

## Protective Effect of Glycine and Tri-Methyl Glycine (Betaine) Against Heavy Metals-Induced Oxidative Stress in Liver-Derived Post-Nuclear Supernatant (PNS)

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### Abstract

Heavy metals are environmental pollutants, which pose toxicity toward biological systems. Most organs are susceptible to heavy metals-induced toxicity. Hence, finding protective agents against heavy metals-induced toxicity is valuable. The post-nuclear supernatant (PNS) has been accepted as an *in vitro* model for assessing xenobiotics-induced toxicity toward biological systems. Monitoring the toxic effects of a large number of xenobiotics in a short time is one of the superiorities of PNS system. The goal of the present study was to validate the PNS as an *in vitro* model for investigating the effect of heavy metals (Cd, Co, Cu, Fe, As, Hg, Cr, and Pb)-induced toxicity and evaluating the potential protective effects of glycine and betaine. Markers of oxidative stress including ROS formation, lipid peroxidation, and glutathione content in addition of to succinate dehydrogenase activity (MTT test) were monitored in the presence of heavy metals alone or in combination with glycine (1 mM) and betaine (100  $\mu$ M). Our results suggest that PNS preparations can be used as an appropriate model for future investigation of xenobiotics-induced toxicity and estimation of the protective properties of different agents. Indeed, further evaluations in other experimental models could reveal the protective properties of betaine and glycine against heavy metals-induced organ injury.

**Keywords:** Amino acids, Environmental toxicology, Hepatotoxicity, Hepatoprotection, Organ Injury.

### 1. Introduction

Heavy metals are environmental pollutants, whose exposure is associated with a wide range of health problems in humans. Cancer, neurological diseases, diabetes, and many other disorders might directly associate with heavy metal over-exposure (1-8). Several mechanisms have been proposed for metal-induced toxicity toward

biological systems. Among these mechanisms, oxidative stress and its subsequent complications seem to play a primary role (7, 8). Oxidative stress is an impairment in the balance of production and detoxification of reactive oxygen species (ROS). Therefore, administration of antioxidant protective molecules might prevent heavy metal toxicity.

Finding experimental models for evaluation and monitoring of the toxic effects of xenobiotics has a great value. Among the experimental modes, those with higher efficacy of monitoring

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a large number of xenobiotics in a short time and using fewer laboratory animals are of considerable attention. The post-nuclear supernatant (PNS) is an *in vitro* model for evaluation of a large number of xenobiotics and give a reasonable estimation of their toxicity for further assessment in other experimental models (9).

## 2. Materials and methods

### 2.1. Chemicals

Glycine and Betaine (Trimethylglycine), were purchased from Sigma (St. Louis, MO, USA). 4,2 hydroxyethyl,1-piperazineethane-sulfonic acid (HEPES), 3-(N-morpholino) propane sulfonic acid (MOPS), dimethyl sulfoxide (DMSO), D-mannitol, fatty acid free bovine serum albumin (BSA, Fraction V), thiobarbituric acid (TBA), chobalte chloride, iron sulfate, cadmium chloride, copper sulfate, trichloroacetic acid (TCA), 3-[4,5dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), rhodamine123, coomassie brilliant blue, ethylene glycol-bis (2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), sodium succinate, hydroxymethyl aminomethane hydrochloride (Tris-HCl), and ethylenediaminetetraacetic acid (EDTA) were purchased from Merck (Darmstadt, Germany). All salts for preparing buffer solutions (analytical grade) were obtained from Merck (Darmstadt, Germany).

### 2.2. Animals

Male Sprague-Dawley rats (250-300 g) were obtained from Animal Breeding Center of Shiraz University of Medical Sciences, Shiraz Iran. Rats were housed in cages with wood-chip bedding at a temperature of  $24 \pm 1$  °C and relative humidity of  $\approx 40\%$ . Animals had free access to a standard rodents chow diet (Behparvar<sup>®</sup>, Tehran, Iran) and tap water. Animals were handled in compliance with the guidelines of the laboratory animal care and use approved by the institutional ethics committee of Shiraz University of Medical Sciences, Shiraz, Iran (#11630/11576).

### 2.3. Post-nuclear supernatant (PNS) preparation from liver tissue

Liver-derived PNS was prepared based on the differential centrifugation method (9). Briefly,

rats were anesthetized (Thiopental 80 mg/kg, *i.p.*) and the liver was excised and washed with ice-cooled sodium phosphate buffer (0.1 M, 4 °C). Liver tissue was homogenized in phosphate buffer (1: 10 w:v tissue: buffer ratio, 4 °C). Afterward, tissue homogenate was centrifuged (1000 g, 20 min, 4 °C). The supernatant was collected, and the centrifugation process was repeated. Finally, the supernatant was collected and used as PNS. For evaluation of heavy metals toxicity, PNS (10 ml) was incubated with different concentrations of the investigated heavy metals for 1 h at 37 °C in a shaker incubator. Betaine and glycine were added 15 min before PNS exposure to heavy metals. The protein content of samples was measured based on the Bradford method for standardization of the obtained data.

### 2.4. Reactive oxygen species formation

Reactive oxygen species (ROS) formation in PNS samples was estimated using 2', 7' dichlorofluorescein diacetate as the fluorescence probe (10-15). Briefly, PNS samples (100  $\mu$ L) were mixed with Tris-HCl buffer (1 mL; pH=7.4 4 °C) and DCF (final concentration 10  $\mu$ M) (16, 17). The mixture was incubated at 37 °C (15 min, in the dark). Finally, the fluorescence intensity (FI) of samples was assessed using a FLUOstar Omega<sup>®</sup> multifunctional microplate reader (BMG Labtech, Germany) with  $\lambda_{\text{excitation}}=485$  nm and  $\lambda_{\text{emission}}=525$  nm (10, 18).

### 2.5. Brain tissue glutathione content

2.5 mL of the PNS samples were added to 2 ml of distilled water (4 °C) and 1 ml of trichloroacetic acid (50% w/v; 4 °C) (19-21). Samples were mixed well and centrifuged (10,000 g, 4 °C, 25 min). Afterward, 1 mL of the supernatant was mixed with 4 mL of Tris buffer (pH=8.9; 4 °C), and 100  $\mu$ l of DTNB (10 mM in methanol) (16, 17). The absorbance of the developed color was measured at  $\lambda=412$  nm using a FLUOstar Omega<sup>®</sup> multifunctional microplate reader (BMG Labtech, Germany) (22).

### 2.6. Lipid peroxidation

The thiobarbituric acid reactive substances (TBARS) test was used as a method to assess

lipid peroxidation in the brain tissue (22-25). The reaction mixture was consisted of 500  $\mu\text{L}$  of PNS, 1 mL of thiobarbituric acid (0.375%, w/v), and 3 mL of metaphosphoric acid (1% w/v, pH=2). Samples were mixed well and heated (100  $^{\circ}\text{C}$ ; 45 min). Then, the mixture was cooled, and 2 mL of n-butanol was added. Samples were vigorously vortexed and centrifuged (10,000 g for 5 min). Finally, absorbance of the developed color in n-butanol phase was read at  $\lambda=532$  nm using a FLUOstar Omega<sup>®</sup> multifunctional microplate reader (BMG Labtech, Germany) (22).

### 2.7. Ferric reducing antioxidant power (FRAP) of the brain tissue

FRAP assay is a method to measure the formation of a blue colored  $\text{Fe}^{2+}$ -tripyridyl-triazine compound from the colorless oxidized  $\text{Fe}^{3+}$ , which is formed by the action of electron-donating antioxidants (26, 27). In the current study, the working FRAP reagent was prepared by mixing 10 volumes of acetate buffer (300 mmol/L, pH=3.6), with 1 volume of TPTZ (10 mmol/L in 40 mmol/L hydrochloric acid) and 1 volume of ferric chloride (20 mmol/L). All solutions were freshly prepared. Then, 50  $\mu\text{L}$  of PNS sample and 150  $\mu\text{L}$  of deionized water were added to 1.5 mL of the FRAP reagent (28). The reaction mixture was incubated at 37  $^{\circ}\text{C}$  for 5 min. in the dark. Finally, samples were centrifuged (13000 g, 5 min., 4  $^{\circ}\text{C}$ ) and absorbance of the developed color was measured at  $\lambda=595$  nm by a FLUOstar Omega<sup>®</sup> multifunctional micro-

plate reader (BMG Labtech, Germany) (11, 29).

### 2.8. Succinate dehydrogenase activity (MTT test)

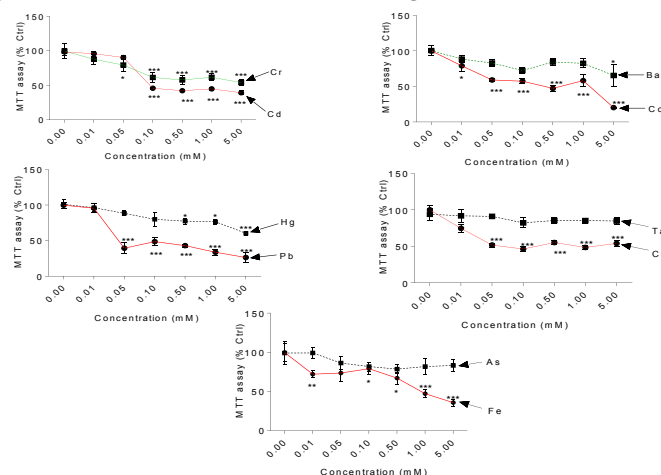
A colorimetric method based on the reduction of the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was applied for the determination of mitochondrial dehydrogenases activity (30-33). Briefly, 1 mL of PNS was incubated with 40  $\mu\text{L}$  of MTT (0.4% w:v; 37  $^{\circ}\text{C}$  for 30 min, in the dark) (32, 34). Then, samples were centrifuged (10,000 g, 5 min) and the pellet of purple formazan crystals was dissolved in 1 mL of dimethyl sulfoxide (DMSO). Afterward, 0.1 ml of the dissolved formazan was added to a 96 well plate. Finally, the optical density (OD) at  $\lambda=570$  nm was measured with an EPOCH plate reader (BioTek<sup>®</sup> Instruments, Highland Park, USA) (30, 31).

### 2.9. Statistical analysis

Data are given as the Mean $\pm$ SD. Data comparison was conducted by the one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests as the post hoc. Differences were considered statistically significant when  $P<0.05$ .

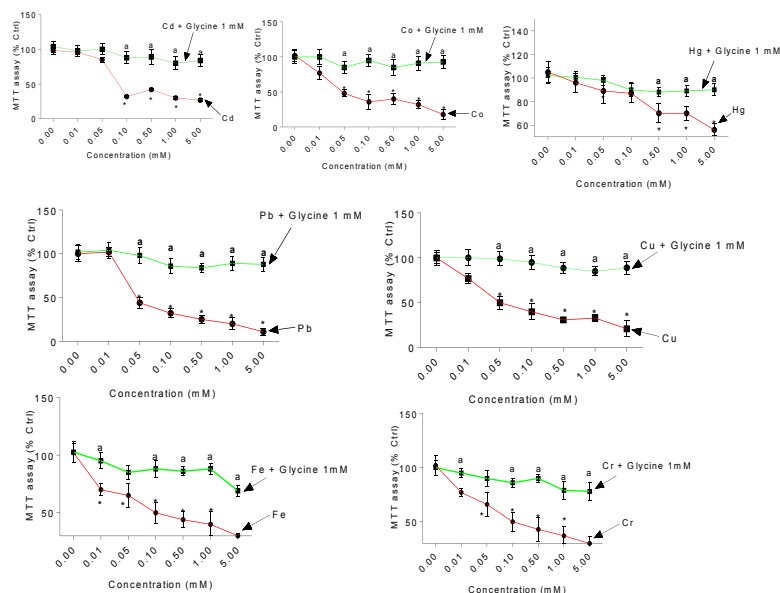
## 3. Results

Monitoring the toxic effects of heavy metals in the liver-derived PNS was performed by MTT assay (Figure 1). It was found that increasing concentrations of Fe, Co, Cd, Pb, Hg, Cr, and



**Figure 1.** Methyl tetrazolium assay for concentration-response of the heavy metals toxicity. Data are given as Mean $\pm$ SD (n=8).

\*Indicates significantly different as compared with control (concentration of 0 mM) ( $P<0.05$ ).



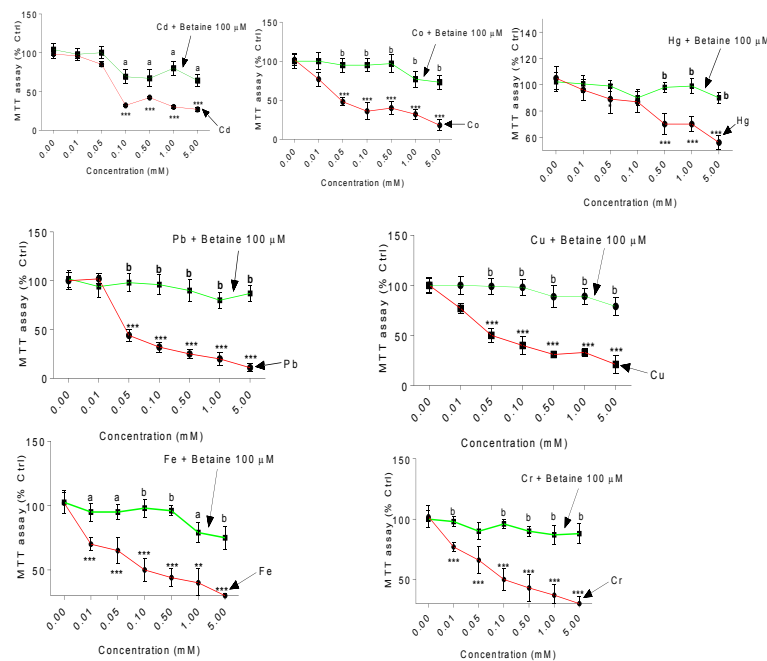
**Figure 2.** Effect of glycine on the heavy metals toxicity (MTT assay) in liver-derived post- nuclear supernatant. Data are given as Mean±SD (n=8).

\*Indicates significantly different as compared with control ( $P<0.05$ ).

<sup>a</sup>Indicates significantly different as compared with the heavy metal-treated group ( $P<0.05$ ).

Cu significantly decreased dehydrogenases activity in comparison with control PNS (Figure 1). On the other hand, it was found that pre-incubation of liver PNS with betaine (100  $\mu$ M) and glycine (1

mM) prevented heavy metal-induced decrease in dehydrogenases activity (MTT test) (Figures 2 and 3). It was also found that PNS exposure to Tl, As, and Ba caused no significant changes in the MTT

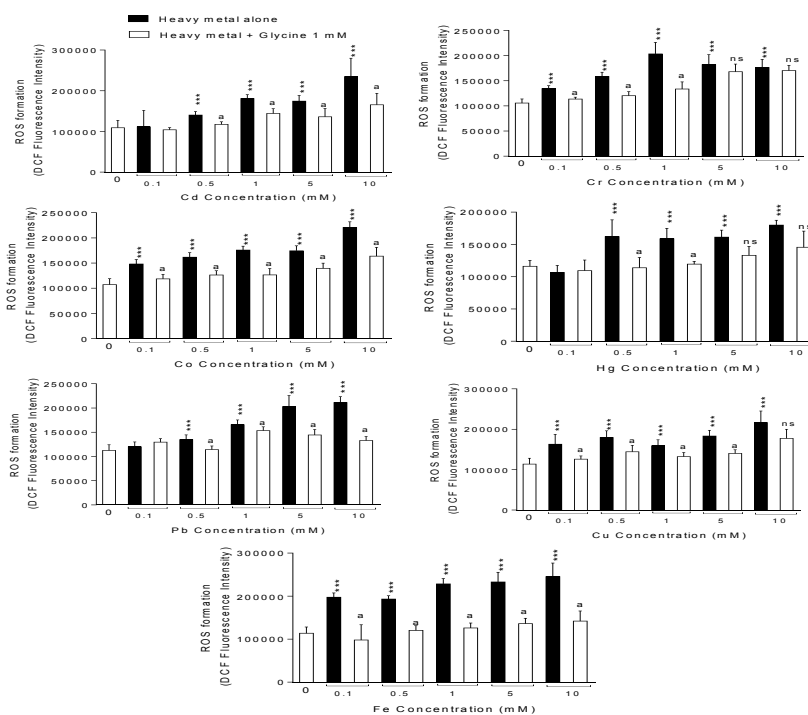


**Figure 3.** Effect of betaine on the heavy metals toxicity (MTT assay) in the liver post-nuclear supernatant. Data are given as Mean±SD (n=8).

\*Indicates significantly different as compared with control ( $P<0.05$ ).

<sup>a</sup>Indicates significantly different as compared with the heavy metal-treated group ( $P<0.05$ ).

<sup>b</sup>Indicates significantly different as compared with the heavy metal-treated group ( $P<0.01$ ).

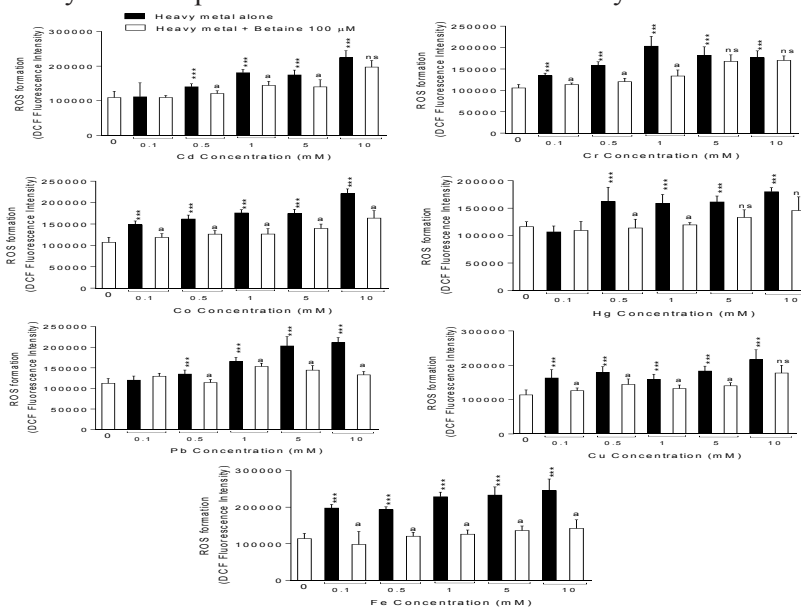


**Figure 4.** Heavy metals-induced oxidative stress in the liver post-nuclear supernatant and the effect of glycine supplementation. Liver-derived post-nuclear supernatant was treated with heavy metals for 60 min. and the DCF fluorescence intensity was assessed. Data are given as Mean±SD (n=8). DCF: Dichlorofluorescein. \*\*\*Indicates significantly different as compared with control ( $P<0.001$ ). <sup>a</sup>Indicates significantly different as compared with the heavy metal-treated group ( $P<0.01$ ).

test (Figure 1).

Significant elevation in ROS formation was detected in heavy metal-exposed liver PNS

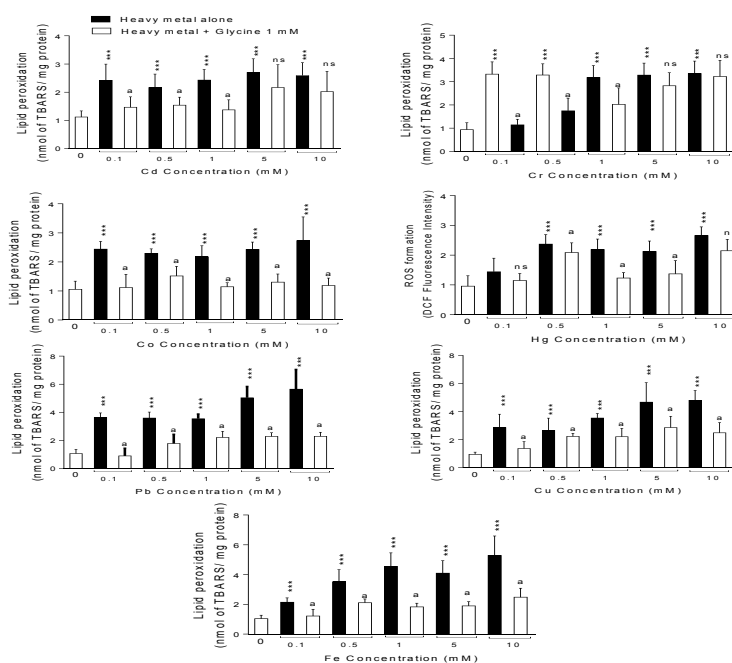
(Figures 4 and 5). It was found that betaine (100 μM) and glycine (1 mM) supplementation decreased heavy metal-induced ROS formation in



**Figure 5.** Effect of betaine treatment on the heavy metals-induced oxidative stress in the liver post-nuclear supernatant. Liver-derived post-nuclear supernatant was treated with heavy metals for 60 min. and the DCF fluorescence intensity was assessed. Data are given as Mean±SD (n=8). DCF: Dichlorofluorescein.

\*\*\*Indicates significantly different as compared with control ( $P<0.001$ ).

<sup>a</sup>Indicates significantly different as compared with the heavy metal-treated group ( $P<0.01$ ).



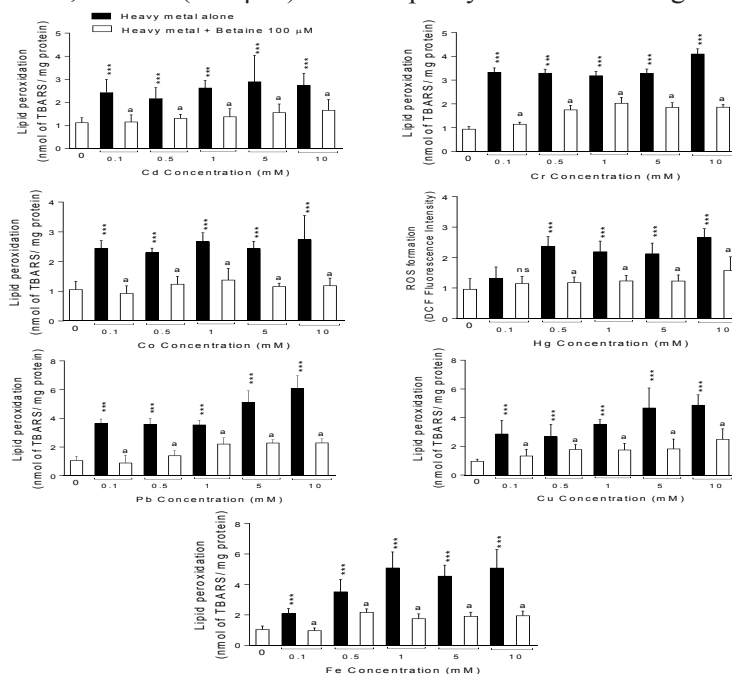
**Figure 6.** Heavy metals-induced lipid peroxidation in the liver post-nuclear supernatant and the effect of glycine treatment. Data are given as Mean±SD (n=8). \*\*\*Indicates significantly different as compared with control ( $P<0.001$ ). <sup>a</sup>Indicates significantly different as compared with the heavy metal-treated group ( $P<0.01$ ).

the liver-derived PNS (Figures 4 and 5).

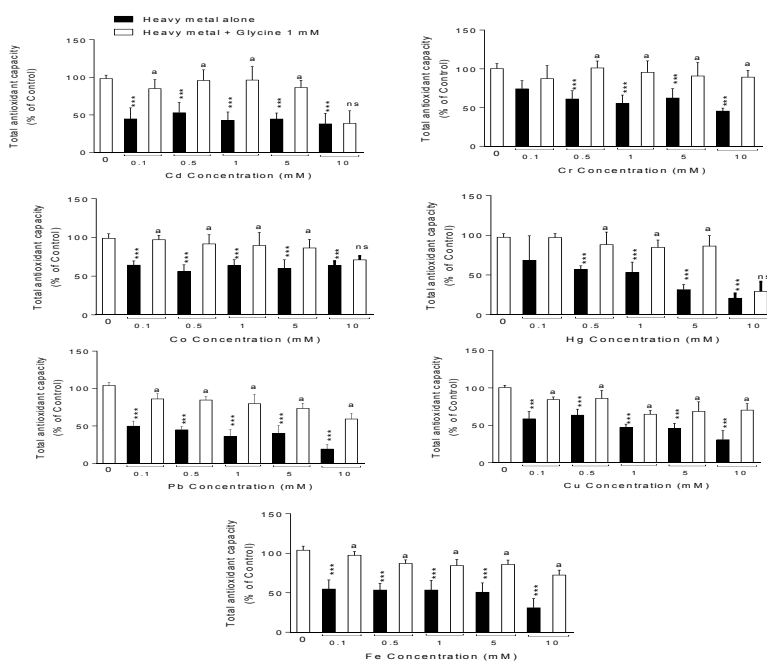
Heavy metals caused significant lipid peroxidation in liver PNS preparations (Figures 6 and 7). On the other hand, betaine (100  $\mu$ M) and

glycine (1 mM) treatment decreased heavy metal-induced lipid peroxidation (Figures 6 and 7).

It was found that the total antioxidant capacity of PNS was significantly decreased when



**Figure 7.** Effect of betaine treatment on the heavy metal-induced lipid peroxidation in the liver post-nuclear supernatant. Data are given as Mean±SD (n=8). \*\*\*Indicates significantly different as compared with control ( $P<0.001$ ). <sup>a</sup>Indicates significantly different as compared with the heavy metal-treated group ( $P<0.01$ ).



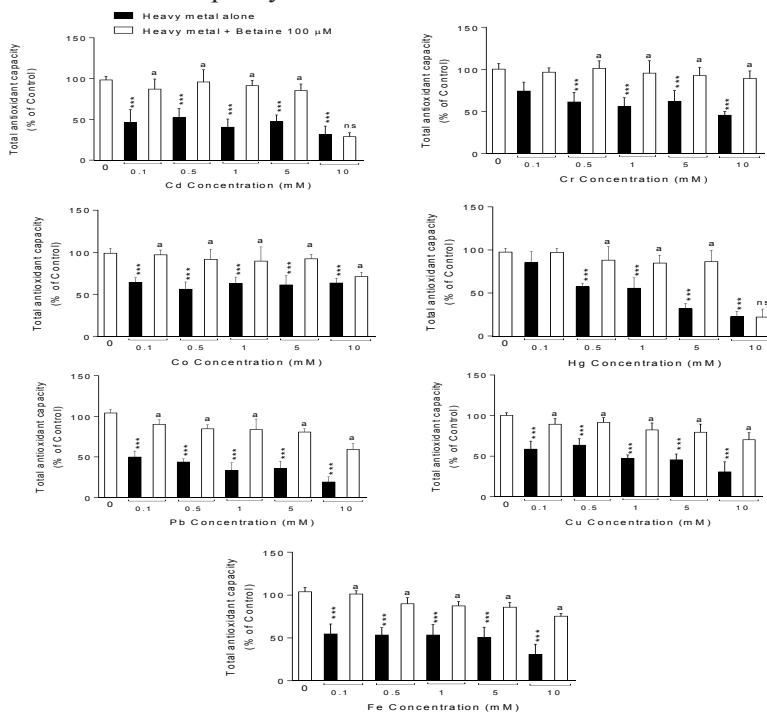
**Figure 8.** Effect of glycine treatment on the total antioxidant capacity of liver-derived post-nuclear supernatant exposed to heavy metals. Data are given as Mean±SD (n=8).

\*\*\*Indicates significantly different as compared with control ( $P<0.001$ ).

<sup>a</sup>Indicates significantly different as compared with heavy metal-treated group ( $P<0.01$ ).

heavy metals were added (Figures 8 and 9). Administration of betaine (100 μM) and glycine (1 mM) preserved PNS antioxidant capacity in the

presence of the investigated heavy metals (Figures 8 and 9).



**Figure 9.** Effect of betaine on the total antioxidant capacity of liver-derived post-nuclear supernatant exposed to heavy metals. Data are given as Mean±SD (n=8).

\*\*\*Indicates significantly different as compared with control ( $P<0.001$ ).

<sup>a</sup>Indicates significantly different as compared with the heavy metal-treated group ( $P<0.01$ ).

#### 4. Discussion

Heavy metals are environmental pollutants with a wide range of adverse effects in different organs (8, 35). Mechanistically, oxidative stress and its related complications play an essential role in heavy metals-induced organ injury (7, 8). Therefore, administration of antioxidant agents might have protective value. In the current study, the oxidative stress-mediated injury induced by heavy metals was monitored in an *in vitro* model of liver-derived post-nuclear supernatant (PNS). It was found that glycine (1 mM) and betaine (100  $\mu$ M) supplementation could provide protective properties against heavy metals oxidative stress and toxicity.

A wide range of diseases is attributed to heavy metals overexposure (8, 35, 36). Heavy metals could induce deleterious complications such as cancer and/or debilitating neurological diseases (37-40). Hence, finding protective agents against metal-induced toxicity has significant clinical value. Glycine and its methylated form, betaine, are abundantly found in different food sources (41-44). These chemicals are safe even at high doses (41, 42). Therefore, they could be good candidates as protective agents against xenobiotics (e.g., heavy metals)-induced injury toward biological systems.

Glycine is the simplest amino acid with several pharmacological properties (11, 32, 45, 46). Anti-inflammatory, antioxidant, and cytoprotective properties of glycine have been reported in previous investigations (11, 32, 45-50). On the other hand, glycine is a constituent of the glutathione (GSH) molecule as the primary cellular antioxidant system. In the current study, it was found that glycine supplementation could ameliorate heavy metals-induced injury by modulating oxidative stress and its associated complications. The cytoprotective properties of betaine also have been

reported in different experimental models (23, 51-55). Betaine supplementation effectively mitigated oxidative stress and its associated events (54, 56-58). Hence, betaine could protect biological targets such as antioxidant enzymes and biomembrane lipids against ROS. In the current investigation, we found that betaine supplementation (100  $\mu$ M) alleviated biomarkers of oxidative stress in heavy metal-exposed PNS. All these data could imply the potential protective properties of betaine and glycine against heavy metal toxicity in other experimental models.

PNS is a convenient experimental tool to estimate the toxicity of different xenobiotics (e.g., drugs, heavy metals, *etc.*). Rapid monitoring of a large number of xenobiotics is one of the major superiorities for the PNS system.

In conclusion, our data indicate that PNS could be a good experimental tool to monitor heavy metal-induced oxidative stress and evaluate the protective properties of different agents against this complication. Indeed, PNS system-derived data give an estimation of the cytotoxicity/cytoprotective effects of different chemicals. Therefore, further studies in other experimental models are needed to confirm the data obtained from the PNS preparations.

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#### Conflict of Interest

None declared.

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