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Original Article

Cadmium exposure-induced oxidative stress; delay in sexual maturation and impaired hormones in developing rat ovary

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Cadmium; Ovary; Sexual maturation

Abstract
Cadmium (Cd) is a common environmental pollutant by discharge from industrial processes. Cd one of the elements found to damage antioxidant systems in mammals. The major route of Cd exposure for the general population is oral intake. However, excess Cd exposure is associated with various pathological conditions including reproductive dysfunction. Cd can traverse the placental barrier and cause wide range of abnormalities in fetal development. Therefore, the present study was carried out to determine the toxic effects of lactational Cd exposure on ovary in developing female Wistar rats. Two different doses of cadmium (50 and 200 ppm) were given to Wistar rats aged 45 and 65 days. Specific activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) were estimated. Hydrogen peroxide (H_2O_2), lipid peroxidation (LPO) and serum gonadotropins *viz* luteinizing hormone (LH) and follicle stimulating hormone (FSH) were also assayed. Specific activities of SOD, CAT, GPx, GR and GST were decreased while H_2O_2 and LPO increased. These results suggest that lactational Cd exposure induces oxidative stress in rat ovary by decreasing antioxidant enzymes, which were associated with delayed puberty and altered steroids and gonadotropin levels.

INTRODUCTION

Environment is defined as the totality of circumstances surrounding an organism or group of organisms, especially, the combination of external physical conditions that affects and influences the growth, development and survival of organisms [1]. Cadmium (Cd) may be released to water by natural weathering processes, by discharge from industrial processes, by discharge from industrial facilities or sewage treatment plants, atmospheric deposition, by leaching from landfills or soil, or phosphate fertilizers [2]. The dominant use of cadmium is in active electrode materials in Ni-Cd batteries (83% of total cadmium use) [3]. Cadmium chloride is used in photography, photocopying, dyeing, calico printing, vacuum tube pigment manufacture, galvanoplasty, lubricants, ice-nucleation agents, and in the manufacture of special mirrors. Cadmium sulfate solution is used in standard Weston cells [4].

Cadmium was found to result in inducing oxygen free radical production [5-7]. Oxidative stress affects multiple physiological processes, from oocyte maturation to fertilization, embryo development and pregnancy [8]. Piasek *et al* [9] evaluated the direct effects of *in vitro* Cd exposure on steroid genesis in rat ovaries. It may also cause severe damage to embryos and the reproductive organs in adults including the ovary and testes, which are sensitive to Cd toxicity [10]. Cd is a known endocrine disruptor by affecting the synthesis and/or regulation of several hormones [7, 11]. Lipid peroxidation (LPO) is one of the main manifestations of oxidative damage, which plays an important role in the toxicity of many xenobiotics [12]. Cd causes a significant increase of LPO in liver and kidney of rats, since it causes LPO in numerous tissues both *in vivo* and *in vitro* [13]. Cd induces oxidative stress by producing hydroxyl radicals [14].

Reactive oxygen species (ROS) act on the cellular molecules of the embryo and may block or retard early embryonic development [15]. The rodents have been widely used as a model system to study the hormonal interactions. The ovary is an important organ shown to be controlled by steroid hormones. In an attempt to understand the adverse effects of Cd on female reproductive system, we studied the status of antioxidants in the rat ovary. In the background of the existing information, it is hypothesized that post-parturition exposure to Cd would disrupt ovarian function by inducing oxidative stress.

MATERIALS AND METHODS

Experimental design

Ninety-day-old female albino rats of Wistar strain (*Rattus norvegicus*) obtained from the National Institute of Nutrition, Hyderabad, India weighing 140 ± 10 g were used for the present investigation. The rats were maintained in a temperature controlled animals' quarter with 12:12 h dark:light schedule and were fed standard rat pellet diet (Broke Bond, Lipton India Ltd, Kolkata, India) and drinking water *ad libitum*. The animals were dewormed with albendazole (10 mg/kg b.wt, orally; Bendex-400, Protec Cipala Ltd, Mumbai, India) before the initiation of the experiment. The females were mated with males at a ratio of 2:1. Cohabitation began at approximately 16.30 h on each mating day. On the following morning the females were removed from the mating cages and smeared individually for the presence of sperm in the vaginal lavage. The presence of sperm in the vaginal lavage is indicative that the females mated and those were selected for further studies. The pregnant animals were then allowed to give birth. The mother animals with female pups were divided into the following groups:

-***Group I:*** control

-***Group II:*** 50 ppm Cd

-***Group III:*** 200 ppm Cd

The minimum (50 ppm) and maximum (200 ppm) effective doses of Cd were selected [16], and the mother rats along with female pups were treated with Cd in the form of cadmium chloride 0 day post-parturition (pp) to 65 days pp.

-***Subgroup I:*** 45-day-old rat; puberty occurred

-***Subgroup II:*** 65-day-old rat; full growth of ovary

Each group consisted of a minimum of six animals. Pups of different age of ovarian developmental milestones were killed on the morning. Ovaries were separated. Trunk blood was collected and sera was separated out and used for hormone assays.

Antioxidant enzymes, tissue preparation

Ovaries were separated. One milligram of rat ovary was homogenized in 3 ml of 20 mM phosphate buffer (pH 7.4) by using homogenizers (Heidolph Instruments, Schwabach, Germany) with a Teflon pestle. 10 µl 0.5 M 2,6-bis(1.1-dimethylethyl)-4-methylphenol (BHT) in acetonitrile was added to 1 ml of tissue homogenate to prevent sample oxidation. The precipitate was removed by centrifugation (2000 rpm). An aliquot of the sample was removed and the sample was freezed immediately at -70°C or kept on ice prior to testing. 0.2 ml of the homogenate was used for assay.

Antioxidant enzymes

Superoxide dismutase (SOD) was assayed according to the method of Marklund and Marklund [17]; the enzyme activity was expressed as units (U)/mg protein. The activity of catalase (CAT) was assayed by the method of Sinha [18] and expressed in U/mg protein (1 U is the amount of enzyme that utilizes 1 µmol of hydrogen peroxide/min). The activity of glutathione peroxidase (GPx) was determined by the method of Rotruck *et al* [19]; the enzyme activity was expressed as U/mg protein (1 U is the amount of enzyme that converts 1 µmol reduced glutathione [GSH] to oxidized glutathione [GSSG] in the presence of hydrogen peroxide/min). Glutathione S-transferase (GST) was assayed by the method of Habig *et al* [20]; the enzyme activity was expressed as U/mg protein (1 U is the amount of enzyme that converts 1 µmol GSH to GSSG in the presence of hydrogen peroxide/min).

Sexual maturation and estrous cycle

Vaginal opening was observed in rats from postnatal day (PND) 22, every 24 h to determine the sexual maturation, as this was used as an index to assess the onset of puberty. After the puberty, vaginal smears were examined every morning as described previously [21-23].

Reactive oxygen species

Tissue lipid peroxidation was measured by the method of Devasagayam and Tarachand [24]. The malondialdehyde (MDA) content of the samples was expressed as nanomoles of MDA formed per mg protein. Hydrogen peroxide production was assessed by the spectrophotometric method of Holland and Storey [25]. The oxidation of ferrocytochrome c, of the samples was expressed as nmoles/minute/mg protein.

Radio-iodination of steroid hormones

Serum levels of estradiol, progesterone and testosterone were estimated by radioimmunoassay (RIA) as described earlier [21]. The maximum binding of the estradiol antibody was 37-40%, and the sensitivity of the assay was 0.3 pg/ml. The concentration of estradiol in serum is expressed as pg/ml. The percentage of cross-reactivity of the antibody with other steroids was

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0.32% with estriol, 1% with estrone and progesterone and 0.0001% with testosterone. Inter- and intra-assay coefficients of variation were 4.5-8% and 3-4.8%, respectively. The percentage binding of the testosterone antibody was 36% and the sensitivity of the assay was 0.3 pg testosterone/ml. Testosterone level in serum is expressed as ng/ml. Cross reactivity of the antibody with other steroids was 0.0001% for cortisol, 14% for 5α-dihydrotestosterone, 0.8% for androstenedione and 2.1% for 5α-androstanediol. Inter- and intra-assay coefficients of variation were 6-8% and 4-6%, respectively. The maximum binding of progesterone antibody was 40% and sensitivity of the assay was 0.3 pg/ml. Progesterone level is expressed as ng/ml. The cross-reactivity of this antiserum to corticosterone, 17α-hydroxy progesterone and testosterone was 0.4%, 0.3% and non-detectable, respectively. The intra-assay coefficients of variation were 3.5% and inter-assay was 3.5-4%.

Radio-iodination of peptide hormones

Serum levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH) were assayed by RIA [21]. All assays were carried out in duplicate. Values of peptide hormones are expressed as ng/ml. The maximum binding of the LH antibody was 31.36%, and the cross-reactivity to other peptide hormones was 0.02% for FSH, 0.071% for TSH and 0.01% for GH and PRL. The intra- and inter-assay coefficients of variation were 4.9-8.48% and 9.9%, respectively. Sensitivity of the LH assay was 0.2 ng/ml. The maximum binding of FSH antibody was 31%, and the cross-reactivity to other peptides was 0.5% for LH, 0.2% for TSH, 0.01% for PRL and GH. The intra- and inter-assay coefficients of variation were 5.7-8.9% and 12.2%, respectively. Sensitivity of the FSH assay was 0.2 ng/ml.

Statistical analysis

All data were presented as means ± standard error of the mean (SEM). Statistical significance was calculated using ANOVA to test the significance of individual variations. The value of probability was obtained from the degree of freedom by using standard table value, given by Fischer and Yates [26]. The level of significance was assessed at $P < 0.05$.

RESULTS

Lactational Cd exposure leads to age-dependent changes in the specific activities of antioxidant enzymes, free radicals and hormones in postnatal rat ovary.

Body and ovary weight

Lactational Cd exposure significantly decreased the body weight and ovary weight in PND 45 and PND 65 of Cd-treated rats (Table 1).

Sexual maturation

Vaginal opening has been used as an index of sexual maturation. To evaluate the effect of Cd on the sexual maturation, we have examined the vaginal opening every 24 h in the experimental rats from PND 22 onwards (Fig.1). Cd exposure significantly delayed the sexual maturation in postnatal rats.

Estrous cyclicity

In order to evaluate the effect of Cd on the duration of estrous cycle, vaginal cytology was determined daily (Fig.2). Cd exposure significantly extended the estrous cycle in diestrous stage when compared to control rats. Cd exposure did not alter proestrous, estrous and metestrous phases of estrous cycle.

Antioxidant enzymes

Fig.3 represents the effect of Cd on the activities of different antioxidant enzymes in rat ovary. The specific activity of SOD, CAT, GPx and GST showed a dose-dependent significant decrease in ovary of both treatment groups.

Lipid peroxidation and hydrogen peroxide

Fig.4 represents the concentrations of LPO and H_2O_2 generation in control, 50 and 200 ppm Cd treated rat's ovary. Increased concentrations of LPO and H_2O_2 generation were observed in Cd treated rats.

Serum hormones

Table 2 represents circulating levels of steroid and pituitary hormones in rats exposed to Cd. Both doses of Cd treatment decreased steroids hormones studied in both the age groups. Pituitary hormones LH and FSH showed a varied pattern, FSH decreased in both age groups, while 50 ppm Cd decreased LH level.

Table 1. Effect of lactational exposure to Cd on body and ovary in developing rats (PNDs 45 and 65)

	Body weight (g)			Ovary weight (mg)		
Age	**Control**	**Cd (50 ppm)**	**Cd (200 ppm)**	**Control**	**Cd (50 ppm)**	**Cd (200 ppm)**
PND 45	0.57 ± 0.05	0.46 ± 0.02	0.32 ± 0.02	0.73 ± 0.01	0.56 ± 0.01	0.43 ± 0.01
PND 65	0.98 ± 0.05	0.83 ± 0.09	0.62 ± 0.06	0.94 ± 0.01	0.84 ± 0.008	0.71 ± 0.01

Each value represents the mean and SEM of 30 female rats (from 6 mothers). Cd: Cadmium chloride, PND: postnatal day.

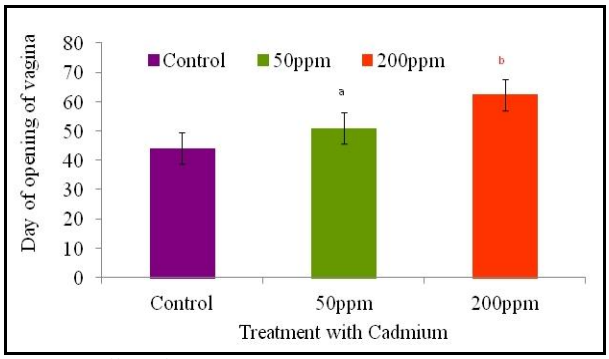

Figure 1. Effect of lactational exposure to cadmium (Cd) on pubertal onset in developing rats. Each bar represents the mean and the vertical line above denotes the SEM of 30 female rats (from 6 mothers). Statistical significance of difference among groups at $P < 0.05$; [a]control *vs* experiment, [b]50 *vs* 200 ppm.

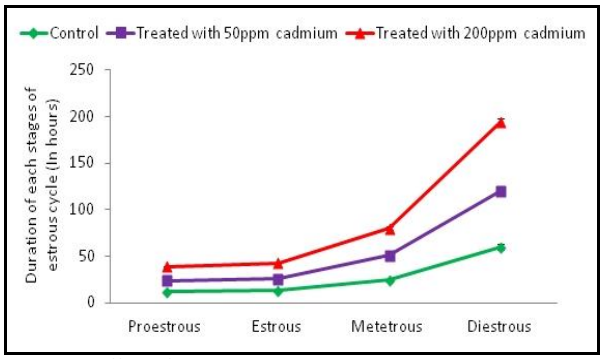

Figure 2. Effect of lactational exposure to cadmium (Cd) on estrous cyclicity in developing rats. Each line represents the mean and the vertical line above denotes the SEM of 30 female rats (from 6 mothers).

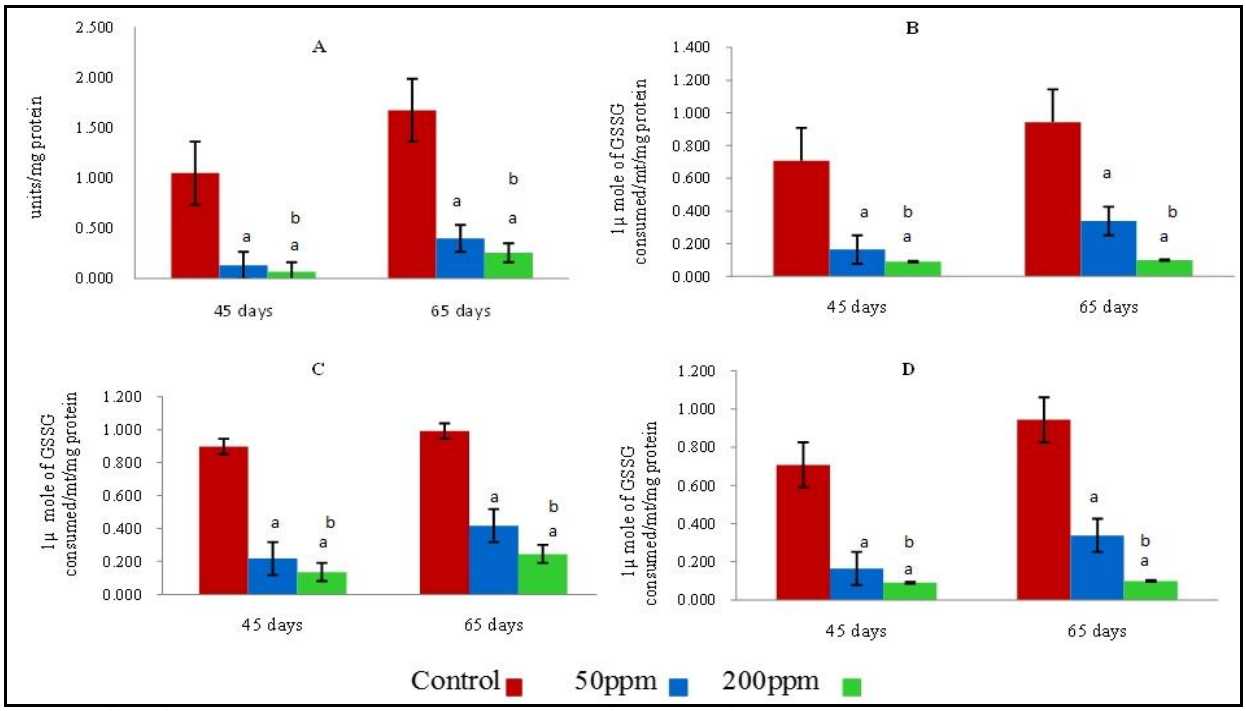

Figure 3. Effect of lactational exposure to cadmium (Cd) on the specific activities of uterine superoxide dismutase (SOD; **A**), catalase (CAT; **B**), glutathione peroxidase (GPx; **C**) and glutathione S-transferase (GST; **D**). Each bar represents the mean and the vertical line above denotes the SEM of 30 female rats (from 6 mothers). Statistical significance of difference among groups at $P < 0.05$; [a]control *vs* experiment, [b]50 *vs* 200 ppm.

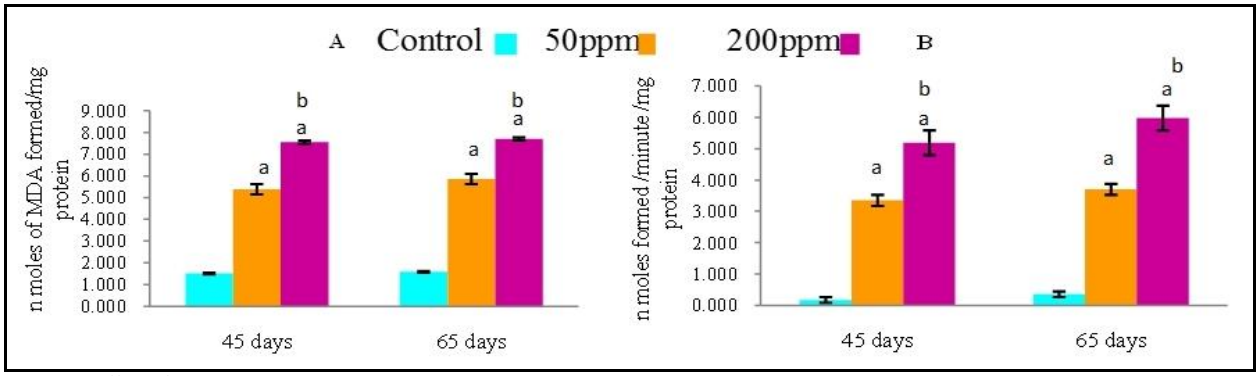

Figure 4. Effect of lactational exposure to cadmium (Cd) on lipid peroxidation (LPO; **A**) and hydrogen peroxide (H_2O_2; **B**). Each bar represents the mean and the vertical line above denotes the SEM of 30 female rats (from 6 mothers). Statistical significance of difference among groups at $P < 0.05$; [a]control *vs* experiment, [b]50 *vs* 200 ppm.

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Table 2. Effect of lactational exposure to cadmium (Cd) on circulating levels of steroid and pituitary hormones in developing rats (postnatal days [PND] 45 and 65)

Hormone	Age	Control	50 ppm	200 ppm
Testosterone	PND 45	0.91 ± 0.06	0.16 ± 0.06[a]	0.01 ± 0[ab]
	PND 65	1.12 ± 0.008	0.19 ± 0.08[a]	0.01 ± 0.02[ab]
Estradiol	PND 45	40.73 ± 0.69	31.14 ± 0.15[a]	21.03 ± 0.53[ab]
	PND 65	44.96 ± 0.41	34.08 ± 0.83[a]	25.36 ± 0.21[ab]
Progesterone	PND 45	7.12 ± 0.09	4.40 ± 0.18[a]	2.10 ± 0.06[ab]
	PND 65	8.46 ± 0.25	5.22 ± 0.56[a]	3.82 ± 0.23[ab]
LH	PND 45	4.75 ± 0.09	3.82 ± 0.12[a]	2.42 ± 0.1[ab]
	PND 65	3.74 ± 0.08	2.78 ± 0.03[a]	1.17 ± 0.18[ab]
FSH	PND 45	10 ± 1.12	5.70 ± 0.08[a]	2.18 ± 0.1[ab]
	PND 65	7.3 ± 0.75	6.72 ± 0.05[a]	2.05 ± 0.01[ab]

Each value represents the mean and SEM of 30 female rats (from 6 mothers). Statistical significance of difference among groups at $P < 0.05$; [a]control *vs* experiment, [b]50 *vs* 200 ppm.

DISCUSSION

There is growing concern about the increasing prevalence of various abnormalities of the reproductive system in humans. Since the reproductive process is critical for perpetuation of any organism, factors or agents that alter or disrupt this process can have devastating consequences. Cadmium also affects reproductive organs [10]. Its action may be either direct, affecting the gonads and accessory organs, or indirect via interference with the hypothalamus-pituitary-gonadal axis [27] The purpose of this study was to investigate the toxic effects of Cd in major female reproductive organs, *viz* ovary. In the present study, body and ovary weight were decreased significantly which may be due to the decreased availability and production of steroids. Sorell and Graziano [28] found reductions in both fetal and maternal weight following maternal consumption of drinking water containing Cd at 50 or 100 ppm. Ovarian weight of pregnant rats were significantly reduced following oral exposure doses of 0.1 and 10 mg, but not 1 mg Cd/kg b.wt/day of 6 weeks prior to mating and gestation [29]. In the present study, Cd exposure exhibited a pubertal delay in a dose-dependent manner compared to control rats with an extended estrous cycle.

Ronis *et al* [30] found out that pre-pubertal lead exposure resulted in significantly delayed vaginal opening in female animals. There was a significant increase in the length of estrous cycle in female rats exposed for 13 weeks to cadmium oxide aerosols at a concentration of 1 mg/m^3 [31]. Data on antioxidant and free radicals clearly establish the development of oxidative stress in the uterus of Cd treated rats, as there was a significant increase in the concentration of H_2O_2 and LPO and subnormal activity of most of the antioxidant enzymes tested. Reactive oxygen and nitrogen species are essential to energy supply, detoxification, chemical signaling and immune function. Ognjanovic *et al* [32] reported that Cd stimulated ROS thus causing oxidative damage in various tissues. When there is an over-production of these species, an exposure to external oxidant substances or a failure in the defense mechanisms, damage to valuable biomolecules (DNA, lipids and proteins) may occur [33]. Cadmium causes a significant increase of LPO in liver and kidney of rats, since it causes LPO in numerous tissues both *in vivo* and *in vitro* [13]. The increase in H_2O_2 production in uterus of Cd-exposed rats might be responsible for the observed increase in the LPO. The increase in H_2O_2 might have induced the peroxidation of polyunsaturated fatty acids and lead to the formation of MDA, one of the by-products of LPO. Since MDA has got high reactivity towards amino groups, it inhibits the synthesis of nucleic acids and proteins and also deactivates the enzymes [22]. Cadmium exposure led to a marked data on antioxidant and free radicals. This associated with reduction of the antioxidant system, *e.g.* GSH and GPx [34]. Estrogen deficiency is also shown to be associated with oxidative stress [35]. Thus, the impaired level of serum steroids may lead to increased level of LPO. Increased LPO may due to decreased activities of SOD and CAT, the free radical scavenging enzymes. Long-term exposure to Cd increased LPO and caused inhibitions of SOD activity indicating oxidative damages in organs [36]. SOD has proven a useful probe for studying the participation of free radicals in reactions involving oxygen, since it acts as a defense against oxidative tissue damage by the dismutation of superoxide radicals [32]. Similarly, when GPx fails to eliminate H_2O_2 from the cell, the accumulated H_2O_2 has been shown to cause inactivation of SOD [37]. The decrease in SOD activity in animals exposed to high dose of metals is associated with increased superoxide

radicals which have been shown to inhibit CAT. Along with CAT, GPx is also involved in the scavenging of H_2O_2. GPx in particular plays a significant role in scavenging peroxides such as H_2O_2 and protects cell membranes from LPO [38]. In the present study, we reported significant decrease in the levels of antioxidant enzymes viz SOD, CAT, GST, GPx in the ovary of Cd treated rats.

Numerous reports in animal models have depicted that Cd intoxication significantly increased the MDA and GPx [39]. Contrary to the previous report, GPx and GST activity were reduced to cadmium toxicity in the presence study. Perhaps the reason was the dysfunctioning of protein synthesizing machinery that was affected by Cd toxicity. The GST enzyme has an important role in detoxification of xenobiotics, drugs and carcinogens and thus protects the cells against redox cycling and oxidative stress [40, 41]. Thus with increased Cd toxicity there is enhanced production of H_2O_2 in the ovary of developing rats which is accompanied by minimal activity of GPx and GST activity. This could result in oxidative stress in the ovary of these animals.

The pituitary gland and the hypothalamus are considered to be classical targets for reproductive toxicants. During the antral follicular development, FSH and LH receptors are present on granulose and theca cells, respectively. During follicular phase, E2 synthesis increases in response to FSH and LH levels. In the present study, Cd alters LH and FSH levels in an age- and dose-dependent manner; this, along with the decreased steroid levels was also observed in previous studies [16, 21]. The previous study reported the accumulation of chromium (Cr) in serum and ovary of rats exposed to hexavalent chromium and the decrease in the ovarian follicle number [21].

Considering our observations collectively, Cd decreases the specific activities of SOD, CAT, GPX and GST, while serum titers of FSH and LH were decreased both in 50 and 200 ppm Cd treated rats in an age-dependent manner. In additon, Cd treatment to female Wistar rats induced the action of LPO and H_2O_2. Thus, the present study on ovary clearly points out the poor reproductive health after Cd exposure. It may be concluded that Cd affects the female reproductive health in rats. Our results support the hypothesis that Cd-induced changes in the ovarian function are associated with oxidative stress and delayed puberty. Studies at the histology could further strengthen the hypothesis proposed.

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