



*J. Serb. Chem. Soc.* 78 (2) 179–195 (2013)  
JSCS–4407

## The effects of ethanol on paracetamol-induced oxidative stress in mice liver

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(Received 24 July, revised 21 November 2012)

**Abstract:** The aim of this study was to investigate the effects of binge drinking on paracetamol-induced oxidative stress in mice liver. Male Swiss mice were divided into groups: control; ethanol-treated group (E) in five sequential doses of 2 g kg<sup>-1</sup>, administered through an orogastric tube; paracetamol-treated group (P) in a dose of 300 mg kg<sup>-1</sup>, intraperitoneally and a group that received paracetamol 12 h after the last dose of ethanol (PE). Blood and liver samples were collected for the determination of oxidative stress parameters 6, 24 and 48 h after treatment. Prior binge drinking potentiated the paracetamol-induced increase in the liver malondialdehyde level 48 h after treatment in comparison with the P and E groups (17.14±1.98 vs. 13.14±0.82 and 12.99±1.18 μmol L<sup>-1</sup>, respectively, *p* < 0.01). Ethanol and paracetamol in combination induced a more pronounced decrease in the liver GSH level than either of the individual substances alone at all time intervals (*p* < 0.01). Total liver superoxide dismutase (SOD) activity was significantly lower in PE 48 h after treatment in comparison with the P and E groups (251.73±80.63 vs. 707.62±179.92 and 1179.62±147.94 U mg<sup>-1</sup> protein, respectively, *p* < 0.01). The lowest MnSOD activity in the PE group was detected 48 h after treatment (86.52±28.31; 41.13±11.07 and 23.16±5.18 U mg<sup>-1</sup> protein in the P, E and PE groups, respectively, *p* < 0.05). Prior binge ethanol drinking potentiates paracetamol-induced reduction of antioxidative capacity of hepatocytes due to GSH depletion and SOD activity reduction, simultaneously increasing lipid peroxidation caused by paracetamol.

**Keywords:** ethanol; paracetamol; oxidative stress; liver; mice.

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doi: 10.2298/JSC120724127M

## INTRODUCTION

Paracetamol (acetaminophen), a widely used antipyretic and analgetic, is found to exert hepatotoxic effects in humans and rodents. When used in high doses, it produces centrilobular hepatic necrosis that can be fatal.<sup>1,2</sup> The mechanisms of paracetamol hepatotoxicity are still poorly understood. The main mechanism responsible for its toxicity is considered to be its metabolic activation in the liver.<sup>3</sup> The toxic metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI), produced by the CYP2E1 isoenzyme of cytochrome P450-dependent monooxygenase, induces liver injury by forming acetaminophen–protein adducts<sup>1,4</sup> and by inducing mitochondrial permeability transition.<sup>5</sup> A reduced glutathione content in the liver potentiates the toxic effects of paracetamol since glutathione is a major component involved in NAPQI detoxication.<sup>6</sup> Additional mechanisms apparently involved in paracetamol toxicity are oxidative,<sup>7–11</sup> nitrosative stress<sup>12–15</sup> and activation of Kupffer cells with the release of various pro-inflammatory cytokines.<sup>16</sup> It has been postulated that paracetamol increases the production of superoxide anion and hydrogen peroxide, as well as nitrotyrosine formation in the liver.

Ethanol, when chronically ingested, is known to induce liver injury. Among various mechanisms involved in ethanol-induced liver injury are the production of reactive oxygen species, the formation of acetaldehyde–protein adducts that induce an immune response and the release of gut endotoxin.<sup>17–19</sup> The main source of reactive oxygen species is CYP2E1, an inducible enzyme involved in ethanol metabolism.<sup>17–19</sup> In the presence of iron, which is increased during chronic ethanol consumption, more powerful oxidants, including hydroxyl radicals, ferryl species and 1-hydroxyethyl radical, are produced.<sup>20</sup> An additional source of reactive oxygen species in the liver are Kupffer cells, activated by increased release of endotoxin from gut lumen into portal circulation.<sup>21</sup> In contrast to chronic effects, the mechanisms of acute ethanol-induced liver injury are still not fully understood.

It has been suggested that chronic ethanol consumption potentiates paracetamol hepatotoxicity.<sup>22</sup> Various studies indicated that chronic alcoholics are more susceptible to therapeutic doses of paracetamol than non-alcoholics.<sup>22–24</sup> Most studies regarding these interactions were focused on the metabolic interaction between ethanol and paracetamol. It was suggested that ethanol potentiates paracetamol-induced liver injury by CYP2E1 and CYP3A induction, which are enzymes involved in NAPQI production,<sup>25,26</sup> However, another study did not confirm these interactions.<sup>27</sup>

The effects of acute ethanol intoxication on paracetamol hepatotoxicity are even more blurred. These effects are of great importance, since it is usual for adolescents to take paracetamol or other non-steroidal anti-inflammatory drugs for the treatment of headache caused by binge drinking. Some clinical studies

suggest a protective effect of ethanol pretreatment on paracetamol hepatotoxicity.<sup>28</sup> Interactions between paracetamol and ethanol during acute toxicity cannot be entirely explained by metabolic changes in the liver, since ethanol does not induce CYP2E1 acutely. Possible interactions should be explained by other mechanisms involved in their toxicity. Since reactive oxygen species are postulated to mediate hepatotoxic effects of ethanol and paracetamol, the aim of our study was to investigate the effects of binge drinking on paracetamol-induced oxidative stress in rat liver.

## EXPERIMENTAL

### *Animals*

The experiment was performed on adult male Swiss mice weighing 25–30 g, raised at the Military Medical Academy, Belgrade. The animals were kept under standard laboratory conditions (temperature  $22\pm 2$  °C, relative humidity  $50\pm 10$  %, 12/12 light–dark cycle with lights turned on at 9 a.m.) and had free access to tap water and standard pelleted LM2 food (Veterinary Institute “Subotica”, Subotica, Serbia). The diet, which had a metabolizable energy of at least  $11.5$  MJ  $\text{kg}^{-1}$ , was composed of a maximum of 7 % cellulose and a minimum of 19 % protein. On the day prior to sacrifice, the mice were fasted overnight. The study was performed according to the Guidelines for Animal Study No. 282-12/2002 and was approved by the Ethic Committee of the Military Medical Academy for animal experiments.

All animals ( $n = 96$ ) were randomly divided into the following groups: 1) control, saline-treated group (0.9 % NaCl) ( $n = 24$ ); 2) ethanol-treated group (E;  $n = 24$ ) in five subsequent doses of  $2$  g  $\text{kg}^{-1}$ , administered every 12 h by an orogastric tube; 3) paracetamol-treated group (P;  $n = 24$ ) in a dose of  $300$  mg  $\text{kg}^{-1}$  intraperitoneally (i.p.); 4) group that received paracetamol and ethanol (PE;  $n = 24$ ). Ethanol was administered to the PE group in five repetitive doses in the same manner as it was administered to the E group. Paracetamol was i.p. administered to these animals in a dose of  $300$  mg  $\text{kg}^{-1}$ , 12 h after the last dose of ethanol. Animals from P and E group received saline instead of ethanol and paracetamol, respectively. For oral administration, the ethanol was dissolved in distilled water in concentration of 30 % v/v. Paracetamol was dissolved in saline (0.9 % NaCl) before i.p. administration.

The mice were sacrificed by cervical dislocation 6, 24 and 48 h after paracetamol administration (or saline for E and the control group). Eight animals from each group were sacrificed at all time intervals. Blood samples for the determination of the oxidative stress parameters were collected from the right side of the heart. For the same purpose, the livers were excised and stored as described below.

### *Analysis*

Liver samples for biochemical analysis were homogenized on ice, in cold buffered 0.25 M sucrose medium (Serva, Heidelberg, New York), 10 mM phosphate buffer (pH 7.0) and 1 mM EDTA (Sigma, St. Louis, USA). The homogenates were centrifuged at  $2000\times g$  for 15 min at 4 °C. The crude sediments were dissolved in a sucrose medium and centrifuged. The supernatants were transferred into the tubes and centrifuged at  $3200\times g$  for 30 min at 4 °C. The obtained sediments were dissolved in deionized water. After one hour of incubation, the samples were centrifuged at  $3000\times g$  for 15 min at 4 °C, and the supernatants were stored at  $-70$  °C. The contents of proteins were determined by the Lowry method using bovine serum albumin as the calibrant.<sup>29</sup>

Biochemical evaluation of liver injury was performed by quantifying the serum activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Their activities were determined spectrophotometrically using Sigma test kits (Sigma, St. Louis, USA) on a BTS-330 photometer, according to the manufacturer's instructions.

The total superoxide dismutase (EC1.15.1.1.; SOD) activity in the liver was measured spectrophotometrically, as the inhibition of epinephrine auto-oxidation at 480 nm. After addition of 10 mM epinephrine (Sigma, St. Louis, USA), the analysis was performed in sodium carbonate buffer (50 mM, pH 10.2; Serva, Heidelberg, New York) containing 0.1 mM EDTA (Sigma, St. Louis, USA). Samples for manganese SOD were previously treated with 8 mM KCN (Sigma, St. Louis, USA) and then analyzed as previously described.<sup>30</sup>

Lipid peroxidation in the plasma and liver homogenates was measured as malondialdehyde (MDA) production, assayed in the thiobarbituric acid reaction as described by Girotti *et al.*<sup>31</sup> The results are expressed as  $\mu\text{mol L}^{-1}$  in plasma or  $\text{nmol mg}^{-1}$  protein in the liver homogenates.

The concentration of nitrites + nitrates ( $\text{NO}_x$ ), as a measure of nitric oxide (NO) production, was determined using the Griess reagent. After reduction of the nitrates, the total nitrites were reacted with sulfanilamide and *N*-(1-naphthyl)ethylenediamine to produce an azo dye, which was measured spectrophotometrically at 492 nm.<sup>32</sup>

Reduced glutathione (GSH) was determined by reaction of aliphatic thiol compounds in Tris-HCl buffer (0.4 mol, pH 8.9) with 5,5-dithiobis-2-nitrobenzoic acid (DTNB, 36.9 mg in 10 mL of methanol), thereby producing the yellow-colored *p*-nitrophenol anion that was measured at 412 nm. The intensity of the formed chemical compound was proportional to the GSH concentration.<sup>33</sup>

#### Chemicals

All reagents and chemicals were of analytical grade or higher purity. Ethanol was purchased from Merck (Darmstadt, Germany). Paracetamol was obtained from Sigma (St. Louis, MO, USA).

#### Statistical analysis

Results are expressed as means  $\pm$  SD. Since the normal distribution of the data was confirmed by the Kolmogorov-Smirnov test, multifactorial analysis of variance (multifactorial ANOVA) with Tukey's *post hoc* test were used for testing the difference among the groups. The difference was considered statistically significant if  $p < 0.05$ . The SPSS15.0 program was used for the statistical analysis.

## RESULTS

The activities of the serum aminotransferases (ALT and AST) were significantly elevated at all times after paracetamol administration ( $p < 0.01$ ). Additionally, ethanol induced a significant increase in their activities when administered either alone or in combination with paracetamol ( $p < 0.01$ ) (Table I). The study also showed that the serum MDA concentration was significantly higher in all groups that received either ethanol or paracetamol at all times when blood samples were collected (Table I). A significant rise in the MDA concentration was found 6 h after paracetamol administration in the control group ( $p < 0.01$ ). A similar increase was found after ethanol administration ( $4.78 \pm 0.69 \mu\text{mol L}^{-1}$ ) ( $p < 0.01$ ). Concomitant administration of ethanol and paracetamol did not induce a

significant change in serum MDA concentration in comparison with the ethanol- and paracetamol-treated groups ( $p > 0.05$ ). Neither was a significant change found within 48 h after treatment ( $p > 0.05$ ). However, a significant increase in the liver MDA concentration was found in the PE group 48 h after paracetamol administration, in comparison with the P and E groups ( $p < 0.01$ ). A progressive increase in the liver MDA levels was also found within 48 h in all the experimental groups (Table I). The highest level of liver MDA was measured 48 h after paracetamol or ethanol administration in comparison with the same group of animals 6 and 24 h after treatment ( $p < 0.01$  and  $p < 0.05$ , respectively).

TABLE I. Activities of serum aminotransferases and parameters of lipid peroxidation in the plasma and liver of the experimental animals. Results are expressed as means  $\pm$  SD. For statistical evaluation, multifactorial ANOVA with Tukey's post hoc test were used (\* $p < 0.05$ , \*\* $p < 0.01$  vs. the control group, # $p < 0.05$ , ## $p < 0.01$  in comparison with the group on the same experimental protocol 6 (a) and 24 h (b) after treatment); abbreviations: C<sub>6</sub>, C<sub>24</sub>, C<sub>48</sub>, control group 6, 24 and 48 h after treatment; P<sub>6</sub>, P<sub>24</sub>, P<sub>48</sub>, paracetamol-treated group 6, 24 and 48 h after administration; E<sub>6</sub>, E<sub>24</sub>, E<sub>48</sub>, ethanol-treated group 6, 24 and 48 h after treatment; PE<sub>6</sub>, PE<sub>24</sub>, PE<sub>48</sub>, group that received paracetamol and ethanol 6, 24 and 48 h after treatment

Group	Serum ALT U L <sup>-1</sup>	Serum AST U L <sup>-1</sup>	Conc. serum MDA $\mu$ mol L <sup>-1</sup>	Liver MDA level nmol mg <sup>-1</sup> prot.
C <sub>6</sub>	41 $\pm$ 5	52 $\pm$ 10	3.33 $\pm$ 0.71	7.67 $\pm$ 1.65
C <sub>24</sub>	39 $\pm$ 3	55 $\pm$ 7	3.13 $\pm$ 0.64	7.86 $\pm$ 0.87
C <sub>48</sub>	43 $\pm$ 7	51 $\pm$ 7	3.86 $\pm$ 1.73	7.31 $\pm$ 0.82
P <sub>6</sub>	1924 $\pm$ 74**	2197 $\pm$ 102**	4.83 $\pm$ 1.05**	9.45 $\pm$ 0.81*
P <sub>24</sub>	2025 $\pm$ 68**	2297 $\pm$ 97**	5.25 $\pm$ 1.39**	11.50 $\pm$ 0.38** a#
P <sub>48</sub>	2028 $\pm$ 81**	2274 $\pm$ 80**	5.10 $\pm$ 1.34*	13.14 $\pm$ 0.82** b#
E <sub>6</sub>	1789 $\pm$ 57**	2562 $\pm$ 93**	4.78 $\pm$ 0.69**	9.56 $\pm$ 0.32*
E <sub>24</sub>	1785 $\pm$ 78**	2734 $\pm$ 107**	4.89 $\pm$ 0.94**	11.47 $\pm$ 0.80** a#
E <sub>48</sub>	1702 $\pm$ 71**	2672 $\pm$ 76**	5.21 $\pm$ 0.50*	12.99 $\pm$ 1.18** b#
PE <sub>6</sub>	1942 $\pm$ 134**	2431 $\pm$ 86**	5.11 $\pm$ 2.04**	9.23 $\pm$ 0.30*
PE <sub>24</sub>	2145 $\pm$ 101**	2497 $\pm$ 94**	4.25 $\pm$ 1.66**	11.31 $\pm$ 0.77** a#
PE <sub>48</sub>	2108 $\pm$ 96**	2503 $\pm$ 111**	4.95 $\pm$ 1.25*	17.14 $\pm$ 1.98** b##

The liver NO<sub>x</sub> concentration was significantly higher in the paracetamol-treated group (1.24 $\pm$ 0.41  $\mu$ mol L<sup>-1</sup>) than in the control group (0.34 $\pm$ 0.06  $\mu$ mol L<sup>-1</sup>) 6 h after treatment ( $p < 0.01$ ) (Fig. 1). After this period, its concentration showed a progressive rise and maximal values were attained 48 h after paracetamol administration (2.86 $\pm$ 0.21  $\mu$ mol L<sup>-1</sup>). Similar to paracetamol, ethanol had also induced a rise in the liver NO<sub>x</sub> concentration 6 h after treatment (1.04 $\pm$ 0.33  $\mu$ mol L<sup>-1</sup>) ( $p < 0.01$ ). However, this level remained relatively unchanged within 24 h after administration of the last dose of ethanol (0.97 $\pm$ 0.24  $\mu$ mol L<sup>-1</sup>) ( $p > 0.05$ ). After this period, ethanol induced an additional increase in the NO<sub>x</sub> concentration, and its level was significantly higher 48 h after treatment (2.90 $\pm$ 0.32  $\mu$ mol L<sup>-1</sup>) than after 6 h ( $p < 0.01$ ). When administered sequentially, ethanol and para-

cetamol also induced a progressive rise in the  $\text{NO}_x$  concentration within the first 48 h with the highest level being observed at the end of this interval ( $2.98 \pm 0.05 \mu\text{mol L}^{-1}$ ) (Fig. 1). However, no significant change in the  $\text{NO}_x$  concentration was found in the PE vs. the P group at all times ( $p > 0.05$ ).

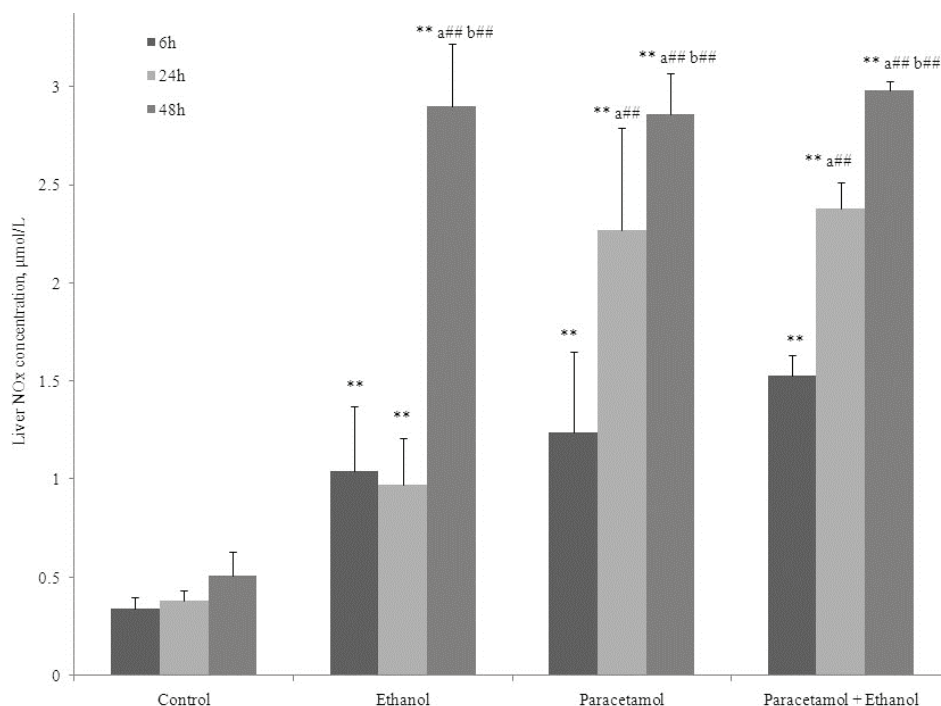


Fig. 1. Liver concentration of nitrites and nitrates ( $\text{NO}_x$ ) in the experimental groups. The  $\text{NO}_x$  values were determined at 6, 24 and 48 h after treatment. For statistical evaluation, multi-factorial ANOVA with the Tukey's *post hoc* test were used (\*\* $p < 0.01$  vs. control group, ## $p < 0.01$  in comparison with the group on the same experimental protocol 6 (a) and 24 h (b) after treatment). Abbreviations: C – control group, E – ethanol-treated group, P – paracetamol-treated group, PE – group that received paracetamol 24 h after the last dose of ethanol.

The liver GSH content was significantly lower in ethanol- and paracetamol-treated groups ( $0.58 \pm 0.05$  and  $0.75 \pm 0.06 \text{ nmol mg}^{-1}$  protein, respectively) in comparison with control group ( $1.03 \pm 0.12 \text{ nmol mg}^{-1}$  protein) 6 h after treatment ( $p < 0.01$ ), Fig. 2. Within the next 18 h, its content returned to the control range in the ethanol-treated group ( $0.99 \pm 0.09 \text{ nmol mg}^{-1}$  protein) and remained approximately constant within the next 24 h ( $1.05 \pm 0.11 \text{ nmol mg}^{-1}$  protein) ( $p > 0.05$ ). In contrast to ethanol, paracetamol had induced a significant increase in the liver GSH content 24 h after its administration ( $1.57 \pm 0.18$  vs.  $1.04 \pm 0.10 \text{ nmol mg}^{-1}$  protein in the P and control group, respectively,  $p < 0.01$ ). After this period,

a progressive decline in the GSH content was observed in the P group and 48 h after paracetamol administration, its content ( $0.56 \pm 0.05$  nmol  $\text{mg}^{-1}$  protein) was significantly lower in comparison with that of the control group ( $1.11 \pm 0.14$  nmol  $\text{mg}^{-1}$  protein) and P group 24 h after treatment ( $p < 0.05$  and  $p < 0.01$ , respectively). Subsequent administration of paracetamol to ethanol-treated animals induced a more pronounced decline in the liver GSH content than administration of paracetamol or ethanol alone ( $p < 0.01$ ). The lowest level of liver GSH in the PE group was detected 6 h after paracetamol administration ( $0.12 \pm 0.01$  nmol  $\text{mg}^{-1}$  protein). After this period, its level gradually increased but was still significantly lower than in P and E groups within 48 h ( $0.24 \pm 0.03$  nmol  $\text{mg}^{-1}$  protein), Fig. 2.

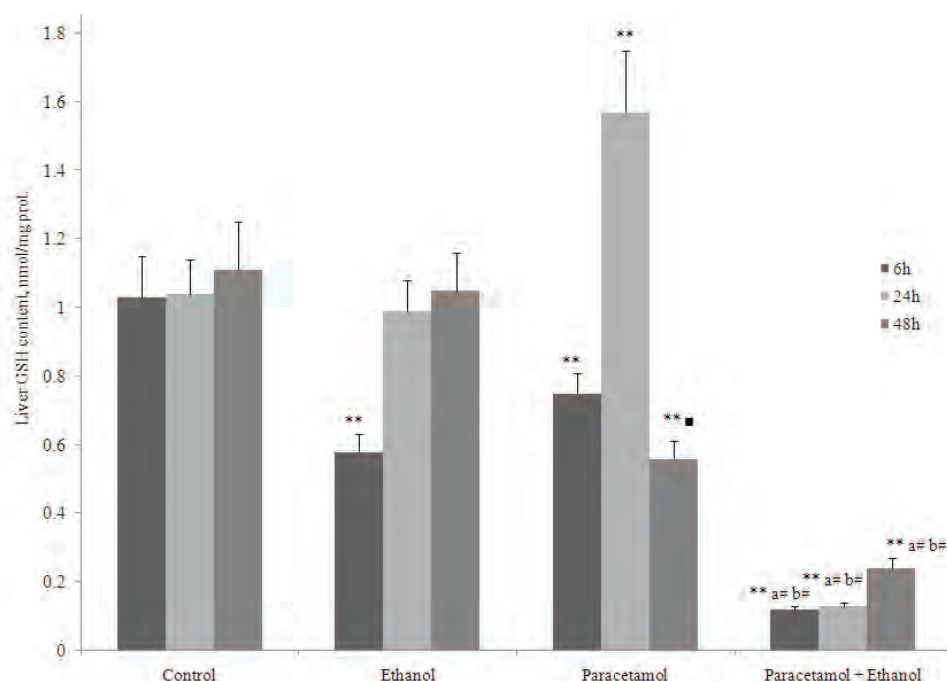


Fig. 2. The level of the reduced glutathione (GSH) in liver samples of treated animals. The GSH levels were determined 6, 24 and 48 h after the appropriate treatment. For statistical evaluation, multifactorial ANOVA with Tukey's *post hoc* test were used (\*\* $p < 0.01$  vs. the control group, # $p < 0.01$  vs. the paracetamol-treated group 24 h after its administration,  $p < 0.01$  in comparison with ethanol- (a) and paracetamol-treated group (b) at the corresponding times). Abbreviations as in Fig. 1.

Ethanol and paracetamol were found to induce a decrease in the liver SOD activity at all time intervals when administered alone or in combination (Fig. 3). However, the most intensive decrease was observed in the PE group. Prior ethanol administration did not significantly potentiate the paracetamol-induced decrease in SOD activity within the first 24 h after treatment ( $723.75 \pm 102.86$  and

784.61±70.19 U mg<sup>-1</sup> protein after 6 h, 645.35±70.56 and 768.88±143.31 U mg<sup>-1</sup> protein after 24 h in the PE and P groups, respectively). However, the total SOD activity was significantly lower 48 h after treatment in the PE group (251.73±±80.63 U mg<sup>-1</sup> protein) in comparison with the P (707.62±179.92 U mg<sup>-1</sup> protein) and E groups (1179.62±147.94 U mg<sup>-1</sup> protein,  $p < 0.01$ ).

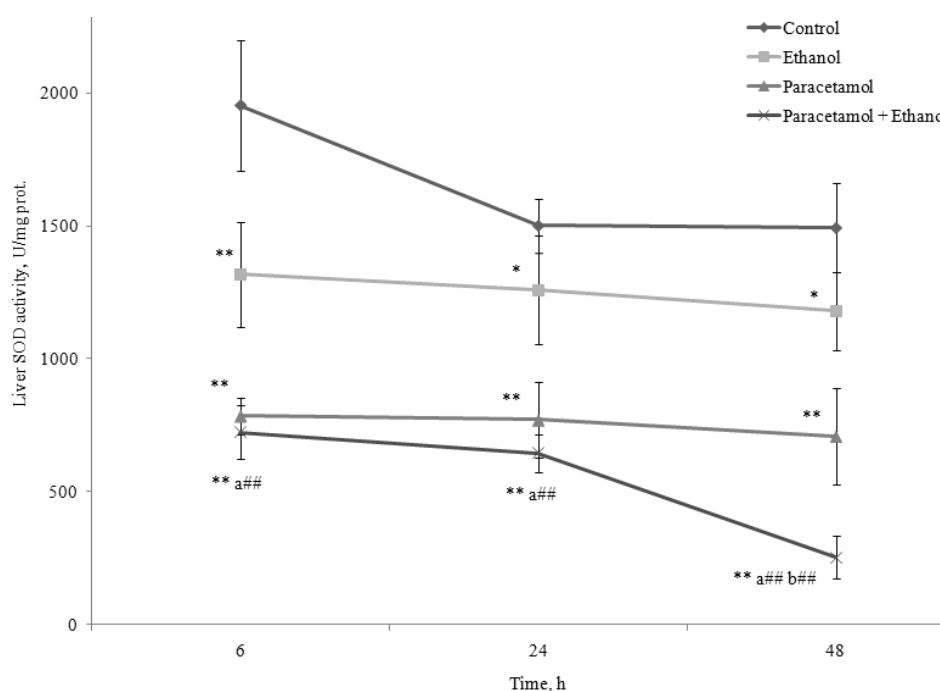


Fig. 3. Total superoxide dismutase (SOD) activity in the liver of treated animals. Liver samples for measuring SOD activity were collected 6, 24 and 48 h after the appropriate treatment. For statistical evaluation, multifactorial ANOVA with Tukey's *post hoc* test were used ( $*p < 0.05$ ,  $**p < 0.01$  vs. the control group,  $##p < 0.01$  in comparison with the ethanol-treated group (a) and the paracetamol-treated group (b) at the same time intervals).

In contrast to the total SOD activity, the mitochondrial SOD activity was higher after 6 h in all treated groups in comparison with the control group (Fig. 4). Subsequently, the MnSOD activity began to fall and reached its lowest level 48 h after treatment in all experimental groups. The MnSOD activity was significantly lower in the PE group in comparison with the P and E groups at all time when its activity was measured. The lowest MnSOD activity was detected 48 h after treatment (86.52±28.31, 41.13±11.07 and 23.16±5.18 U mg<sup>-1</sup> protein in the P, E and PE groups, respectively,  $p < 0.05$ ).



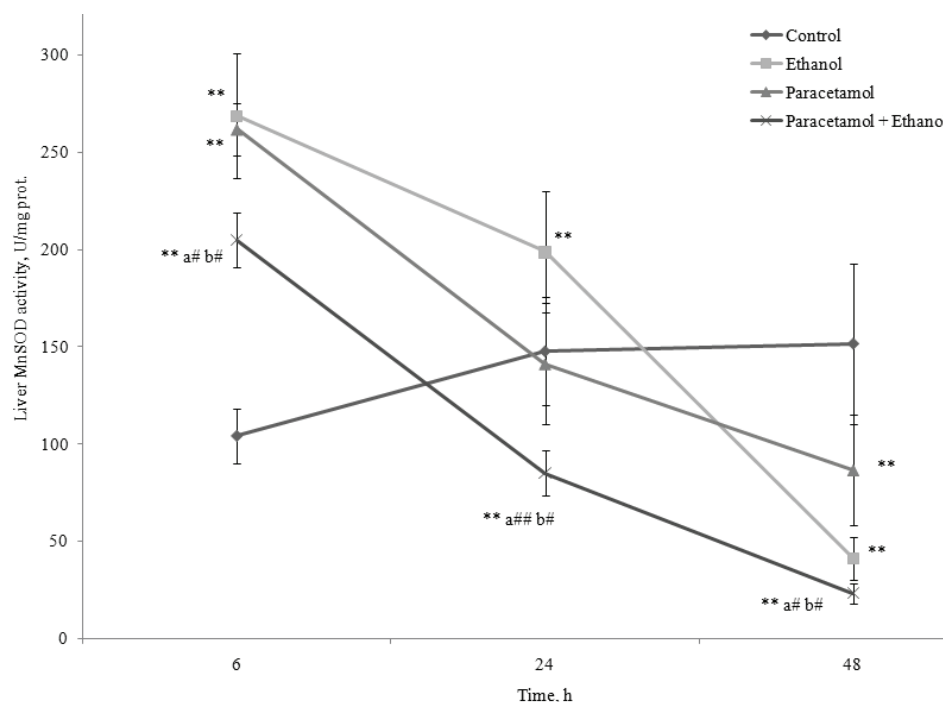


Fig. 4. Activity of mitochondrial (manganese) superoxide dismutase (MnSOD) in the liver of the experimental animals. The MnSOD activity was measured in the mitochondrial liver fraction 6, 24 and 48 h after the experimental protocols. For statistical evaluation, multi-factorial ANOVA with Tukey's *post hoc* test were used (\*\* $p < 0.01$  vs. the control group, # $p < 0.05$ , ## $p < 0.01$  in comparison with animals treated with ethanol (a) or paracetamol (b) alone).

#### DISCUSSION

The obtained results showed that the serum aminotransferase activity was significantly elevated after paracetamol or ethanol administration. This indicates that paracetamol and ethanol induce liver injury in the early phase of acute intoxication and that this injury persists for at least 48 h. In addition, the serum and liver MDA concentrations were significantly higher in all groups treated with paracetamol or ethanol in comparison with those of the control group. Based on these data, it could be suggested that lipid peroxidation may be an important mechanism of paracetamol- and ethanol-induced hepatotoxicity, even when administered acutely. Lipid peroxidation was confirmed to be an important mechanism of paracetamol-induced liver injury at different doses in various studies.<sup>7,8,34-36</sup> Paracetamol was found to increase the serum MDA level 4 h after administration<sup>35</sup> and this elevation persisted for 7 days.<sup>34</sup> The role of lipid peroxidation was also confirmed by the protective effects of L-carnitine and some medicinal plants on hepatocyte injury caused by paracetamol.<sup>8,34,35</sup> This pro-

tection can, at least partly, be explained by their ability to inhibit MDA production. Similarly, earlier studies showed that a vitamin E-rich diet reduces lipid peroxidation in hepatocytes due to paracetamol toxicity.<sup>37</sup> Vitamin E was found to protect animals on a Wendel diet against paracetamol hepatotoxicity,<sup>37,38</sup> while it had no effect on injury in animals on a standard diet.<sup>39</sup>

It is well known that oxidative stress is a major contributor of ethanol-induced liver injury. Lipid peroxidation, as an important consequence of oxidative stress, was detected in various models of acute and chronic ethanol intoxication.<sup>40–43</sup> Previous binge drinking was found to potentiate paracetamol-induced lipid peroxidation 48 h after paracetamol treatment. These results suggest that ethanol, when acutely administered, has synergistic effects with paracetamol related to lipid peroxidation and this can be one potential mechanism of aggravation of paracetamol-induced liver injury by ethanol.

Liver nitrites and nitrates, markers of NO production, were increased in all groups treated with either paracetamol or ethanol. Their maximal level was reached 48 h after paracetamol or ethanol administration. These findings suggest that reactive nitrogen species are also involved in acute paracetamol- and ethanol-induced liver injury. The role of nitrosative stress in acute paracetamol intoxication, detected as an increase in nitrotyrosine production, was suggested in various studies.<sup>12,14,15,44</sup> Nitrotyrosine immunoreactivity was found to be increased 4 h after paracetamol treatment in mouse liver.<sup>12,44</sup> Similarly, it was found that ethanol is also an inductor of NO synthesis,<sup>45</sup> although its role in ethanol-induced liver injury is not completely understood. In low concentrations NO exerts hepatoprotective effects, while in high concentrations, NO in the presence of superoxide anion leads to peroxynitrite formation, which is known to be a potent oxidant in cells.<sup>41,46</sup> Normally, peroxynitrite is detoxified by GSH/GSH peroxidase, especially in the mitochondria.<sup>47</sup> It has been postulated that GSH depletion induced by paracetamol or ethanol leads to peroxynitrite accumulation, thus aggravating nitrosative stress.<sup>48</sup> The present results are in accordance with these data, since a significant GSH depletion was found in the P and E groups 48 h after treatment, at the same time when the liver nitrite + nitrate level reached its maximal value. However, the increase in the liver nitrite + nitrate level was accompanied by GSH elevation 24 h after treatment. This suggests that ethanol- and paracetamol-induced NO production surpassed the GSH capacity to detoxify reactive nitrogen species in first 24 h.

In the present study, it was found that the liver nitrite + nitrate concentration was not significantly different in the PE group to that in the P group. Based on these results, it appears that prior binge drinking exerts neither a synergistic nor a protective effect on paracetamol-induced nitrite/nitrate production. On the contrary, studies in mice using a chronic alcoholism model found that alcohol pre-treatment increased nitrotyrosine formation induced by paracetamol.<sup>25</sup> However,

within a few hours after treatment, the nitrotyrosine formation was found to be decreased when the animals had been pretreated with ethanol.<sup>25</sup> This indicates that interaction between ethanol and paracetamol depends on the exposition time.

The liver GSH content was found to be decreased in the ethanol-treated animals 6 h after its administration. After this period, its level tended to rise and reached the control level within 24 h. This indicates that GSH depletion could represent a contributory mechanism to acute ethanol-induced oxidative injury within a few hours after intoxication. The role of GSH depletion in ethanol-induced liver injury is supported by other studies that confirmed a protective role of *N*-acetylcysteine and L-2-oxothiazolidine-4-carboxylic acid in animal models of alcoholic liver disease.<sup>49,50</sup> Recent studies showed that silymarin and betaine, also exerted protective effects on binge-drinking mice.<sup>40,42</sup> Among the various mechanisms involved in their protective effects, reduction of GSH depletion was suggested to play an important role.

In the present study, paracetamol was found to have decreased the GSH content in the liver 6 h after its administration. Various studies suggested that GSH depletion plays a contributory role in paracetamol hepatotoxicity.<sup>13,51</sup> *N*-Acetylcysteine, a GSH precursor, was found to reduce paracetamol toxicity in mice and this effect was pronounced in the first hours after paracetamol treatment.<sup>52</sup> Similarly, pretreatment with  $\alpha$ -lipoic acid reduced GSH depletion induced by high doses of paracetamol (2.5 g kg<sup>-1</sup>) and protected markedly against its hepatotoxicity.<sup>53</sup> Studies in mice showed that the GSH level was decreased by approximately 90 % at 1- and 2-hour intervals after paracetamol treatment.<sup>13</sup> In addition, *in vitro* studies confirmed a profound GSH depletion after a paracetamol overdose.<sup>54</sup> This is not surprising since the major mechanism involved in early paracetamol hepatotoxicity is inactivation of sulfhydryl groups of various cellular compounds due to NAPQI detoxification.<sup>44</sup> Glutathione-*S*-transferase (GST) is an enzyme that enables detoxification of NAPQI, as well as other electrophilic substrates (products of xenobiotic metabolism) by binding to GSH.<sup>55</sup> However, the contribution of this enzyme to GSH depletion and liver injury in paracetamol intoxication remains controversial. *In vitro* studies suggested that  $\pi$  class GST consumes the majority of GSH for NAPQI detoxication in mice and possibly ameliorates liver injury.<sup>56</sup> Although  $\pi$  class GST was found at high levels in mice liver, *mGstp1/2*<sup>-/-</sup> mice (knockout mice for  $\pi$ -GST gene) challenged with an overdose of paracetamol exhibited a markedly decreased, not increased hepatotoxicity when compared to *mGstp1/2*<sup>+/+</sup> mice.<sup>57</sup> The role of GST in mediating the toxic effects of paracetamol in humans is even more blurred, since  $\pi$  class GST is absent or expressed at low levels in the human liver.<sup>55</sup>

After an initial decline, the GSH level returned or became closer to the normal values depending on the administered dose within the next few hours.<sup>13</sup> In the present study, a significant increase in the GSH level in the liver was found

24 h after paracetamol administration in comparison with the control group. Higher doses of paracetamol were found not to induce any rise in the liver GSH content.<sup>58</sup> The increase found in the present study may possibly be explained by an increased expression of glutamate cysteine ligase, the key enzyme in the synthesis of GSH, which is known to be induced by oxidative stress *via* the nuclear factor kappaB (NF-κB), activator protein-1 (AP-1) and other transcription factors.<sup>59</sup> After this period, GSH level fell again and within the following 24 h reached its lowest level, even lower than in the first 6 h. This decrease may be a result of GSH consumption in ROS/RNS detoxification in the liver. According to the results of the present study, it could be suggested that paracetamol induces a triphasic change in the liver GSH level. This finding is supported by the two-phase theory of paracetamol hepatotoxicity. According to this theory, in the first metabolic phase, NAPQI, a reactive metabolite of paracetamol, leads to GSH depletion and covalent binding to various proteins.<sup>60,61</sup> This mechanism may have been involved in the initial fall in the GSH level. In the second oxidative phase, a mitochondrial permeability transition occurs that contributes to the toxic effects of paracetamol. The ROS involved in mitochondrial permeability transition may also be involved in the late GSH consumption.<sup>62</sup>

It was found in some studies that prior ethanol binge drinking in five doses of 4 g kg<sup>-1</sup> potentiated paracetamol-induced GSH depletion in mice liver.<sup>62</sup> According to the present study, even smaller doses of ethanol (5×2 g kg<sup>-1</sup>) appeared to potentiate this effect of paracetamol. This potentiation was clearly observed at all time intervals. Additionally, an adaptive increase in GSH level was not observed in the PE group. This indicates that paracetamol and ethanol exert synergistic effects on the liver GSH level.

Liver SOD activity was decreased after administration of either paracetamol or ethanol at all time intervals. Paracetamol was found to induce a more pronounced decrease in SOD activity than ethanol. However, this effect was not the same for both fractions of SOD. While Cu/Zn SOD activity was lower in both paracetamol- and ethanol-treated groups, the activity of mitochondrial MnSOD was higher in the P and E groups 6 h after treatment compared with the control group. After this period, the MnSOD activity progressively decreased and reached its lowest level 48 h after treatment. According to other studies, the effects of ethanol on the SOD activity are inconclusive and depended on dose and route of administration. Some studies found that ethanol induced MnSOD after acute administration.<sup>63,64</sup> A single dose of ethanol was found to increase liver MnSOD activity by about 30 % three hours after administration, with a further increase up to about 50 % in the subsequent 6 h.<sup>63</sup> In the present study, an even more pronounced increase in its activity of approximately by 150 % six hours after binge drinking was found. This increase may be a result of an adaptive response to the increased production of ROS. Since mitochondria are the major source of ROS,<sup>41</sup>

it is not surprising that mitochondrial SOD isoenzyme activity was increased after binge drinking. The subsequent decrease in the MnSOD activity indicates the consumption of this enzyme in the detoxification of ROS. Similar to the present results, the Cu/Zn SOD activity and its immunoreactivity in the liver were found to decrease in various studies using the enteral alcohol model.<sup>65,66</sup>

Paracetamol was found to decrease liver SOD activity in various experimental models.<sup>34,67</sup> Even smaller doses ( $150 \text{ mg kg}^{-1}$ ) were found to reduce its activity by about 30 % sixteen hours after paracetamol administration.<sup>67</sup> The precise role of SOD in paracetamol-induced hepatotoxicity is not completely understood. Although it is to be expected that SOD overexpression would have a protective antioxidant effect in the liver, some studies found that deletion of Cu/Zn SOD (SOD1) genes decreased susceptibility to paracetamol.<sup>68</sup> This finding was explained by the fact that SOD1 deletion reduces hepatic protein nitration and CYP2E1 activity by approximately 50 %.<sup>68</sup> In the present study, paracetamol induced a drastic decrease in Cu/Zn SOD activity, which could represent a possible autoprotective mechanism in acute paracetamol overdose. However, many studies suggest a protective role of SOD in acetaminophen-induced liver injury.<sup>67,69,70</sup> In contrast to the total SOD activity, paracetamol had induced a significant increase in the MnSOD activity 6 h after treatment, thus suggesting an adaptive response of the hepatocyte mitochondria to increased production of ROS. On the other hand, Agarwal *et al.*<sup>71</sup> found that the MnSOD activity was decreased 1 h and remained significantly decreased at 2 h in mice that had been treated i.p with  $300 \text{ mg kg}^{-1}$  of paracetamol. These decreases in MnSOD activity were explained by an increased production of ROS in the mitochondria, which react with nitric oxide, leading to increased levels of reactive nitrogen species.

Paracetamol and ethanol were found to have synergistic effects on the SOD activity in the present study. Within the first 24 h, the liver SOD activity in the PE group was similar to its activity in the P group, thus indicating that prior binge drinking did not potentiate paracetamol-induced reduction in the total SOD activity at this time interval. However, within the next 24 h, ethanol and paracetamol induced a more pronounced decline in the SOD activity when administered together than either of the substances alone. This suggests that reduction in hepatic SOD activity is not an early event in possible synergistic hepatotoxic effects of ethanol and paracetamol. Within the first 6 h, the MnSOD activity was increased to a lesser extent in the PE group than in the P and E groups. Additionally, subsequent administration of paracetamol after ethanol caused a more pronounced decrease in the MnSOD activity than either of these substances alone within 48 h after treatment. This indicates that ethanol and paracetamol induce oxidative stress synergistically and the more pronounced decrease in MnSOD and SOD activity may be explained by increased consumption of this enzyme in ROS detoxication.

## CONCLUSIONS

According to this study, it can be concluded that prior binge ethanol drinking may potentiate the paracetamol-induced decrease in the antioxidative capacity of hepatocytes due to GSH depletion and reduction in SOD activity. In addition, paracetamol-induced lipid peroxidation is aggravated by prior ethanol administration within 48 h. However, the formation of reactive nitrogen species is not influenced by paracetamol ingestion. Based on the obtained results, a possible protective effect of ethanol on paracetamol hepatotoxicity cannot be attributed to alleviation of oxidative stress. Other mechanisms involved in this possible protective effect should be further investigated.

*Acknowledgement.* This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Grant No. 175015.

## ИЗВОД

ДЕЈСТВО ЕТАНОЛА НА ОКСИДАТИВНО ОШТЕЋЕЊЕ ЈЕТРЕ МИШЕВА  
ИЗАЗВАНО ПАРАЦЕТАМОЛОМ

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Циљ наше студије је био да се испита ефекат викенд пијанства на оксидативно оштећење јетре мишева изазвано парацетамолом. У експерименту су коришћени мишеви, мужјаци *Swiss* соја подељени у следеће групе: 1. контрола; 2. животиње третиране етанолом (5 појединачних доза од по 2 g kg<sup>-1</sup> путем орогастричне сонде) (Е); 3. група третирана парацетамолом (појединачна доза од 300 mg kg<sup>-1</sup> интраперитонеално) (П); 4. животиње које су примиле парацетамол 12 сати после последње дозе етанола (ПЕ). За одређивање параметара оксидативног стреса узимани су узорци крви и јетре, 6, 24 и 48 сати након третмана. Када су администрирани у комбинацији, етанол и парацетамол изазивају значајно повећање нивоа малондиалдехида (MDA) у јетри 48 сати након третмана у поређењу са П и Е групом (17,14±1,98 vs. 13,14±0,82 и 12,99±1,18 nmol mg<sup>-1</sup> protein, *p* < 0,01). Такође, етанол и парацетамол у комбинацији доводе до значајног смањења нивоа глутатиона у јетри у свим временским интервалима, у поређењу са П и Е групом (*p* < 0,01). Укупна активност супероксид-дисмутазе (SOD) била је значајно смањена у ПЕ групи 48 сати након третмана у поређењу са П и Е групама (251,73±80,63; 707,62±179,92 и 1179,62±147,94 U mg<sup>-1</sup> protein, *p* < 0,01). Најнижа активност манган SOD (MnSOD) нађена је у ПЕ групи 48 сати након третмана (86,52±28,31; 41,13±11,07 и 23,16±5,18 U mg<sup>-1</sup> protein у П, Е и ПЕ групама, *p* < 0,05). На основу наших резултата може се закључити да акутна администрација етанола потенцира дејство парацетамола путем смањења антиоксидативног капацитета хепатоцита, услед смањења нивоа глутатиона и активности SOD, уз истовремено повећање липидне пероксидације изазване парацетамолом.

(Примљено 24. јула, ревидирано 21. новембра 2012)

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