

Lead and Cadmium Co-exposure Mediated Toxic Insults on Hepatic Steroid Metabolism and Antioxidant System of Adult Male Rats

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Received: 26 December 2008 / Accepted: 17 July 2009 / Published online: 4 August 2009
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Abstract The redox status and steroid metabolism of liver of adult male rat exposed to lead (Pb) and cadmium (Cd) either alone or in co-exposure (0.025 mg/kg body weight intraperitoneally/15 days) was studied. Pb and Cd significantly accumulated in the liver. The activity of steroid metabolizing enzymes 17- β -hydroxysteroid oxidoreductase and uridine diphosphate–glucuronyltransferase were decreased in experimental animals. 17- β -Hydroxysteroid dehydrogenase was reduced to 33%, 38%, and 24% on treatment of Pb, Cd, and co-exposure (Pb+Cd). Furthermore, the activity of uridine diphosphate–glucuronosyltransferase was significantly reduced to 27% (Pb exposure), 36% (Cd exposure), and 25% (co-exposure of Pb+Cd). Cd exposure exhibited more toxic effect than Pb, while co-exposure demonstrated the least. The activities of antioxidant enzymes, superoxide dismutase, catalase, glutathione reductase, and glucose-6-phosphate dehydrogenase decreased and glutathione peroxidase increased in mitochondrial and post-mitochondrial fractions. The level of lipid peroxidation increased, and cellular glutathione concentration decreased. Hepatic DNA was decreased, whereas RNA content and the activity of alanine transaminase remained unchanged. Histological studies revealed that only Cd-exposed groups exhibited cytotoxic effect. These results suggest that when Pb and Cd are present together in similar concentrations, they exhibited relatively decreased toxic effect when compared to lead and cadmium in isolation with regard to decreased steroid metabolizing and antioxidant enzyme activities. This seems that the toxic effect of these metals is antagonized by co-exposure due to possible competition amongst Pb and Cd for hepatic accumulation.

Keywords Pb · Cd · Antioxidant enzyme · Reactive oxygen species (ROS)

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Introduction

Constantly increasing environmental pollutants due to increased urbanization, industrialization, and through the scientific and technical advancement has stimulated interest in studying toxic substances and its effects on biological system. Effect of Pb and Cd on hepatic tissue has been reported by several groups of researchers [1, 2].

The liver has several mechanisms for hormone biotransformation. Direct conjugation, in which the steroid is conjugated to glucuronic acid, produces a more water-soluble product that can then be excreted in bile and urine [3, 4]. Steroid hydroxylation accomplishes the same goal by stereo-selectively and regio-specifically attaching hydroxyl groups to the steroid [3, 5], which also provides sites for subsequent conjugation reactions. Sex-specific differences in steroid hydroxylation, uridine diphosphate–glucuronosyltransferase (UDPGT), enzyme has been investigated and well documented in mammals, birds, and reptiles [4, 6]. Oxido-reduction of testosterone to androstenedione, dihydrotestosterone, and/or androstanediols is another hepatic biotransformation pathway that influences circulating levels of testosterone. We have earlier reported a decrease in the hepatic estradiol metabolism in adult female rats exposed to lead and cadmium [7]. In addition, we have reported the effects of exposure to lead and cadmium either alone or in combination during pregnancy and lactation on hepatic estradiol-metabolizing enzymes in pups (PND21) and fetus [8].

Though specific differences in the toxicities of Pb and Cd may be related to differences in their solubilities, absorbability, transport, chemical activity, and the complexes that are formed within the body, evidences suggest that one of the basic mechanisms involved in metal induced toxicity might be via reactive oxygen species (ROS) [9, 10]. ROS such as super oxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydrogen radical (OH) are generated in vivo from exposure to environmental agents such as radiation and redox cycling agents [11]. Oxidative stress occurs when the production of ROS exceeds the body's natural antioxidant defense mechanisms, causing damage to macromolecules, such as DNA, proteins, and lipids [12]. Moreover, both Pb and Cd are known sulfhydryl reactive metals [13], and therefore, depletion of GSH increases susceptibility of cells to free radical-induced toxicity [14].

In majority of the animal and human studies to date, exposure to single metal has been elucidated in high concentration. However, in environment, the population receives continuous simultaneous multiple exposures, indicating the need for experimental work with combinations of substances. Previous studies from our laboratory in these lines have reported a decrease in phase I and II enzyme activity with co-exposure to Pb and Cd in non-pregnant female rats [15]. Also the suggestive mechanism of oxidative stress for such an effect has been demonstrated in female reproductive [16] as well hepatic tissue [8]. The objective of the present study was to examine the co-exposed effects of Pb and Cd on hepatic steroid metabolism and antioxidant system of adult male rats.

Materials and Methods

Chemicals

Dehydroisoandrosterone, β -estradiol, sodium salts of $NADP^+$, glucose-6-phosphate (G6P), and ter-butyl hydroperoxide (t-BOOH) were obtained from Sigma, USA. Bovine serum albumin fraction V, nicotinamide adenine dinucleotide (NAD) and 5,5'-dithio-bis-(2-

nitrobenzoic acid) were obtained from SRL, India. Reduced glutathione (GSH) was a product of Hi Media, India. All other chemicals were of the highest purity grade and were purchased locally.

Animals and Treatment

Adult Charles foster male rats weighing 200–220 g of body weight were maintained as per the national guidelines and protocols, approved by the institutional ethical committee. There were four groups of five animals each in the study: group 1, animals exposed with sodium acetate as control; group 2, Pb acetate; group 3, Cd acetate, and group 4, received Pb acetate and Cd acetate in combination. The route of treatment is intraperitoneal with 0.025 mg/kg body weight of metal, per day for 15 days. The co-exposure consists half of Pb and Cd for a total dose of 0.025 mg/kg. The dose was selected on the basis of previous studies on the effect of simultaneous exposure of Pb and Cd on hepatic estradiol metabolism [7]. The animals were killed by decapitation; the procedure was completed within 5–10 s to avoid stressors.

Metal Analysis

Pb and Cd concentrations were determined in liver samples. The samples were digested in reagent grade nitric acid/perchloric acid (2:1) mixture. The digestion was continued until samples became colorless. Then, the acid mixture was evaporated, and the precipitate thus obtained was dissolved in a few drops of concentrated HCl. The sample was diluted to 1 ml with distilled water. Pb and Cd levels were determined using thermo atomic absorption spectrophotometer by acetylene-air flame. Sensitivities of the assays were 0.06 and 0.009 µg/ml for Pb and Cd, respectively.

Biochemical Analyses

Hepatic steroid-metabolizing enzyme 17-βhydroxysteroid oxidoreductase (17-βHSOR) [17] and UDPGT [18] were determined. DNA [19] and RNA [20] content were determined in liver. Alanine transaminase (ALT) activity was carried out using method given by Reitman and Frankel [21]. Determination of lipid peroxidation (LPO) was according to published method [22]. Content of glutathione (GSH) was measured by the method described earlier [23]. Superoxide dismutase (SOD) activity was determined according to the methods described by Marklund and Marklund [24]. Catalase activity was assayed by following the decrease in H₂O₂ at 240 nm [25]. Glutathione peroxidase (GPx) activity was determined by the procedure described by Hafeman et al. [26]. Glutathione reductase (GR) activity was assayed [27]. Glucose-6-phosphate dehydrogenase (G6PDH) activity was determined by following the reduction of NADP⁺ spectrophotometrically at 340 nm [28]. Protein was estimated by the method of Lowry et al. with bovine serum albumin used as the standard [29].

Histology

Liver was removed and fixed in Bouins fixative. Histological examination of liver was carried out by standard histological techniques. Sections of 5-µm thickness were cut and stained with heamatoxylin/eosin. Histological observations were made using five samples of each group under the light microscope.

Statistical Analyses

Statistical analyses of data was done by one-way analysis of variance, and all groups were compared by means of Dunnett's test (post hoc tests), with significance set at $p < 0.05$. All values represent the mean \pm SEM.

Results

There were no significant changes in the body weight of rats exposed with Pb and Cd in isolation and combination as compared to controls (Table 1). Weights of the liver remained unchanged in all exposed groups as compared with the corresponding groups of control animals (Table 1). Table 2 summaries the results on the effect of Pb and Cd on hepatic DNA and RNA content and on the alanine transaminase activity. The hepatic DNA content was decreased significantly in the Cd- and Pb-exposed groups, while no significant changes was observed in RNA content compared to control. The specific activity of alanine transaminase, a marker enzyme for hepatic injury remained unchanged.

Histological observations of liver after various doses of Pb and Cd treatment are shown in Fig. 1. The Pb and co-exposed groups did not show marked alteration compared to control. Cd treatment caused marked changes in liver such as massive fatty degeneration in hepatocytes. Nucleus was pycnotic in appearance and necrotic regions could be seen.

The hepatic concentration of Pb and Cd in the adult male rats is shown in Figs. 2 and 3, respectively. Both metals were accumulated in the liver in amounts higher than in control group after exposure of 0.025 mg/kg body weight for 15 days. The concentration of Pb in Pb and co-exposed groups was higher (99% and 26% increase, respectively) while diminished in Cd-exposed group (33% decrease) than in control group. The significant accumulation was observed only in Pb-exposed group. Similarly Cd concentration was higher in Cd (1,270% increase; $p < 0.01$) and co-exposed (87% increase; not significant) groups than in control while diminished in Pb (91% decrease; 0.01) exposed group than in control. In addition, the amount of Pb found in control is higher than the one found in rats exposed with Cd acetate (Fig. 2; not significant). Similarly, the amount of cadmium found in control is higher than the one found in rats exposed with Pb acetate (Fig. 3; $p < 0.01$). When Pb and Cd present together, accumulations of both metals were decreased compared to individual-exposed groups, and the decrease in accumulation is higher in case of Cd ($p < 0.05$) than Pb ($p < 0.05$).

We assayed both 17- β HSOR (Fig. 4) and UDPGT (Fig. 5) enzyme activities as the steroidogenic catabolism in liver. Cd-exposed group demonstrated highest reduction in both

Table 1 Effect of Pb and Cd in Isolation and Combination Co-exposure for 15 Days (0.025 mg/kg Body Weight) on Body Weight Gain and Relative Liver Weight (g)

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Body weight gain(g)	53.2 \pm 1.0	54.9 \pm 1.83	48.8 \pm 2.8	51.0 \pm 1.8
Relative liver weight (g)	3.49 \pm 0.19	3.45 \pm 0.19	3.99 \pm 0.25	3.36 \pm 0.23

Values are expressed as mean \pm SEM ($n=5$ in each group)

Table 2 Effect of Pb and Cd in Isolation and Co-exposure on Hepatic DNA, RNA Content, and Alanine Transaminase Activity of Adult Male Rats

Group	DNA ($\mu\text{g/g}$)	RNA ($\mu\text{g/g}$)	ALT (IU)
Control	322 \pm 37	0.076 \pm 0.007	32.55 \pm 3.15
Lead	214 \pm 11*	0.065 \pm 0.001	31.94 \pm 5.08
Cadmium	182 \pm 24**	0.063 \pm 0.002	35.13 \pm 1.51
Lead+cadmium	277 \pm 37	0.071 \pm 0.005	34.90 \pm 3.76

Values are expressed as mean \pm SEM ($n=5$ in each group)

* $P<0.05$, ** $P<0.01$ vs. control group

enzyme activities followed by Pb, while co-exposure exhibited antagonist effect, hence least toxic. 17- β HSOR was found to decrease by 33%, 38%, and 24% in Pb, Cd, and co-exposed group, respectively. Similarly, the UDPGT activity was significantly reduced to 27% (Pb exposure), 36% (Cd exposure), and 25% (Pb+Cd exposure).

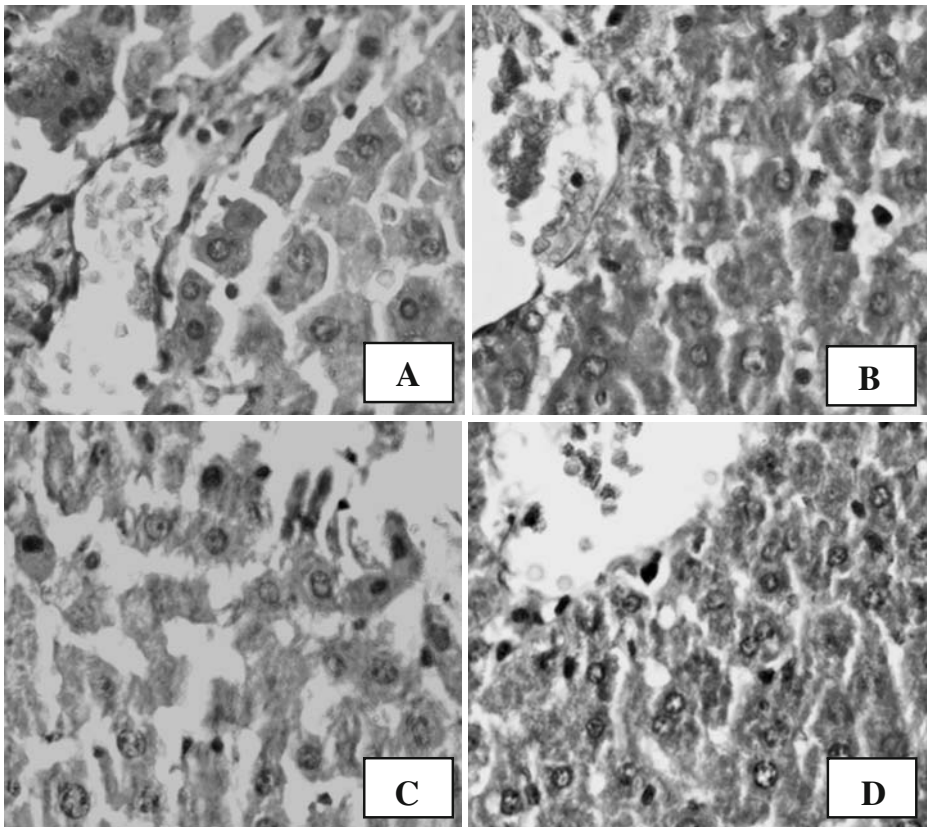
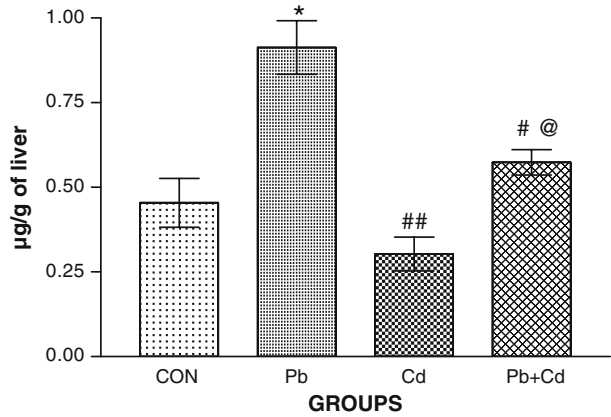


Fig. 1 a Liver of control ($\times 40$), b Pb-exposed group ($\times 40$), c hepatocytes showing pycnotic nuclei with moderate degenerative changes and large vacuoles after exposure to 0.025 mg/kg body weight dose of Cd for 15 days ($\times 40$), and d Pb+Cd-exposed ($\times 40$) group rat showing normal hepatocytes

Fig. 2 Pb levels in the liver (microgram per gram of liver) of male rats exposed to Pb acetate and Cd acetate alone and in co-exposure for 15 days (0.025 mg/kg body weight per day). Values are expressed as mean±SEM ($n=5$ in each group). * $P<0.05$ vs. control; # $P<0.05$; ### $P<0.01$ vs. Pb and @ $P<0.05$ vs. Cd group



The cellular damage induced by ROS was estimated by monitoring LPO, a well-known indicator of cellular damage from oxidative stress [30]. In our experiments, elevated levels of LPO were observed highest in Cd-exposed group followed by Pb- and co-exposure-exposed groups. On metal exposure, GSH content was decreased; the changes observed in Cd-exposed rats were more as compared to Pb and co-exposed groups. Increase LPO and decrease GSH content, suggesting the generation of ROS in hepatic mitochondrial and post-mitochondrial fraction (Table 3).

To evaluate the activities of antioxidant defense system in liver affected by Pb and/or Cd exposure, we assayed hepatic SOD, catalase, GPx, GR, and G6PDH. Except GPx, the activities of all antioxidant enzymes decreased in mitochondrial and post-mitochondrial fraction of liver when compared with the corresponding group of control animal. In Pb and Cd metal-exposed groups, the activities of antioxidant enzymes were decreased at greater extent, where the co-exposure groups seems to be least effective. The GPx activity increases in all exposed groups, and co-exposed group showed highest increase followed by Pb- and Cd-exposed groups (Table 3).

Fig. 3 Cd levels in the liver (microgram per gram of liver) of male rats exposed to Pb acetate and Cd acetate alone and in co-exposure for 15 days (0.025 mg/kg body weight per day). Values are expressed as mean±SEM ($n=5$ in each group). * $P<0.01$ vs. control; # $P<0.01$; vs. Pb and @ $P<0.01$ vs. Cd group

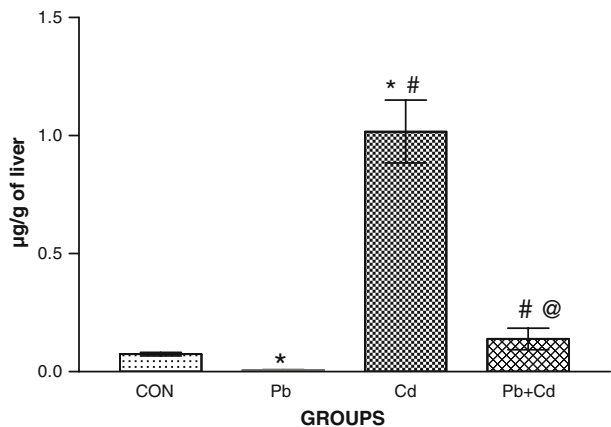
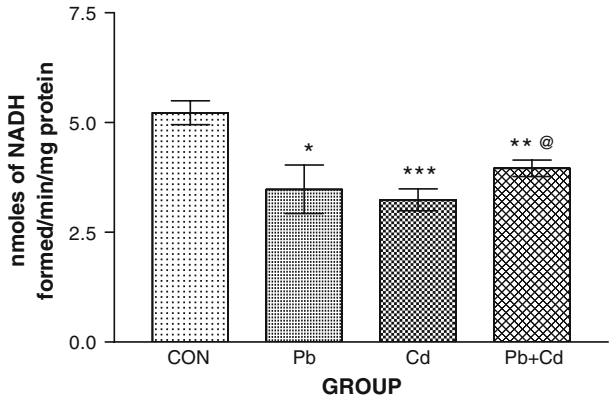


Fig. 4 Effect of Pb and Cd alone and in co-exposure on 17 β -hydroxy steroid oxidoreductase activity in adult male rats liver exposed with a dose of 0.025 mg/kg body weight daily for 15 days. Values are expressed as mean \pm SEM ($n=5$ in each group). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. control group and @ $P<0.05$ vs. Cd group



Discussion

The results from the present study indicate that exposure to Pb and Cd (0.025 mg/kg body weight per day for 15 days, intraperitoneally), resulted in alteration on steroid metabolic enzymes and antioxidant system in liver of adult male rats. Body weight and the weight of liver in Pb- and Cd-exposed rats did not show significant change, indicating that the general metabolic condition of the animals was within normal range. The specific activity of ALT, a marker enzyme for hepatic injury remained unchanged, indicating absence of tissue injury in Pb and Cd in isolation and co-exposed rats. However, histological studies demonstrated cytotoxic effect in Cd-exposed group. It is possible that more massive damage in Cd-exposed animal will reflect the change in ALT activity. Accumulation of Pb and Cd in Pb- and Cd-exposed groups are 100% and 1,270% higher, respectively, than control group. When both metals given together accumulation of Pb declined by ~4-fold, while Cd content declined by ~13-fold. This suggests that higher percentage elimination of more toxic metal, i.e., Cd, could be one of the reasons for co-exposed group being lowest toxic in all parameters studied. Decreased amount of Pb and Cd in Cd- and Pb-exposed rats, respectively, compared to control further supports the competitive nature amongst the metals.

Fig. 5 Effect of Pb and Cd alone and in co-exposure on UDPGT activity in adult male rats liver exposed with a dose of 0.025 mg/kg body weight daily for 15 days. Values are expressed as mean \pm SEM ($n=5$ in each group). * $P<0.05$, ** $P<0.01$ vs. control group

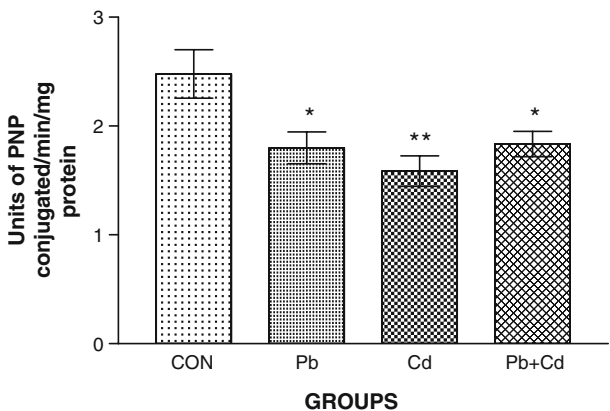


Table 3 Effect of Pb and Cd in Isolation and Co-exposure on Level of GSH, Lipid Peroxidation, and Antioxidant Enzymes Activities in Liver of Adult Male Rats

Parameters	Groups			
	Control	Pb	Cd	Pb + Cd
Reduce glutathione ^a				
Mitochondrial	13.51±1.35	8.570±1.65	7.269±1.1*	9.762±1.3
Post-mitochondrial	11.76±0.83	9.474±0.89	8.093±0.64**	11.37±0.65*****
LPO ^b				
Mitochondrial	1.365±0.15	2.266±0.3*	2.353±0.28*	2.027±0.2
Post-mitochondrial	1.404±0.008	2.319±0.15*	2.530±0.28*	1.994±0.32
SOD ^c				
Mitochondrial	1.771±0.284	0.845±0.175*	0.5281±0.086**	0.8310±0.09*
Post-mitochondrial	3.346±0.327	1.635±0.167**	1.213±0.09**	1.454±0.26**
Catalase ^d	226.8±24.10	148.6±11.19*	111.2±8.025**	210.3±16.6*****
GPx ^e				
Mitochondrial	4,114±341.6	4,848±233.5	4,370±259.4	6,377±389*****
Post-mitochondrial	1,415±74.5	1,643±121.2	1,523±111.4	1,761±69.91*
GR ^f				
Mitochondrial	71.38±5.21	63.40±1.71	34.46±5.35*****	63.87±6.66*****
Post-mitochondrial	26.22±1.24	22.00±1.72	9.708±3.3*****	20.69±2.01*****
G6PDH ^g	6.039±0.27	4.449±0.5*	3.809±0.34**	4.573±0.27

The data are presented as mean±SEM of five independent observations

* $P < 0.05$, ** $P < 0.01$ vs. control; *** $P < 0.05$, **** $P < 0.01$ vs. lead; ***** $P < 0.05$, ***** $P < 0.01$ vs. cadmium group

^a Picomole per milligram protein at 37°C

^b Nanomole malondialdehyde per milligram protein at 37°C

^c Amount of enzyme required for 50% inhibition of pyrogallol auto oxidation at 37°C

^d Micromole H₂O₂ decomposed per minute per milligram protein at 37°C

^e Δlog [GSH] per minute per milligram protein at 37°C

^f Micromole NADPH reduced per minute per milligram protein at 37°C

^g Micromole NADPH formed per minute per milligram protein at 37°C

The present study demonstrated that exposure to Pb and Cd, either alone or in combination, decreased the activities of steroid metabolizing enzymes, 17-βHSOR and UDP glucuronyl transferase. Since the active site of 17-βHSOR contains lysine and tyrosine residues, the changes observed in the enzyme activity can be due to the binding of the divalent metal ions to these amino acid residues [18]. The inhibition in the activity of UDP glucuronyl transferase can be explained by a similar mechanism, since the enzyme has methionine at its active site. Also our earlier work on female rats showed similar kind of inhibition [7]. Among the two metals used, the percentage decrease in the steroid metabolized enzyme was higher in Cd- than Pb-exposed group. This is due to the fact that Cd is more hepatotoxic than Pb [31], as shown by histological studies. Also the distribution pattern obtained in the current study demonstrated that Cd is more retained in liver than Pb, when both the metals given individually. This observation supports the fact that the Cd half-life in human body is more than 30 years [32]. The decreased activity of catabolizing

enzyme could also be attributed to decreased availability of steroids. We have earlier reported that co-exposure of Pb and Cd caused decreased in bioavailable steroids in non-pregnant female rats [33].

We were interested in investigating whether Pb- and Cd-induced changes in estradiol metabolism are due to oxidative damage to the liver. We observed that Pb and Cd, even with low level exposure for 15 days, led to higher levels of LPO in the rat liver and decreased level in GSH content. Since Pb and Cd interact with cell membranes, elevated levels of LPO may be direct consequence of membrane damage [34, 35]. Our earlier studies have reported changes in membrane fluidity with both Pb and Cd [15]. One of the mechanism for the observed decrease in GSH content in the present study could be the binding of these divalent metals with -SH groups [36, 37]. The GSH depletion and enhanced LPO in the present study indicates failure of antioxidant defense mechanism, which otherwise prevents the formation of excess free radical.

In mitochondrial and/or post-mitochondrial fraction of liver, the activities of antioxidant enzymes SOD, catalase, GR, and G6PDH were decreased, whereas the activity of GPx was increased. SOD generally dismutates the super oxide anion radical (O_2^-) into hydrogen peroxide (H_2O_2), which is degraded by catalase and GPx. The decreased SOD activity is due to replacement of the manganese and zinc of the SOD molecule in mitochondrial and post-mitochondrial fraction, respectively, by Pb [38] and Cd [39, 40]. Reduction in the activity of catalase may reflect inability of liver to eliminate H_2O_2 produced after 15 days of metal exposure. This may also be due to enzyme inactivation caused by excess ROS production [41]. Thus, the major route of H_2O_2 elimination would seem to be via GPx, as can be noted from the data (Table 3) after 15 days of metal exposure. The increased activity of GPx may indicate a stress response of liver to Pb and Cd toxicity. The decrease GR activity correlates with decrease GSH content. Non-availability of GSH in the liver is further compounded by low G6PDH activity. Although NADPH, the substrate for GSH reduction can be generated by systems other than G6PDH, such as isocitrate dehydrogenase or glutamate dehydrogenase [42]. It is not clear at this stage whether these alternate systems are also affected by 15-day metal exposure. Hence, an imbalance between O_2^- and H_2O_2 might occur in the liver of Pb- and Cd-exposed rats; the altered antioxidant enzymes activity thus might indirectly lead to an increase in oxidative stress, causing ROS-induced damage to macromolecules such as DNA, proteins, and key enzymes involved in hepatic steroid metabolism. Earlier, in a similar kind of metal-exposed study, the decreased activities of antioxidant enzyme in pituitary [43] and ovary [16] were also reported in female rats.

The changes in the various biochemical parameters observed might result from independent effect of Pb and Cd in Pb- and Cd-exposed groups, respectively, and due to competitive nature in co-exposed group. The observed effect may involve changes in metal accumulation and concentration of various essential elements such as Zn, Cu, and Fe in the serum and liver. In the present investigation, co-exposure of Pb and Cd showed antagonistic results in various parameters studied. In most of the studies reporting on co-exposure to metals, researchers have used the same concentrations of the metals both in individual and co-exposed group [44]. The results from such studies showed additive effect in the co-exposed group as the concentration of the metals are increased, whereas in the present study, the total concentration of metals in the co-exposed group is the same as that in the individual-metal-exposed group. This pattern avoids multiple stresses in the co-exposed group.

In conclusion, the effects produced by the co-exposed group of metals are antagonist. The present study reveals that low doses of Pb and Cd in isolation and in co-exposed group

generate oxidative stress by decreasing the mitochondrial and post-mitochondrial activities of antioxidant enzymes, altering redox metabolites and altered steroid metabolism, which further may eventually result into altered circulating sex-steroid hormone homeostasis. Most of the alteration are similar to that demonstrated in female rats and thus did not demonstrate any sexual dimorphism with Pb and Cd exposure.

Acknowledgment We are thankful to Dr. S. Sharma (Geology department, M.S. University of Baroda) for his help in metal estimation. Authors thank Dr. S. Gupta (M.D. Pathologist, Vadodara) for the help in histological observations.

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