su konzumirali bilo kada tijekom prethodnih 12 mjeseci, ali više ne konzumiraju) i sadašnji konzumenti. Prema indeksu tjelesne mase (BMI / kg m^{-2}) bolesnici su bili pothranjeni (BMI<18,5), normalno uhranjeni (18,5<BMI<25) i prekomjerno uhranjeni (BMI≥25).

Analiza podataka

Za istraživanje kašnjenja izračunani su medijan i 75. percentil ukupnog kašnjenja te svake ispitivane varijable. Hi-kvadrat testom su unutar svake varijable uspoređene frekvencije ispitanika s kašnjenjem manjim ili jednakim medijanu kašnjenja (ili 75. percentilu) s kašnjenjem većim od medijana (ili 75. percentila). Prediktivni čimbenici za kašnjenje analizirani su univarijantnom logističkom regresijom rabeći medijan, odnosno 75. percentil kao prijelomnu točku te su izračunani omjeri izgleda (OR) i 95 %-tni

Tablica 1 *Kašnjenje u otkrivanju i početku liječenja tuberkuloze prema demografskim i socioekonomskim osobitostima*

Osobitost	Broj	Medijan / dani	Bolesni s dugim kašnjenjem[a] / broj (%)	Bolesni s ekstremnim kašnjenjem[b] / broj (%)
Spol	Ukupno 240			
Muški	134	69	67 (50,0)	36 (26,9)
Ženski	106	67	52 (49,1)	20 (18,9)
Dobna skupina	Ukupno 240			
15 do 34	48	69,5	24 (50,0)	8 (16,7)
35 do 64	131	68	65 (49,6)	34 (26,0)
65+	61	67	30 (49,2)	14 (23,0)
Bračni status	Ukupno 240			
S partnerom	137	71	46 (44,7)	25 (24,3)
Bez partnera	103	67	73 (53,3)	31 (22,6)
Država rođenja	Ukupno 240			
Hrvatska	183	67	30 (52,6)	14 (24,6)
U inozemstvu	57	75	89 (48,6)	42 (23,0)
Najviši završeni stupanj obrazovanja	Ukupno 240			
Bez završene osnovne škole i s njom	116	90	61 (52,6)	39 (33,6) [c]
Srednja škola	101	67	44 (43,1)	16 (15,8)
Više i visoko obrazovanje	23	64	10 (43,5)	1 (4,3)
Radni status	Ukupno 240			
Zaposlen	90	67	43 (47,8)	17 (18,9)
Nezaposlen	42	71,5	21 (50,0)	13 (31,0)
Umirovljenik/kućanica/student	108	70	55 (50,9)	26 (24,1)
Ukupan obiteljski mjesečni prihod po osobi [d]	Ukupno 210			
≤Minimalna plaća	108	69	54 (50,0)	27 (25,0) [c]
Minimalna do prosječna plaća	71	75	40 (56,3)	21 (29,6) [c]
Iznad prosječne plaće	31	64	12 (38,7)	2 (6,5)

[a] *duže od medijana ukupnog kašnjenja (68 dana)*
[b] *duže od 75.percentila ukupnog kašnjenja (120 dana)*
[c] *p≤0,05 u univarijantnoj logističkoj regresiji rabeći 75. percentil (120 dana) kao prijelomnu točku*
[d] *prema podacima Državnog zavoda za statistiku*

Tablica 2 *Kašnjenje u otkrivanju i početku liječenja tuberkuloze prema osobitostima rizičnoga životnog stila*

Osobitost	Broj	Medijan / dani	Bolesnici s dugim kašnjenjem[a] broj (%)	Bolesnici s ekstremnim kašnjenjem[b] broj (%)
Kućni kontakt s tuberkuloznim bolesnikom	Ukupno 240			
Da	47	67	21 (44,7)	8 (17,0)
Ne	193	70	98 (50,8)	48 (24,9)
Kontakt izvan kućanstva s tuberkuloznim bolesnikom	Ukupno 240			
Da	83	90	47 (56,6)	25 (30,1)
Ne	157	67	72 (45,9)	31 (19,7)
Pušačke navike	Ukupno 239			
Nepušač	76	67	34 (44,7)	12 (15,8)
Bivši pušač	80	73	43 (53,8)	20 (25,0)
Pušač	83	68	41 (49,4)	24 (28,9)[c]
Konzumacija alkohola u posljednjih 12 mjeseci	Ukupno 240			
Ne	103	70	55 (53,4)	25 (24,3)
Prije da, sada ne	28	91	15 (53,6)	9 (32,1)
Da, sada	109	67	49 (45,0)	22 (20,2)
Pijanstvo	Ukupno 240			
Da, često	24	109,5	14 (58,3)	8 (33,3)
Da, rijetko	84	67	38 (45,2)	22 (26,2)
Nikad	132	70	67 (50,8)	26 (19,7)
Uporaba droga	Ukupno 240			
Da	13	97	11 (84,6)[d]	4 (30,8)
Ne	227	67	108 (47,6)	52 (22,9)

[a] *duže od medijana ukupnog kašnjenja (68 dana)*
[b] *duže od 75. percentila ukupnog kašnjenja (120 dana)*
[c] *p≤0,05 u univarijantnoj logističkoj regresiji rabeći 75. percentil (120 dana) kao prijelomnu točku*
[d] *≤0,05 u univarijantnoj logističkoj regresiji rabeći medijan (68 dana) kao prijelomnu točku*

intervali pouzdanosti (CI). Kako bi se dobile varijable nezavisno povezane s dugim i ekstremnim kašnjenjem, napravljena je multivarijantna (višestruka) stupnjevita logistička regresija u koju su uključene sve varijable koje su bile značajne u univarijantnoj logističkoj regresijskoj analizi, zajedno sa spolom i dobnim skupinama kao uobičajenim pristranostima ili „zbunjujućim" varijablama. Pritom se rabila strategija gradnje modela kakvu su predložili Hosmer i Lemeshow (10). Razinom statističke značajnosti smatrala se vrijednost od P≤0,05. Podaci su analizirani računalnim programom Statistica 8.0 (StatSoft Inc., Tulsa, SAD).

REZULTATI

U istraživanju je sudjelovalo 240 tuberkuloznih bolesnika, od kojih su 134 (56 %) bili muškog spola, a 106 (44 %) ženskoga. Medijan kašnjenja u otkrivanju i liječenju tuberkuloze iznosio je 68 dana, dok je ekstremno kašnjenje (75. percentil) iznosilo 120 dana (5. i 95. percentil iznosili su 14 i 285 dana).

Ukupno kašnjenje može se podijeliti ovako: 16,7 % ispitanika započelo je liječenje unutar mjesec dana od početka simptoma, 23,8 % u drugome mjesecu, 23,3 % u trećem mjesecu, 12,9 % u četvrtome mjesecu, dok je u 23,3 % ispitanika liječenje započelo više od četiri mjeseca nakon početka simptoma.

Deskriptivne informacije o statistički značajnim i ostalim epidemiološki relevantnim varijablama, kao što su demografska i socioekonomska obilježja (tablica 1), rizični životni stil (tablica 2) te karakteristike zdravstvenog stanja (tablica 3) prikazane su u navedenim tablicama.

Uporaba droga bila je statistički značajno povezana s dugim kašnjenjem (p=0,021, OR=6,06; 95 %-tni CI=1,31 do 27,96), dok se kod ekstremnog

Tablica 3 *Kašnjenje u otkrivanju i početku liječenja tuberkuloze prema osobitostima zdravstvenog stanja*

Osobitost	Broj	Medijan / dani	Bolesnici s dugim kašnjenjem[a] broj (%)	Bolesnici s ekstremnim kašnjenjem[b] broj (%)
Komorbiditet	Ukupno 240			
Da	101	75	55 (54,5)	30 (29,7) [c]
Ne	139	67	64 (46,0)	26 (18,7)
Kaverne na rendgenskoj snimci pluća	Ukupno 240			
Da	64	69,5	32 (50,0)	17 (26,6)
Ne	176	67	87 (49,4)	39 (22,2)
Uzorak	Ukupno 240			
Iskašljaj	197	68	97 (49,2)	46 (23,4)
Drugo	43	75	22 (51,2)	10 (23,3)
Mikroskopski pozitivan razmaz iskašljaja	Ukupno 240			
Da	165	67	77 (46,7)	37 (22,4)
Ne	75	75	42 (56,0)	19 (25,3)
Kašalj	Ukupno 240			
Da	176	70	92 (52,3)	42 (23,9)
Ne	64	64.5	27 (42,2)	14 (21,9)
Kašalj s iskašljavanjem krvi	Ukupno 240			
Da	42	67	20 (47,6)	11 (26,2)
Ne	198	69	99 (50,0)	45 (22,7)
Povišena temperatura	Ukupno 240			
Da	115	67	51 (44,3)	23 (20,0)
Ne	125	75	68 (54,4)	33 (26,4)
Gubitak tjelesne težine	Ukupno 240			
Da	137	75	74 (54,0)	36 (26,3)
Ne	103	67	45 (43,7)	20 (19,4)
Noćno znojenje	Ukupno 240			
Da	111	67	53 (47,7)	27 (24,3)
Ne	129	70	66 (51,2)	29 (22,5)
Nestašica zraka	Ukupno 240			
Da	73	75	39 (53,4)	21 (28,8)
Ne	167	67	80 (47,9)	35 (21,0)
Umor	Ukupno 240			
Da	155	70	80 (51,6)	40 (25,8)
Ne	85	67	39 (45,9)	16 (18,8)
Indeks tjelesne mase na dan intervjua	Ukupno 236			
Pothranjenost	31	67	15 (48,4)	11 (35,5)
Normalna uhranjenost	155	70	79 (51,0)	35 (22,6)
Prekomjerna uhranjenost	50	67	23 (46,0)	10 (20,0)

[a] *duže od medijana ukupnog kašnjenja (68 dana)*
[b] *duže od 75. percentila ukupnog kašnjenja (120 dana)*
[c] *p≤0,05 u univarijantnoj logističkoj regresiji rabeći 75. percentil (120 dana) kao prijelomnu točku*

kašnjenja značajnost uočila kod najnižeg stupnja obrazovanja (p=0,021; OR=11,14; 95 %-tni CI=1,45 do 85,75), minimalnog (p=0,039; OR=4,83; 95 %-tni CI=1,08 do 21,61) te minimalnog do prosječnog obiteljskog prihoda (p=0,020; OR=6,09; 95 %-tni CI=1,33 do 27,87), sadašnjeg pušenja (p=0,050; OR=2,17; 95 %-tni CI=1,00 do 4,72) i komorbiditeta (p=0,048; OR=1,84; 95 %-tni CI=1,00 do 3,36).

Tablica 4 *Logistička regresijska analiza[a] ekstremnog kašnjenja[b] u otkrivanju i početku liječenja tuberkuloze*

Osobitost	Univarijantna analiza		Multivarijantna analiza	
	p-vrijednost	OR (95%-tni CI)	p-vrijednost	OR (95%-tni CI)
Spol				
Muški	0,147	1,58 (0,85 do 2,93)	0,914	1,04 (0,47 do 2,31)
Ženski		1		1
Dobne skupine (godine)				
15-34	0,419	0,67 (0,56 do 1,76)	0,990	0,98 (0,29do 3,38)
35-64	0,655	1,18 (0,58 do 2,40)	0,634	1,27 (0,47 do 3,43)
65+		1		1
Najviši završeni stupanj obrazovanja				
Bez završene osnovne škole i s njom	0,021	11,14 (1,45 do 85,75)	0,033	10,28 (1,20 do 87,49)
Srednja škola	0,179	4,14, (0,52 do 32,95)	0,305	3,06 (0,36 do 25,79)
Više i visoko obrazovanje		1		1
Ukupan obiteljski mjesečni prihod po osobi[c]				
≤Minimalna plaća	0,039	4,83 (1,08 do 21,61)	0,357	2,10 (0,43 do 10,22)
Minimalna do prosječna plaća	0,020	6,09 (1,33 do 27,87)	0,055	4,67 (0,97 do 22,54)
Iznad prosječne plaće		1		1
Pušačke navike				
Pušač	0,050	2,17 (1,00 do 4,72)	0,017	2,97 (1,21 do 7,31)
Bivši pušač	0,157	1,78 (0,81 do 3,95)	0,045	2,53 (1,02 do 6,27)
Nepušač		1		1
Komorbiditet				
Da	0,048	1,84 (1,00 do 3,36)	0,160	1,667 (0,82 do 3,40)
Ne		1		1

[a] varijable značajne u univarijantnoj analizi na razini p≤0,05 zajedno sa spolom i dobnim skupinama, uvrštene su u multivarijantnu analizu
[b] duže od 75. percentila ukupnog kašnjenja (120 dana)
[c] prema Državnom zavodu za statistiku
OR=omjer izgleda, CI=interval pouzdanosti

U multivarijantnome modelu dugo je kašnjenje ostalo povezano s uporabom droga (p=0,021; OR=6,06; 95 %-tni CI=1,31 do 27,96), dok su se najniži stupanj obrazovanja (p=0,033; OR=10,25; 95 %-tni CI=1,20 do 87,49), sadašnje (p=0,017; OR=2,97; 95 %-tni CI=1,21 do 7,31) i bivše pušačke navike (p=0,045; OR=2,53; 95 %-tni CI=1,02 do 6,27) pokazali značajno povezanima s ekstremnim kašnjenjem (tablica 4).

RASPRAVA

U ovom istraživanju pokazalo se da je medijan razdoblja od nastupa simptoma do početka antituberkulozne terapije 68 dana, dok je ekstremno kašnjenje (75. percentil) 120 dana. Ti su rezultati usporedivi s rezultatima iz drugih industrijaliziranih zemalja (12-15) i mnogo manji od onih iz zemalja visoke incidencije (16-18).

Za razliku od drugih istraživanja (13, 18-21), nije uočeno da postoji razlika po spolovima vezana uz kašnjenje. Jednako pravo i pristup zdravstvenim uslugama jedno je od prava zajamčenih Ustavom, što se reflektira i u ovim rezultatima.

Podatak da bolesnici koji imaju više od osam godina formalnog školovanja manje kasne od onih koji imaju samo završenu osnovnu školu ili su bez nje ne čudi jer je pronađen i u drugim radovima (9). U jednom istraživanju iz Hrvatske (22) pokazalo se da je bolje znanje o tuberkulozi povezano s višim stupnjem obrazovanja, nakon što su se isključili drugi demografski i socioekonomski čimbenici. Zaključeno je da su najmlađe dobne skupine ispitanika i oni koji su najlošije obrazovani ciljne skupine za intervencijske strategije (22).

Zanimljivo je da se socioekonomski čimbenici nisu pokazali povezanima s dugim ili ekstremnim kašnjenjem, za razliku od drugih radova (23). Vjerujemo da se to može objasniti činjenicom da su dijagnostika i liječenje tuberkuloze u Hrvatskoj besplatni. To vrijedi za sve osobe, bez obzira na to jesu li zdravstveno osigurane ili nisu, premda je više od 98 % hrvatske populacije u vrijeme provođenja istraživanja imalo zdravstveno osiguranje (24).

Među čimbenicima vezanim uz rizični životni stil pokazalo se da bolesnici koji su često bili alkoholizirani te oni koji uzimaju drogu imaju najduži medijan (5, 9, 23, 25). Ti podaci ne čude s obzirom na to da takve osobe obično ne vode brigu o vlastitom zdravlju. Više od polovice bolesnika imalo je neku od popratnih bolesti, što se pokazalo statistički značajno povezanim s ekstremnim kašnjenjem, u skladu s rezultatima drugih istraživanja (12).

Plućna tuberkuloza s pozitivnim razmazima iskašljaja dijagnosticirana je u 69 % ispitanika. Postojanje negativnih nalaza razmaza bilo je povezano s dužim medijanom kašnjenja, što upućuje na moguću slabu točku u dijagnostici tuberkuloze i naglašava potrebu za novim testovima za brzu dijagnozu, čemu u prilog govore i drugi radovi (14, 21, 26). S druge strane, ta razlika nije bila statistički značajna. To bi se moglo objasniti činjenicom da se, prema Naputku za suzbijanje i sprečavanje tuberkuloze (27), liječenje može započeti već na osnovi kliničke sumnje prije nalaza kulture. Propisano je i da laboratoriji trebaju na vrijeme javiti nalaze mikobakterioloških pretraga, i to mikroskopske nalaze unutar 24 sata, kultivacijske nalaze unutar 14 dana te identifikaciju *M. tuberculosis* unutar 21 dana (27).

Kao i u drugim radovima kašalj, gubitak tjelesne težine, povišena temperatura i umor bili su najčešće prijavljeni simptomi (6, 9, 12, 23, 25). Međutim pokazalo se da postojanje bilo kojeg simptoma nije povezano s kraćim kašnjenjem, što upućuje na činjenicu da ti simptomi nisu pobudili sumnju (oprez) ni samih ispitanika, a ni liječnika. Isto tako je moguće da ovi simptomi nastanu i kao rezultat uznapredovale bolesti koja nije liječena, odnosno da su posljedica kašnjenja.

Kašalj može biti povezan s pušenjem, s obzirom na to da su više od dvije trećine ispitanika bili pušači, bilo sadašnji ili bivši, koji mogu imati „pušački“ kašalj. Međutim gubitak težine, povišena temperatura i umor mogu biti pokazatelji niza potencijalno teških bolesti, zanemarivanje kojih može dovesti do uznapredovale bolesti i veće smrtnosti.

U Hrvatskoj je uobičajeno da primarnu zdravstvenu zaštitu osoba iznad 7 godina pružaju liječnici opće/obiteljske medicine. Mreža takvih liječnika, kao i razvijena mreža od 14 mikobakterioloških laboratorija smatra se dovoljnom za pružanje odgovarajuće dijagnostičke obrade i skrbi o tuberkuloznim bolesnicima (8, 28, 29). Ovakva organizacija sigurno smanjuje ograničenja u pristupu zdravstvenim uslugama poput udaljenosti i dugog vremena putovanja do zdravstvene ustanove, kako je opisano u drugim radovima (23, 30).

Stoga unatoč dostupnosti zdravstvene zaštite zabrinjava činjenica da više od polovice bolesnika ne započne liječenje više od dva mjeseca nakon pojave simptoma, dok je značajan dio bolesnika (23,1 %) puna četiri mjeseca nakon pojave simptoma još uvijek izvor zaraze u populaciji.

Rezultate ovog istraživanja treba tumačiti u svjetlu nekih ograničenja. Prvo, činjenica da se bolesnici moraju prisjetiti trajanja simptoma prije traženja liječničke pomoći može biti uzrokom pogreške sjećanja. Određivanje točnog datuma pojave simptoma ograničenje je svih sličnih istraživanja (21, 23, 25). To se nastojalo izbjeći na više načina. Istraživanje je posebno osmišljeno za ovu svrhu. Bolesnici su uzastopno uključivani u istraživanje te su potrebni podaci dobiveni od njih intervjuom, a ne samo pregledom medicinske dokumentacije. Svi su intervjui napravljeni netom nakon što je dijagnoza postavljena uz iznimne napore da se definira točan datum početka simptoma. Smatramo da je ovaj pristup pridonio točnosti podataka.

Jakost ovog istraživanja jesu strogi kriteriji uključivanja i isključivanja bolesnika. Jedino su zarazni slučajevi bolesti, potvrđeni kultiviranjem, bili dijelom istraživanja, dok su se isključili bolesnici kod kojih je bolest slučajno pronađena budući da je to moglo dovesti do podcjenjivanja kašnjenja. Županije u kojima se provelo istraživanje odabrane su slučajno; stoga su populacije s različitim stopama incidencije, kao i liječnici s različitim iskustvom i sumnjom na tuberkulozu bili sudionici istraživanja.

ZAKLJUČAK

Kako bi se smanjio razorni učinak tuberkuloze na osobnoj i obiteljskoj razini te razini zajednice, tuberkuloza bi se trebala otkriti što je ranije moguće, i ranim javljanjem bolesnika kad primijete simptome i ranim otkrivanjem od strane zdravstvenog sustava.

Prikazano kašnjenje u otkrivanju i liječenju tuberkuloze moglo bi se smanjiti zdravstvenim prosvjećivanjem opće populacije ne samo o tuberkulozi nego i općenito o zdravlju i stavovima o prevenciji i ranom liječenju. Javnost treba naučiti kako prepoznati simptome bolesti i kako razviti model ponašanja u kojem se rano traži liječnička pomoć kontinuiranom edukacijom o zdravlju.

U isto vrijeme edukacija o tuberkulozi među zdravstvenim radnicima mora se intenzivirati. Procijenjeno je da svaki liječnik opće/obiteljske medicine ima u prosjeku jednog tuberkuloznog bolesnika svake dvije godine (29). U takvim okolnostima razumno je pretpostaviti da mnogi važni postupci vezani uz nadzor nad tuberkulozom možda nisu ispravno provedeni zbog nedostatnog iskustva i znanja o tuberkulozi.

Zbog nespecifične prirode mnogih simptoma i znakova tuberkuloze, zajedno sa sporim, ali kontinuiranim smanjivanjem stopa incidencije tuberkuloze, nerealno je očekivati da svi slučajevi tuberkuloze budu brzo dijagnosticirani bez dobrog znanja o tuberkulozi i prisutne sumnje na nju u diferencijalnoj dijagnozi (19, 26).

Kašalj, kao najčešći simptom tuberkuloze, ima različito značenje ovisno o prevalenciji tuberkuloze u promatranoj populaciji. Za razliku od zemalja visoke incidencije, u zemljama niske ili srednje incidencije kašalj je češće uzrokovan drugim respiratornim bolestima (19). Bez obzira na to, pasivno otkrivanje slučajeva treba biti brzo, dok aktivno pronalaženje slučajeva treba biti intenzivnije. Trenutačno se aktivnim traženjem među kontaktima tuberkuloznih bolesnika i među rizičnim skupinama otkrije 5 %, odnosno 2 % od ukupno prijavljenih tuberkuloznih bolesnika (27). Novi naputak za suzbijanje i sprječavanje tuberkuloze može biti koristan jer pruža dijagnostički postupnik te informacije o brzoj mikrobiološkoj dijagnostici tuberkuloze (27).

Javnozdravstvene intervencije su potrebne kako bismo se brže i uspješnije usmjerili eliminaciji tuberkuloze, što uključuje i intenzivne napore na različitim razinama od mijenjanja ponašanja usmjerenog traženju zdravstvene usluge, unaprjeđivanja svijesti o tuberkulozi i povećavanja dijagnostičke sumnje zdravstvenih radnika, kao i usavršavanja njihova znanja o tuberkulozi.

Zahvale

Zahvalni smo svim epidemiolozima koji su sudjelovali u prikupljanju podataka jer su znatno pridonijeli uspješnomu vođenju ovog istraživanja.

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Summary

DELAYS IN DIAGNOSING AND TREATING TUBERCULOSIS IN CROATIA

The aim of this study was to determine factors causing delay in tuberculosis diagnosis and treatment in Croatia. It included 240 adults with pulmonary tuberculosis, who were interviewed for demographics, socioeconomic, lifestyle, and personal health data. Total delay was defined as a number of days from the onset of symptoms to the initiation of therapy. The median and the 75th percentile of the total delay were 68 and 120 days, respectively: 16.7 % of the patients initiated treatment within the first month , 23.8 % within the second month, 23.3 % within the third month, 12.9 % within the fourth month, and 23.3 % more than four months after the symptoms appeared. Long delay (exceeding median delay) was strongly associated with drug abuse (p=0.021). Extreme delay (75th percentile of delay) was significantly associated with the lowest level of education (p=0.021), below minimal income (p=0.039), minimal to average income (p=0.020), current smoking (p=0.050), and co-morbidity (p=0.048). In the multivariate model, long delay remained associated with drug abuse, while extreme delay was associated with the lowest level of education (p=0.033) and current (p=0.017) and ex-smoking (p=0.045).
In a setting with decreasing TB incidence, the reported delay can be reduced by increasing health education, not only about tuberculosis *per se*, but about health in general and attitudes towards prevention and early care. It is also important to increase tuberculosis knowledge among healthcare workers as well as their diagnostic skills.

KEY WORDS: *alcohol use, co-morbidity, drug abuse education, health care, prevention, smoking*

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Review

OBESITY: GENOME AND ENVIRONMENT INTERACTIONS

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Obesity has become one of the major threats for public health in industrialised world among adults, but also among adolescents and children. It is influenced by the interaction of genes, nutrition, environment, and lifestyle. Environmental and lifestyle risk factors include foetal and lifelong environment, nutrient quality, chemical and microbial exposure, and psychical stress, all of which are important contributing influences. Removing or limiting chemical and pharmaceutical obesogens from human environment could make a difference in the growing epidemic of obesity.
Additionally, nutrigenomics describes how modifications in individual diets can improve health and prevent chronic diseases, as well as obesity, by understanding the effects of a genetic profile in the interaction between food and increase in body weight. Furthermore, individual genetic variations in genome represent an individual′s predisposition for obesity. Therefore, the use of individual genetic information, avoiding obesogens, and a healthy lifestyle could help to improve the management of obesity and maintain a healthy weight.

KEY WORDS: *genes, nutrigenomics, nutrition, obesogens*

The growing prevalence of obesity and the fact that its management is mostly ineffective are opening novel fields of research - investigation of genes, environment, and their interactions with the intention to create a concept of personalised medicine and nutrition (1).

Obesity and its implications for human health decrease the quality of life and life expectancy considerably, as they increase the risk of a great number of chronic diseases (2-10; table 1). Excess weight and obesity are the consequences of a higher energy intake and lower energy expenditure, which results in a positive energy balance (11). Several biological changes in our body are related with the state of overweight and obesity (Figure 1).

Obesity is a disease which affects not only the individual, but also the public health. It is influenced by many factors such as genetic and epigenetic predisposition, metabolic, hormonal, environmental, behavioural, social, and cultural aspects (12). This is why it is important to better understand obesity and its aetiology and find more effective prevention and treatment tools.

Genetic aspect of obesity

Individual human differences and genetic variations influence the risk of becoming obese (11). At present, over 100 genes are under discussion for their effect on obese phenotypes. The latest update of the Human Obesity Gene Map, which was created in 2005, focuses on 253 groups of genes related to obesity (13). This catalogue of obesity genes shows that putative loci affecting obesity-related phenotypes are situated on all chromosomes except Y. Table 2 displays five categories of the most investigated obesity-related genes in recent years (13-16, Figure 1).

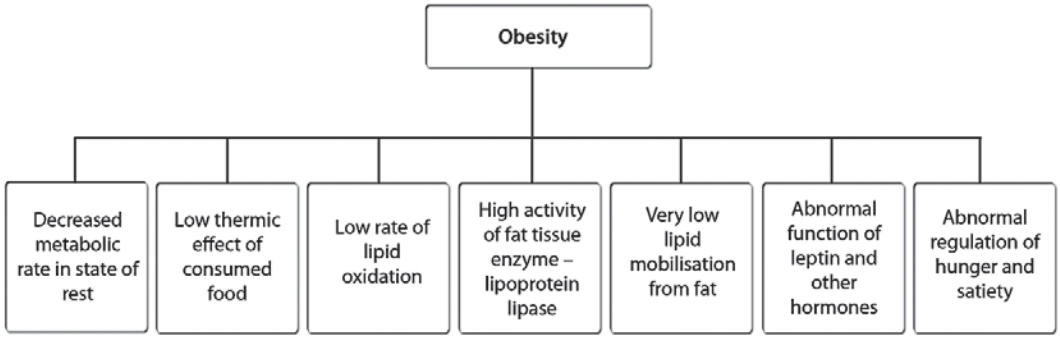

Figure 1 *The physiological changes associated with obesity*

The studies on both humans and animals showed that different genes play roles in different responses to weight gain or weight loss, and that body defence against losing fat/weight is greater than that against gaining fat/weight (17). Also, the weight loss response to (dietary) interventions varies. Many genes involved in the regulation of energy balance, appetite, lipid metabolism, and adipogenesis have been reported to affect the risk of dietary intervention failure in some individuals. Some of these genes have already been mentioned: β-adrenergic receptor (ADBR), uncoupling proteins (UCPs), leptin (LEP), leptin receptor (LEPR), melanocortin receptor 3 (MC3R), pro-opiomelanocortin (POMC), and interleukin 6 **(**IL-6) (18, Table 2).

In studies involving adult monozygotic twins (19, 20) who were overfed and whose energy intake or exercise were restricted (21-23), it was shown that the predisposition to increase or decrease body fat was genetically based (1).

Mammes et al. (2001) reported that a G>A transition at position -2549 in the promoter region of the LEP gene is associated with obesity and that carriers of the -2549A allele have higher leptin levels and lower weight loss as a response to low calorie intake (24, 25). Furthermore, carriers of a variant C allele in the LEPR gene [Ser (T) 343Ser (C)] have lost more weight in response to a low calorie intake than the noncarriers (26).

In a study of the Lys656Asn variant within LEPR, homozygotes for the Lys656 allele had a considerable loss of body weight, fat mass, waist circumference, and a decrease in body mass index (BMI), systolic blood pressure, and leptin levels, compared to the carriers of the Asn656 allele (27). Some studies have investigated the effect of a polymorphism in the peroxisome proliferator-activated receptor γ (PPARγ) gene, Pro12Ala. Lindi et al. (28) showed that Ala12 carriers increased body weight considerably in a period of 10 years. However, Ala12Ala homozygotes had, in the same period of time, lower levels of fasting plasma insulin, regardless of the increased body weight. In another study, Ala12 carriers on a hypocaloric diet lost almost the same body weight as Pro12 homozygotes during a period of six months, but unlike Pro12 homozygotes, they gained more weight after the hypocaloric treatment was completed (29).

Candidate gene variants for polygenic obesity appear to disrupt pathways involved in the regulation of energy intake and expenditure and include adrenergic receptors, uncoupling proteins, PPARγ, POMC, melanocortin receptor 4 (MC4R), and a set of single nucleotide polymorphisms in the fat mass and obesity (FTO) locus. Obviously, obesity polygenic character involves complex gene-gene and gene-environment interactions and their mutual interactions that result in multi-factorial obese phenotypes (for reviews see 30-32).

Environmental obesogens

Human diseases are entirely or partially caused by environmental chemicals, which introduce genetic and/or epigenetic changes in the genome. Heritable changes in gene expression exclude any modification of the primary DNA sequence. However, they lead to chromatin remodelling through DNA methylation, a complex set of histone modifications, and the influence of non-coding RNAs. Besides the changes in the epigenetic status (33), lifestyle, dietary intakes, and environmental chemicals affect cell transcriptional factors (34), hormones (35, 36), inflammatory mechanisms (37), and gut microbiome in certain organisms (38-41), all of which are contributing factors for obesity epidemic (42-44, Figure 1). The

most important molecular and/or cellular changes that are reflected on obesity phenotypes appearance are further described.

Obesogens (Figure 1) are foreign endocrine disrupting chemicals (EDCs) that inappropriately alter normal development and/or homeostasis of lipid metabolism, adipogenesis, fat storage, obesity, and type 2 diabetes (4, 45-48). EDCs can interfere with normal functions of the endocrine system by disrupting the balanced system of hormones that regulate vital body functions such as growth, stress response, sex development, behaviour, ability to reproduce, production and utilisation of insulin, and metabolic rate. Recent experiments on animals confirmed that EDCs can disrupt the gene-controlled, normal signalling systems that determine every aspect of foetal development. Furthermore, epidemiology studies indicate that exposure to EDCs during the development of a foetus is associated with excess weight and obesity later in life (49).

It has been proposed that dangerous environmental obesogens can be categorised into the following groups of compounds: endocrine disrupting chemicals organotins and bisphenol A (BPA), perfluorooctanoic acid, diisobutyl phthalate, and pharmaceutical obesogens such as thiazolidinediones: rosiglitazone and pioglitazone (45, 47, 48).

Organotins are widespread persistent environmental organic pollutants with potent endocrine-disrupting properties in both vertebrates and invertebrates. They are used as fungicides and pesticides in crops, antifungal agents in wood treatments, slimicides in

Table 1 *Obesity related diseases or disorders*

Disease / disorder	Relation with obesity	Source/publication
Type II diabetes	80 % correlation of type II diabetes and obesity 85 % of children with type II diabetes are obese Term "diabesity" underscores the strong connection	Lau, 2008 (2)
Cardiovascular diseases	Correlation with obesity in 70 % of cases	Lau, 2008 (2)
Metabolic syndrome Hypertension Elevated plasma insulin concentrations Insulin resistance Hyperglycaemia Hyperlipidaemia	Higher risk associated with intraabdominal visceral fat tissue	Kopelman, 2007 (3)
Gout, liver disease, asthma and pulmonary problems, gall bladder disease, kidney disease, osteoarthritis	Strong connection with obesity and excess weight	Newbold, 2010 (4) Chlebowski, 2005 (5)
Cancer	Connection with colon and breast cancer 42 %	Lau, 2008 (2)
Breast cancer	Obesity associated with breast cancer recurrence and mortality through low physical activities, high calorie and fat intake, and changes in hormones (high oestrogen production in adipose tissue)	Chlebowski, 2005 (5), Sauter et al., 2008 (6), Rose and Vona-Davis, 2010 (7)
Prostate cancer	More aggressive forms are diagnosed in men with higher BMI (by 15 % to 21 % higher risk of fatal prostate cancer or biochemical recurrence) 12 % to 20 % of prostate cancer deaths connected with obesity	Strom et al.,2005 (8), Cao and Ma, 2011 (9)
Chronic musculoskeletal problems, lumbago, skin problems, obstructive sleep apnoea	Decreased physical activity, BMI >30	Kanasaki and Koya, 2011 (10)

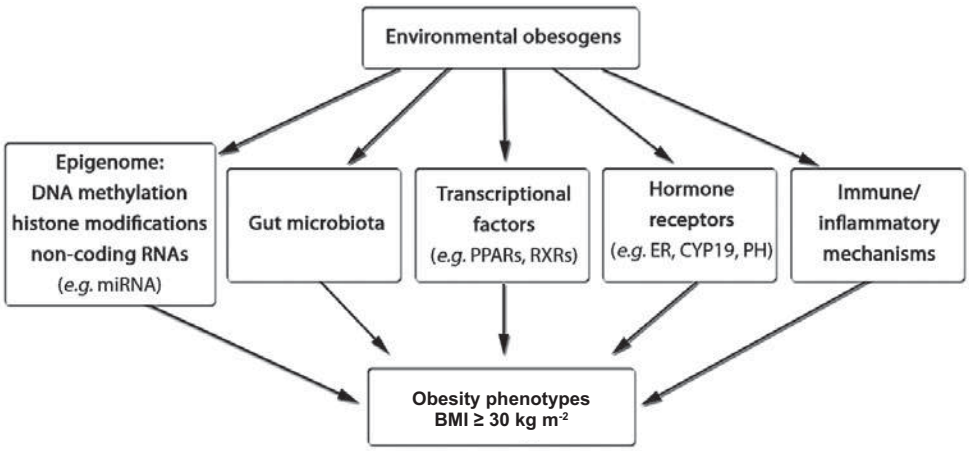

Figure 2 *Possible targets of obesogens. Environmental obesogens influence a variety of molecular and/or cellular targets that may act either alone or with each other creating a complex network that affects gene expression and results in obesity phenotypes (abbreviations: miRNA - microRNA, PPARs - peroxisome proliferator-activated receptors, RXRs- retinoid X receptors, ER - oestrogen receptor, CYP19 - Cytochrome aromatase p450, PH – peptidergic hormones, BMI - body mass index).*

industrial water systems, marine antifouling agents, in polyvinylchloride plastics, and in the textile industry (45, 47, 48). Contaminated drinking water, agricultural products, seafood, and leaching from plastics are the most frequent sources of organotins (50-52). Organotins such as tributyltin (TBT) and triphenyltin are potent activators of nuclear hormone receptors retinoid X receptor (RXRα, -β, and -γ) and peroxisome proliferator-activated receptor γ which regulate adipocyte number, size, and function (53). Moreover, the organotin compound tributyltin, an agonist of both retinoid X receptor and peroxisome proliferator-activated receptor γ, alters the fate of stem cell compartment by sensitising multipotent stromal stem cells to differentiate into adipocytes (49). More recently, studies have confirmed that TBT and tetrabromobisphenol A modify hypothalamic gene regulations resulting in hypothalamic dysregulations (54).

Bisphenol A as a component of polycarbonate plastics is widely used in numerous products such as polycarbonate baby bottles, beverage containers, the linings of food cans, dental composites, and sealants (4). BPA has a potential to bind to the nuclear oestrogen receptor and interact with a variety of other targets in mammalian cells. In addition to acting as an androgen receptor antagonist, BPA interacts with thyroid hormone receptors (55). A variety of abnormalities in the female and male reproductive and mammary gland tissues were found after perinatal exposure to low doses of BPA (55). Exposure to low doses of BPA during prenatal and neonatal periods affected both mice and rats insomuch as their body weight increased (57-60).

Perfluorooctanoic acid and phthalates can negatively affect the adipose homeostasis and lipids. These classes of chemicals include various perfluoroalkyl compounds and phthalate plasticisers that are widely used as surface repellents and surfactants.

Pharmaceutical obesogens, rosiglitazone and pioglitazone (thiazolidinediones) are PPARγ agonists, which improve serum triglycerides and glycaemic control for type 2 diabetes. However, side effects mediated by PPARγ include weight gain in diabetics when such agonists are used for a prolonged time (61, 62), an increase in cardiovascular risks and, for rosiglitazone, an increased risk of acute myocardial infarction, stroke, and heart failure (63).

EDCs can probably affect multiple target mammalian cells through a variety of mechanisms. The most likely mechanism involves direct binding to nuclear receptors such as oestrogen receptor α and/or PPARγ acting as agonists. Another possibility would be their binding to nuclear receptors and acting as antagonists. The indirect EDCs′ effect involves disrupting hormone levels through the inhibition of enzymatic activity or the activation of expression of P450 enzymes (4).

All proposed mechanisms may also interact with each other creating a complex network. However, in the future, when research studies will have clarified the network of reactions in the cell, a complete picture of interactions will be available.

Nutrigenomics: nutrient and gene interaction

Nutrigenomics investigates the effect of interactions between lifestyle, genes (individual genetic variations in single-nucleotide polymorphisms), and food on different/unique responses to food among individuals and consequently, the relationship of that response with the aetiology of common chronic diseases (64, 65).

Evolution has brought about minor changes of human genome but these are able to influence an individual′s metabolism and response to food (66).

Nutrigenomics is related to the human genome and, as already mentioned, it tries to describe the cause of some chronic diseases (obesity, diabetes mellitus, cancer) through the interactions between genes and the environment (food, pesticides, and pharmaceuticals) (67). It also attempts to create a personalised nutrition for every individual, according to a unique genetic profile (68, 69).

The individual advice regarding nutrition and lifestyle changes should be based on an analysis of genes, in order to achieve a better health status (70). When managing multifactorial diseases (including obesity), it is crucial to point out that some genotypes (haplotype combinations) are susceptible to nutrition treatments and that genetic differences among individuals could influence the occurrence of obesity and its health implications (71).

It is important to emphasize that some nuclear transcription factors (e.g. PPARγ) are more sensitive to nutrition treatment/interventions than others. For example, Kim et al. (72) selected 31 genes in the liver of obese C57BL/6J mice and showed that genes involved in fatty acid beta-oxidation, fatty acid synthesis, and gluconeogenesis were upregulated, but genes involved in sterol biosynthesis, insulin signalling, and oxidative stress defence system were downregulated with a high-fat diet.

Hence, nutrigenomics aims to develop a diet based on individual genetic variants (73) through the research of the influence of such variants on the connections between food and aetiology of obesity (74). It has been shown that individuals with high genetic predisposition for obesity will easily gain weight and will have a different response to weight treatment compared to those with low genetic predisposition (11). Results from studies show that high-fat diets may lead to obesity. One study compared the body mass indexes of individuals according to how much fat they ate and showed that the number of obese individuals was higher among those on a high-fat diet compared with those on a low-fat diet. Also, their body weight changed differently in response to the restriction of calories from fat (1, 75).

It is known that interactions between genes, gender, and the environment alternate the development of a disease. In the Framingham Heart Study, interactions between a promoter polymorphism at the apolipoprotein A1 gene, gender, and dietary poly-unsaturated fatty acid intake modulated plasma concentrations of high-density lipoprotein cholesterol (76), high levels of which protect from cardiovascular diseases.

In another study, Sotos-Prieto et al. (77) showed that the rs1466113 polymorphism in the somatostatin receptor 2 gene was associated with anthropometric variables in the Mediterranean population with differences in food intake.

Interactions between genes and the environment have also been associated with a decrease in the levels of hormone adiponectin. These changes in adiponectin

Table 2 *The list of obesity genes*

Genotype in obesity	Genes
Thriftiness (low metabolic rate, inadequate thermogenesis)	β-2-adrenergic receptor and β-3 (ADRB2; ADRB3), uncoupling protein 1, 2, and 3 (UCP1, UCP2, UCP3)
Hyperphagia (abnormal regulation of hunger and satiety)	dopamine receptor D2 (DRD2); 5-hydroxytryptamine (serotonin) receptor 2C (HTR2C); leptin (LEP); leptin receptor (LEPR); melanocortin receptor 4 (MC4R); nuclear receptor subfamily 3, group C, member 1 (NR3C1)
Low rate of lipid oxidation	angiotensin-converting enzyme (ACE), adiponectin (ADIPOQ), guanine nucleotide binding protein, β -3 subunit (GNB3), hormone sensitive lipase (LIPE), low density lipoprotein receptor (LDLR)]
Adipogenesis (fat storage)	peroxisome proliferator-activated receptor γ (PPARγ); vitamin D receptor (VDR), resistin (RETN), interleukin-6 (IL6); tumour necrosis factor α (TNF)]
Low physical activity	dopamine receptor D2 (DRD2); melanocortin receptor 4 (MC4R)

level can cause obesity. Ntalla et al. (78) investigated whether variants of adiponectin gene interacted with food/specific components of food and influenced the levels of adiponectin. The results of the study, conducted on healthy school-aged children, showed that a single nucleotide polymorphism rs1501299 and fibre interaction was significantly associated with adiponectin levels.

Consumption of refined carbohydrates contributes to the development of obesity and type 2 diabetes mellitus. Most genes of the metabolic pathways of carbohydrates are associated with quantitative trait loci for obesity and many for type 2 diabetes mellitus. It is significant to underline that metabolic pathway genes have a role in the development of a disease and different appearance of such disease among individuals, which results in different risks for a specific disease (79).

Inflammation plays an important role in the development of health implications of obesity (80) and fat tissue is essential, as obesity is associated with inflammation (81): adipocytes, or fat cells, secrete pro- and anti-inflammatory adipokines (82-84). Reduced adiponectin (85, 86) and increased C-reactive protein (87, 88) concentrations are associated with cardiovascular disease and type 2 diabetes. A decrease in inflammation can prevent health complications associated with obesity. Some food has anti-inflammatory effects (89) and has been associated with the decrease in the prevalence of some chronic diseases related to dietary and lifestyle habits (90, 91). In line with this, Bakker et al. (92) conducted a dietary intervention with antioxidative substances such as resveratrol, green tea extract, α-tocopherol, vitamin C, omega-3 polyunsaturated fatty acids, and tomato extract, and showed that these compounds influenced inflammatory processes, oxidative stress, and metabolism in individuals.

How to maintain a healthy weight after low calorie/low energy treatment is the issue of many studies (93). Peripheral blood mononuclear cells (PBMCs) can help to investigate the response to nutrition interventions (94), and Goyenechea et al. (93) demonstrated the role of pro-inflammatory status in weight changes in obese subjects receiving a low-calorie diet (LCD) during a six-month weight maintenance period. This could help to create individual dietary treatments for maintaining healthy weight. In another study, Goyenechea et al. (95) analysed the expression of two interacting genes (RIPK3 and RNF216) in obese subjects receiving LCD and the authors concluded that the expression of these two genes in PBMCs could identify those obese subjects who will regain more weight after a successful initial weight loss.

Personalised nutrition

Nutrigenetics and personalised nutrition can give every individual an advice on a diet that is in line with personal genetic profile (96). Arkadianos et al. (70) showed that nutrigenetically (gene-nutrition) tailored diets result in better compliance of patients, longer reduction of body weight, and improvement in glucose blood levels.

Negative public opinion on genetic testing in order to create a unique personalised diet, may influence the success of nutrigenomic intervention. Steward-Knox et al. (97) conducted a study in order to determine the opinion of people in Europe on genetic testing and individually tailored nutrition. Individuals who were willing to undertake a genetic test for creating personalised dietary interventions in most cases had high blood cholesterol levels, central obesity, and/or high levels of stress, whereas individuals who were not obese were not willing to have a nutrigenomic intervention.

To summarise, obesity involves an interaction between genetic and environmental factors (98). This is the reason why it is important to include gene–environment information in the management and prevention of excess weight. However, some issues of weight management are still crucial (15): identification of markers that can predict the results of dietary interventions; development of methods for identifying biomarkers; simple and available measurement of markers; and responsible use of individual genetic information.

CONCLUSION

Obesity is caused by the combined impact of genetic, environmental, and lifestyle factors. It represents a risk factor for cardiovascular and metabolic diseases (hypertension, type 2 diabetes, insulin resistance, hyper-insulinaemia, gout, liver disease, gall bladder disease), kidney disease, reproductive problems, sleep apnoea, osteoarthritis, and multiple types of cancer. Overall, it is the culprit for many current health problems in the population of western countries. As the prevalence of obesity continues to increase, it is important to better understand the genetic aspect of obesity and the

importance of the interactions between genes, genome, epigenome, and the environment obesogens. The disputable and known pharmaceutical and environmental obesogens, such as thiazolidinediones, organotins, perfluorooctanoic acid, diisobutyl phthalate, and bisphenol A, are still used today and should be banned and removed from the environment.

Also, although nutritional interventions can reduce body mass, the process of gaining weight can be affected by an individual genetic profile. Nutrigenomic approach allows us to explore these interactions and apply them in the management of obesity. Many genes in which polymorphisms can affect the development of obesity are identified, but further investigations are still necessary; especially in the development of new diagnostic methods, categorisation of obesity based on specific genotype, creation of personalised nutrition for treating excess weight, and the most important, effective prevention tools. To conclude, the future of nutrigenomics science brings many challenges for scientists, practitioners, caregivers, and individuals. Investigating obesity and its significant health implications is and will continue to be important, as more effective ways need to be found to resolve this public health problem.

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Sažetak

PRETILOST – MEĐUDJELOVANJE GENOMA I OKOLINE

U industrijaliziranom svijetu među odraslim osobama, adolescentima i djecom pretilost je postala jedna od glavnih prijetnja za javno zdravlje ljudi. Njezina je pojavnost pod utjecajem međudjelovanja gena, prehrane, okoliša i načina života. Važni čimbenici rizika vezani su uz okolinu i način života, uključujući čimbenike prisutne već u okruženju fetusa te one prisutne tijekom cijeloga života kao što su kvaliteta prehrane, izloženost kemikalijama, mikroorganizmima i psihičkom stresu. Uklanjanje ili ograničavanje kemijskih tvari i lijekova koji uzrokuju pretilost iz ljudske okoline moglo bi utjecati na opadanje epidemije pretilosti.
Dodatno, nutrigenomika opisuje kako se promjenama u prehrani pojedinca može poboljšati zdravstveno stanje i spriječiti razvoj kroničnih bolesti, uključujući i pretilost, a pritom je potrebno poznavati utjecaj genskog profila na međudjelovanje hrane i porasta tjelesne mase. Nadalje, genske varijacije u genomu pojedinih osoba stvaraju i njihovu predispoziciju za razvoj pretilosti. Stoga se zahvaljujući informacijama o genskom profilu pojedinca, izbjegavanjem tvari koje uzrokuju pretilost i zdravim načinom života može poboljšati kontrola pretilosti i održavati optimalna tjelesna masa.

KLJUČNE RIJEČI: *geni, nutrigenomika, prehrana, tvari koje uzrokuju pretilost*

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Review

BIOLOGICAL INDICATORS IN RESPONSE TO RADIOFREQUENCY/MICROWAVE EXPOSURE

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Over the years, due to rapid technological progress, radiation from man-made sources exceeded that of natural origin. There is a general concern regarding a growing number of appliances that use radiofrequency/microwave (RF/MW) radiation with particular emphasis on mobile communication systems. Since non-thermal biological effects and mechanisms of RF/MW radiation are still uncertain, laboratory studies on animal models, tissues, cells, and cell free system are of extraordinary importance in bioelectromagnetic research. We believe that such investigations play a supporting role in public risk assessment. Cellular systems with the potential for a clear response to RF/MW exposures should be used in those studies. It is known that organism is a complex electrochemical system where processes of oxidation and reduction regularly occur. One of the plausible mechanisms is connected with generation of reactive oxygen species (ROS). Depending on concentration, ROS can have both beneficial and deleterious effects. Positive effects are connected with cell signalling, defence against infectious agents, and proliferative cell ability. On the other hand, excessive production, which overloads antioxidant defence mechanism, leads to cellular damage with serious potential for disease development. ROS concentration increase within the cell caused by RF/MW radiation seems to be a biologically relevant hypothesis to give clear insight into the RF/MW action at non-thermal level of radiation. In order to better understand the exact mechanism of action and its consequences, further research is needed in the field. We would like to present current knowledge on possible biological mechanisms of RF/MW actions.

KEY WORDS: *interactions, macromolecules, non-ionizing, non-thermal, radiation, reactive oxygen species*

For centuries human population has been exposed to natural electromagnetic sources such as radiation from sun, space, and earth. Today, besides this natural radiation and owing to rapid technological progress we are more than ever exposed to man-made electromagnetic radiation (EM). Various products and applications in our everyday life make use of some form of electromagnetic energy (1). Connection between technological development and increase in EM radiation doubtlessly exists. Levels of radiation have rapidly increased and man-made sources at specific frequencies now by far exceed those of natural origin. There is a particular concern regarding an increase in the number of appliances which use RF/MW radiation. Some of them have become an integral part of our lives like mobile phones, television, microwave ovens, medical devices, radars, and satellites (2). Besides the obvious benefits, there is also a widespread public concern about the potential health effects this technology can have on biological system and living beings. EM radiation can be described as the propagation of energy through space in the form of waves or particles (photons) and it is characterised by specific wavelength, frequency, and energy. Because of thermal agitation of charged particles every single object is continuously generating

electromagnetic field. Energy of the field is directly proportional to its frequency, with higher frequency meaning greater energy (3). Minimum energy capable of causing ionisation by breaking the intermolecular bonds and releasing electrons from an atom or molecule is considered to be 10 eV (1). Based on their ability to cause ionisation, we can distinguish two types of electromagnetic radiation; ionising and non-ionising radiation. Non-ionising radiation includes three frequency ranges; static (0 Hz) and extremely low frequency range (<300 Hz), intermediate frequency range (300 Hz-10 MHz), and radiofrequency range including RF and microwaves (10 MHz to 300 GHz) (2). Over the years, rapid increase in technology, especially telecommunication, has raised great concerns regarding possible adverse health effects of excessive RF/MW exposure. This is not surprising if we know that charged ions, molecules, and structures inside the body contribute to electromagnetic processes that are crucial for the normal functioning of every living organism. These processes are characterised by specific frequencies usually found in the microwave region of electromagnetic field (4).

Passing through a biological system, RF radiation can be reflected, transmitted, refracted or absorbed (1). Absorption of RF energy stirs up the motion of charged particles and rotation of molecules, primarily water, inevitably rising the temperature (5). Measure of absorbed energy within the organism is defined as the specific absorption rate (SAR) and is usually expressed in watts per kilogram (W kg^{-1}) (6). For general public, SAR limit for the whole body is 0.08 W kg^{-1} and for people occupationally exposed to RF energy limit is 0.4 W kg^{-1} (7). Based on the amount of absorbed energy within the biosystem, effects can be divided into thermal and non-thermal. Certainly the most acceptable mechanism of RF interaction is tissue heating. Thermal effects occur with the temperature increase exceeding 1 °C causing cellular and intracellular changes particularly at molecular level (1). The amount of heat depends on the radiation intensity, electrical properties of exposed tissue and body′s thermoregulatory mechanism (7). The World Health Organization stated that many biological effects of acute exposure to non-ionising radiation are consistent with responses to induced heating, resulting either in the rises in tissue or body temperature by about 1 °C or more, or in responses to minimising the total heat load. However, during low intensity radiation thermal homeostasis remains stable due to activated thermoregulation processes. The body temperature might rise by up to 1 °C without accumulation. Based on this statement basic exposure limits for occupational and general population have been established and are known as exposure standards (8). Consequently, the problem of a prolonged exposure to low intensity radiation and non-thermal biological effects derived from RF/MW sources remains open to science.

Biological mechanisms of RF/MW action

A number of published papers point at different modifications and biological effects caused by RF/MW radiation, while at the same time others lack significance. These contradictions are not surprising given the variety of factors influencing experimental procedure. Some of them include polarisation, duration of exposure, continuous or pulsed wave exposure, biological factors of exposed system and many others. All these factors have to be considered so that research results can be reproducible as it was proposed by Belyaev et al. (9) in 2000 and 2005 (10).

The exact mechanism of non-thermal electric and magnetic field interactions with biological structures is still unknown and many different theories suggest various explanations. Detectible changes can only be observed if the applied RF/MW radiation exceeds thermal noise caused by constant random movement of charges at temperatures above absolute zero. Minimum energy required for these processes should exceed 26 meV, the average energy of thermal noise measured at body temperature. Considering low energy level of RF/MW radiation, it is believed that changes could only be detected when biological system is resonantly sensitive to applied frequency (11).

Fröhlich was the first to propose a model based on vibration interactions between large molecules and components of biological systems. Trying to explain possible biological effects he suggested a particular coherent state of vibrations and the existence of specific frequencies within the RF/MW band of electromagnetic spectrum in which energy could be absorbed (12, 13). Under special conditions during vibration, electrically polar structures generate EM field. However significant effects can only be detected when these structures are in resonance with external electric field having the same frequency (11). Because of its ability to detect and amplify small signals against the background noise, organism could be easily compared with a radio receiver. Based on this mechanism, Hyland (4) also suggested the possibility

of biological effects occurring at frequencies of mobile phones. These effects could only be observed if certain biological structures had the same vibration frequencies as the applied EM field (12, 14). Cell membranes were first considered a possible source of cellular vibrations until the discovery of cytoskeleton when much attention was given to microtubules. Microtubule reassembly was related with the peak of cellular EM field emission during replication suggesting the crucial role in their generation. Interestingly microtubules have the ability to vibrate in kHz to GHz frequency region (11).

Pokorný et al. (15, 16) proposed a model based on microtubule vibrations. However there were few remarks regarding potential vibration damping given the highly viscous medium surrounding them. Taking this into account, Adair (17) calculated and concluded that because of damping effect vibrations were too small to cause any biological effect. On the other hand, Pokorný (18) showed that damping effect on microtubules could be minimised due to a slip layer formed at the boundary of microtubules and cytosol. Changes in microtubule structure were also observed in several papers (19-22).

Bohr and Bohr (23, 24) suggested that some protein conformations had very similar energies and that transition between them corresponded to the frequencies of approximately few GHz. Based on the experimental results they concluded that MW radiation has the ability to change protein conformation by enhancing the kinetics of protein folding and denaturation, which could eventually lead to detrimental biological effects. De Pomerai et al. (25) also showed that aggregation of protein bovine serum albumin could be enhanced by microwave radiation. Other different approaches have been considered regarding the interactions between RF/MW radiation and biological systems. One of them is based on the interactions with small particles of ferromagnetic material magnetite. Magnetite is found not only in certain bacteria but also in human beings, especially in the brain. It strongly absorbs radiation at frequencies between 500 MHz and 10 GHz and converts the energy into acoustic vibrations dissipating it in cellular structures at microwave frequencies. These findings also led Kirschvink (26) to propose magnetite as a possible mechanism of interaction. Besides magnetic nanoparticles, there are other possible targets for magnetic field interactions like spinning magnetic moments in radical pairs or long-lived rotational states of some molecules (27, 28).

Radical pair mechanism is considered the most probable mechanism in explaining the interactions between cellular and static or extremely low EM field (ELF). At the same time researchers suggest that the proposed mechanism could also explain biological effects of radiofrequency radiation (1, 29). Radical pairs are formed during normal metabolic processes and although have very short lifetime they react with each other or other molecules and generate new radicals. They contain one or more unpaired electrons and their magnetic properties are related to electron spin. Electron spins of two radicals can be in either singlet or triplet state i.e. parallel or opposite from one another. Oscillations between two states depend on magnetic interactions of unpaired electrons with each other, nuclear spins, and external magnetic field (30). Stable molecules are formed only when two spins are opposite one from the other, while parallel spins first require a spin change. External magnetic field slows down the processes of spin change, causes radical pair separation and increases their lifetime and concentration. The process is even more expressed when radicals are attached to cellular structures such as cell membrane (31). A biophysical model for the effect of oscillating electric fields on cells has been given by Panagopoulos et al. in 2002. This model is based on forced-vibration of free ions on the surface of a plasma membrane caused by an external oscillating field. It was shown that coherent vibration of electric charge was able to affect electro sensitive channels on the membrane which could lead to the disruption of cell′s electrochemical balance and function. The proposed model theoretically proved that pulsed EM fields could be twice as effective biologically as the continuous ones (32). Furthermore there have been a number of papers connecting exposure of RF/MW radiation with excessive formation of free radicals (67-74). Action of free radicals is additionally enhanced in the presence of oxygen which further causes the formation of new radicals. These free radicals are also known as reactive oxygen species (33).

Reactive oxygen species

Under physiological conditions, ROS are generated everyday as a result of various metabolic processes. They are known to have both beneficial and deleterious effects. At low concentrations they are often involved in defence against infectious agents, cell signalling, and mitogenic response. On the other hand, overload of ROS concentration and antioxidant deficiency leads

to cellular damage including membranes, lipids, proteins, and even DNA. Several diseases are associated with adverse effects caused by reactive oxygen species such as diabetes, atherosclerosis, chronic inflammation, malignant and neurodegenerative diseases, and many others (34, 35).

Balance between these effects is accomplished by redox regulation (34, 36). ROS include oxygen radicals like superoxide radical ($O_2^{\cdot-}$), hydroxyl ($HO^{\cdot}$), peroxyl ($RO_2^{\cdot}$), and alkoxyl ($RO^{\cdot}$) radicals as well as nonradical derivatives of oxygen like hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), and ozone (O_3) (37).

Even oxygen molecule, having two unpaired electrons found in triplet state, qualifies as a free radical, or to be precise - biradical. However, because of parallel spins oxygen is able to accept only one electron at a time which consequently slows down its′ reactivity (Figure 1) (38).

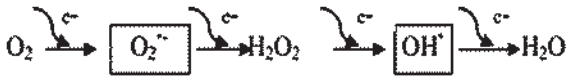

Figure 1 *One electron reduction of oxygen and formation of superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) with water as a final electron acceptor.*

One of the major sources of ROS in the cell is mitochondrion (35). Out of total oxygen consumed, 1 % to 2 % is converted to oxygen free radicals, mostly at the level of complex I and complex III of respiratory chain (39, 40). Besides mitochondrion, there are several other sources of ROS in the cell. Some of them include peroxisomes, enzymes like xanthine oxidase, cytochrome P450, neutrophils, eosinophils, and macrophages (41, 42). Superoxide radicals are mostly generated in mitochondria as a result of electron leakage from electron transport chain and premature reduction of oxygen. They are known as the primary ROS able to generate "secondary" ROS by further reacting with other molecules. Superoxide radicals extract iron from iron-containing molecules and enable Fe^{2+} to participate in the Fenton reaction creating hydroxyl radical (Eq. 2). They also participate in the Haber-Weiss reaction (Eq. 3), which combines the reduction of iron and the Fenton reaction (Eq. 1 and 2) (43).

$$Fe^{+3} + O_2^{\cdot-} = O_2 + Fe^{+2} \quad (1)$$

$$Fe^{+2} + H_2O_2 = Fe^{+3} + HO^{\cdot} + OH^{-} \quad (2)$$

Equation 1, 2 - the Fenton reaction

$$O_2^{\cdot-} + H_2O_2 = O_2 + HO^{\cdot} + OH \quad (3)$$

Equation 3 – the Haber-Weiss reaction

Hydroxyl radicals can also be produced in reaction with other metals like copper, chromium or cobalt (44). However, reaction catalysed by iron is considered to be the major mechanism in the generation of highly reactive hydroxyl radicals (45). Because of a very short lifetime, these radicals are known to react in close proximity of the site of formation (43). Superoxide can also generate hydrogen peroxide in a reaction of dismutation. In biological systems, this reaction is additionally accelerated by superoxide dismutase (SOD) (46). Although not a radical, hydrogen peroxide is considered to be a reactive oxygen metabolite. It can easily pass the cell membrane and directly or indirectly cause damage to macromolecules even at a low concentration. Because of its lipid solubility H_2O_2 can also generate $HO^{\cdot}$ radical at localised sites containing iron or copper (47).

Excessive level of free radicals can cause severe damage to biological systems, which could eventually lead to disease development. Because of their high level of polyunsaturated fatty acids (PUFAs), cell membranes are particularly sensitive to oxidative damage. During the three-stage process of lipid peroxidation, aldehydes are formed as a final product (48). One of the most abundant products of lipid peroxidation is certainly malondialdehyde (MDA), which is also used as a biomarker of oxidative stress. MDA is a known mutagen and carcinogen. It reacts with DNA and forms adducts with deoxyguanosine, deoxyadenosine, and deoxycytidine (49, 50). Besides lipids, proteins are also highly sensitive to free radical exposure. They are involved in different biological functions and even the slightest change in protein structure or conformation could result with their loss of function. Modifications of amino acids, fragmentation of polypeptide chains or protein-protein cross-linkage are some of the changes that could be induced as a result of protein interactions with free radicals (51). Considering that different modifications are numerous, there are also various methods for detecting and quantifying them. Certainly the most measurable products of protein oxidation are protein carbonyls (52). They are characterised by carbonyl group generated at the protein side chains. Because of their stability, they can be easily detected (53). ROS can also react with nucleic acids, especially DNA, and cause different modifications, even single or double strand breaks. Guanine is most susceptible to oxidative damage and in reaction with ROS forms highly mutagenic and carcinogenic 8-oxoguanine (43). Because of a high level of ROS present in mitochondria

and limited repair mechanism, mitochondrial DNA is particularly sensitive to free radicals (54).

Cell antioxidant defence system

Given the variety of possible damage that could be caused by free radicals, organisms have developed a whole spectrum of different defence mechanisms. They include preventive and repair mechanisms as well as physical and antioxidant defence. Antioxidant defence includes enzymes like SOD, glutathione peroxidase (GPx), and catalase (CAT), while non-enzymatic antioxidants include glutathione (GSH), vitamin E and C, carotenoids, flavonoids, and many others (34). Certainly one of the most significant compounds of antioxidant defence is glutathione. Glutathione is a low molecular weight antioxidant capable of preventing oxidative damage by reacting directly or indirectly with ROS (47). It is found in reduced (GSH) or oxidised (GSSG) form with their ratio often used as a measure of toxicity or antioxidant capacity (55). GSH can react directly with free radicals. This results in GSH becoming a radical itself that forms GSSG reacting with another GSH radical. Accumulation of GSSG causes disturbance of redox balance, which affects metabolic processes, homeostasis, and cellular integrity (56, 57). GSH is also known to act as a cofactor for several enzymes, participates in the transport of amino acids and helps the regeneration of vitamin E and ascorbic acid (47, 43).

As previously mentioned, SOD helps to remove superoxide radicals by producing hydrogen peroxide which is later removed by the activity of GPx and CAT. There are three SOD isoforms that exist in animal tissues; cytosolic CuZnSOD, MnSOD, and extracellular SOD (ECSOD), each dealing with decomposition of $O_2^{\cdot-}$ in different compartments (58).

DISCUSSION

It is known that altering magnetic field produces an electric field and vice versa, a varying electric field generates a magnetic field. Because of this interdependence, both fields jointly are considered a single entity - the electromagnetic field (59). Alternating electric fields have a wide range of effects on living systems. At extremely low frequencies, electric fields stimulate excitable tissues through membrane depolarisation, stimulate bone growth, and accelerate fracture healing (60-63). As the electric field frequency increases, the stimulatory effect disappears, ending in the so-called thermal effect, when tissue heating becomes a dominant event (64). In-between the desired stimulatory and undesired heating effects, there is this mostly unexplored biological potential of RF/MW radiation at a non-thermal level of exposure.

Since the expanded mobile phone use has caused a serious apprehension worldwide, along with the fact that relationship between low intensity RF/MW radiation and its undesirable bioeffects are very close, we attempted to recognise reasonable indicators which might give an insight into the mechanism of action. It seems that such approach has become more important than ever.

Considering the Fröhlich hypothesis, Pokorný at al. (65) investigated the non-thermal cellular effects of radiation in the microwave range. It was concluded that the observed intracellular changes are a consequence of macromolecular chain resonance caused by external radiation. In electrobiological entity such as a single cell, internal electromagnetic field created by own polar intracellular structures enters into a coherent state with external field, which means that oscillating electric field of low strength could cause significant non-thermal biological changes. As it was mentioned previously, it occurs when the wavelength of radiation is in the range of the properties of biological tissue. In the state of coherence, external field could cause attenuation or amplification of internal frequency field leading to disruption of homeostatic balance. Further, RF/MW radiation seems to induce electric oscillations that disturb cell membrane proteins, activating enzyme cascades that may transfer cell surface signals to the intracellular system (66).

Several investigations point at a possible connection between RF/MW exposure and ROS generation. Results of conducted researches are often contradictory and inconclusive because many factors have to be considered during the experiment. One of the reasonable pathways of RF/MW intracellular action is based on the generation of reactive oxygen species (ROS). Increased levels of ROS, antioxidants, and cellular damage were found in different types of investigations including cell cultures, laboratory animals, and even in the humans after RF/MW exposure (67-74). Xu et al. (75) exposed human lens epithelial cells to 1.8 GHz RF field at different SAR values. After 2 h of intermittent exposure, only the

group exposed to smallest SAR value of 1 W kg^{-1} didn't cause increase in ROS concentration, while other groups of 2 W kg^{-1}, 3 W kg^{-1} and 4 W kg^{-1} did. In order to show the effects of cellular phone radiation on the brain and blood of guinea pigs, Meral et al. (76) exposed animals to 890 MHz to 915 MHz radiation for 12 h per day for 30 days. The brain concentration of MDA increased, while CAT and GSH decreased. On the other hand, the blood levels of MDA, CAT, vitamins A, D3, and E increased and GSH decreased. A study conducted by Moustafa et al. (49) examined the effect of acute exposure to 900 MHz radiofrequency field on 12 male volunteers. Results showed significant increase in plasma lipid peroxides and reduction in the SOD and GPx activity in erythrocytes supporting the interaction of radiofrequency fields with biological systems. However, numerous studies were unable to connect generation of ROS and oxidative damage with RF/MW exposure (77-80). In their study, Ferreira et al. (81) exposed rats to 834 MHz radiation to determine the effects on non-enzymatic antioxidant defence, as well as lipid and protein damage. After measuring the level of MDA and protein carbonyls in rat brain tissue, they concluded that there was no significant difference compared to the sham exposed group. Combining the effects of EM radiation and chemicals known to induce free radicals, researchers also tried to investigate the possibility that RF/MW radiation alters the effect of chemical agents. Zmysloný et al. (82) tested the effect of iron ions on rat lymphocytes exposed to continuous wave (CW) EM field. The authors compared two groups of lymphocytes, one treated with $FeCl_2$ and the other treated with $FeCl_2$ and exposed to 930 MHz. They noticed that after acute exposure for 5 min and 15 min ROS level was significantly higher in the exposed group. Similar experiments and results were published by Höytö (83) and Luukkonen et al. (84). It could be concluded that existence of non-thermal effects, including oxidative damage caused by RF/MW radiation, must not be denied. Thereafter, the International Agency for Research on Cancer (IARC) classified RF electromagnetic fields as possibly carcinogenic to human (85). Given the electromagnetic nature of living beings and a growing number of man-made sources of non-ionising radiation, there is also a constant need for better understanding the exact mechanism of interaction between radiation and cellular systems. What makes it particularly difficult is certainly the highly dynamic and complex organisation and function of every organism. A variety of factors contribute to research results, which is the reason why *in vitro* studies are frequently used in this kind of research. However, despite precisely defined and controlled studies, some results are still open to doubt. Future research should pay more attention to determining a possible connection between RF/MW radiation and generation of free radicals *in vitro*. Special consideration should be given not only to the concentration but also to the type of ROS, their origin and the changes in the antioxidant defence system, as well as to macromolecular changes.

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Sažetak

BIOLOŠKI POKAZATELJI KAO ODGOVOR NA IZLOŽENOST RADIOFREKVENCIJSKOMU/ MIKROVALNOM ZRAČENJU

Zbog ubrzanoga tehnološkog napretka tijekom godina zračenje iz umjetnih izvora mnogostruko je nadmašilo zračenje iz prirodnih izvora. Rastući broj uređaja koji rabe radiofrekvencijsko/mikrovalno (RF/MW) zračenje, osobito mobilni komunikacijski sustav, izaziva zabrinutost opće i znanstvene javnosti. S obzirom na to da su učinci i mehanizmi netermalnoga biološkog djelovanja RF/MW zračenja dvojbeni, laboratorijske studije u kojima se rabe životinjski modeli, tkiva, stanice i bestanični sustavi smatraju se izuzetno važnima u bioelektromagnetskim istraživanjima. Vjerujemo da takva istraživanja podupiru procjenu opasnosti od zračenja za živi organizam. Zbog mogućnosti jasnog odgovora stanice na RF/MW izloženost preporučuje se uporaba staničnog sustava. Zna se da je organizam složen elektrokemijski sustav, gdje se među mnogim drugima redovito događaju oksidacijsko-redukcijski procesi. Jedan od mogućih mehanizama djelovanja povezuje se sa stvaranjem reaktivnih kisikovih spojeva (ROS). Ovisno o koncentraciji ROS-ovi mogu štetiti, ali i djelovati korisno na biosustav. Povoljni učinci ROS-ova povezuju se s diobenom sposobnosti stanice, staničnim signalnim putovima i obranom protiv infektivnih čimbenika. Međutim prekomjerno stvaranje koje preopterećuje antioksidacijski obrambeni sustav dovodi do oštećenja stanica i mogućnosti razvoja bolesti. Čini se da je hipoteza o povišenju koncentracije ROS-ova u stanici zbog zračenja biološki valjana, jer može pojasniti mehanizam netermalnih učinaka RF/MW zračenja. S ciljem boljeg razumijevanja točnog mehanizma, kao i njegovih posljedica potrebna su dodatna istraživanja. Ukratko su prikazane postojeće spoznaje o mogućim biološkim mehanizmima djelovanja RF/MW zračenja.

KLJUČNE RIJEČI: *makromolekule, neionizirajuće zračenje, netermalno zračenje, reaktivni kisikovi spojevi*

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ANNOUNCEMENT

EUROANALYSIS XVII
Varšava, Poljska, 25. - 29. kolovoza 2013.

Sedamnaesti međunarodni skup iz područja analitičke kemije održat će se na Tehnološkom fakultetu Sveučilišta u Varšavi.

Znanstveni program skupa podijeljen je u petnaest tema: Bioanalitička kemija, Elektroanalitička kemija, Priprava uzoraka, Tehnike odijeljivanja u analitici, Spektroskopija u analitici, Analitika u biologiji i medicini, Analitika lijekova, Mikro- i nanoanalitika, Bio- i kemosenzori, Vezane tehnike, Analiza okoliša i hrane, Analitička kemija u kulturnom nasljeđu, Kemometrija i kvaliteta u analitičkoj metodologiji, Razvoj analitičkih instrumenata i Suvremeni alati u podučavanju analitičke kemije. Predviđena su plenarna predavanja koja će održati pozvani predavači, usmena priopćenja i posterske prezentacije.

Sažeci se mogu prijaviti do 15. svibnja 2013. godine, a do 15. srpnja 2013. moguća je registracija po sniženoj cijeni.

Više informacija o međunarodnom skupu dostupno je na web-adresi: http://www.euroanalysis2013.pl.

Irena Brčić Karačonji

REPORTS
Arh Hig Rada Toksikol 2012;63:A24-A29

REPORTS

NACIONALNA KONFERENCIJA O SIGURNOSTI PČELINJIH PROIZVODA
Opatija, Hrvatska, 13. travnja 2012.

„Nacionalna konferencija o sigurnosti pčelinjih proizvoda“ održana je u Opatiji 13. travnja 2012. u organizaciji Katedre za zdravstvenu ekologiju Medicinskog fakulteta Sveučilišta u Rijeci, Nastavnog zavoda za javno zdravstvo Primorsko-goranske županije, „Biopčele“, Udruge za promociju i primjenu ekološke pčelarske etike te Centra za brdsko-planinsku poljoprivredu.

Ovo je bio prvi skup na nacionalnoj razini na kojem se, sa znanstvene, stručne, zakonodavne i proizvođačke razine, raspravljalo o sigurnosti pčelinjih proizvoda i njihovoj zdravstvenoj ispravnosti na tržištu Republike Hrvatske te proizvodnim i zakonodavnim aspektima koji definiraju odgovornost u ovom izuzetno važnom sektoru. O važnosti koja je pridana ovom skupu, govori činjenica da je Konferenciji dodijeljeno višestruko institucionalno pokroviteljstvo najrelevantnijih državnih tijela i institucija – Ministarstva zdravlja Republike Hrvatske, Ministarstva znanosti, obrazovanja i sporta Republike Hrvatske, Ministarstva poljoprivrede Republike Hrvatske, Primorsko-goranske županije te Turističke zajednice Grada Opatije.

Ono što posebno značajno valja istaknuti jest podrška koju je kroz institucionalno pokroviteljstvo dodijelila tzv. proizvođačka i stručna „baza“ odnosno Hrvatsko apiterapijsko društvo, Hrvatski pčelarski savez te domaćinska, Pčelarska udruga „Učka“ iz Opatije.

Konferencijom su okupljeni predavači iz redova ponajboljih stručnjaka, pripadnika akademske i stručne zajednice, koji se po različitim osnovama bave valorizacijom sigurnosti i zdravstvene ispravnosti pčelinjih proizvoda. Predavači su iznijeli vlastita iskustva i najnovije spoznaje vezane uz utvrđivanje sigurnosti i zdravstvene ispravnosti pčelinjih proizvoda iz višestruko različitih aspekata. Pri tome su posebno značajan doprinos dali i cijenjeni stručnjaci u ovom području iz susjednih zemalja Europske unije – Slovenije i Italije, a kao trajni pisani trag s Konferencije ostao je Zbornik radova „Nacionalne konferencije o sigurnosti pčelinjih proizvoda”. Konferencija je potaknula vrlo dinamičnu raspravu o raznim pitanjima vezanim uz valorizaciju sigurnosti i analitičke i proizvodne aspekte zdravstvene ispravnosti pčelinjih proizvoda, ali i njihova učinka na zdravlje. Vodeći se vlastitim (ali i inozemnim) iskustvima, uz puno uvažavanje stručnih i zakonodavnih odrednica, pokazalo se da valorizacija sigurnosti i zdravstvene ispravnosti pčelinjih proizvoda podrazumijeva multidisciplinarnost i sveobuhvatnost u pristupu te cjelovitu mobilizaciju i odgovornost hrvatskoga stručnog, znanstvenog i proizvođačkog potencijala. Kako bi se osiguralo da sigurnost pčelinjih proizvoda u budućnosti dobije jasne odrednice, sudionici „Nacionalne konferencije o sigurnosti pčelinjih proizvoda” konsenzusom su donijeli sljedeće zaključke:

1. Uočena je potreba uspostave baze standarda kojima će se jasno definirati odrednice sigurnosti i zdravstvene ispravnosti pčelinjih proizvoda u Republici Hrvatskoj;

2. Istaknuta je potreba za kontinuiranim praćenjem zdravstvene ispravnosti pčelinjih proizvoda u Republici Hrvatskoj;

3. Naglašena je potreba provedbe edukacije o sigurnosti i zdravstvenoj ispravnosti pčelinjih proizvoda u Republici Hrvatskoj na svim razinama – od proizvođačke i potrošačke do stručne i znanstvene razine;

4. Istaknuta je potreba za podizanjem kulture konzumacije pčeljinjih proizvoda građana Republike Hrvatske;

5. Uočena je potreba za promocijom ekoloških aspekata u pčelarskoj proizvodnji Republike Hrvatske;

6. Istaknuta je potreba za povećanjem razine analitičkog praćenja sigurnosti i zdravstvene ispravnosti pčelinjih proizvoda u Republici Hrvatskoj;

7. Naglašena je potreba za provedbom revizije statističkih podataka o potrošnji pčelinjih proizvoda u Republici Hrvatskoj;

8. Uočena je potreba za promocijom apiterapijskih aspekata temeljenih na egzaktnim znanstvenim dokazima i potvrđenim jasnim pravilima struke;

9. Istaknuta je potreba za jasnijim određenjem prema suzbijanju pojave patvorina pčelinjih proizvoda na tržištu Republike Hrvatske.

S obzirom na nacionalni karakter skupa kao i odgovornosti koja proizlazi iz dodijeljene multiinstitucionalne potpore, zaključci „Nacionalne konferencije o sigurnosti pčelinjih proizvoda" održane u Opatiji, 13. travnja 2012. postavljaju nove odrednice kojima će se u budućnosti morati graditi sigurnost i zdravstvena ispravnost pčelinjih proizvoda u Republici Hrvatskoj.

Dražen Lušić

NANOSAFETY CONGRESS-TURKEY
Kemer, Antalya, Turska, 26. – 29. travnja 2012.

Pod pokroviteljstvom triju velikih projekata, MARINA (Managing Risks of Nanomaterials), NanoLINEN (NANOtoxicology Link between INdian and European Nations) i NanoValid (Developing Reference Methods for Nanomaterials) organiziran je kongres u Turskoj (Kemer Resort Hotel) kojem je tema bila nanosigurnost. Sva tri projekta su djelomično ili u potpunosti financirani iz Europske unije i FP7 projekata kojima je cilj bio povezati što više istraživača iz europskih zemalja (sedam), Tursku i Indiju u zajedničke projekte u kojima bi se zajedničkim snagama utvrdila štetnost utjecaja nanomaterijala i našli mogući pokazatelji kojima bi se mogla mjeriti izloženost, zaštita i poboljšala sigurnost te proširila upotreba nanomaterijala u raznim životnim područjima. MARINA projekt je smješten u Institutu za medicinu rada u Velikoj Britaniji te uključuje 10 FP europskih projekata te istraživače iz Rusije i Kine. MARINA 3 i 4 projekti imaju zadatak proučiti fizičko-kemijska svojstva i pripremiti harmonizirane i validirane protokole za disperziju i karakterizaciju sljedećih nanomaterijala: dioksida titana (TiO_2), silicija, cerija te cinka i višestraničnih ugljičnih nanocjevčica (MWCNT-od engl. *multi-wall carbon nanotubes*), srebrnih nanočestica te bentonita. Osim toga, trebaju proučiti modele bioakumulacije, transporta te utjecaja na životni ciklus živih organizama, odrediti dozno-odzivne krivulje za svaku vrstu kontakta (inhalacija, oralno ili površinski – kožno) te odrediti strategije za redukciju rizika u ljudi i ostalih živih organizama.

Tema kongresa bila je sigurnost u upotrebi i dizajniranju nanomaterijala, u koju je sudionike uvelo i uvodno pozvano predavanje Güntera Oberdörstera : *The Issues Surrounding Safety Assessment of Engineered Nanomaterials*. Kongres je također obuhvatio i dvije radionice. Prva naziva *Workshop on Safety Assessment of the Nanomaterials: New Paradigms* bavila se raspravom o nepoznanicama između istraživačkog dijela nanomaterijala te utjecaja tih materijala na okoliš i na sigurnost živih organizama. Tu je bilo govora o procjeni toksičnosti PEi chitosan (kitozan)/DNA nanomaterijala koji bi se mogli koristiti u *in vitro* i *in vivo* uvjetima kao vektori koji bi utjecali na ekspresiju gena, o utjecaju umjetnih nanomaterijala na niže organizme ekosustava, o utjecaju TiO_2 i MWCNT nanočestica na nazalne i bronhalne epitelne stanice pacijenata s nazalnim polipima i kroničnom opstruktivnom pulmonarnom bolešću, o utjecaju nanomaterijala na reproduktivnost i razvoj, o toksičnosti metalo-oksidnih nanočestica na stanice i u *in vivo* uvjetima, o utjecaju na neuronske stanice u staničnoj liniji, o biomarkerima oksidativnog stresa koji bi se mogli koristiti u istraživanju u sklopu lipidomike (oksidirani fosfolipidi su biomarkeri oksidativnog stresa, njima se može mjeriti pulmonarna peroksidacija nakon inhalacije ugljičnih nanocjevčica), te je bilo govora o sigurnosti radnika koji rade u proizvodnji i upotrebi nanočestica. Što se tiče izloženosti radne populacije, još uvijek nije razjašnjeno koji bi se biomarkeri koristili i koliko bi bile duge studije praćenja te kako utvrditi koje radnike treba pratiti i koji su pod povećanim rizikom od izlaganja. Druga radionica *naziva Workshop on Genotoxicity Tests to Assess Human Toxicity* više se okrenula metodama kojima bi se moglo proučavati utjecaj okolišnih faktora na zdravlje ljudi, odnosno u procjeni genotoksičnosti ne samo nanomaterijala već i formaldehida i stirena, zatim novim tehnikama u procjeni genotoksičnosti te problema na koje se može naići u velikim epidemiološkim studijama. Na kongresu je bilo 100 znanstvenika iz cijeloga svijeta, izložene su 22 posterske prezentacije, a osim njih 6 prezentacija je bilo izabrano za oralnu prezentaciju. Ovakav sastanak je pokazao koliko se zapravo malo zna o utjecaju nanočestica na okoliš, a budući da je to područje dobilo na važnosti u mnogim proizvodima s različitom primjenom, pokazano je koliko je bitno dalje proučavati ove utjecaje, te je oformljena radna

skupina koja bi 2014. godine, opet u Turskoj, trebala organizirati međunarodni kongres o utjecaju nanočestica i njihovoj sigurnosti s predviđenim brojem od preko 500 uzvanika iz cijeloga svijeta.

Mirta Milić

13TH INTERNATIONAL CONGRESS OF THE INTERNATIONAL RADIATION PROTECTION ASSOCIATION (IRPA13)
Glasgow, Škotska, 13. – 18. svibnja 2012.

Međunarodno društvo za zaštitu od zračenja ("International Radiation Protection Association" – IRPA) udruga je nacionalnih ili regionalnih društava za zaštitu od zračenja kojeg je od 1992. godine punopravni član i Hrvatsko društvo za zaštitu od zračenja (HDZZ). Svake četiri godine IRPA organizira svjetske kongrese iz područja zaštite od zračenja te se tako ove godine održao trinaesti kongres u Glasgowu (Škotska, Velika Britanija) od 13. do 18. svibnja.

Na kongresu IRPA13 sudjelovalo je više od 1500 sudionika iz 77 zemalja. Ovakvi svjetski kongresi predstavljaju najveće i najkompetentnije okupljanje svih znanstvenika i stručnjaka iz različitih područja vezanih uz zaštitu od zračenja. Glavna tema ovoga kongresa bila je „Living with Radiation – Engaging with Society". Jedanaest područja i pridruženih podtema prate ICRP strukturu „Planned, Existing and Emergency Exposure Situations", a dodano je i specifično područje koje pokriva sve aspekte nesreće u Fukushimi. Profesionalno izlaganje osoblja, zatim stanovništva i pacijenata uključeni su u određene teme. Obuhvaćena su sljedeća područja: *Biological and Health Effects of Ionising Radiation, Measurements and Dosimetry, Radiation Protection System Development and Implementation, Stakeholder Engagement and Involvement, Non-Ionising Radiation, Planned Exposure Situations: Industry and Research, Planned Exposure Situations: Medicine, Planned Exposure Situations: Radioactive Waste Management, Emergency Exposure Situations, Existing Exposure Situations, Protection of the Environment* i *Fukushima*.

Rad kongresa odvijao se u šest paralelnih sekcija s plenarnim predavanjima i usmenim priopćenjima te u dvije dvodnevne sekcije s posterskim priopćenjima. U jutarnjim satima održano je i 25 tzv. *Refresher courses* s vrlo zanimljivim temama. Posebna je pozornost ove godine bila posvećena nesreći u nuklearnoj elektrani u Fukushimi (Japan, 2011. godine), njenim posljedicama na okoliš, globalnim posljedicama za nukleranu energetiku te na percepciju nuklearne energije u javnosti. Osim plenarnih predavanja, usmenih izlaganja i postera organizirane su i sekcija posvećene diskusiji o ključnim temama od interesa za zaštitu od zračenja i razne radionice.

Mladi stručnjaci imali su prilike natjecati se za nagrade. Ove godine je u natjecanju sudjelovalo 17 kandidata iz cijeloga svijeta, među kojima i Branko Petrinec s Instituta za medicinska istraživanja i medicinu rada u Zagrebu, kojega je odabralo i sponzoriralo Hrvatsko društvo za zaštitu od zračenja. Svi su kandidati održali usmene prezentacije u svojim odgovarajućim sekcijama, a međunarodni žiri na čelu s Alfredom Hefnerom (Austrija) ocjenjivao je izlaganja prema sljedećim kriterijima: važnost rezultata za područje zaštite od zračenja (0 do 25 bodova), znanstvena/stručna kvaliteta (0 do 35 bodova), kreativnost (0 do 30 bodova).

Sažeci svih radova objavljeni su u zborniku sažetaka (na USB memorijskoj kartici i na internetu, http://www.irpa13glasgow.com/2012/05/irpa13-downloads-page/). Svi prezentirani posteri i sva predavanja također su dostupni na službenim stranicama kongresa http://www.irpa13glasgow.com/.

Na IRPA13 kongresu sudjelovalo je 11 članova HDZZ-a: Saveta Miljanić, Ines Krajcar Bronić, Đurđica Milković, Marina Poje, Branko Petrinec, Branko Vekić, Jelena Popić Ramač, Ivica Prlić, Dario Faj, Slaven Jurković i Mladen Novaković.

S. Miljanić je održala predavanje *Peripheral Doses in Children Undergoing Gamma Knife Radiosurgery* (autori: S. Miljanić, H. Hršak, Ž. Kneževic, Z. Heinrich i B. Vekić) koje je polučilo dosta interesa uz pitanja i diskusije poslije predavanja i nakon sekcije. B. Petrinec je u okviru natjecanja za *Young Professional Award* održao predavanje ^{137}Cs *inventory in South Adriatic* (autori B. Petrinec i Z. Franić).

Ostala priopćenja prikazana su kao posteri (abecednim redoslijedom):

1. J. Barešić, I. Krajcar Bronić, N. Horvatinčić, B. Obelić, A. Sironić, J. Kožar-Logar: Interlaboratory comparison of tritium electrolytic enrichment systems at RBI (Zagreb) and JSI (Ljubljana) (P02-200, sekcija 2: Measurements and Dosimetry)

2. S. Jurković, M. Švabić, A. Diklić, Đ. Smilović Radojčić, M. Kasabašić, A. Ivković, D. Faj: Upgrading QA/QC Programm in Radiation Therapy in Croatia: Results of the IAEA CRO 6008 Project. (P07-17, sekcija: Planned Exposure Situations: Medical)

3. I. Krajcar Bronić, B. Obelić, N. Horvatinčić, A. Sironić, J. Barešić, A. Rajtarić, B. Breznik, A. Volčanšek: Impact of refuelling of the Krško Nuclear Power Plant on the ^{14}C in the atmosphere and plants (P11-32, sekcija 11: Protection of the environment)

4. Milković Đ., Beck N., Miljanić S., Knežević Ž., Ranogajec-Komor M., Garaj V., Flegar L. Radiation Protection of Children During Chest X Ray Depending on Human Factor. (P07-151, sekcija: Planned Exposure Situations: Medical)

5. Popić Ramac, J; Knežević, Z; Hebrang, A; Vidjak, V. Radiation Dose Reduction by using low dose CT Protocol of Thorax. (P07-132, sekcija: Planned Exposure Situations: Medical)

6. I. Prlić, M. Surić, M. Hajdinjak, Z. Cerovac: Investigation of Specific Local Ecosystem Arised on the Tenorm Slag and Ashes. (P11-08, sekcija: Protection of the Environment)

7. V. Radolić, I. Miklavčić, M. Poje, D, Stanić, B. Vuković: Radon Levels in Manita Pec Cave (Croatian NP Paklenica) and Assessment of Effective Dose Received by Visitors and Tourist Guides. (P10-56, sekcija: Existing Exposure Situations)

8. Špirić, Z; Vekić, B; Barišić, D; Frontasyeva, M; Kusan, V. Moss Biomonitoring in Radiation Exposure Assessment (P09-49)

9. Szántó, P., Apáthy, I., Bodnar, L., Deme, S., Keri, A., Miljanić, S, Pázmándi, T., Vekić, B. Investigation of the dosimetric parameters of the PorTL thermoluminescent dosimetry system. (P02-235, sekcija Measurements and Dosimetry)

10. Vilić M., Aladrović J., Beer Ljubić B., Gottstein Z., Pejaković J., Miljanić S., Kraljević P. Antioxidant Status in Chicken Embrio Liver after Low Dose Gamma Irradiation. (P01-10, sekcija 01: Biological and health effect of ionising radiation)

Tijekom IRPA13 kongresa održana je i Generalna skupština IRPA-e u kojoj HDZZ predstavlja 4 delegata (od ukupno 195 delegata). Delegaciju je predvodila predsjednica HDZZ-a Saveta Miljanić, a članovi su još Ines Krajcar Bronić, tajnica HDZZ-a, te Đurđica Milković i Marina Poje. Na skupštini se biralo novo rukovodstvo IRPA-e, glasovalo se o promjenama statuta, a također je odlučeno da se IRPA-in 15. kongres 2020. godine održi u Južnoj Koreji. Prije četiri godine odlučeno je da se IRPA-in 14. kongres 2016. godine održi u Cape Townu (Južna Afrika).

I. Krajcar Bronić je prisustvovala skupu predstavnika društava za zaštitu od zračenja, Associate Societies Forum (ASF). Predstavila je aktivnosti našega društva u protekle dvije godine u usmenom izlaganju naslova "CRPA activities 2010 – 2012". Među najvažnijim predstavljenim aktivnostima bila je organizacija 8. simpozija HDZZ-a s međunarodnim sudjelovanjem, Krk, 13. – 15. travnja 2011., a najavljeno je i održavanje 9. simpozija 2013. godine.

Na inicijativu Austrijskog društva za zaštitu od zračenja održan je sastanak mladih (do 35 godina starosti) članova IRPA-e i dogovoreno je osnivanje grupe mladih unutar europskih društava za zaštitu od zračenja (*Young Members' group within the European IRPA societies*). Na osnivačkom sastanku su sudjelovali Branko Petrinec i Marina Poje, koja je dogovorno imenovana kao predstavnica HDZZ-a u toj grupi. O načinu rada i zadacima te grupe bit će više govora na 9. sastanku europskih društava za zaštitu od zračenja u Veneciji 22. listopada 2012.

Ines Krajcar Bronić i Saveta Miljanić

37TH INTERNATIONAL SYMPOSIUM ON ENVIRONMENTAL ANALYTICAL CHEMISTRY (ISEAC 37)
Antwerpen, Belgija, 22. – 25. svibnja 2012.

ISEAC je simpozij koji objedinjuje najnovije spoznaje u razvoju i primjeni analitičkih metoda prikladnih za karakterizaciju materijala iz okoliša i istraživanju srodnih problema. Ovogodišnji, 37. po redu međunarodni simpozij održan je u organizaciji Kemijskog odsjeka Sveučilišta u Antwerpenu te u suradnji s Odsjekom za analitičku i anorgansku kemiju Sveučilišta u Hamburgu i Međunarodnim udruženjem o okolišnoj analitičkoj kemiji (*International Association of Environmental Analytical Chemistry*, IAEAC).

Simpoziju se odazvalo oko 200 sudionika iz područja obrazovanja, znanosti i industrije iz različitih zemalja svijeta. Program je uključivao 9 pozvanih predavanja, 43 usmena i 137 pismenih priopćenja, od kojih su dva najbolja bila nagrađena nagradom „Roland W. Frei". Sažeci svih priopćenja objavljeni su u zborniku sažetaka.

Na ovom simpoziju aktivno su sudjelovale dvije znanstvenice iz Instituta za medicinska istraživanja i medicinu rada s posterskim priopćenjima. Sljedeći simpozij (ISEAC 38) održat će se 17.-20. lipnja 2014. u Laussanei, Švicarska.

Marija Sambolec

11TH INTERNATIONAL MEETING ON CHOLINESTERASES
Kazan, Rusija, 4. – 9. lipnja 2012.

Znanstveni skup *11th International Meeting on Cholinesterases* održan je u Kazanu, Rusija, od 4. do 9. lipnja 2012. u organizaciji sljedećih institucija: Russian Academy of Sciences, Kazan (Volga Region) Federal University, Lomonosov Moscow State University, Emanule Institute of Biochemical Physics, Kazan Scientific Center of Russian Academy of Sciences, Arbuzov Institute of Organic and Physical Chemistry, Kazan State Medical University, Kazan Institute of Biochemistry and Biophysics i Institute of Physiologically Active Compounds. Organizatori sastanka bili su Sergei Varfolomeev, Evgeny Nikolovsky i Patrick Masson, dok je tajnica skupa bila Sofya Lushchekina. Međunarodni savjetodavni odbor činio je 31 renomirani znanstvenik iz Izraela, SAD-a, Švedske, Njemačke, Švicarske, Ujedinjenog Kraljevstva, Slovenije, Čilea, Hrvatske, Francuske i Kine. U raznovrsnom znanstvenom programu Skupa održana su 2 plenarna predavanja, 57 pozvanih predavanja i 46 kratkih predavanja te je prikazano 95 postera. Na samom početku Skupa održano je predavanje posvećeno preminuloj Elsi Reiner „Tribute to Elsa Reiner" koje je održala Zrinka Kovarik iz Hrvatske. Plenarna predavanja održali su Joel Sussman (EMBO plenarno predavanje) iz Izraela i Oksana Lockridge iz SAD-a. Na Skupu je sudjelovalo oko 180 istraživača iz 22 države koji su prezentirali svoje najnovije spoznaje iz područja molekularne biologije, kinetike, kristalografije, analitičke kemije te molekulskog modeliranja. Skupu su prisustvovale četiri predstavnice iz Hrvatske, iz Instituta za medicinska istraživanja i medicinu rada, i to Zrinka Kovarik kao pozvani predavač te Anita Bosak, Maja Katalinić i Suzana Berend s posterskim priopćenjima. Sve prezentacije bile su raspoređene u 8 sekcija: *Struktura i dinamika kolinesteraza i srodnih proteina α/β-strukture, Interakcije kolinesteraza sa supstratima, inhibitorima i reaktivatorima, Antikolinesteraze: mehanizmi toksičnosti, detekcija i analitičke metode, dijagnoza izlaganja, detoksifikacija i terapija te strategija u borbi protiv terorizma, Stehiomtrijska i katalitička biološka čistila od antikolinesteraznih spojeva; nanobiotehnologija za kolinesteraze i srodni terapeutski aspekti, Enzimi koji nisu kolinesteraze, a reagiraju s antikolinesteraznim spojevima, Molekularna i stanična biologija kolinesteraza i alternativne funkcije kolinesteraza, Bolesti povezane s kolinesterazama i terapija kolinesteraznim inhibitorim*a te 3D sekcija.

Sažeci svih prezentacija objavljeni su u Knjižici sažetaka (urednica S. Lushchekina) u izdanju Kazan State University. Predavači su pozvani da svoja predavanja objave u Specijalnom izdanju časopisa *Chemico-Biological Interactions* izdavača Elsevier. Sljedeći, dvanesti po redu, sastanak o kolinesterazama bit će u Alicanteu, u Španjolskoj početkom lipnja 2015. ili u listopadu/studenom 2014. Informacije o sljedećem sastanku dostupne su na web-stranici: http://tox.umh.es/12thche/index.html.

Anita Bosak

FEBS3+ MEETING „FROM MOLECULES TO LIFE AND BACK"
Opatija, Hrvatska, 13. – 16. lipnja 2012.

Hrvatsko društvo za biokemiju i molekularnu biologiju je po prvi put dobilo priliku da u sklopu Programa FEBS3+ Europskog udruženja biokemijskih društava (*Federation of European Biochemical Societies*, FEBS) u suradnji s Mađarskim biokemijskim društvom i Slovenskim biokemijskim društvom organizira znanstveni sastanak iz područja molekularnih znanosti o životu, s ciljem povezivanja znanstvenika iz ovih zemalja, ostvarivanja znanstvene suradnje te uspostavljanja kontakata radi što uspješnijeg zajedničkog prijavljivanja budućih međunarodnih projekata. Ovaj FEBS3+ sastanak pod nazivom „From molecules to life and back" održan je od 13. do 16. lipnja 2012. godine u Opatiji.

Program sastanka je u 14 tematskih sekcija obuhvaćao prezentacije novih znanstvenih spoznaja iz područja biokemije, molekularne biologije i biomedicine. Kroz paralelne sekcije programa održano je 35 predavanja, 43 kratke prezentacije te je predstavljeno 148 posterskih prezentacija. Poseban doprinos znanstvenoj važnosti sastanka dala su četiri plenarna predavanja. Prvo plenarno predavanje na temu „Life expectancy – wishes, predictions and reality" u sklopu svečanosti otvaranja sastanka održala je Ada Yonath (Izrael), dobitnica Nobelove nagrade iz područja kemije za 2009. godinu. Tijekom sastanka plenarno predavanje održao je Kai Simons, Njemačka („Lipids organizing cell membranes"), Josef Jiricny, Švicarska („Non-canonical mismatch repair") te na samom zatvaranju sastanka Sandra Oršulić, SAD („Parallel universes in cancer"). Također, znanstveni

program obogaćen je i dvama predavanjima na temu znanost i društvo: „What it takes to succeed in science – and how Europe's institutions could help" (Gottfried Schatz, Švicarska) i „Genetically modified plants: Are they useful and safe?" (Jacques-Henry Weil, Francuska). U suradnji s Hrvatskim društvom fiziologa organizirana je i posebna sekcija posvećena novim spoznajama u istraživanju membranskih transportera.

Iznimno uspješnom FEBS3+ sastanku prisustvovalo je oko 300 sudionika, među kojima je bio velik broj doktoranata i postdoktoranata. Upravo je sudjelovanje mladih znanstvenika dodatno potaknuto dodjeljivanjem pedesetak stipendija kako nacionalnih biokemijskih Društava tako i samog FEBS-a. Uz bogat znanstveni program organizatori su sudionicima priredili i niz društvenih događanja, kada su sudionici imali priliku upoznati se s poviješću glagoljice te tajnama Istre kao *Terrae Icognitae*. Sastanak su svojom nazočnošću uveličali i Israel Pecht, generalni tajnik FEBS-a te Jacques-Henry Weil, predsjednik FEBS-ova Odbora za znanost i društvo, koji su zahvalili organizatorima i znanstvenom odboru na uspješnom sastanku dajući im punu potporu za organizaciju nekog od sljedećih susreta biokemijskih Društava ove regije.

Maja Katalinić