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## OXIDATIVE STRESS BY ACUTE ACETAMINOPHEN ADMINISTRATION IN MOUSE LIVER

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**Abstract**—Acetaminophen was given to mice at a single dose of 375 mg/kg. In situ liver chemiluminescence,  $H_2O_2$  steady-state concentration, and the liver concentrations of total and oxidized glutathione were measured 15, 30, and 60 min after acetaminophen administration. Increases of 145% and 72% in spontaneous chemiluminescence and  $H_2O_2$  concentration were observed 15 min after the injection, respectively. Total glutathione was decreased by acetaminophen administration at all the times studied. The maximal decrease, 83%, was found 60 min postinjection. The ratio GSH/GSSG was found significantly decreased at all the times studied. Microsomal superoxide production was increased by 2.4-fold by addition of acetaminophen. The activities of the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase were determined. Catalase was slightly inhibited (30%) 15 min after acetaminophen administration. No significant changes were found in superoxide dismutase activity. Se and non-Se glutathione peroxidase activities were decreased by 40% and 53% respectively, 15 min after acetaminophen administration. The decrease in catalase and glutathione peroxidase would result in an increased steady state level of  $H_2O_2$  and hydroperoxides, contributing to cell injury. Damaged hepatocytes were observed, and severe lesions and necrosis appeared 60 min after acetaminophen administration. Our results indicate the occurrence of oxidative stress as a possible mechanism for acetaminophen-induced hepatotoxicity.

**Keywords**—Acetaminophen, Hepatotoxicity, Oxidative stress, Chemiluminescence, Lipid peroxidation, Free radicals

### INTRODUCTION

It is well known that acetaminophen, a widely used analgesic-antipyretic drug, produces centrilobular liver necrosis and renal failure, at relatively high doses.<sup>1</sup> The use of acetaminophen, phenacetin (a precursor for acetaminophen), and aspirin has been correlated epidemiologically with a 2-fold increased risk of renal pelvic cancer.<sup>2</sup>

A large number of metabolites are produced by acetaminophen in biological systems; some of them can form covalent adducts with cellular macromolecules, and others can generate free radicals and superoxide. Formation of such reactive metabolites is thought to be critical to the toxicity, mutagenicity, and potential carcinogenicity of these compounds. High concentrations of metabolic intermediates of phenacetin and acetaminophen induce morphological transformation of C3H/10T1/2 mouse embryo cells.<sup>3</sup>

Acetaminophen is mainly metabolized by cytochrome P450 to form an electrophilic metabolite, *N*-acetyl *p*-benzoquinone imine, which is primarily inactivated by conjugation with glutathione.<sup>4,5</sup> At high doses, the detoxification pathways become saturated, and the intermediate metabolite accumulates and causes liver damage by covalent binding to tissue molecules.<sup>6,7</sup>

Acetaminophen hepatotoxicity appears to be critically dependent on the depletion of cellular glutathione. Numerous studies showed the effectiveness of sulfhydryl compounds in reducing the degree of covalent binding of the reactive metabolite of acetaminophen.<sup>8</sup> Methionine and *N*-acetylcysteine have been successfully used in the clinical treatment of acetaminophen overdose.<sup>9</sup>

Reduced glutathione functions as a reductant in the metabolism of both hydrogen peroxide and various organic hydroperoxides. This reaction is catalyzed by the glutathione peroxidases present in the cytosol and mitochondria of various cells.<sup>10</sup> A relatively high reduction in the intracellular level of reduced glutathione leads to a situation of oxidative stress.

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Alternatively, the one-electron oxidation of acetaminophen by P450 may generate reactive oxygen species, and the subsequent thiol depletion via oxidation may lead to an alteration in calcium homeostasis and cause hepatotoxicity.<sup>11</sup> It has been suggested that an alkylating acetaminophen metabolite causes  $\text{Ca}^{2+}$  deregulation in the nucleus, leading to activation of a  $\text{Ca}^{2+}$ -sensitive endonuclease, fragmentation of DNA, and cell death.<sup>12</sup> Evidence was provided that nuclear DNA fragmentation is an early step in acetaminophen-induced hepatocellular damage *in vitro*.<sup>13</sup>

In this study, we evaluated the occurrence of mouse liver oxidative stress by acetaminophen acute treatment through measurements of chemiluminescence and hydrogen peroxide steady-state concentration. Superoxide production by acetaminophen was determined in liver microsomes supplemented with NADPH. Glutathione content, the activities of antioxidant enzymes, and morphological parameters of tissue damage were also measured.

## MATERIALS AND METHODS

### *Animals and treatment*

Female Swiss mice weighing 20–25 g were supplied with water and laboratory animal food *ad libitum* and housed at 22–24°C on a 12-h light–dark cycle. Acetaminophen was dissolved in 0.9% NaCl solution at 40°C and injected to the animals at a dose of 375 mg/kg of body weight.

### *Liver chemiluminescence*

Control and treated mice were anesthetized with urethane (1.5 g/kg of weight *ip*). The liver surface was exposed by laparotomy, and chemiluminescence was measured with a Johnson Foundation photon-counter (Johnson Research Foundation, University of Pennsylvania, Philadelphia, PA).<sup>14</sup> Chemiluminescence is expressed in counts per second per unit of liver surface ( $\text{cps}/\text{cm}^2$ ).

### *Sample handling*

Liver fragments (0.5–1 g) obtained from the left lateral lobule of control and treated mice were hemisectioned for biochemical and histopathological determinations.

### *Intracellular steady-state concentration of hydrogen peroxide*

Tissue slices 0.1 mm thick were incubated 10 min in 120 mM NaCl, 30 mM phosphate buffer (pH 7.4)

at 30°C and at a tissue/medium ratio of 1/20. Samples of the incubation medium were diluted 1/2.5 with 100 mM phosphate buffer (pH 7.4) containing 2.8 U/ml horseradish peroxidase and 40  $\mu\text{M}$  p-hydroxyphenylacetic acid as hydrogen donor and fluorescence intensity was measured at 317–414 nm.  $\text{H}_2\text{O}_2$  concentration was determined by a standard calibration curve and calculated by subtracting the value of a sample treated with 0.1  $\mu\text{M}$  catalase from the value of an untreated sample.<sup>15</sup>

### *Glutathione content*

Total and oxidized glutathione were determined 15, 30, and 60 min after acetaminophen administration. The sum of the reduced and oxidized forms of glutathione was determined using 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), NADPH, and glutathione reductase in a kinetic assay at 412 nm. Oxidized glutathione (GSSG) was determined using NADPH and glutathione reductase, at 340 nm. Glutathione concentration is expressed in  $\mu\text{mol}/\text{g}$  organ.<sup>16</sup>

### *Superoxide production*

The superoxide dependent oxidation of epinephrine to adrenochrome is followed spectrophotometrically at 480 nm with a sensitive single-wavelength spectrophotometer ( $\epsilon = 4.0 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ ) in a reaction medium containing 50 mM glycine buffer pH 8.5, 1 mM epinephrine, 100  $\mu\text{M}$  NADPH, and 1 mg liver microsomes.<sup>17</sup>

### *Enzyme assays*

Mouse liver homogenates were prepared in a medium consisting of 120 mM KCl and 30 mM potassium buffer (pH 7.4) and centrifuged at 600 g for 10 min to discard nuclei and cellular debris. The supernatant, a suspension of preserved organelles, was termed "homogenate."

Superoxide dismutase (SOD) was determined in the homogenates by measuring the inhibition of the rate of autocatalytic adrenochrome formation and expressed in U/g liver.<sup>18</sup>

Catalase activity was determined in the homogenates following the decrease in absorption at 240 nm<sup>19,20</sup> and expressed in nmol catalase heme/g liver ( $\mu\text{M}$ ).

Glutathione peroxidase (GPx) was measured in the homogenates following NADPH oxidation at 340 nm in the presence of GSH, glutathione reductase, and *tert*-butyl hydroperoxide or  $\text{H}_2\text{O}_2$  as described by Flohé and Gunzler.<sup>21</sup> The activity of glutathione peroxidase

is expressed in U/g liver, and the enzyme content is also expressed as nmol enzyme/g liver ( $\mu\text{M}$ ). A significant effect of hepatic residual acetaminophen in inhibiting glutathione peroxidase activity of liver homogenates was ruled out by appropriate controls.

#### Histopathological studies

Hemisections of liver fragments obtained from control and treated mice were fixed in 10% formol buffer pH 7.4 for 24 h, included in paraffin, and stained with hematoxylin-eosine. Morphological damage was evaluated in slices 5  $\mu\text{m}$  thick, by examining 50 nonconsecutive high power fields ( $\times 400$ ). Liver damage was scored using a scale of 0 to 3 points based on the degree of two structural parameters: cell swelling and vacuolar degeneration. Necrotic cells were also evaluated according to their presence (3 points) or absence (0 points). Those cells with nuclear alterations (picnosis, fragmentation, or lysis), cytoplasmic rarefaction with cell boundaries lost were considered as necrotic. The total score of lesion per sample was obtained from the sum of the scores given to each morphological type of damage including necrosis (maximal score per high power field examined = 9 points). If the score per each examined field were the maximal (9 points), the total score of lesions per sample would be 450 points. It was assumed that this score represented 100% of total damage.

#### Marker enzymes of liver damage

Serum samples were assayed for lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) using commercial laboratory kits. Values are given in units per liter of serum.

#### Statistics

Values are expressed as mean values  $\pm$  SEM. The significance of differences between mean values were analyzed by ANOVA and Tukey test.

### RESULTS

#### Spontaneous chemiluminescence and $\text{H}_2\text{O}_2$ steady-state concentration

In situ liver chemiluminescence was measured at different times after acute acetaminophen administration. Spontaneous chemiluminescence (control value =  $109 \pm 13$  cps/cm<sup>2</sup>) was transiently increased by 145% 15 min after acetaminophen administration. Chemiluminescence values were not significantly different from control ones in animals measured 60 min after acetaminophen admin-

istration (Fig. 1). The intracellular  $\text{H}_2\text{O}_2$  steady-state concentration was also measured in liver slices of control and acetaminophen-treated animals.  $\text{H}_2\text{O}_2$  steady-state concentrations showed a similar pattern to the one of chemiluminescence, being transiently increased by 72% 15 min after acetaminophen administration and returning to normal values at 30 and 60 min postinjection. The control value was  $0.09 \pm 0.01$   $\mu\text{M}$  (Fig. 1).

#### Glutathione content

Total glutathione was decreased by acetaminophen administration at all the studied times. The maximal effect was observed 60 min after the injection (83% decrease). The control value was  $8.1 \pm 0.7$   $\mu\text{mol/g}$  liver (Table 1). Oxidized glutathione (GSSG) was increased by 70% 15 min after acetaminophen administration, but at longer periods (30 and 60 min postinjection) decreases of 45% and 65% were observed, respectively, as compared with control values (Fig. 2). Reduced glutathione (GSH) content values were calculated and are shown in Figure 2 and Table 1. There is a maximal decrease of 83% in GSH content 60 min after acetaminophen administration, as compared with control values. The ratio GSH/GSSG was found significantly decreased at all the times studied (Table 1).

#### Superoxide production

The addition of 8 mM acetaminophen produced a marked increase of approximately 2.4-fold in the rate of superoxide production by liver microsomes supplemented with NADPH. The effect depended on the acetaminophen

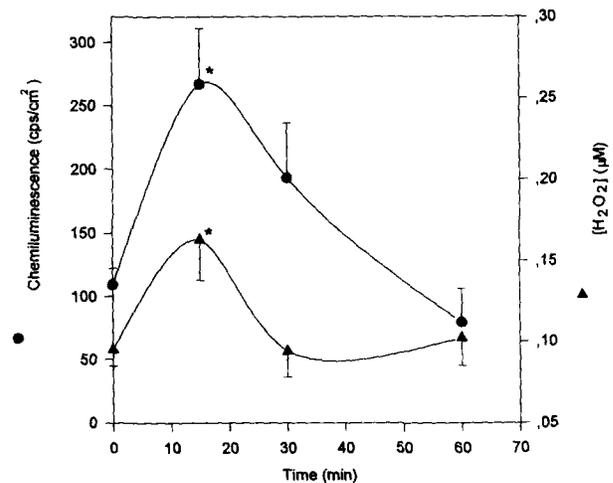


Fig. 1. Spontaneous chemiluminescence (●) and  $\text{H}_2\text{O}_2$  steady-state concentration (▲) in the liver of acetaminophen-treated mice. Symbols indicate mean values from six animals and bars indicate S.E.M. \* $p < 0.05$ .

Table 1. Effect of Acetaminophen Administration on Mouse Liver Antioxidant Enzyme Activities and Glutathione Content

Parameter	Control	15 min	60 min
A—Enzyme activity			
SOD (U/g)	600 ± 187	561 ± 77	561 ± 44 (85%)
Catalase (μM)	1.2 ± 0.1	0.8 ± 0.2	1.1 ± 0.1 (92%)
Se-GPx (U/g)	2.5 ± 0.4	1.5 ± 0.2	1.3 ± 0.2* (52%)
(μM)	0.13 ± 0.02	0.08 ± 0.03	0.07 ± 0.01
non Se-GPx (U/g)	2.20 ± 0.20	0.30 ± 0.06*	0.70 ± 0.10* (32%)
B—Glutathione			
GSH + 2GSSG (μmol/g)	8.1 ± 0.7	5.9 ± 0.8	1.4 ± 0.5* (17%)
GSSG (μmol/g)	0.20 ± 0.03	0.34 ± 0.01*	0.07 ± 0.02* (35%)
GSH (μmol/g)	7.7 ± 0.5	5.2 ± 0.6	1.3 ± 0.1* (17%)
GSH/GSSG	38.50 ± 0.20	15.40 ± 0.60*	17.40 ± 0.04* (45%)

Values are expressed as mean ± SEM of 4–6 animals. Numbers between parenthesis are percent of control values.

\*  $p < 0.05$ .

concentration (Fig. 3). The rate of microsomal NADPH oxidation was also increased by acetaminophen in a concentration-dependent manner. Adrenochrome formation was inhibited by addition of 0.2 μM superoxide dismutase, both in the presence or absence of acetaminophen, giving specificity to the assay. The effect of 8 mM acetaminophen increased NADPH oxidation in 4.7 nmol/min·mg and superoxide production in 7.2 nmol/min·mg, indicating a stoichiometry of 1.5 O<sub>2</sub><sup>•-</sup>/NADPH with a highly efficient O<sub>2</sub><sup>•-</sup> production in the acetaminophen-stimulated NADPH oxidation. In the absence of acetaminophen, the stoichiometry was 0.6 O<sub>2</sub><sup>•-</sup>/NADPH.

#### Antioxidant enzyme activities

Superoxide dismutase, catalase, and glutathione peroxidase activities were measured in liver homoge-

nates at 15 and 60 min after acetaminophen administration. Superoxide dismutase was not significantly affected by acetaminophen treatment. Catalase was inhibited by 30% 15 min after injection. Se-glutathione peroxidase showed a decrease of 40% 15 min after acetaminophen administration, while non-Se glutathione peroxidase was decreased by 53% as compared with control values (Table 1).

#### Histopathological studies

Indexes of liver damage were high in the experimental treated groups with respect to the control group (Table 2). The index of liver damage increased with the time of drug administration. A minor degree of vacuolar degeneration was observed in the control group, as it is shown in Figure 4. More severe forms

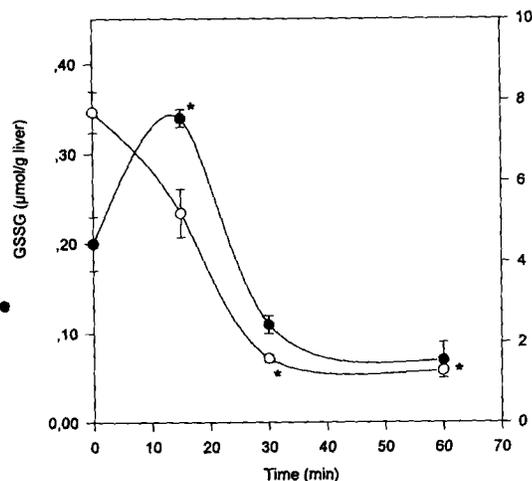


Fig. 2. Effect of acetaminophen administration on GSH (○) and GSSG (●) content. Bars represent mean values of 4–6 animals ± S.E.M. \*  $p < 0.05$ .

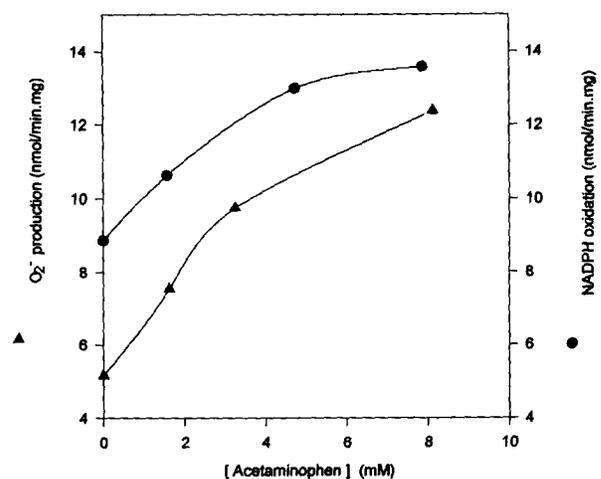


Fig. 3. Effect of added acetaminophen concentrations on superoxide production (▲) and NADPH oxidation (●) in liver microsomes.

Table 2. Index of Liver Damage (%) in Acetaminophen-Treated Animals

Time (min)	Index of Liver Damage (%)
0	4 ± 1
15	39 ± 7*
30	39 ± 2*
60	89 ± 2*

Values are expressed as mean ± SEM of 4–6 animals.

\*  $p < 0.05$ .

of hepatocellular degeneration were observed in the treated groups, mainly in Zones 2 and 3 of the hepatic acinus. Necrosis was more markedly observed in the acetaminophen-treated group 60 min postinjection of the drug. The most damaged zone of the acinus was the perivenular area (Fig. 5).

**Marker enzymes of liver damage.** Lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were determined in mouse serum samples, 30 and 60 min after acetaminophen injection. LDH was increased by 42% and 136% 30 and 60 min after drug administration. ALT showed increases of 72% and 100% 30 and 60 min after acetaminophen administration. AST was not altered after

30 min of acetaminophen injection but was increased 62% after an hour of treatment (Table 3).

## DISCUSSION

Increased in situ liver chemiluminescence has been associated with the development of cell injury and liver necrosis in different experimental situations: chronic and acute alcohol administration,<sup>22</sup> vitamin E- and selenium deficiency,<sup>23</sup> tumor-bearing animals,<sup>24</sup> and mitoxantrone treatment.<sup>25</sup> Recent studies have shown in two models of lung toxicity—paraquat injection and exposition to hyperbaric oxygen—that lung chemiluminescence is an earlier marker of lung damage because it precedes the decrease in survival and is related to polymorphonuclear cell migration.<sup>26</sup> Increased chemiluminescence has also been postulated as the earliest marker of the septic syndrome in rat liver and muscle, preceding the increase of serum levels of marker enzymes of liver and muscle damage.<sup>27</sup> In our study, the increase in liver photoemission observed 15 min after acetaminophen administration indicates that the drug induces a situation of oxidative stress in liver tissue with increases in the steady-state concentrations of hydrogen peroxide and singlet oxygen.<sup>28</sup> Slight morphological changes were observed 15 min after acetaminophen administration, during maximal emission, while 60 min after acetaminophen injection more seri-



Fig. 4. Control mouse liver. Portal space and hepatocytes with normal morphological appearance. (Hematoxylin-eosin ×250). HA: hepatic artery; PV: portal vein; BD: biliary duct; Z<sub>1</sub>: zone 1 = periportal area.

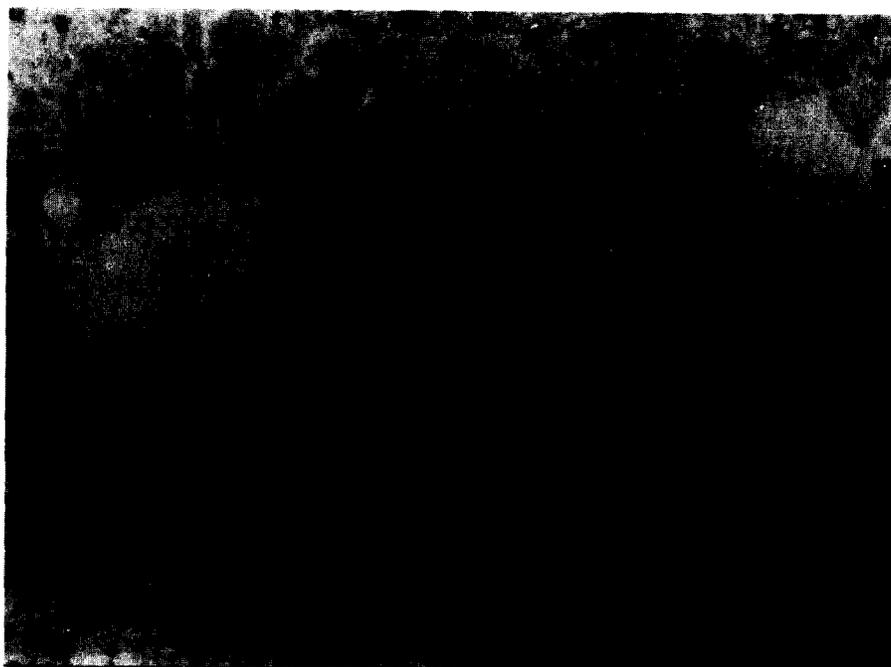


Fig. 5. 60 min acetaminophen-treated mouse liver. Perivenular hepatocytes with marked tumefacted cytoplasm and vacuolar degeneration. Necrotic cells can also be observed. Periportal hepatocytes with normal morphological appearance (Hematoxylin-eosin  $\times 250$ ).  $Z_2$ : Zone 2 = medioacinar area;  $Z_3$ : Zone 3 = perivenular area; VD: hepatocytes with vacuolar degeneration; HN: hepatocellular necrosis; THV: terminal hepatic venule.

ous damage and coagulation necrosis were observed. Necrosis results in a leakage of transaminases (ALT and AST) and LDH into the serum, indicating destabilization of the hepatocyte cellular membrane. These results suggest that oxidative stress induced by acetaminophen in mouse liver precedes the appearance of necrosis.

$H_2O_2$  steady-state concentration increased 15 min after acetaminophen administration. Intracellular  $O_2^{\cdot-}$  and  $H_2O_2$  concentration lead to lipid peroxidation chain reactions with the generation of excited species responsible for photoemission.

Assuming that the most important fraction of the total emission detected corresponded to bimolecular singlet oxygen de-excitation, while only a minor fraction would consist of green-blue light (400–500 nm) coming from excited carbonyls, a quadratic relation-

ship between chemiluminescence (Chl) and  $H_2O_2$  was found (eq. 1).<sup>29</sup>

$$\text{Chl} = K \times [H_2O_2]^2 \quad (1)$$

In our study, such quadratic relationship between chemiluminescence and  $H_2O_2$  concentration could be verified in 15 min treated animals ( $t$ ) in relation to control ones (eq. 2).

$$\text{Chl}_t/\text{Chl}_c = [H_2O_2]_t^2/[H_2O_2]_c^2 \equiv 2-3 \quad (2)$$

It is generally accepted that acetaminophen hepatotoxicity is critically dependent on glutathione depletion. From several studies it has been estimated that loss of more than 70% is critical to the tissue. Evidences have been provided that oxidative stress actually decreases the  $[GSH]/[GSSG]$  ratio.<sup>30</sup> In our model, the decrease in glutathione content 15 min after injection occurs parallel to the increase in chemiluminescence (Fig. 1), suggesting that GSH depletion leads to an increase in the steady-state level of  $H_2O_2$  (as previously described) and of lipid peroxides. The slight decrease in GSH content observed 15 min after acetaminophen administration together with the increase in GSSG level reflects the initial oxidation of GSH to

Table 3. Effect of Acetaminophen Administration on Marker Enzymes of Liver Damage

Time (min)	LDH (U/l)	ALT (U/l)	AST (U/l)
0	374 $\pm$ 20	39 $\pm$ 2	26 $\pm$ 5
30	530 $\pm$ 117	67 $\pm$ 13	23 $\pm$ 4
60	881 $\pm$ 44	78 $\pm$ 27	42 $\pm$ 1

GSSG, probably due to direct interaction of GSH with free radical intermediates.

Glutathione peroxidase was markedly inhibited by acetaminophen treatment, the decrease in non-Se glutathione peroxidase being particularly important. The partial inactivation of catalase, Se-glutathione peroxidase, and non-Se glutathione peroxidase leads to a situation in which  $H_2O_2$  and hydroperoxides formation cannot be matched by their corresponding utilization by the metabolizing enzymes resulting in an increased steady-state level of  $H_2O_2$  and hydroperoxides.

The mechanism by which acetaminophen produces hepatocyte necrosis is still unclear. Several metabolites of acetaminophen are suggested to be implicated as the ultimate toxic species. The main pathway of acetaminophen transformation by cytochrome P450 involves the formation of the intermediate: *N*-acetyl *p*-benzoquinone-imine. The mechanism proposed by Vries involves the reduction of the oxy-ferrocyclochrome-acetaminophen complex by electron transfer from NADPH via the cytochrome *c* reductase, followed by the oxidation of acetaminophen to the semiquinone. The semiquinone radical intermediate of acetaminophen might undergo a cyclic oxidation-reduction process consisting of the oxidation of the semiquinone to the quinonimine by molecular oxygen, with the generation of superoxide, followed by the reformation of the semiquinone by microsomal NADPH-cytochrome *c* reductase.<sup>31</sup> Recent reports have shown that silymarin, a 3-oxyflavone with antioxidant properties, protects against acetaminophen-induced lipid peroxidation and liver damage, even at low levels of GSH, possibly acting as a scavenger of superoxide and alkoxy radicals.<sup>32</sup> Our results show that superoxide anion is produced during acetaminophen metabolism by hepatic cytochrome P450, and NADPH is consumed as a result of the process. Depletion of glutathione level to a threshold value of 5 nmol/mg protein is associated with a significant conversion of xanthine dehydrogenase to reversible xanthine oxidase. This would be another possible mechanism for the stimulation of superoxide production during glutathione depletion.<sup>33</sup>

The results of our study indicate that oxidative stress induced by acetaminophen administration in mouse liver would play a role in drug hepatotoxicity. The data presented here provide evidence for the microsomal production of  $O_2^{\cdot-}$ -forming acetaminophen intermediate, postulated by Vries, and also for the in situ liver oxidative stress, which is consistent with the physiological production of *N*-acetyl *p*-benzoquinone-imine. The antioxidant depletion (glutathione and the antioxidant enzymes) produced by acetaminophen would lead to an increased steady-state level of active oxygen species reflected in in situ liver chemiluminescence. Further studies will help to

elucidate the contribution of other metabolizing pathways and molecular mechanisms to acetaminophen-induced liver damage.

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