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Original scientific paper

## The effect of toluene on oxidative processes in rat blood

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**Abstract:** This study was designed to investigate the effects of toluene treatment on oxidative stress in rat blood. Since toluene metabolism produces reactive oxygen and nitrogen species, it was hypothesized that the toluene treatment would: 1) provoke changes in the activities of antioxidant enzymes, 2) impair the integrity of the cell membrane and 3) induce structural changes in the plasma proteins. Female Wistar rats were treated with toluene intraperitoneally, at a daily dose of 0.38 mmol/kg body weight for 12 days, and 5 mmol/kg body weight for 6 days, respectively, with propylene glycol as the carrier. Toluene significantly increased superoxide dismutase activity at low doses, catalase activity at high doses and the level of erythrocytes malondialdehyde in both treated groups when compared to the control group. The nitrite ( $\text{NO}_2^-$ ) level in both treated groups was not different from that in the control animals. Toluene caused oxidative modification of plasma proteins and, consequently, changes in the concentration of glycoproteins and lipoproteins when compared to the control group. The observed alterations indicate that toluene treatment might be involved in free radical processes.

**Keywords:** toluene; free radicals; antioxidant enzymes; protein modification.

### INTRODUCTION

Toluene is an organic solvent widely used in industry. During the process of its biotransformation, reactive intermediary products (aryl oxides),<sup>1–3</sup> reactive oxygen species (ROS), including the superoxide anion ( $\text{O}_2^{\bullet-}$ ), the hydroxyl radical ( $\text{OH}^\bullet$ ) and hydrogen peroxide<sup>2</sup> and reactive nitrogen species (RNS), including the nitrosyl radical ( $\text{NO}^\bullet$ ),<sup>4</sup> could be formed.  $\text{O}_2^{\bullet-}$  in reaction with NO generates peroxynitrite ( $\text{ONOO}^-$ ), which can cause extensive cell damage through peroxidation of lipids.<sup>5</sup> The reactive  $\text{O}_2^{\bullet-}$  is converted into less toxic  $\text{H}_2\text{O}_2$  by superoxide dismutase (SOD).  $\text{H}_2\text{O}_2$  may be converted to  $\text{H}_2\text{O}$  either by catalase

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(CAT) or glutathione peroxidase. It may generate the highly reactive free HO• *via* a Fenton reaction, which is believed to be responsible for oxidative damage.<sup>6</sup> HO• leads to peroxidation of polyunsaturated fatty acids (PUFAs), the constituents of cell and organelle membranes. In the terminal phase of lipid peroxidation (LPO), malondialdehyde (MDA) is produced, as one of the products of degradation of PUFAs.<sup>7,8</sup> Exposure of proteins to HO•, O<sub>2</sub><sup>•-</sup> or both leads to gross structural modifications.<sup>9</sup> Oxidative damage of plasma proteins commonly occurs *via* reaction with small reactive aldehydes or with aldehydes derived specifically from LPO, such as MDA,<sup>8</sup> leading to the accumulation of heterogeneous protein modifications, classified as advanced glycation end-products (glycoproteins) or advanced lipidation end products (lipoproteins).<sup>10,11</sup>

Toluene promotes the state of oxidative stress,<sup>4</sup> by exhaustion of the anti-oxidative defense enzymes. Hence, it was hypothesized that toluene treatment would: 1) provoke changes in the activities of the anti-oxidant enzymes, 2) induce the process of LPO and 3) lead to oxidative modifications of plasma proteins.

Previous studies suggest that toluene may be toxic to humans at concentrations lower than the toluene threshold limit value (*TLV*) (50 ppm) recommended by the American Conference of Governmental Industrial Hygienists (ACIGH).<sup>12</sup> It was, therefore, important to investigate the influence of toluene at the concentrations lower and higher than the *TLV*. In this study, the resulting processes in the blood of rats exposed to toluene were investigated.

#### EXPERIMENTAL

All the used chemicals were of reagent grade, purchased from Merck (Darmstadt, Germany).

##### *Animals*

Adult female Wistar rats (180–220 g) were housed in stainless steel grid-bottom cages, with free access to food and water. They were maintained under constant conditions (12 h light–dark cycles, temperature 22±2 °C). The study was conducted in compliance with the EEC Directive 86/609 and was approved by the Ethics Committee of the Faculty of Veterinary Medicine, the University of Belgrade.

##### *Toluene treatments*

Twelve rats were randomly assigned to each group. The animals were treated daily, at 9 am, by injection of propylene glycol intraperitoneally (i.p.) (vehicle control, group I), or toluene, at a dose of 0.38 mmol/kg body weight (bw) for 12 days (low dose, group II), a concentration lower than the toluene *TLV*, and at a dose of 5 mmol/kg bw for 6 days (high dose, group III), a concentration higher than the toluene *TLV*. The i.p. route enables the maximal absorption of toluene. The rats were killed by diethyl ether inhalation 24 h after the last administration.

Blood (6–8 ml) was obtained from the *aorta abdominalis* and collected in tubes containing Na citrate (3.8 % w/v) as anticoagulant. The erythrocytes were separated by centrifugation (3000 rpm) and washed three times in saline solution, followed by the immediate assessment of the enzyme activities.

### *Erythrocyte enzymes activities*

The CAT activity was determined by the method of Aebi.<sup>13</sup> The decrease in H<sub>2</sub>O<sub>2</sub> was measured spectrophotometrically at 240 nm (Cecil CE 2021 UV/Vis). One unit of CAT activity was defined as the activity required to degrade 1.0 μmol H<sub>2</sub>O<sub>2</sub> in 60 s at 25 °C and pH 7.0. The activity is expressed as 10<sup>4</sup> μM min<sup>-1</sup> (g Hb)<sup>-1</sup>.

The SOD activity was determined using a Superoxide Assay Kit (Calbiochem), which utilizes 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzyl[*c*]fluorene. This reagent undergoes alkaline auto-oxidation, which is accelerated by SOD. The auto-oxidation of this reagent was measured at 525 nm (Cecil CE 2021 UV/Vis). The SOD activity is expressed as U (g Hb)<sup>-1</sup>.

The SOD isoenzymes (SOD1 and SOD2) were separated on discontinuous polyacrylamide gels according to Beauchamp and Fridovich.<sup>14</sup>

The LPO in erythrocytes was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) by the method of Stock and Dormondy,<sup>15</sup> and is expressed in nmol MDA (g Hb)<sup>-1</sup>.

The total Hb content was measured by the cyanmethemoglobin method of Salvati and Tentori.<sup>16</sup>

The NO<sub>2</sub><sup>-</sup> levels were analyzed by ELISA assay, using Griess reagent by the method of Guevara.<sup>17</sup> The absorbance was measured using a microplate reader (Plate reader, Mod. A1, Nubenco Enterprises, Inc.) at a wavelength of 545 nm. The results are expressed as μM L<sup>-1</sup>.

### *Native polyacrylamide gel electrophoresis (PAGE)*

Plasma proteins were analyzed by native PAGE (8 %) according to the method of Laemmli.<sup>18</sup> The native PAGE (8 %) of glycoproteins and lipoproteins were performed according to Hames and Rickwood<sup>19</sup> and Laemmli.<sup>18</sup> The glycoproteins and lipoproteins bands were stained using Pas Schiff's reagent<sup>19,20</sup> and Amido Black,<sup>21</sup> respectively. The electrophoretic analyses were performed on a vertical device Mini Ve Hoffer (LKB 2117, Bromma, Uppsala, Sweden).

The band intensities of the isoenzymes of SOD, plasma proteins, glycoproteins and lipoproteins were estimated using Scion Image Beta 4.02 software (<http://www.scioncorp.com>).<sup>22</sup> The density of each band was estimated with respect to the total area. Data are expressed as percentages of the total protein area.

The plasma protein concentration was determined spectrophotometrically according to Lowry.<sup>23</sup>

### *Statistical analyses*

Data are expressed as the means ± standard deviation (*SD*). Statistical significance was tested by the one-way Anova, followed by Dunnett's *t*-test. The minimum level of statistical significance was set to *p*<0.05.

## RESULTS

The changes in the SOD and CAT activities and the MDA levels in the erythrocytes are presented in Figs. 1–3, respectively. Toluene treatment at the low dose (0.38 mmol/kg bw) significantly increased the total SOD activity (Fig. 1a), as well as the isoenzyme SOD1 and SOD2 activity, 50.90, 52.21 and 130 %, respectively (Fig. 1b and 1c), when compared to the control group. The MDA levels also significantly increased 43.69 % upon treatment (*p* < 0.05). There is a tendency towards an increase in the CAT activity. The high dose of toluene (5 mmol/kg bw) induced the CAT activity (*p* < 0.001) and MDA levels (*p* < 0.05)

by 34.07 and 50.96 %, respectively. No differences in activity of the total SOD, SOD1, and SOD2 were observed among treated animals in comparison with the control group.

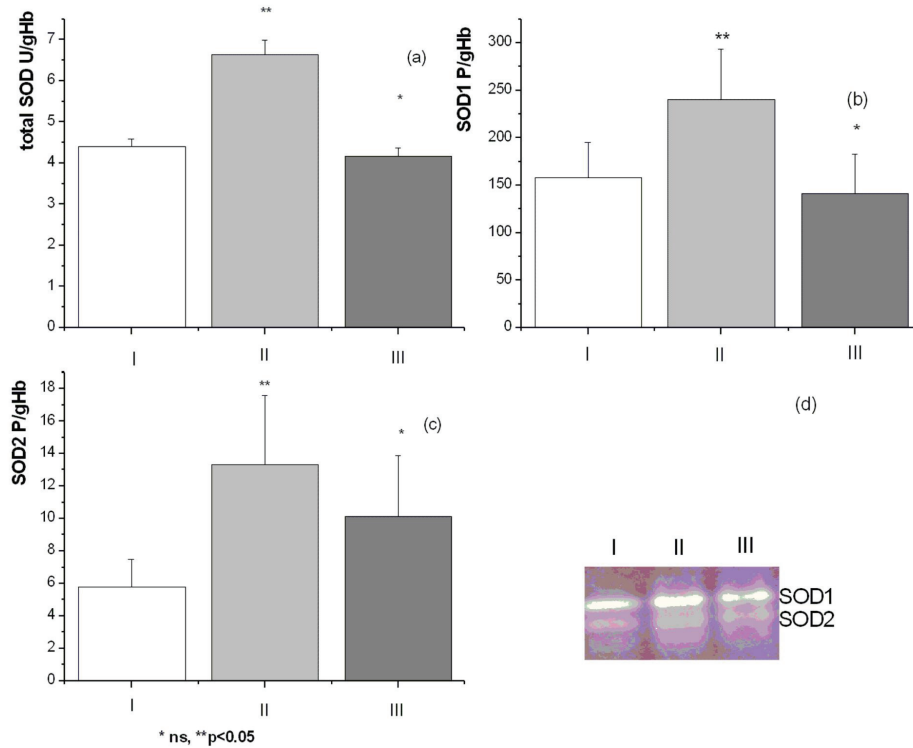


Fig. 1. Activity of SOD: total activity of SOD (a), activity of isoenzyme SOD1 (b), SOD2 (c) and activity of SOD on native PAGE (d); I – control group, II – rats treated i.p. with toluene at doses of 0.38 mM/kg bw and III – rats treated i.p. with toluene at doses of 5.00 mM/kg bw.

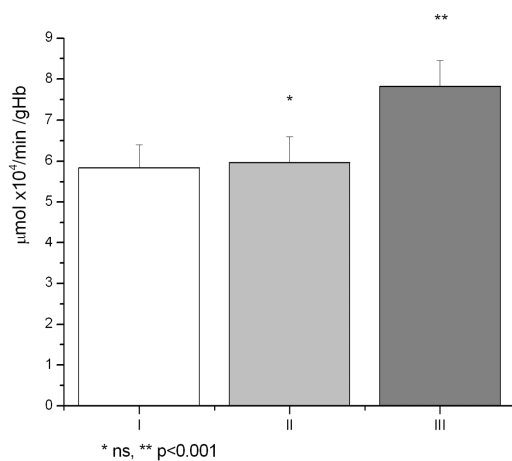


Fig. 2. CAT activities: I – control group, II – rats treated i.p. with toluene at doses of 0.38 mM/kg bw and III – rats treated i.p. with toluene at doses of 5.00 mM/kg bw.

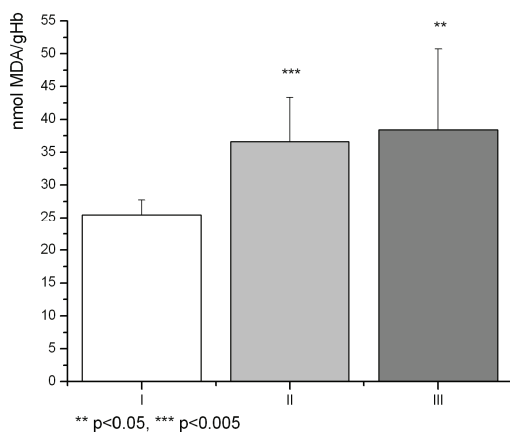


Fig. 3. Levels of erythrocytes MDA: I – control group, II – rats treated i.p. with toluene at doses of 0.38 mM/kg bw and III – rats treated i.p. with toluene at doses of 5.00 mM/kg bw.

The changes in the  $\text{NO}_2^-$  levels and total protein content of the control and the exposed groups of rats in blood plasma are presented in Figs. 4 and 5, respectively. Toluene treatment at the low dose decreased the  $\text{NO}_2^-$  levels ( $p > 0.05$ ) and total protein content ( $p < 0.05$ ) by 8.10 % compared to the non-treated animals. The high dose of toluene significantly decreased the total protein content ( $p < 0.05$ ) by 9.46 % compared to the control group. The observed decrease in the  $\text{NO}_2^-$  levels was not statistically significant.

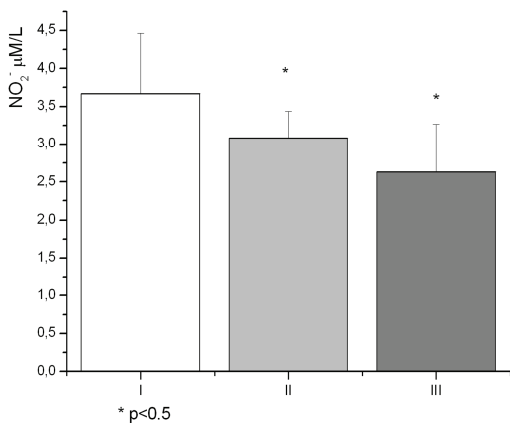


Fig. 4. Content of  $\text{NO}_2^-$ : I – control group, II – rats treated i.p. with toluene at doses of 0.38 mM/kg bw and III – rats treated i.p. with toluene at doses of 5.00 mM/kg bw.

The plasma proteins, glycoproteins and lipoproteins were separated by native PAGE and the results of the quantitative analysis of the gel bands are given in Tables Ia, Ib and Ic as percentages of the total area. Seven major protein fractions of plasma proteins are presented in Table Ia. The content of glycoproteins and lipoproteins are given in Table Ib and Ic, respectively.

Toluene treatment induced changes in the concentrations of plasma proteins (Table Ia). Toluene treatment at the low dose significantly increased the concentration of alpha-1 glycoprotein ( $p < 0.001$ ) and decreased the concentration of albumin ( $p < 0.001$ ) by 37.05 and 12.02 %, respectively, compared to the control

group. Toluene treatment at the high dose significantly increased the content of alpha-1 ( $p < 0.05$ ), alpha-2 glycoprotein and haptoglobin ( $p < 0.001$ ) by 23.11, 67.96 and 101 %, respectively, and decreased albumin ( $p < 0.001$ ) by 27.27 % compared to the values for the control animals. The albumin to globulin ratio (A/G) decreased in both groups ( $\approx 1.36$  fold for the low dose group, and  $\approx 1.91$  fold for the high dose group) compared to the control group.

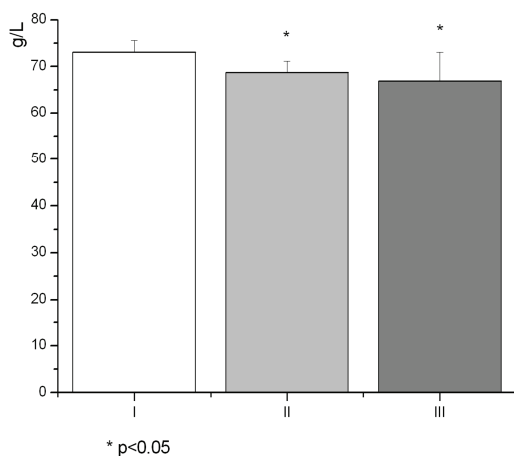


Fig. 5. Total content of plasma proteins: I – control group, II – rats treated i.p. with toluene at doses of 0.38 mM/kg bw and III – rats treated i.p. with toluene at doses of 5.00 mM/kg bw.

TABLE Ia. The results of quantitative analysis of gel bands of plasma proteins expressed as mean  $\pm$ SD for: I – control group, II – rats treated i.p. with toluene at doses of 0.38 mM/kg bw, and III – rats treated i.p. with toluene at doses of 5.00 mM/kg bw

Group	Total area, % (mean $\pm$ SD)							A/G
	Albumin	Alpha-1	Alpha-2	Transferrin	Haptoglobin	Gamma 1	Gamma2	
I	55.16 $\pm 3.8$	48.53 $\pm 1.07^a$	40.19 $\pm 3.72^a$	8.16 $\pm 1.49$	6.37 $\pm 0.79$	4.40 $\pm 0.73$	3.91 $\pm 1.06$	1.28 $\pm 0.52$
II	11.12 $\pm 1.26$	15.24 $\pm 1.73^a$	13.69 $\pm 0.66^b$	9.08 $\pm 0.70$	6.77 $\pm 1.27$	4.81 $\pm 1.47$	3.43 $\pm 0.83$	0.94 $\pm 0.15$
III	5.15 $\pm 1.62$	6.65 $\pm 1.35$	18.65 $\pm 2.36^a$	9.12 $\pm 0.85$	12.81 $\pm 2.28^a$	4.44 $\pm 1.50$	3.26 $\pm 0.91$	0.67 $\pm 0.22$

TABLE Ib. The results of quantitative analysis of gel bands of glycoproteins expressed as mean  $\pm$ SD for: I – control group, II – rats treated i.p. with toluene at doses of 0.38 mM/kg bw, and III – rats treated i.p. with toluene at doses of 5.00 mM/kg bw

Group	Total area, % (mean $\pm$ SD)					IgG
	Albumin	Alpha-1 and alpha-2	Transferrin	Beta region	Haptoglobin	
I	17.05 $\pm$ 8.40	7.79 $\pm$ 4.18	6.53 $\pm$ 3.82	16.62 $\pm$ 3.84 <sup>b</sup>	21.28 $\pm$ 6.62	19.75 $\pm$ 1.30
II	11.41 $\pm$ 8.72	13.81 $\pm$ 4.28	23.03 $\pm$ 4.59 <sup>b</sup>	22.65 $\pm$ 4.51 <sup>a</sup>	25.58 $\pm$ 6.28	13.69 $\pm$ 3.63 <sup>b</sup>
III	5.49 $\pm$ 1.59 <sup>b</sup>	11.16 $\pm$ 4.59	20.73 $\pm$ 2.65 <sup>a</sup>	24.18 $\pm$ 3.33 <sup>a</sup>	33.13 $\pm$ 6.58 <sup>b</sup>	6.11 $\pm$ 2.70 <sup>a</sup>

TABLE Ic. The results of quantitative analysis of gel bands of lipoproteins expressed as mean  $\pm$ SD for: I – control group, II – rats treated i.p. with toluene at doses of 0.38 mM/kg bw, and III – rats treated i.p. with toluene at doses of 5.00 mM/kg bw

Group	Total area, % (mean $\pm$ SD)			ApoA-HDL/ApoB-LDL
	Albumin	ApoA-HDL	ApoB-LDL	
I	19.40 $\pm$ 1.45	17.79 $\pm$ 2.05	26.04 $\pm$ 5.79b	1.75 $\pm$ 0.64
II	23.96 $\pm$ 3.51	17.62 $\pm$ 5.94	5.43 $\pm$ 2.39a	3.41 $\pm$ 0.90
III	41.87 $\pm$ 5.46	60.09 $\pm$ 6.61a	45.02 $\pm$ 4.63b	8.34 $\pm$ 0.52

<sup>a</sup> $p < 0.001$ ; <sup>b</sup> $p < 0.05$

Toluene treatment induced changes in the content of glycoproteins (Table Ib) and lipoproteins (Table Ic). Statistical analysis of the glycoproteins revealed a significantly increased transferrin content ( $p < 0.05$ ,  $\approx 65\%$ ) and the amount of proteins of the beta region ( $p < 0.001$ ,  $>85\%$ ) and decreased the IgG content in both groups compared to the control group (Table Ib). Toluene treatment with high dose increased the alpha-1 and alpha-2 glycoproteins and haptoglobin ( $p < 0.05$ ) by 113 and 55.69%, respectively, and decreased albumin ( $p < 0.05$ ) by 67.80% in comparison to the control group.

The low dose of toluene given along a period of 12 days significantly increased the level of the apoB-LDL lipoprotein fraction ( $p < 0.001$ ) by 43.52% and non-significantly decreased the level of the ApoA-HDL lipoprotein fraction ( $p < 0.1$ ), whilst toluene treatment with the high dose significantly increased level of ApoB-LDL and albumin ( $p < 0.05$ ) by 7.52 and 36.00%, respectively, and decreased the level of ApoA-HDL ( $p < 0.001$ ) by 77.34%, compared to the control group. The ratio ApoA-HDL/ApoB-LDL increased in both groups ( $\approx$  two-fold for the low dose and  $\approx$  five-fold for the high dose group) compared to the control group (Table Ic).

## DISCUSSION

Toluene is a lipophilic agent and therefore absorbed and retained well by the lipid rich areas in organisms.<sup>24</sup> Toluene and its metabolites cause a significant elevation in the rate of ROS generation.<sup>2</sup> The interplay between ROS and antioxidant defense in living aerobic organisms is connected with a series of intracellular antioxidant enzymes, the roles of which are to intercept and inactivate reactive radicals. CAT scavenges an excess of free radicals *via* enzymatic and chemical mechanisms, which results in depletion of H<sub>2</sub>O<sub>2</sub>. This study showed significantly increased CAT activities in rats treated with toluene at the high dose. This increase could be attributed to the production of ROS provoked by toluene. The ROS scavenging activity of SOD is effective only when it is followed by the action of CAT, since the dismutase activity of SOD generates H<sub>2</sub>O<sub>2</sub> from O<sub>2</sub><sup>-•</sup>.<sup>25</sup> The increased concentration of H<sub>2</sub>O<sub>2</sub> and CAT activity in rats treated with toluene at the high dose, which appears to inhibit some protective enzymes including SOD, allows the production of HO<sup>•</sup>, formed in Fenton and Haber-

-Weiss reactions.<sup>26</sup> The HO• radicals lead to peroxidation of PUFAs and MDA is produced in the terminal phase of LPO. MDA has been mainly employed as a marker of oxidative stress in both *in vitro* and *in vivo* studies.<sup>7,8,27</sup> In the present study, toluene significantly increased the erythrocytes MDA levels in both treated groups.

Organic solvents (toluene and benzene) and their metabolites induced the formation of RNS<sup>28,29</sup> and enhanced the expression of inducible nitric oxide synthase (iNOS), which can lead to the formation of excessive NO•.<sup>30</sup> The present results showed that there were no significant changes in the level of NO<sub>2</sub><sup>-</sup> in plasma of both toluene treated groups. The reason possibly lies in the difference in the toluene concentration and, consequently, the difference in NO• release and the higher reactivity of NO• with toluene metabolites<sup>31</sup> and cellular macromolecules. The combination of NO• and O<sub>2</sub><sup>-</sup> also results in the rapid generation of the highly reactive molecule ONOO<sup>-</sup>.<sup>32</sup> The marker for ONOO<sup>-</sup> formation is nitration of tyrosine residues in proteins (Tyr(NO<sub>2</sub>)). Tyr(NO<sub>2</sub>) is a post-translational modification associated with oxidative stress.<sup>29</sup>

Oxidative damage of plasma proteins can be induced directly *via* H<sub>2</sub>O<sub>2</sub>, *via* xenobiotics such as CCl<sub>4</sub>, through reduced transition metals (Fe<sup>2+</sup>) and ionizing radiation,<sup>33</sup> indirectly *via* reaction with small reactive aldehydes, such as glyoxal, methylglyoxal, or with aldehydes derived specifically from LPO, such as MDA,<sup>8</sup> leading to the accumulation of heterogeneous protein modifications, which are classified as advanced glycation end products (AGEs)<sup>10,11</sup> or advanced lipidation end products (ALEs).<sup>10,11</sup> The total plasma proteins were analyzed on native PAGE to study the changes in individual protein component and changes in the A/G ratio. The level of albumin decreased but alpha-1, alpha-2 and haptoglobin increased upon toluene treatment compared to the control group. These proteins are classified as acute-phase proteins. Ceron *et al.*<sup>34</sup> showed that concentration of acute-phase proteins changes with inflammation, tissue damage, infection, certain cancers and xenobiotics. Kaukianinen found a positive correlation between blood glucose levels and toluene exposure.<sup>35</sup> An increased level of glucose leads to increased glycosilation of some of the plasma proteins (alpha-1 and alpha-2, transferrin, proteins of beta region, and haptoglobin),<sup>36</sup> as the present results also show.

The two lipoprotein subfractions responsible for cholesterol transport are low density lipoproteins (LDLs) and high density lipoproteins (HDLs).<sup>37</sup> Knezević *et al.*<sup>38</sup> showed that human exposure to toluene and xylene increases the level of plasma LDL cholesterol but substantially decreases the HDL concentration, thereby increasing the LDL/HDL ratio. The present results are in accordance with these findings. LDL binds receptors *via* protein ApoB (ApoB-LDL). Receptor-bound LDL is internalized by endocytosis into the cell. Oxidative damage of LDL could arise from a number of different causes, including free radicals, such as O<sub>2</sub><sup>-</sup> and NO•.<sup>39</sup> LDL oxidation is a progressive process leading at



first to the formation of mildly oxidized LDL, which is defined by a low content of lipid-peroxidation derivatives and slight ApoB modifications, and later to extensively oxidized LDL, which contains high levels of lipid-peroxidation products and severe ApoB alterations.<sup>40-42</sup> These severe ApoB alterations lead to the failure to bind and internalize LDL, which is probably the reason for the increased LDL levels in the plasma of the animals treated in this study. HDL particles contain molecules of apolipoprotein A1 (ApoA1-HDL). In addition to its role in reverse cholesterol transport (from peripheral tissues to the liver),<sup>43,44</sup> HDL exhibits a protective effect against the cytotoxicity of oxidized LDL, by inhibiting LDL oxidation induced by cells and inhibiting the cytotoxicity of oxidized LDL.<sup>45,46</sup> To the best of our knowledge, the mechanism of the protective effect of HDL at the cellular level is still unclear. This protective effect is impaired on account of decreased HDL levels.

#### CONCLUSIONS

The results of this study show that toluene treatment of rats (low and high dose) leads to oxidative stress caused by ROS and RNS, and consequently to the: 1) changed antioxidant enzyme activity, 2) increased LPO and impaired integrity of the cell membrane and 3) structural changes in the plasma proteins. Further studies are required to evaluate the possible molecular mechanisms of the toxicity induced by toluene exposure.

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#### ИЗВОД

#### УТИЦАЈ ТОЛУЕНА НА ОКСИДАТИВНЕ ПРОЦЕСЕ У КРВИ ПАЦОВА

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У овом раду је испитиван утицај толуена на оксидативни стрес у крви пацова. С обзиром на то да метаболизам толуена доводи до стварања реактивних кисеоничних и азотових врста, претпостављено је да третирање пацова различитим концентрацијама толуена доводи до 1) промене у активности ензима антиоксидативне одбране, 2) нарушавања интегритета ћелијске мембране и 3) структурних промена код плазма протеина. Женке пацова соја Wistar су подељене у три групе: I група – контролна, II и III група – пацови третирани i.p. толуеном свакодневно 12 и 6 дана, дозом од 0,38 mmol/kg телесне масе и 5,0 mmol/kg телесне масе. Толуен је статистички значајно повећавао активност SOD у II групи и активност CAT у III групи, док је садржај MDA био значајно повећан у обе групе у поређењу са контролном групом. Снижење NO<sub>2</sub><sup>-</sup> у обе третиране групе није било статистички значајно у односу на контролну групу. Толуен је довео до оксидативних промена плазма протеина, а самим тим и до промене концентрације глико- и липопротеина у односу на контролну групу. Доказане промене указују на то да третирање толуеном изазива слободно-радикалске процесе.

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