

Amelioration Effect of Zinc and Iron Supplementation on Selected Oxidative Stress Enzymes in Liver and Kidney of Cadmium-Treated Male Albino Rat

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ABSTRACT

Cadmium (Cd) is a highly toxic, nonessential heavy metal with many industrial uses that can contribute to a well-defined spectrum of diseases in animals as well as in humans. The present study examines the effect of zinc (Zn) and iron (Fe) supplementation on oxidative stress enzymes in Cd-treated rats. Wistar strain male albino rats were treated with cadmium chloride (CdCl₂) at a dose of 1/10th LD₅₀/48 h, that is, 22.5 mg/kg body weight for 7, 15, and 30 days (d) time intervals. The 15d Cd-treated rats were divided into three groups. The first group received Zn (12 mg/kg), second group Fe (40 mg/kg) alone, and third group supplemented with both Zn and Fe and observed for 7, 15, and 30d. After the specific time intervals, rats were decapitated and oxidative stress enzymes like catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST) were assayed in liver and kidney. Simultaneously lipid peroxidation (LPO) levels were also measured. A significant elevation in LPO levels with decreased activity levels of CAT, SOD, GPx, and GST were observed during Cd intoxication. With Zn and/or Fe supplementation, a significant reversal in the oxidative stress enzymes was observed. Our study reveals that combination of Zn and Fe supplementation is effective in detoxifying the Cd body burden from the test tissues.

Key words: Cadmium, kidney, liver, oxidative stress, rat, trace elements supplementation

INTRODUCTION

Cadmium (Cd) is one of the most dangerous occupational and environmental pollutants, arising primarily from battery, electroplating, pigment, plastics, fertilizer industries, and cigarette smoke. It is an earth's crust natural element and found in drinking water, atmospheric

air, and even in food (Moulis and Thevenod, 2010; Obaiah *et al.*, 2013). Cd exposure has been recognized to result in a wide variety of cellular responses, oxidative stress, and body weight loss. Over the past 2 centuries, anthropogenic and industrial activities have led to high emissions of Cd into the environment at concentrations significantly exceeding those originating from natural sources (Jarup and Akesson, 2009; Cuypers *et al.*, 2010; Dzugan *et al.*, 2011; Nakamura *et al.*, 2012). It has a

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serious toxicity in humans and animals and causes Itai-Itai disease (Nad *et al.*, 2005) and also induces the onset of anemia, carcinogenicity, testicular atrophy, decreases red blood cell count, and hemoglobin concentration (Hartwig, 2010; Honda *et al.*, 2010). Cd has an extremely long half-life (20–30 years) in the human body (Flora *et al.*, 2008) and is highly cumulative, especially in the liver and kidney (Nordberg *et al.*, 2007; Nawrot *et al.*, 2008; Munga *et al.*, 2010; Johri *et al.*, 2010; Włostowski *et al.*, 2010; Nakamura *et al.*, 2012; Guirlet and Das, 2012).

It is a ubiquitous toxic metal and induces oxidative damage by disturbing the prooxidant–antioxidant balance in the tissues (Ognjanovic *et al.*, 2008). Intake of Cd results in consumption of glutathione and protein binding sulfhydryl groups, and subsequently increases the levels of free radicals such as hydrogen peroxide (H_2O_2), hydroxide (OH^-), and superoxide (O_2^-) anions. One of the most important effects of free radicals is oxidation of polyunsaturated fatty acids (PUFAs). As a result of free radical attack, lipids are oxidized; and hence membranes are damaged (Stohs *et al.*, 2001; Ashraf and Kamar, 2007). Cd is readily distributed in tissues after ingestion and it inhibits oxidative stress enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione S-transferase (GST) (Asagba and Eriyamremu, 2007). As a result of this inhibition, the electron transport chain becomes highly reduced; electrons are transferred directly to available oxygen leading to enhanced formation of reactive oxygen species (ROS) (Tatrai *et al.*, 2001). ROS may increase oxidative stress in tissues, cellular damage, peroxidation of membrane lipids, and loss of membrane bound enzymes (Liu *et al.*, 2000). However, ROS may be used as an indicator of cell membrane injury. Cd interferes with intracellular signaling network and gene regulation at multiple levels, induces lipid peroxidation (LPO) and alterations in activity of oxidative stress enzymes (Wang *et al.*, 2004; Sladan *et al.*, 2005). At the cellular level, Cd induces oxidative stress in many organisms (Thevenod, 2009; Bertin and Averbeck, 2006), which might result in histological changes and physiological damage to different organs (Nawrot *et al.*, 2008; Jarup and Akesson, 2009; Cuyper *et al.*, 2010; Salinska *et al.*, 2012). Antioxidants/oxidative stress enzymes prevent free radical-induced tissue damage by preventing the formation of radicals or scavenging them or by promoting their decomposition.

Trace elements supplementation has protective effect against Cd accumulation and toxicity in rats fed inorganic Cd salt (Matek *et al.*, 2002; Piasek *et al.*, 2004). The complex interrelationships between Cd and some essential trace elements have not been elucidated. Several essential trace elements like selenium (Se), copper (Cu), zinc (Zn), and iron (Fe) participate in controlling various metabolic and signaling pathways. *In vitro* studies suggests that there is a competition for transport mechanism between Cd and

some essential trace elements like Zn and Cu and Fe in rats (Peraza *et al.*, 1998; Hakan *et al.*, 2001) and Zn and Se in Japanese quails (Nad *et al.*, 2005) and calcium in suckling rats (Saric *et al.*, 2002; Deepthi *et al.*, 2010). Zn occurs in all living cells as a constituent of metalloenzymes involved in major metabolic pathways. Zn controls several enzymes of intermediary metabolism, DNA and RNA synthesis, gene expression, immunocompetence, and plays a significant role in homeostasis of hormones (Dzukan *et al.*, 2012). Fe plays an essential role in biological processes. It is also a vital component of several enzymes and proteins (Cairo and Recalcati, 2007; Muckenthaler *et al.*, 2008; Qureshi *et al.*, 2010). Fe supplementation reduces Cd retention and Cd-induced anemia in young rats (Schumann *et al.*, 1996). Fe is a central element of the heme molecule, which is a critical part of hemoglobin and essential for oxygen transport. Heme is also a part of many essential enzymes such as CAT and cytochrome series (Brewer, 2007). In several studies, dietary Fe appeared to decrease Cd toxicity, but the exact mechanism of this interaction is unknown (Martinez *et al.*, 2001; Park *et al.*, 2002; Kwong *et al.*, 2011). Hence, we made an attempt to study the interactions of Zn and/or Fe against Cd-induced toxicity in liver and kidney of a mammalian model, rat.

MATERIALS AND METHODS

Chemicals

Cd as cadmium chloride (CdCl_2), Zn as zinc chloride (ZnCl_2), and Fe as ferric chloride anhydrous (FeCl_3) were purchased from Merck (Dormstadt, Germany). All other chemicals which were used in the present study were obtained from the standard chemical companies like Sigma Chemical Co. (St Louis, MO, USA) and SD Fine Chemicals, India. The chemicals used in this study were of the highest purity.

Animals

Three-months-old Wistar strain male albino rats weighing 180 ± 20 g were chosen for the present study. The animals were obtained from Sri Venkateswara Traders, Bangalore, Karnataka, India and were kept in stainless steel mesh cages, housed under standard laboratory conditions ($23 \pm 2^\circ\text{C}$, $50 \pm 20\%$ relative humidity, 12-h light-dark cycle) with standard rat chow (Sai Durga Feeds and Foods, Bangalore, India) and drinking water *ad libitum*. The rats were acclimatized to the laboratory conditions for 10 days. The protocol and animal use has been approved by the Institutional Animal Ethics Committee (Resol. No. 10(ii)/a/CPCSCA/IAEC/SVU/AUR-JO dt 22-12-2008), Sri Venkateswara University, Tirupati, Andhra Pradesh, India.

Experimental design

After acclimatization, the rats were divided into two groups, namely control and experimental. Control rats received only

deionized water without Cd. The experimental rats were treated with Cd as CdCl₂ at a dose of 1/10th LD₅₀ 48h, that is, 22.5 mg/kg body weight over a period of 7, 15, and 30 days (d) time intervals. Then 15d Cd-treated rats were divided into three groups. Group I received supplementation of Zn (12 mg/kg) for 7, 15, and 30d. Group II received Fe supplementation (40 mg/kg) and Group III animals were supplemented with both Zn and Fe at the above said doses for 7, 15, and 30d long sojourn.

Isolation of tissues

After specific time intervals, the control and experimental rats were decapitated and tissues such as liver and kidney were quickly isolated under ice cold conditions and weighed to their nearest mg using Shimadzu electronic balance. After weighing, tissues were immediately used for the assay of oxidative stress enzymes like CAT, SOD, GST, GPx, and the levels of LPO and were stored at -80°C for future use.

Assay of oxidative stress enzymes

LPO

The LPO was determined by the thiobarbituric acid (TBA) method of Ohkawa *et al.*, (1979). The tissues were homogenized in 1.5% KCl (20% W/V). To 1ml of tissue homogenate, 2.5 ml of 20% trichloroacetic acid (TCA) was added and the contents were centrifuged at 3,500 g for 10 min and the precipitate was dissolved in 2.5 ml of 0.05 M sulfuric acid. To this, 3 ml of TBA was added and the samples were kept in a hot water bath for 30 min. The samples were cooled and malonaldehyde (MDA) was extracted with 4 ml of n-butanol and the color was read at 530 nm in an ultraviolet (UV) spectrophotometer (Hitachi U-2000) against the reagent blank. Trimethoxy pentane (TMP) was used as external standard. Values are expressed in micromoles of MDA formed/gram tissue/hour.

CAT (EC: 1.11.1.6)

CAT activity was measured by a slightly modified method of Aebi (1984) at room temperature. The tissue was homogenized in ice-cold 50 mM phosphate buffer (pH 7.0) containing 0.1 M Methylene diamine tetra acetic acid (EDTA) to give 5% homogenate (W/V). The homogenates were centrifuged at 10,000 rpm for 10 min at 4°C in cold centrifuge. The resulting supernatant was used as enzyme source. A 10 µl of 100% ethyl alcohol (EtOH) was added to 100 µl of tissue extract and then placed in an ice bath for 30 min. After 30 min, the tubes were kept at room temperature followed by the addition of 10 µl of Triton X-100 RS. In a cuvette containing 200 µl of phosphate buffer, 50 µl of tissue extract and 250 µl of 0.006 M H₂O₂ (in phosphate buffer) were added and the decrease in optical density was measured at 240 nm for 60s in a UV spectrophotometer (Hitachi U-2000). The molar extinction coefficient of 43.6 M cm⁻¹ was used to determine

CAT activity. One unit of activity is equal to the moles of H₂O₂ degraded/mg protein/min.

SOD (EC: 1.15.1.1)

SOD activity was determined according to the method of Misra and Fridovich (1972) at room temperature. The tissue was homogenized in ice-cold 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA to give 5% homogenate (W/V). The homogenates were centrifuged at 10,000 rpm for 10 min at 4°C in cold centrifuge. The supernatant was separated and used for enzyme assay. A 100 µl of tissue extract was added to 880 µl (0.05 M, pH 10.2, containing 0.1 mM EDTA) carbonate buffer; 20 µl of 30 mM epinephrine (in 0.05% acetic acid) was added to the mixture, and the optical density values were measured at 480 nm for 4 min using UV spectrophotometer (Hitachi U-2000). Values are expressed in superoxide anion reduced/milligram protein/minute.

GST (EC: 2.5.1.18)

GST activity was measured with its conventional substrate 1-chloro, 2,4-dinitrobenzene (CDNB) at 340 nm as per the method of Habig *et al.*, (1974). The tissues were homogenized in 50 mM Tris-HCl buffer pH 7.4 containing 0.25 M sucrose and centrifuged at 4,000 g for 15 min at 4°C and the supernatant was again centrifuged at 16,000 g for 1 h at 4°C. The pellet was discarded and the supernatant was used as the enzyme source. The reaction mixture in a volume of 3 ml contained 2.4 ml of 0.3 M potassium phosphate buffer pH 6.9, 0.1 ml of 30 mM CDNB, 0.1 ml of 30 mM glutathione, and the appropriate enzyme source. The reaction was initiated by the addition of glutathione and the absorbance was read at 340 nm against reagent blank and the activity was expressed as micromoles of thioether formed/milligram protein/minute.

GPx (EC: 1.11.1.9)

GPx was determined by a modified method of Flohe and Gunzler (1984) at 37°C. A 5% (W/V) of tissue homogenate was prepared in 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA. The homogenates were centrifuged at 10,000 g for 10 min at 4°C in cold centrifuge. The resulting supernatant was used as enzyme source. The reaction mixture consisted of 500 µl of phosphate buffer, 100µl of 0.01 M GSH (reduced form), 100 µl of 1.5 mM NADPH, and 100 µl of glutathione reductase (GR, 0.24 units). The 100 µl of tissue extract was added to the reaction mixture and incubated at 37°C for 10 min. Then 50 µl of 12 mM t-butyl hydroperoxide was added to 450 µl of tissue reaction mixture and measured at 340 nm for 180 s. The molar extinction coefficient of 6.22 × 10³ M cm⁻¹ was used to determine the activity. The enzyme activity was expressed in micromoles of NADPH oxidized/milligram protein/minute.

Estimation of protein content

Protein content of the tissues was estimated by the method of Lowry *et al.*, (1951). One percent (W/V) homogenates of the tissues were prepared in 0.25 M ice-cold sucrose solution. To 0.5 ml of homogenate, 1 ml 10% TCA was added, and the samples were centrifuged at 1,000 g for 15 min. Supernatant was discarded and the residues were dissolved in 1 ml of 1N sodium hydroxide. To this, 4 ml of alkaline copper reagent was added followed by 0.4 ml of Folin-phenol reagent (1:1folin: H₂O). The color was measured at 600 nm in a UV spectrophotometer (Hitachi U-2000) against reagent blank. The protein content of the tissues was calculated using a standard protein (bovine serum albumin (BSA)) graph.

Data analysis

The data was subjected to statistical analysis such as mean, standard deviation (SD), and analysis of variance (ANOVA) using standard statistical software, Statistical Package for Social Sciences (SPSS; version 16). All values are expressed as mean \pm SD of six individual samples. Significant differences were indicated at $P < 0.05$ level.

RESULTS

The data on the alterations in the oxidative stress enzymes such as CAT, SOD, GST, and GPx as well as LPO in Cd-treated rat liver and kidney both before and after supplementation with Zn and/or Fe are tabulated in Tables 1-4. The decreased enzyme levels (CAT, SOD, GST, and GPx) in the Cd-treated rats were statistically significant ($P < 0.05$). The supplementation of Zn and/or Fe significantly reversed the Cd-induced alterations in the oxidative stress enzymes.

Our results revealed that LPO levels were increased markedly in both liver and kidney of Cd-treated rats at all the test periods when compared to the controls. The MDA levels were increased with the time intervals of Cd treatment compared to controls and were maximum for 30 d kidney (71.083 ± 2.113 μ mole of MDA

formed/g tissue/h). After supplementation with Zn and/or Fe, the LPO levels were progressively decreased at all time periods in both the test tissues. Maximum depletion was observed in 30d Zn and Fe mixture supplemented rat kidney (38.791 ± 1.278 μ mole of MDA formed/g tissue/h).

CAT activity levels progressively decreased during Cd treatment. However, the activity reversed after supplementation with Zn and/or Fe. CAT activity levels were significantly reduced in 30d Cd-treated kidney (0.982 ± 0.029 μ mole of H₂O₂ metabolized/mg protein/min) when compared to the liver tissue. However, supplementation of both Zn and Fe showed maximum elevation in CAT activity (1.324 ± 0.017 μ mole of H₂O₂ metabolized/mg protein/min) levels for 30d liver tissue.

There was significant decrease in SOD activity during all time intervals of Cd treatment and it was maximum for 30d rat kidney (8.810 ± 0.567 superoxide anion reduced/mg protein/min). However with 30d Zn + Fe supplementation, rat liver showed maximum elevation in its activity (17.067 ± 0.188 superoxide anion reduced/mg protein/min) when compared to other time intervals of supplementation.

GST activity levels also showed a progressive decrement at all the time intervals of Cd treatment with a maximum depletion in 30d rat liver (15.146 ± 0.715 μ mole of thioether formed/mg protein/min). Further supplementation with both Zn and Fe, the GST activity reached to normalcy in 30d rat kidney (30.299 ± 0.288 μ mole of thioether

Table 1: Alterations in the activity levels of selected oxidative stress enzymes in the liver of Cd-treated rats

Enzyme	Control	7 days	15 days	30 days
LPO	30.916 ^a \pm 2.840	41.416 ^b \pm 2.359	52.625 ^c \pm 2.998	66.458 ^d \pm 1.798
CAT	1.335 ^a \pm 0.011	1.303 ^a \pm 0.015	1.246 ^b \pm 0.017	1.159 ^c \pm 0.022
SOD	18.121 ^a \pm 0.193	16.929 ^b \pm 0.520	14.151 ^c \pm 0.586	9.297 ^d \pm 0.580
GST	32.256 ^a \pm 0.710	28.354 ^b \pm 0.560	23.777 ^c \pm 0.525	15.156 ^d \pm 0.715
GPx	1.985 ^a \pm 0.117	1.871 ^a \pm 0.110	1.442 ^b \pm 0.090	0.907 ^c \pm 0.106

All values are expressed as mean \pm SD of six individual samples. Means of the same row followed by different letter (s) differ significantly ($P < 0.05$). CAT=Catalase, SOD=Superoxide dismutase, GPx=Glutathione peroxidase, GST=Glutathione S-transferase, LPO=Lipid peroxidation, SD=Standard deviation

Table 2: Alterations in the activity levels of selected oxidative stress enzymes in the liver of Cd-treated rats after Zn and/or Fe supplementation

Enzyme	Zinc (Zn)			Iron (Fe)			Zn and Fe		
	7d	15d	30d	7d	15d	30d	7d	15d	30d
LPO	49.416 ^a \pm 1.125	44.041 ^b \pm 1.798	36.416 ^c \pm 0.970	49.958 ^a \pm 1.065	43.125 ^b \pm 0.684	36.875 ^c \pm 1.115	49.166 ^a \pm 1.080	42.000 ^b \pm 1.060	35.500 ^c \pm 1.620
CAT	1.258 ^a \pm 0.028	1.270 ^a \pm 0.020	1.321 ^b \pm 0.023	1.260 ^a \pm 0.016	1.276 ^a \pm 0.017	1.322 ^b \pm 0.024	1.259 ^a \pm 0.015	1.283 ^a \pm 0.022	1.324 ^b \pm 0.017
SOD	14.736 ^a \pm 0.249	15.545 ^b \pm 0.261	17.028 ^c \pm 0.203	14.246 ^a \pm 0.266	15.297 ^b \pm 0.232	16.878 ^c \pm 0.246	14.812 ^a \pm 0.265	15.660 ^b \pm 0.232	17.067 ^c \pm 0.188
GST	24.980 ^b \pm 0.183	26.948 ^c \pm 0.281	30.207 ^d \pm 0.208	24.465 ^{ab} \pm 0.409	26.898 ^b \pm 0.333	29.992 ^c \pm 0.340	25.162 ^b \pm 0.252	26.970 ^c \pm 0.298	30.931 ^d \pm 0.265
GPx	1.494 ^a \pm 0.010	1.568 ^b \pm 0.012	1.671 ^c \pm 0.010	1.481 ^a \pm 0.013	1.519 ^a \pm 0.014	1.631 ^b \pm 0.013	1.513 ^a \pm 0.015	1.601 ^b \pm 0.017	1.736 ^c \pm 0.018

All values are expressed as mean \pm SD of six individual samples. Means of the same row followed by different letter (s) differ significantly ($P < 0.05$) over 15d Cd treatment. CAT=Catalase, SOD=Superoxide dismutase, GPx=Glutathione peroxidase, GST=Glutathione S-transferase, LPO=Lipid peroxidation, SD=Standard deviation, d=day

formed/mg protein/min), suggesting the protective role of trace elements Zn and Fe.

GPx activity levels also showed a significant decrease at all the time intervals of Cd treatment with a maximum decrease in 30d kidney (0.822 ± 0.097 μ mole of NADPH oxidized/mg protein/min). However, the aforesaid Cd-inhibited GPx activity levels were markedly elevated in both the test tissues after supplementation with the trace elements Zn and Fe individually as well as in combination. The combination of Zn and Fe at the time interval 30d as supplement was more effective in elevating the GPx activity levels in the liver tissue of rats (1.266 ± 0.008 μ mole of NADPH oxidized/mg protein/min).

DISCUSSION

The results of the present study revealed that Cd induces oxidative stress as evidenced by increase in LPO and alterations in selected antioxidant enzyme status in liver and kidney of male albino rat at specific time intervals. However, supplementation of Zn and/or Fe reverses the Cd-induced changes in the LPO and antioxidant enzyme status.

Several mechanisms have been proposed for Cd-induced various abnormalities, but none have yet been defined explicitly. Disruption of a variety of biochemical processes have been proposed rather than a single mechanism responsible for the toxicity. Oxidative stress has recently

been reported as one of the important mechanisms of toxic effects of Cd (Matovic *et al.*, 2011).

From the results, it is evident that LPO increased markedly in both liver and kidney of Cd-treated rats when compared to the control [Table 1 and 3]. MDA levels in the tissues are used as an index of oxidative stress. In our study, elevated MDA levels were observed in Cd-treated rats as an indication of increased oxidative stress. The increased MDA levels were more prominent in 30 d Cd treated rats when compared to that of other scheduled time intervals. Cd may induce oxidative damage in different tissues by enhancing peroxidation of membrane lipids and altering the antioxidant defense system of the cells. It may also decrease the absorption of trace elements in the tissues. As liver contains more amounts of unsaturated lipids, it undergoes oxidative damage by ROS than the kidney. Cd triggers the generation of ROS in the organism (Lucia *et al.*, 2010; Olalekan *et al.*, 2011; Jovanovic *et al.*, 2012). In the present study, Cd-treated rats showed not only a significant increase in LPO but also significant decrease in the activity levels of antioxidant enzymes such as CAT, SOD, GST, and GPx [Tables 1-4]. These findings are in agreement with earlier reports of Stohs *et al.*, (2001) and Kim *et al.*, (2011) in rats and Carp, respectively, under Cd stress.

During oxidative stress the CAT activity levels progressively decreased due to high accumulation of H₂O₂ in the tissues, and thereby more peroxidation of lipids is favored. This could be the reason for increased LPO levels observed in Cd intoxicated rats. The significant decrease in CAT activity may be due to its inactivation by superoxide radical or due to decrease in the rate of reaction as a result of excess production of H₂O₂ to water and oxygen. It may also be due to the decreased absorption of essential trace element, Fe, required for the activity of this enzyme (Beytut and Aksakal, 2002; Jurczuk *et al.*, 2004). The decrement in the CAT activity levels with Cd treatment indicates inefficient scavenging of hydrogen peroxide due to oxidative inactivation of enzyme.

SOD is an important antioxidant enzyme that inhibits oxyradical formation and is usually used as a biomarker to indicate oxidative stress (Zhang *et al.*, 2004). The decrease

Table 3: Alterations in the activity levels of selected oxidative stress enzymes in the kidney of Cd-treated rats

Enzyme	Control	7 days	15 days	30 days
LPO	36.916 ^a ±2.818	48.083 ^b ±2.411	59.250 ^c ±2.434	71.083 ^d ±2.113
CAT	1.184 ^a ±0.030	1.147 ^a ±0.040	1.100 ^b ±0.030	0.982 ^c ±0.029
SOD	18.843 ^a ±0.247	15.947 ^b ±0.538	13.799 ^c ±0.624	8.810 ^d ±0.567
GST	30.455 ^a ±0.403	27.056 ^b ±0.411	24.178 ^c ±0.476	17.017 ^d ±0.543
GPx	1.887 ^a ±0.132	1.779 ^a ±0.102	1.434 ^b ±0.074	0.892 ^c ±0.097

All values are expressed as mean±SD of six individual samples. Means of the same row followed by different letter (s) differ significantly ($P < 0.05$). CAT=Catalase, SOD=Superoxide dismutase, GPx=Glutathione peroxidase, GST=Glutathione S-transferase, LPO=Lipid peroxidation, SD=Standard deviation

Table 4: Alterations in the activity levels of selected oxidative stress enzymes in the kidney of Cd-treated rats after Zn and/or Fe supplementation

Enzyme	Zinc (Zn)			Iron (Fe)			Zn and Fe		
	7d	15d	30d	7d	15d	30d	7d	15d	30d
LPO	54.416 ^b ±1.428	48.166 ^c ±1.241	39.708 ^d ±1.100	54.083 ^b ±1.044	48.875 ^c ±1.357	40.333 ^d ±0.995	53.85 ^b ±1.036	47.708 ^c ±1.088	38.791 ^d ±1.278
CAT	1.110 ^a ±0.033	1.123 ^b ±0.020	1.148 ^c ±0.023	1.112 ^a ±0.021	1.126 ^b ±0.029	1.156 ^c ±0.021	1.115 ^a ±0.019	1.128 ^b ±0.015	1.162 ^c ±0.021
SOD	14.289 ^a ±0.281	14.918 ^b ±0.311	16.111 ^c ±0.259	13.941 ^a ±0.265	14.779 ^b ±0.319	15.937 ^c ±0.212	14.417 ^a ±0.350	15.031 ^b ±0.347	16.225 ^c ±0.201
GST	25.040 ^a ±0.310	26.965 ^b ±0.993	29.731 ^c ±0.215	25.030 ^a ±0.272	26.364 ^b ±0.404	29.496 ^c ±0.256	25.061 ^a ±0.334	26.921 ^b ±0.205	30.299 ^c ±0.288
GPx	1.491 ^a ±0.016	1.647 ^b ±0.019	1.798 ^c ±0.014	1.481 ^a ±0.015	1.619 ^b ±0.016	1.725 ^c ±0.012	1.508 ^a ±0.018	1.685 ^b ±0.018	1.802 ^c ±0.016

All values are expressed as mean±SD of six individual samples. Means of the same row followed by different letter (s) differ significantly ($P < 0.05$) over 15d Cd treatment. CAT=Catalase, SOD=Superoxide dismutase, GPx=Glutathione peroxidase, GST=Glutathione S-transferase, LPO=Lipid peroxidation, SD=Standard deviation, d=Day

in SOD activity may be due to its inhibition by the excess production of ROS as evidenced by LPO in the present study. Excessive production of ROS may result in alterations in the biological activity of cellular macromolecules. Therefore, the reduction in the activity of SOD may result in a number of deleterious effects due to the accumulation of superoxide radicals. Administration of Cd into rats in the present study may lead to generation of peroxy radical, O_2^- , which is associated with inactivation of SOD and CAT. This may be the reason for the significant reduction in the activities of CAT and SOD in liver and kidney of Cd-treated rats when compared to control. It is in accordance with results from earlier studies in which there was a decrease in SOD activity in rats (Nagaraj *et al.*, 2000) and fish (Obaiah and Usha Rani, 2013) subjected to heavy metal, Cd.

GPx is a hydrogen peroxide degrading enzyme. Its activity was significantly decreased in both liver and kidney under Cd body burden at all the time intervals. The decreased GPx activity in the current study may be due to impairment in GSH homeostasis in liver and kidney tissues. As a result of this, liver and kidney tissue damage might have occurred under Cd insult. Recently, Waisberg *et al.*, (2003), Ognjanovic *et al.*, (2008), and Messaoudi *et al.*, (2009) also reported decreased GPx activity in the liver and kidney tissues of rats under Cd stress. It may be due to either free radical dependent inactivation of enzyme or depletion of its cosubstrate, that is, GSH and NADPH in the Cd-treated rat liver and kidney. Cd-administered rat tissues showed decreased GSH content due to overutilization by the cells in the tissues. Due to nonbioavailability of GSH under Cd burden, decrement in the activity levels of GPx has also been observed in experimental tissues. Depletion of GSH may render in GPx inactivation and/or less activity (Mahendran and Shyamala Devi, 2001).

The reduced GST activity in the tissues may be due to overconsumption of the enzyme GST to escape from the toxicity of peroxides under Cd insult. GST catalyzes the reaction of the thiol (-SH) group of GSH with electrophilic reagents such as those generated by microsomal metabolism of xenobiotics; thereby, neutralizing their electrophilic sites and rendering the products more water soluble (Han *et al.*, 2006). The decrease in GST activity might have resulted with Cd effect on GSH because of its high affinity to this molecule where a sulfhydryl acid, an amino acid, and two carboxylic acid groups, as well as two peptide linkages represent reactive sites for metals. Reactions of metals with glutathione might lead to either the formation of complexes or the oxidation of glutathione. The decreased GST activity in the test tissues is in agreement with El-Missiry and Shalaby (2000) in Cd-treated rat brain and testis. Moreover, the decrease in the activity of each of them would induce increased free radicals, thus injuring the corresponding tissues.

After supplementation with trace elements Zn and Fe individually and in combination there was significant reversal in the Cd-induced alterations in both liver and kidney of rats [Tables 2 and 4]. Zn provides an antioxidant function to cells by different mechanisms and decreases ROS and LPO (Joshi *et al.*, 2004; Formigari *et al.*, 2007; Matovic *et al.*, 2011). The most compelling reason for the protective effects of Zn against Cd toxicity is that Zn induces the production of the metal-binding protein, MT (Bonda *et al.*, 2004; Amara *et al.*, 2008). Zn supplementation could compete with binding sites for Cd uptake. The amelioration effect of Zn may be in part, mediated by induction and synthesis of MT and by providing thio (-SH) groups which can scavenge hydroxyl radicals and singlet oxygen. The protective role of Zn may be due to its ability to maintain normal GSH level and CAT activity. It appears that Zn has a direct antioxidant activity through scavenging of free radicals and indirect effects via prevention of the depletion of blood GSH level and hepatic CAT resulting from Cd treatment or by interfering with Cd transport into the cell rather than its activity to stimulate MT synthesis.

Fe is an essential trace element for many biological processes and it is important to maintain Fe concentration within its narrow normal range in the body (Turgut *et al.*, 2007) and also a vital component of several enzymes and proteins. The nutritional Fe status has a clear effect on Cd absorption (Akesson *et al.*, 2002; Ryu *et al.*, 2004). Fe and Cd most probably compete at the binding sites of the Fe intestinal transfer system (Bhattacharya *et al.*, 2000; Tallkvist *et al.*, 2001; Park *et al.*, 2002). In the present study Cd concentrations were significantly depleted in the test tissues with Fe supplementation suggesting that Fe can reduce the Cd-induced alterations in the oxidative stress enzymes (Obaiah *et al.*, 2009; Usha Rani *et al.*, 2010). In one study, it was observed that the intestinal absorption of dietary Cd in women decreased when the body Fe stores and fiber intake were increased (Berglund *et al.*, 1994). In rats, dietary Fe supplements have shown to decrease the Cd retention after oral Cd exposure (Groten *et al.*, 1992; Schumann *et al.*, 1996).

Zn and Fe most effectively protects against intestinal absorption of Cd from Cd-MT, thereby decreasing Cd uptake by 80% than the other essential elements (Piasek *et al.*, 2004). Further Casalino *et al.*, (1997) reported that Fe supplementation corrects the anemia caused by Cd exposure in rats. It has also been suggested that higher gastrointestinal absorption of Cd is due to lower body Fe stores as measured by the concentrations of serum ferritins (Vahter *et al.*, 1996). The overall discussion suggests that Fe may reduce the Cd body burden in the organisms by eliminating Cd from the tissues. Moreover, in the present study the Cd-induced alterations in the oxidative stress/antioxidant enzymes

were recovered with specific supplements (as mentioned in Materials and Methods) of Zn and Fe individually and in combination. Our work is significant and has got applied value because the intake of trace element supplements like Zn and Fe aids in therapeutic indices of Cd and other heavy metal toxicity.

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Conflicts of interest

There are no conflicts of interest.

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