

POSSIBLE ROLE OF OXIDATIVE STRESS IN ACUTE CADMIUM HEPATOTOXICITY IN RATS

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It is known that cadmium induces a variety of functional disorders, especially liver and kidney dysfunction. The main mechanism involved in cadmium hepatotoxicity is its binding to sulfhydryl groups and initiation of inflammation. Additionally, oxidative stress, due to a decrease in antioxidative capacity, plays a role in chronic cadmium hepatotoxicity. The role of oxidative stress in acute cadmium intoxication is still not clear.

The aim of our study was to investigate the role of reactive oxygen species and efficiency of antioxidant protection in rat liver in acute cadmium intoxication.

Male Wistar rats (n=16) were divided into the following groups: 1. control group (n=7), treated with saline, 2. cadmium-treated (n=9) with a single dose of 2.5 mg/kg intraperitoneally. After administration (24 hours), blood samples from the right side of the heart and liver samples were collected for the determination of oxidative stress parameters.

In our study malondialdehyde concentration was elevated both in plasma and liver after cadmium administration ($p < 0.01$). Moreover, superoxide dismutase activity was increased in the cadmium-treated group, mainly due to increase in copper/zinc superoxide dismutase activity ($p < 0.01$). Reduced glutathione level in liver and plasma and concentration of sulfhydryl groups were not significantly changed by cadmium.

The present study suggests that lipid peroxidation plays an important role in acute cadmium-induced liver injury. Antioxidant capacity of hepatocytes is partly increased due to an adaptive increase of superoxide dismutase activity.

Key words: acute hepatotoxicity, cadmium, oxidative stress, rats

INTRODUCTION

Cadmium is one of the most important toxic metals due to its accumulation in the environment as a result of its industrial, agricultural use and tobacco

smoking (Godt *et al.*, 2006). It has been reported that cadmium may cause injury of various tissues in humans and animals (Godt *et al.*, 2006). Among many toxic effects, this pollutant is known to cause sterility (Bench *et al.*, 1999), renal dysfunction (Cai *et al.*, 2001), liver and pancreas damage (Horiguchi *et al.*, 2000; Shimada *et al.*, 2000). Additionally, cadmium exerts genotoxic and apoptotic effects (Fahmy *et al.*, 2000; Kim *et al.*, 2005), modulates synaptic neurotransmission (Minami *et al.*, 2001) and induces changes in the brain antioxidant status (Carageorgiou *et al.*, 2004).

Cadmium may enter the organism by ingestion, inhalation or by skin resorption (Godt *et al.* 2006). In the bloodstream, cadmium binds to albumin and metallothionein (MT). The latter binds various metals and transports them to the liver. Since the initial site of cadmium accumulation is the liver, the effects of acute cadmium intoxication are most pronounced in this organ. In contrast to acute intoxication, the kidney is the major organ of chronic cadmium accumulation.

Cadmium causes morphological changes in hepatocytes, such as dilatation of the rough endoplasmic reticulum with loss of ribosomes, nuclear condensation and hepatocellular necrosis and apoptosis (Dudley *et al.*, 1984). However, exact mechanisms responsible for cadmium-induced damage are not completely understood. Primary injury of cells resulting from the binding of cadmium to sulfhydryl groups in mitochondria, and secondary injury initiated by the activation of Kupffer cells have been mentioned as possible mechanisms of toxic cadmium effects (Rikans and Yamano, 2000). Inactivation of sulfhydryl groups causes oxidative stress, mitochondrial permeability transition, and mitochondrial dysfunction (Jurczuk *et al.*, 2004). On the other hand, activated Kupffer cells release proinflammatory cytokines and chemokines which stimulate the migration and accumulation of neutrophils and monocytes in the liver. Activated inflammatory cells amplify cadmium-induced primary injury (Dudley *et al.*, 1984). Additionally, it is suggested that hepatocyte injury may be, also, caused by ischemia due to sinusoidal endothelial cell dysfunction. Cadmium has been found to accumulate in endothelial cells leading to its necrosis and denudation of hepatic sinusoids (Dudley *et al.*, 1984).

The role of oxidative stress in chronic cadmium intoxication is well known. It is reported that chronic exposition to cadmium decreases antioxidant capacity of hepatocytes, due to glutathione depletion and sulfhydryl groups inactivation in the liver (Nemmiche *et al.*, 2007). Moreover, chronic cadmium exposition inhibits the activity of antioxidant enzymes, including catalase, manganese-superoxide dismutase, and copper/zinc-superoxide dismutase (Koyu *et al.*, 2006).

However, the role of oxidative stress in acute cadmium intoxication is not completely understood. While *in vitro* studies suggested the important role of superoxide anion and nitric oxide in mediating acute cadmium hepatotoxicity (Misra *et al.*, 1996), results of *in vivo* studies are contradictory (Dong *et al.*, 1998). Based on this background, the aim of our study was to investigate the role of reactive oxygen species and efficiency of antioxidant protection in rat liver in acute cadmium intoxication.

MATERIAL AND METHODS

Animals

The experiment was performed on adult male Wistar rats weighting 220-250 g, raised at the Military Medical Academy, Belgrade. Animals were kept under standard laboratory conditions (temperature 22 ± 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 metabolizable energy of at least 11.5 MJ/kg, 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=16) were randomly divided into two groups: 1. control, saline-treated group (0.9% NaCl) (C; n=7); 2. cadmium-treated group (Cd; n=9) in a dose of 2.5 mg/kg intraperitoneally. Cadmium (Sigma-Aldrich, Co) was dissolved in saline (0.9% NaCl) in concentration 1 mg/kg before intraperitoneal administration.

Rats were sacrificed by cervical dislocation 24 hours after cadmium administration (or saline in the control group). Blood samples for determining parameters of oxidative stress were collected from the right side of the heart. For the same purpose 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, Feinbiochemica, Heidelberg, New York), 10 M phosphate buffer (pH 7.0) and 1 mM EDTA (Sigma chem. co. St. Louis, USA). The homogenates were centrifuged at 2000xg for 15 min at 4°C. Crude sediments were dissolved in a sucrose medium and centrifuged. The supernatants were transferred into tubes and centrifuged at 3200x g for 30 minutes at 4°C. Obtained sediments were dissolved in deionized water. After one hour of incubation, the samples were centrifuged at 3000x g for 15 minutes at 4°C, and supernatants were stored at -70°C. Proteins were determined by the Lowry method using bovine serum albumin as the standard (Lowry *et al.*, 1951).

Total superoxide dismutase (EC1.15.1.1.; SOD) activity in the liver was measured spectrophotometrically, as an inhibition of epinephrine autooxidation at 480 nm. After addition of 10 mM epinephrine (Sigma, St. Louis, USA), analysis was performed in sodium carbonate buffer (50 mM, pH-10.2; Serva, Feinbiochemica, Heidelberg, New York) containing 0.1 mM EDTA (Sigma, St. Louis, USA). Samples for manganese-SOD determination were previously treated with 8 mM KCN (Sigma, St. Louis, USA) and then analysed as described (Sun and Zigman, 1978).

Lipid peroxidation analysis in the plasma and liver homogenates was measured as malondialdehyde (MDA) production, assayed in the thiobarbituric

acid reaction as described by Girotti *et al.* (1991). The results are expressed as $\mu\text{mol/L}$ in plasma or nmol/mg proteins in liver homogenates.

Plasma concentration of total sulfhydryl groups was measured spectrophotometrically at 412 nm in phosphate buffer (0.2 mol+2 mmol EDTA, pH 9) using 5,5-dithiobis-2-nitrobenzoic acid (DTNB, 0.01 M, Sigma) (Elman, 1959).

Liver glutathione (GSH) level was determined by 5,5-dithiobis-2-nitrobenzoic acid (DTNB, 36.9 mg in 10 mL methanol), which reacts with aliphatic thiol compounds in Tris-HCl buffer (0.4 mol, pH 8.9) forming the yellow-coloured p-nitrophenyl anion. Glutathione level was proportional to the yellow colour intensity and was measured spectrophotometrically at 412 nm (Anderson, 1986).

Statistical analysis

Results are expressed as means \pm SD. For testing the difference between groups, Student's t test was used. The difference was considered statistically significant if $p < 0.05$. Statistica 7.0 program was used for statistical analysis.

RESULTS

Results of our study found that cadmium treatment induced significant changes in parameters of oxidative stress and antioxidant capacity in plasma and liver samples of experimental animals. A significant rise in liver MDA level was found 24 h after intraperitoneal administration of cadmium (73.61 ± 11.85 and $19.18 \pm 3.47 \mu\text{mol/mg}$ protein in Cd and C group) ($p < 0.01$) (Fig. 1). Similar to this, plasma MDA concentration was significantly higher in the cadmium-treated group ($196.39 \pm 27.65 \mu\text{mol/L}$) in comparison with the control group ($145.65 \pm 18.96 \mu\text{mol/L}$) ($p < 0.01$) at the same time interval (Fig. 2). In contrast, plasma concentration of sulfhydryl groups was not significantly changed after cadmium treatment (0.46 ± 0.08 and $0.37 \pm 0.10 \mu\text{mol/L}$ in Cd and C group respectively) ($p > 0.05$) (Fig. 3). Liver glutathione level showed a tendency to rise in the cadmium-treated group ($29.41 \pm 4.21 \text{ nmol/mg}$ protein), in comparison with the control group ($26.41 \pm 4.00 \text{ nmol/mg}$ protein), but this difference was not statistically significant ($p > 0.05$) (Fig. 4).

Total liver SOD activity was elevated after cadmium administration ($2204.5 \pm 111 \text{ U/mg}$ protein) in comparison with the control group ($570.5 \pm 27.3 \text{ U/mg}$ protein) ($p < 0.01$) (Fig. 5). Analysis of SOD isoenzymes showed that increase in SOD activity may be attributed mainly to the increase in Cu/Zn SOD activity. Activity of Cu/Zn SOD was approximately fivefold higher in the cadmium-treated group ($1931.9 \pm 111.2 \text{ U/mg}$ protein) in comparison with the control group ($402.7 \pm 6.7 \text{ U/mg}$ protein) ($p < 0.01$) while cadmium administration induced only a twofold increase in MnSOD activity during the same time interval (272.8 ± 19.8 and $167.8 \pm 30.9 \text{ U/mg}$ protein in Cd and C group respectively) ($p < 0.01$) (Fig. 5).

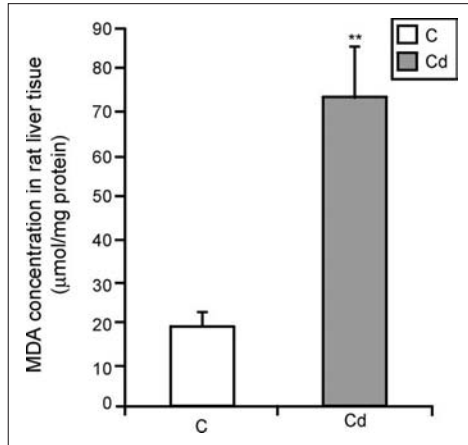


Figure 1. MDA concentration in rat liver tissue. The significance of difference was tested by Student's t test
** $p < 0.01$ in comparison with control group
Legend: C-control;
Cd-cadmium;
MDA-malondialdehyde

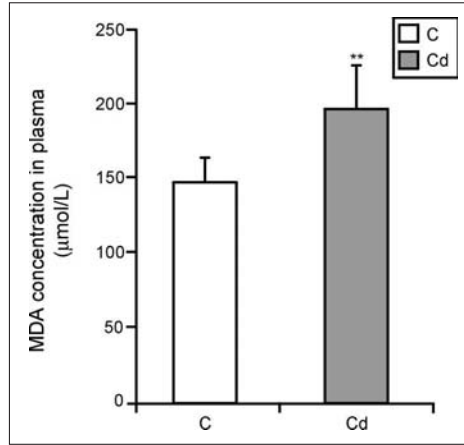


Figure 2. MDA concentration in rat plasma. The significance of difference was tested by Student's t test
** $p < 0.01$ in comparison with control group
Legend: C-control;
Cd-cadmium;
MDA-malondialdehyde

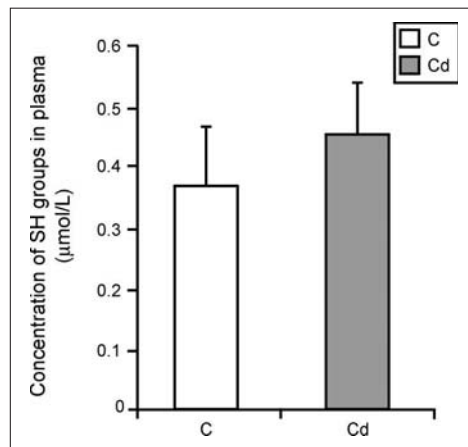


Figure 3. Concentration of SH groups in rat plasma. The significance of difference was tested by Student's t test
No significant difference was found between experimental groups.
Legend: C-control;
Cd-cadmium;
SH-sulfhydryl group

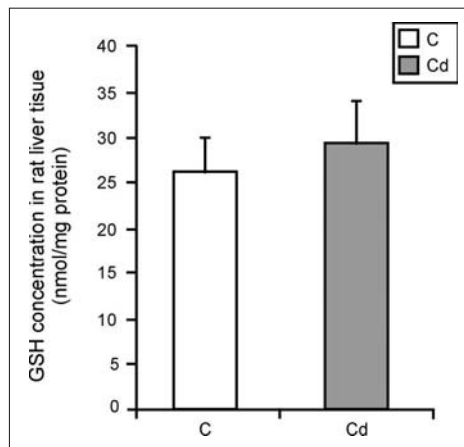


Figure 4. GSH concentration in rat liver tissue. The significance of difference was tested by Student's t test
No significant difference was found between experimental groups.
Legend: C-control;
Cd-cadmium;
GSH-reduced glutathione

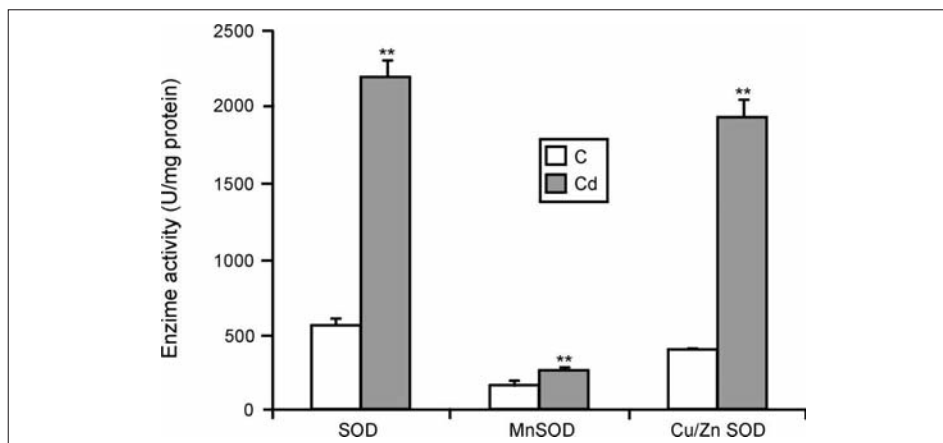


Figure 5. Activity of total SOD and its fractions in rat liver tissue. The significance of difference was tested by Student's t test

** $p < 0.01$ in comparison with control group

Legend: C-control; Cd-cadmium;
 SOD-superoxid dismutase;
 MnSOD-manganese superoxid dismutase;
 Cu/Zn SOD-copper/zinc superoxid dismutase

DISCUSSION

The results of our investigation showed an important role of oxidative stress in acute cadmium-induced liver injury. Our study has shown that plasma and liver MDA concentrations were significantly increased in the cadmium treated group in comparison with the control group 24 h after its administration. Numerous studies suggest that lipid peroxidation is an important mechanism in acute and chronic cadmium intoxication (Yalin *et al.*, 2006). Some studies have shown that production of lipid peroxides in the liver and kidney starts within 6 h after cadmium administration (Casalino *et al.*, 2002) and continues within next 24h (Casalino *et al.*, 2002; Ognjanović *et al.*, 2003). After this period production of lipid peroxides declines (Casalino *et al.*, 2002). The investigation of chronic cadmium effects has shown that repetitive exposure to small doses of cadmium may cause lipid peroxidation (Zikić *et al.*, 1998). The increase in liver MDA was obtained 30 days (Koyu *et al.*, 2006) and 12 weeks after cadmium treatment in rats (Jurczuk *et al.*, 2004). It has been shown that the rise in concentration of substances which react with thiobarbituric acid (TBARS) is less pronounced after previous α -tocopherol administration. Similar effects have also been observed in the plasma and brain (Nemmiche *et al.*, 2007). Moreover, cadmium induced an increase in MDA production in ovariectomized rats (Yalin *et al.*, 2006).

Our results have shown that acute cadmium administration does not change reduced glutathione concentration in the liver within 24h. Glutathione, as a multifunctional, intracellular nonenzymatic antioxidant is the major component of intracellular regulation of the redox state and provides the first line of defense against oxidative injury. In addition, GSH is the important substrate and cofactor in drug metabolism (Nemmiche *et al.*, 2007). Other studies, like ours, did not show the change in liver GSH in acute cadmium intoxication (Siegers *et al.*, 1987). This indicates that GSH depletion is not a primary mechanism of oxidative stress in acute cadmium liver injury. These results may be surprising, since the major direct cadmium effect is its binding to sulfhydryl groups. However, lack of change of liver GSH content can be explained by simultaneous activation of adaptive mechanisms in hepatocytes. Some studies have shown that GSH content in the liver decreases within 4-12h after cadmium administration (Baghci *et al.*, 1996), and subsequently increases within 24h (Yamano *et al.*, 1998). Initially, glutathione depletion is a consequence of cadmium binding to sulfhydryl groups of various peptides. Subsequent rise in its level may be caused by an adaptive response of hepatocytes to increased reactive oxygen species production. Contradictory results may be explained by the use of different routes of cadmium administration. In contrast to intravenous administration, which did not lead to important changes in liver GSH (Nemmiche *et al.*, 2007), it has been shown that subcutaneous cadmium administration causes an increase in liver GSH (Yamano *et al.*, 1998). This difference can be explained by incomplete cadmium absorption from the skin.

In contrast to acute intoxication, the effects of chronic cadmium exposure on liver glutathione is more clear. Studies *in vivo* demonstrated that cadmium caused GSH depletion in the rat liver after a ten-day treatment with cadmium (Nemmiche *et al.*, 2007). It has been shown that previous α -tocopherol administration reduces cadmium-induced GSH depletion in the liver (Nemmiche *et al.*, 2007; El-Demerdash *et al.*, 2004). In contrast, Congiu *et al.* (2000) suggested that prolonged effects of small doses of cadmium can lead to the increase in GSH content in hepatocytes. Similar results were found in some *in vitro* studies (Beyersmann and Hechtenberg, 1997; Tully *et al.*, 2000). This increase is probably due to expression of gamma-glutamylcysteine synthetase, which catalyzes the key reaction of GSH biosynthesis (Griffith, 1999; Shukla *et al.*, 2000; Wild and Mulcahy, 2000).

Similar to GSH, plasma concentration of sulfhydryl groups was not significantly changed within the first 24h after cadmium administration. Since GSH is the most abundant source of sulfhydryl groups in the liver, changes in sulfhydryl groups concentration are in accordance with the changes in liver GSH content.

The results of our study showed that total SOD activity was fourfold increased 24h after cadmium administration. SOD, which is the enzyme involved in the conversion of superoxide anion into a less toxic hydrogen peroxide, is present in hepatocytes in two isoforms, Cu/ZnSOD (cytosolic SOD) and MnSOD (mitochondrial SOD). The activity of both isoforms of this enzyme was found to be increased in response to cadmium treatment. This rise may be interpreted as an adaptive response of hepatocytes to increased reactive oxygen species

production induced by cadmium. However, Cu/Zn SOD activity increased to a greater extent than MnSOD. These results may be surprising, since the major sites of reactive oxygen species production are mitochondria and it is expected that the adaptive response should be more pronounced in mitochondria. However, *in vitro* investigations showed that MnSOD is more vulnerable to the inhibitory effect of cadmium than Cu/Zn SOD (Casalino *et al.*, 2002). Cadmium in a dose of 2.5 mg/kg exerts a greater inhibitory effect on MnSOD than on Cu/ZnSOD activity, thus impeding an additional adaptive increase in MnSOD activity in comparison with cytosolic isoenzyme. Possible mechanisms of greater sensitivity of MnSOD to cadmium may be a substitution of cadmium for manganese at the active site of this enzyme or binding of cadmium to another site at the active centre of the enzyme (Casalino *et al.*, 2002).

Results of other studies related to the influence of cadmium on SOD activity are contradictory. In summary, effects of cadmium depend on dose, exposition time and route of administration. Cadmium in a dose of 0.4 mg/kg was found to induce an adaptive increase in SOD activity (Ognjanović *et al.*, 2003), which is in agreement with our results. In contrast, high doses of cadmium (5 mg/kg) cause a decrease in both mitochondrial and cytosolic SOD activity (Yalin *et al.*, 2006), probably because of overcoming the antioxidative capacity of hepatocytes. Similar effects in the liver were described after administration of other hepatotoxic substances, such as ethanol (Li *et al.*, 2004).

In contrast to acute, chronic cadmium intoxication causes a decrease in the activity of both SOD isoenzymes in the liver and kidney (Jurczuk *et al.*, 2004; Koyu *et al.*, 2006). It has been reported that chronic cadmium exposure causes an increase in SOD activity in rat heart (Ognjanović *et al.*, 2003), but similar changes have not been observed in the liver.

Based on our results, it can be concluded that oxidative stress, among various mechanisms, plays an important role in acute cadmium-induced liver injury in rats. Lipid peroxidation occurs within 24h after cadmium administration. The capacity of the liver antioxidant defense system is only partly increased due to an adaptive increase in SOD activity. Glutathione and sulfhydryl groups do not have an important protective role in early cadmium intoxication.

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MOGUĆA ULOGA OKSIDATIVNOG STRESA U OŠTEĆENJU JETRE PACOVA U AKUTNOJ INTOKSIKACIJI KADMIJUMOM

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SADRŽAJ

Poznato je da kadmijum izaziva niz funkcionalnih poremećaja u organizmu, posebno oštećenje jetre i bubrega. Glavni mehanizmi hepatotoksičnog efekta kadmijuma su njegovo vezivanje za sulfhidrilne grupe i indukcija zapaljenja. Oksi-

dativni stres, kao odraz pada antioksidativnog kapaciteta, igra ulogu u hroničnoj intoksikaciji kadmijumom. Uloga oksidativnog stresa u akutnoj intoksikaciji kadmijumom još uvek nije do kraja razjašnjena.

Cilj našeg rada je bio da se utvrdi uloga slobodnih kiseoničnih radikala, kao i efikasnost antioksidantne zaštite u jetri pacova u akutnoj intoksikaciji kadmijumom.

U eksperimentu su korišćeni pacovi, mužjaci, Wistar soja (n=16) koji su podeljeni u sledeće grupe: 1. kontrolna grupa (n=7) tretirana fiziološkim rastvorom i 2. grupa tretirana kadmijumom (n=9) u jednoj dozi od 2.5 mg/kg intraperitonealno. Uzorci krvi iz desne komore srca i tkiva jetre, u cilju određivanja parametara oksidativnog stresa, uzeti su 24 časa nakon administracije.

Naši rezultati pokazuju porast koncentracije malondialdehida u plazmi i u tkivu jetre nakon administracije kadmijuma ($p < 0.01$). Osim toga, aktivnost superoksid dismutaze je bila povećana u grupi tretiranoj kadmijumom, najvećim delom zbog povećanja aktivnosti bakar/cink superoksid dismutaze ($p < 0.01$). Nivo redukovano glutationa u tkivu jetre i sulfhidrilnih grupa u plazmi nisu bili značajno promenjeni pod uticajem kadmijuma.

Na osnovu dobijenih rezultata se može zaključiti da lipidna peroksidacija igra značajnu ulogu u akutnom oštećenju jetre izazvanom kadmijumom. Antioksidantni kapacitet je delimično povećan, kao odraz adaptivnog porasta aktivnosti superoksid dismutaze.

