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Retraction notice

At the request of the Editor and the Publisher, the following article:

Samuel JB, Stanley JA, Vengatesh G, Princess RA, Muthusami S, Roopha DP, Suthagar E, Kumar KM, Sebastian MS, Aruldhas MM. (2012) Ameliorative effect of vitamin C on hexavalent chromium-induced delay in sexual maturation and oxidative stress in developing Wistar rat ovary and uterus, *Toxicology and Industrial Health* 28(8): 720-733. DOI: 10.1177/0748233711422728

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has been retracted from *Toxicology and Industrial Health* due to redundant publication by the same group of authors in another journal:

Samuel JB, Stanley JA, Roopha DP, Vengatesh G, Anbalagan J, Banu SK, Aruldhas MM. (2011) Lactational hexavalent chromium exposure-induced oxidative stress in rat uterus is associated with delayed puberty and impaired gonadotropin levels, *Human & Experimental Toxicology* 30(2):91-101. DOI: 10.1177/0960327110364638

http://het.sagepub.com/content/30/2/91.abstract

The two papers have used the same data and reporting with the *Toxicology and Industrial Health* manuscript inserting Vitamin C to differ the reporting. Additionally, the *Toxicology and Industrial Health* manuscript does not provide citations to original work by another author.

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Ameliorative effect of vitamin C on hexavalent chromium-induced delay in sexual maturation and oxidative stress in developing Wistar rat ovary and uterus

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Abstract

Hexavalent chromium (CrVI) is a highly toxic metal and major environmental pollutant and is extensively used in more than 50 industries. The major route of CrVI exposure for the general population is oral intake. Chromium is considered an important nutrient responsible for carbohydrate metabolism. However, excess CrVI exposure is associated with various pathological conditions including reproductive dysfunction. CrVI can traverse the placental barrier and cause wide range of abnormalities in fetal development. Cr is transported to offspring through mother's milk in lactating women exposed to CrVI. Therefore, the present study was carried out to determine the toxic effects of lactational CrVI exposure on ovary and uterus and the beneficial role of vitamin C in preventing/ameliorating the toxic effects of CrVI in developing female Wistar rats. Generation of oxidative stress is considered one of the plausible mechanisms behind Cr-induced cellular deteriorations. The present study evidenced a decrease in the specific activities of antioxidants, serum testosterone and progesterone and an increase in the levels of H_2O_2, lipid peroxidation (LPO) and follicle stimulating hormone in rats exposed to CrVI when compared to control. CrVI exposure also delayed the sexual maturation and extended the estrous cycle. Simultaneous administration of vitamin C significantly prevented the increase in LPO and enhanced the antioxidant status. These results suggest the protective effect of vitamin C against the CrVI exposure-induced toxicity and attest the significance of antioxidants in diet.

Keywords

Chromium, ovary, uterus, oxidative stress, sexual maturation, vitamin C

In recent years, contamination of the environment by chromium (Cr) has become a major area of concern. Cr exists in multiple oxidation states. The hexavalent chromium (CrVI) and trivalent chromium (CrIII) states are most important from a biological and an industrial standpoint. CrIII is an important nutrient for the human body; it enhances the action of insulin by increasing the number of insulin receptors, and enhances insulin binding and insulin sensitivity (Anderson, 2000). CrVI is an industrial contaminant of landfills and waterways (Cohen et al., 1993) and used on a large scale in more than 50 industries including metallurgical, electroplating, production

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of paints and pigments, tanning, wood preservation, pulp and paper production (Barceloux, 1999). The tanning industry is the large contributor of Cr pollution in water resources. In India about 2000–3200 tones of elemental Cr escape into the environment annually from the tanning industries, with a Cr concentration ranging between 2000 and 5000 mg L^{-1} in the effluent compared to the recommended permissible limit of 2 mg L^{-1} (Chandra et al., 1997). Sukinda (Orissa), Vapi (Gujarat) and Ranipet (Tamil Nadu) in India were listed as the world's top 10 worst polluted places because their drinking water is heavily contaminated with CrVI (The Blacksmith Institute, 2007). Excessive levels of Cr have been shown to produce various health hazards including reproductive dysfunction (Al-Hamood et al., 1998). The other health hazards caused by Cr include dermatitis bronchitis, lung cancer and nasal ulceration (Burrows, 1978; Halasova et al., 2009).

Occupational exposure to Cr is found among approximately half a million industrial workers in the United States and several millions worldwide; significant contamination with CrVI has been found in approximately 30% of the drinking water sources in California (Salnikow and Zhitkovich, 2008). The deposition of CrVI wastes in landfills and waterways by chromate industries affects millions of people who reside in the vicinity of dangerously polluted sites. Women working in dichromate manufacturing industries and living around Cr-contaminated areas encounter gynecological illness, postnatal hemorrhage and birth complications with high levels of Cr in blood and urine (Greene et al., 2010; Jendryczko et al., 1984; Shmitova, 1980; Zhang et al., 1992).

It is known that environmental chemicals can affect the function of female reproductive organs (Hoyer and Sipes, 1996). CrVI can traverse the feto-placental barrier and also transported to offspring through milk (Bailey et al., 2006; Banu et al., 2008; Junaid et al., 1996; Rodriguez et al., 2008; Samuel et al., 2011b). Earlier studies point out the deleterious effects of CrVI on the structure and function of ovary (Banu et al., 2008; Rao et al., 2009). CrVI exposure results in enhanced formation of reactive oxygen species (ROS) including super oxide anions, hydroxyl radical and nitric oxide, decreased cell viability, increased cellular genomic hepatic DNA fragmentation, enhanced intracellular oxidizing states, membrane damage, apoptotic and necrotic cell death (Bagchi et al., 2002). Previous findings demonstrated that Cr, administrated through drinking water led to abnormal gestational changes in mice and rats, with a decreased number of implantation sites (Junaid et al., 1996; Kamath et al., 1997). Pregnant mice exposed to CrVI through drinking water during gestation resulted in increased pre-implantation and post-implantation loss and decreased litter size (Trivedi et al., 1989). Delayed sexual maturation and reduced fertility were observed in female mice offspring treated with CrVI (Al-Hamood et al., 1998). Earlier, we have reported the ovotoxic nature of CrVI, where lactational CrVI exposure delayed follicular development and impaired ovarian steroidogenesis (Banu et al., 2008). Recently, we have reported the uterotoxic effects of CrVI, where the lactational exposure to CrVI are associated with delayed puberty and oxidative stress (Samuel et al., 2011b).

CrVI can escape from primary contact organs and blood erythrocytes and reach different organs (Barceloux, 1999; Dayan and Paine, 2001). CrVI is a well-known oxidizing agent (Bagchi et al., 1995). In biological systems, after entry into cells, CrVI is rapidly detoxified/reduced to CrIII by intracellular defensive reductant system that includes ascorbate, glutathione (GSH) and cysteine (Valko et al., 2005). Antioxidant compounds have been used to protect cells from various oxidative damages. Vitamin C is known to be an antistress and a powerful reducing agent and plays an important role in attenuating oxidative damage to the cellular membranes (Sugiyama, 1992). Vitamin C accounts for 80% of CrVI metabolism in target tissues such as lung, liver and kidney (Zhitkovich, 2005). In view of the existing background, the present study is aimed to test the hypothesis, 'Vitamin C prevents the lactational CrVI exposure-induced disruption of ovarian and uterine function in Wistar rats.'

Materials and methods

Animals and treatments

Pregnant Wistar rats (*Rattus norvegicus*), 90 days old, were maintained in plastic animal cages in a light controlled room (12-h day light) at 23–25°C and provided with standard rat pellet diet and clean drinking water *ad libitum*.

CrVI doses were selected based on our previous reports (Banu et al., 2008; Samuel et al., 2011b). Pregnant rats were divided into three groups. At birth, male pups were removed and the litters were culled to four female pups per mother rat. In cases where fewer than four female pups were delivered, additional pups were

fostered from other litters. (i) In the control group, the rats received regular drinking water from the day of parturition to day 21 postpartum. (ii) In CrVI-treated group, rats received CrVI (potassium dichromate, 200 mg/L) in drinking water from the day of parturition to day 21 postpartum. (iii) In CrVI + vitamin C group, rats received CrVI (200 mg/L) and vitamin C (ascorbate: 500 mg/L) simultaneously in drinking water from the day of parturition to day 21 postpartum.

During days 1–21 of postnatal life (postnatal day [PND] 1–21), the female pups were exposed to respective CrVI through their mother's milk. After weaning, on PND 45, female pups from 6 mother rats ($n = 24$; 4 pups/mother) from each group (control, CrVI-200 ppm, CrVI-200 ppm + vitamin C) were killed by decapitation; and their blood, ovary and uterus were collected. The remaining pups were maintained separately and fed with regular drinking water and diet. On PND 65, the rest of the rats ($n = 24$) were killed, and their blood, ovary and uterus were dissected, cleansed and used for biochemical assay. All the chemicals used in the present study were purchased from Sigma Chemical Company, St Louis, USA.

Tissue preparation for enzyme assays

Ovary and uterus were dissected out and washed in ice cold physiological saline repetitively, weighed accurately and homogenized in 0.1 mol/l Tris-HCl buffer, pH 7.4. The protein concentrations of the tissue homogenates were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. The detailed methodology for the antioxidant enzymes and ROS assays were described in our earlier article (Samuel et al., 2011b).

Antioxidant enzymes

SOD activity was estimated according to the method of Marklund and Marklund (1974). The enzyme activity was expressed as U/mg protein. Catalase (CAT) activity was quantified colorimetrically (Sinha, 1972) and expressed as mmoles of H_2O_2 utilized/min/mg protein. Glutathione peroxidase (GPx) activity was determined colorimetrically (Rotruck et al., 1973) and expressed as U/mg protein. Glutathione-*S*-transferase (GST) activity was determined by estimating the amount of enzyme that catalyzed the conjugation of a known amount of 1-chloro-2,4-dibenzene with GSH (Habig et al., 1974) and expressed as μmoles of GSH consumed/min/mg protein. Glutathione reductase (GR) activity was assayed colorimetrically (Staal et al., 1969) and expressed as nmoles of NADPH oxidized/min/mg protein.

Reactive oxygen species

Lipid peroxidation (LPO) was measured by the method of (Devasagayam and Tarachand, 1987). The results were expressed as nmoles of malondialdehyde (MDA) formed/mg protein. H_2O_2 production was assessed spectrophotometrically (Holland and Storey, 1981) and the content was expressed as μmoles of H_2O_2/mg protein.

Chromium estimation

Chromium level in ovary and uterus. Cr levels in ovary and uterus of control and CrVI-exposed rats were estimated as described earlier (Banu et al., 2008; Samuel et al., 2011b). To provide enough tissue for analysis, ovary and uterus in a dose group were pooled whenever needed. Fifty milligram of tissue was digested in 1 ml of concentrated HNO_3 and 1 ml of perchloric acid over a sand bath until the solution became pale yellow. If the color of the digest was brown, more HNO_3 and perchloric acid were added and the oxidation was repeated. The digest was made up to 1 ml with deionized water. Aliquots of this were used to estimate Cr, using an atomic absorption spectrometer (Perklin Elmer 2380), at 357.8 nm wavelength. The values are expressed as μg/g tissue.

Chromium level in serum. Serum Cr was directly quantified (Davidson and Secrest, 1972). Serum samples were diluted to the ratio of 1:10 with diluent containing 0.1% Triton and 0.1% HNO_3 in distilled deionized water. Aliquots of this were used to estimate the Cr content, using an atomic absorption spectrometer (Perklin Elmer 2380) at 357.8 nm wavelength. The values are expressed as μg/ml of serum.

Sexual maturation and estrous cycle. Vaginal opening was observed in rats from 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 (Banu et al., 2008; Samuel et al., 2011a, b).

Radioimmunoassay (RIA) of steroid hormones. Serum levels of estradiol, progesterone and testosterone

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

	Body weight (g)			Ovary weight (mg)			Uterus weight (mg)		
Age	Control	CrVI	CrVI + Vit. C	Control	CrVI	CrVI + Vit. C	Control	CrVI	CrVI + Vit. C
PND 45	69 ± 3.6	51 ± 3.8[a]	63 ± 2.7	54 ± 2.2	41 ± 1.9[a]	50 ± 2.4[b]	287 ± 12.4	256 ± 15.9[a]	266 ± 13.7
PND 65	98 ± 7.2	65 ± 5.3[a]	85 ± 5.9	62 ± 3.8	52 ± 4.7[a]	64 ± 5.2[b]	389 ± 20.2	328 ± 26.6[a]	354 ± 21.5

CrVI: hexavalent chromium, PND: postnatal day, Vit. C: vitamin C.
Each value represents the mean and SEM of 24 female rats (from 6 mothers). See Materials and methods section for experimental details; statistical significance of difference among groups at $p < 0.05$.
[a]Control versus experiment.
[b]CrVI versus CrVI + vitamin C.

were estimated by RIA as described earlier (Banu et al., 2008; Samuel et al., 2011a, b). 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 binding of the testosterone antibody was 36%, and the sensitivity of the assay was 0.3 pg testosterone/ml. Testosterone levels in serum are expressed as ng/ml. The maximum binding of progesterone antibody was 40% and the sensitivity of the assay was 0.3 pg/ml. Progesterone level is expressed as ng/ml.

Radioimmunoassay of peptide hormones. Serum levels of luteinising hormone (LH) and follicle stimulating hormone (FSH) were assayed by RIA as described earlier (Banu et al., 2008; Samuel et al., 2011b). The maximum binding of the LH antibody was 31.36% and the sensitivity of the assay was 0.2 ng/ml. The maximum binding of FSH antibody was 31% and the sensitivity of the assay was 0.2 ng/ml. LH and FSH levels were expressed as ng/ml.

Statistical analysis

Data were statistically analyzed using analysis of variance. When the F ratio was statistically significant, the data were subjected to Students Newman-Keul's test. Values were considered significant at $p < 0.05$.

Results

Lactational CrVI exposure leads to age-dependent changes in the specific activities of antioxidant enzymes, free radicals and hormones in postnatal rat ovary and uterus and simultaneous vitamin C treatment prevented the toxic effects of CrVI.

Body, ovary and uterus weight

Lactational CrVI exposure significantly decreased the body weight, ovary weight and uterus weight in both the age groups of CrVI-treated rats. Vitamin C treatment completely prevented the CrVI-induced decrease in body and uterus weight in PND 45 and PND 65 rats; however, vitamin C partially prevented the changes in ovary weight in both PND 45 and PND 65 rats (Table 1).

Chromium content in serum, ovary and uterus

In order to check, whether the decreased body, ovary and uterus weight is associated with the bioavailability of Cr, we have estimated the levels of Cr in serum, ovary and uterus of experimental offspring (Table 2). Cr content was significantly different between serum, ovary and uterus at basal conditions. Serum, ovary and uterus of Cr-exposed animals have higher Cr content than control animals. Vitamin C treatment prevented accumulation of Cr partially in PND 45 rats and completely in PND 65 rats.

Sexual maturation

Vaginal opening has been used as an index of sexual maturation. To evaluate the effect of CrVI on the sexual maturation, we have examined the vaginal opening every 24 h in the experimental rats from PND 22 onwards (Figure 1). CrVI exposure significantly delayed the sexual maturation in postnatal rats, while vitamin C restored the CrVI exposure-induced delay in sexual maturation.

Estrous cyclicity

In order to evaluate the effect of CrVI on the duration of estrous cycle, vaginal cytology was determined daily (Figure 2). CrVI exposure significantly extended the estrous cycle in diestrous stage when compared to

Table 2. Effect of lactational exposure to CrVI on the bioavailability of chromium in the serum, ovary and uterus in developing rats (PNDs 45 and 65)

	Serum level (μg/ml)			Ovary level (μg/g)			Uterus level (μg/g)		
Age	Control	CrVI	CrVI + Vit. C	Control	CrVI	CrVI + Vit. C	Control	CrVI	CrVI + Vit. C
PND 45	0.13 ± 0.02	0.35 ± 0.05[a]	0.29 ± 0.02[a]	0.38 ± 0.04	0.62 ± 0.07	0.43 ± 0.04[b]	0.33 ± 0.03	0.58 ± 0.04[a]	0.43 ± 0.04[a,b]
PND 65	0.11 ± 0.01	0.19 ± 0.01[a]	0.15 ± 0.02[a,b]	0.31 ± 0.04	0.59 ± 0.06[a]	0.33 ± 0.05[b]	0.32 ± 0.05	0.53 ± 0.05[a]	0.39 ± 0.03[b]

CrVI: hexavalent chromium, PND: postnatal day, Vit. C: vitamin C.
Each value represents the mean and SEM of 24 female rats (from 6 mothers). See Materials and methods section for experimental details; statistical significance of difference among groups at $p < 0.05$.
[a]Control versus experiment.
[b]CrVI versus CrVI + vitamin C.

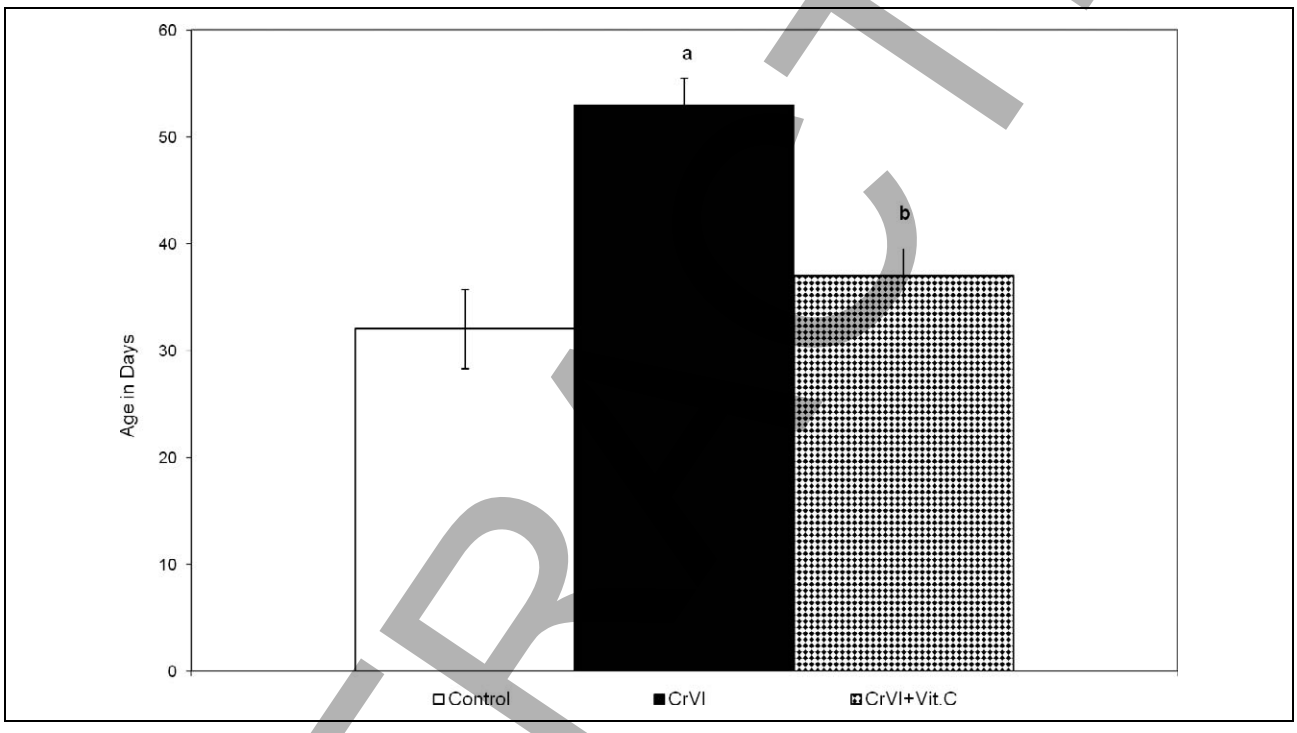

Figure 1. Effect of lactational exposure to hexavalent chromium (CrVI) on sexual maturation in developing rats (postnatal days [PNDs] 45 and 65). Each bar represents the mean and the vertical line above denotes the SEM of 24 female rats (from 6 mothers). See Materials and methods section for experimental details; statistical significance of difference among groups at $p < 0.05$; [a]control versus experiment; [b]CrVI versus CrVI + vitamin C.

control rats. CrVI exposure did not alter proestrous, estrous and metestrous phases of estrous cycle. Vitamin C prevented the extension of estrous cycle in CrVI-exposed rats.

Antioxidant enzymes

Effect of CrVI on the specific activities of different antioxidant enzymes in rat ovary and uterus are described in Figures 3(a–e) and 4 (a–e). CrVI exposure decreased the specific activities of SOD, CAT, GPx, GR and GST in the rat ovary and uterus of PND 45 and PND 65 rats. Vitamin C treatment partially prevented the CrVI exposure-induced decrease in ovarian SOD, CAT, GPx and GR levels in PND 45 rats, while it is completely restored in PND 65 rats. However, CrVI exposure-induced decrease in uterine antioxidants SOD, CAT, GPx, GST and GR were partially prevented by vitamin C treatment in PND 45 and PND 65 rats.

Lipid peroxidation and H_2O_2 concentration

Figures 5 (a, b) and 6 (a, b) represent the levels of MDA and H_2O_2 in control, CrVI with or without

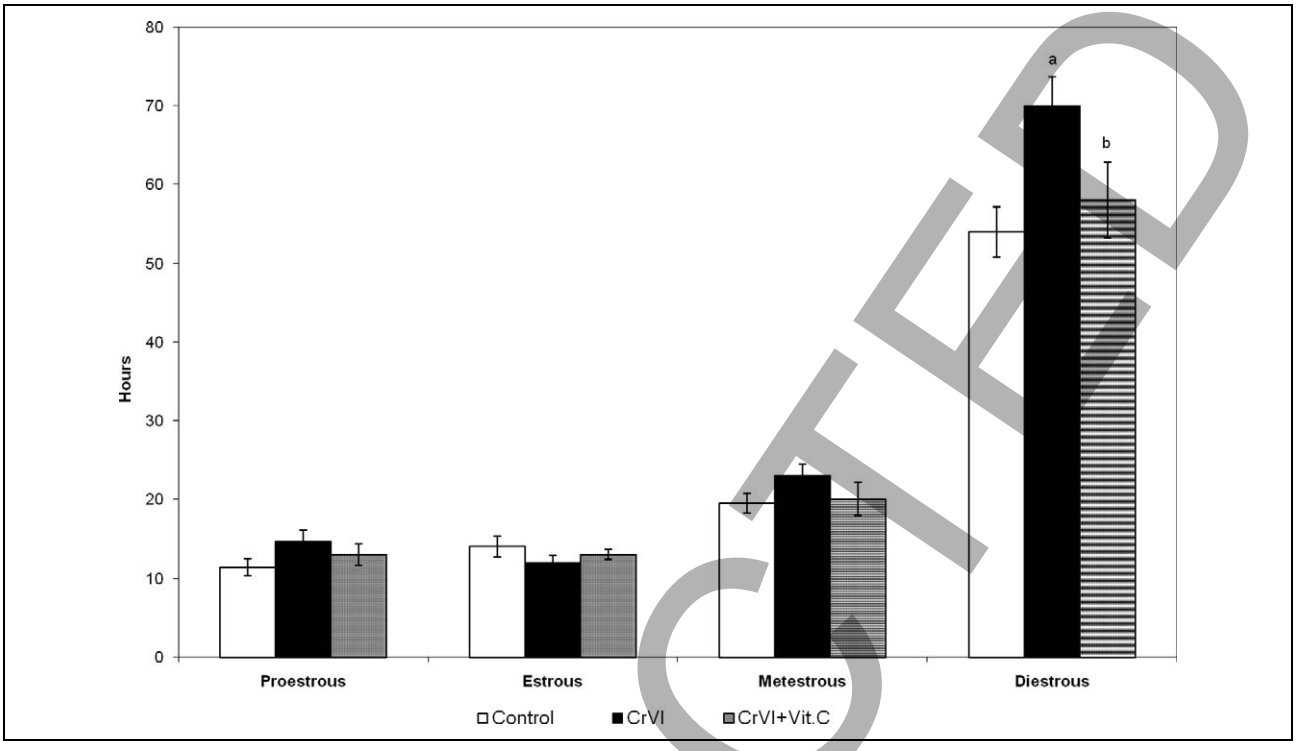

Figure 2. Effect of lactational exposure to hexavalent chromium (CrVI) on estrous cycle in developing rats (postnatal days [PNDs] 45 and 65). Each bar represents the mean and the vertical line above denotes the SEM of 24 female rats (from 6 mothers). See Materials and methods section for experimental details; statistical significance of difference among groups at $p < 0.05$; [a]control versus experiment; [b]CrVI versus CrVI + vitamin C.

vitamin C treated ovary and uterus of PND 45 and PND 65 rats. Increased concentrations of LPO and H_2O_2 generation were observed in CrVI-treated ovaries and uterus of PND 45 and PND 65 rats. However, simultaneous vitamin C treatment partially prevented the CrVI-exposure-induced increase in LPO and H_2O_2 levels in PND 45 and PND 65 uterus and PND 65 ovary, while it is completely prevented in PND 65 rat ovary.

Serum hormones

Table 3 represents circulating levels of steroid and pituitary hormones in postnatal rats exposed to CrVI and CrVI along with vitamin C. CrVI exposure decreased steroid hormones in both PND 45 and PND 65 rats, while vitamin C prevented the CrVI-exposure-induced decrease in steroid hormone levels. Pituitary hormones LH and FSH showed a varied pattern, FSH and LH significantly increased in PND 45 rats, while PND 65 rats did not show any significant difference between control and CrVI-exposed rats. Vitamin C treatment prevented the CrVI-exposure-induced increase in LH and FSH levels.

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. CrVI is the established endocrine disruptor reported to disturb the reproductive process in both males (Aruldhas et al., 2005) and females (Banu et al., 2008; Rao et al., 2009; Rodriguez et al., 2008; Samuel et al., 2011b). The purpose of this study was to investigate the toxic effects of CrVI in two major female reproductive organs, viz., ovary and uterus and to examine the possible beneficial role of vitamin C in preventing CrVI-induced female reproductive toxicity. In the present study, body and organ (ovary and uterus) weight were decreased significantly. This was consistent with our earlier reports (Samuel et al., 2011b) and also with others (Al-Hamood et al., 1998; Rao et al., 2009). The failure of postnatal rats exposed to CrVI through mother's milk to gain body weight may be due to decreased feed intake, malabsorption of nutrients from the gastrointestinal tract and impaired feed

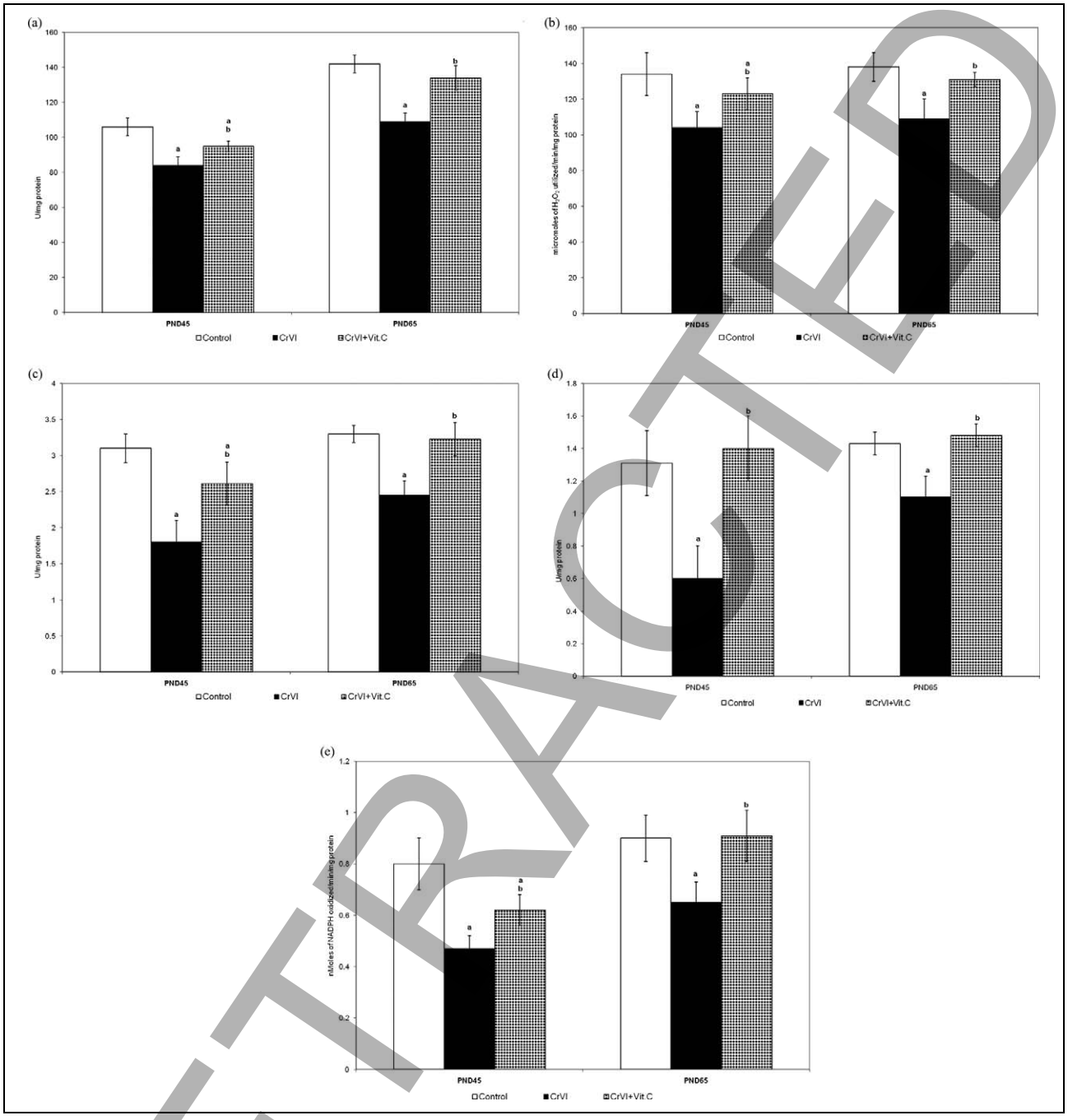

Figure 3. Effect of lactational exposure to hexavalent chromium (CrVI) on the specific activities of ovarian superoxide dismutase (SOD; a), catalase (b), glutathione peroxidase (GPx; c), glutathione-*S*-transferase (GST; d) and glutathione reductase (GR; e). Each bar represents the mean and the vertical line above denotes the SEM of 24 female rats (from 6 mothers). See Materials and methods section for experimental details; statistical significance of difference among groups at $p < 0.05$; [a]control versus experiment; [b]CrVI versus CrVI + vitamin C.

conversion efficiency (Ball and Chhabra, 1981). Cr was shown to be an essential micronutrient required for normal carbohydrate and lipid metabolism and acts as a cofactor for enzymes like cytochrome C, phosphoglucomutase and was involved in nucleic acid metabolism (Mertz, 1993). The observed decrease in body and organ (ovary and uterus) weight may be due to the influence of CrVI on the intermediary metabolism. Vitamin C treatment prevented the CrVI-exposure-induced reduction in

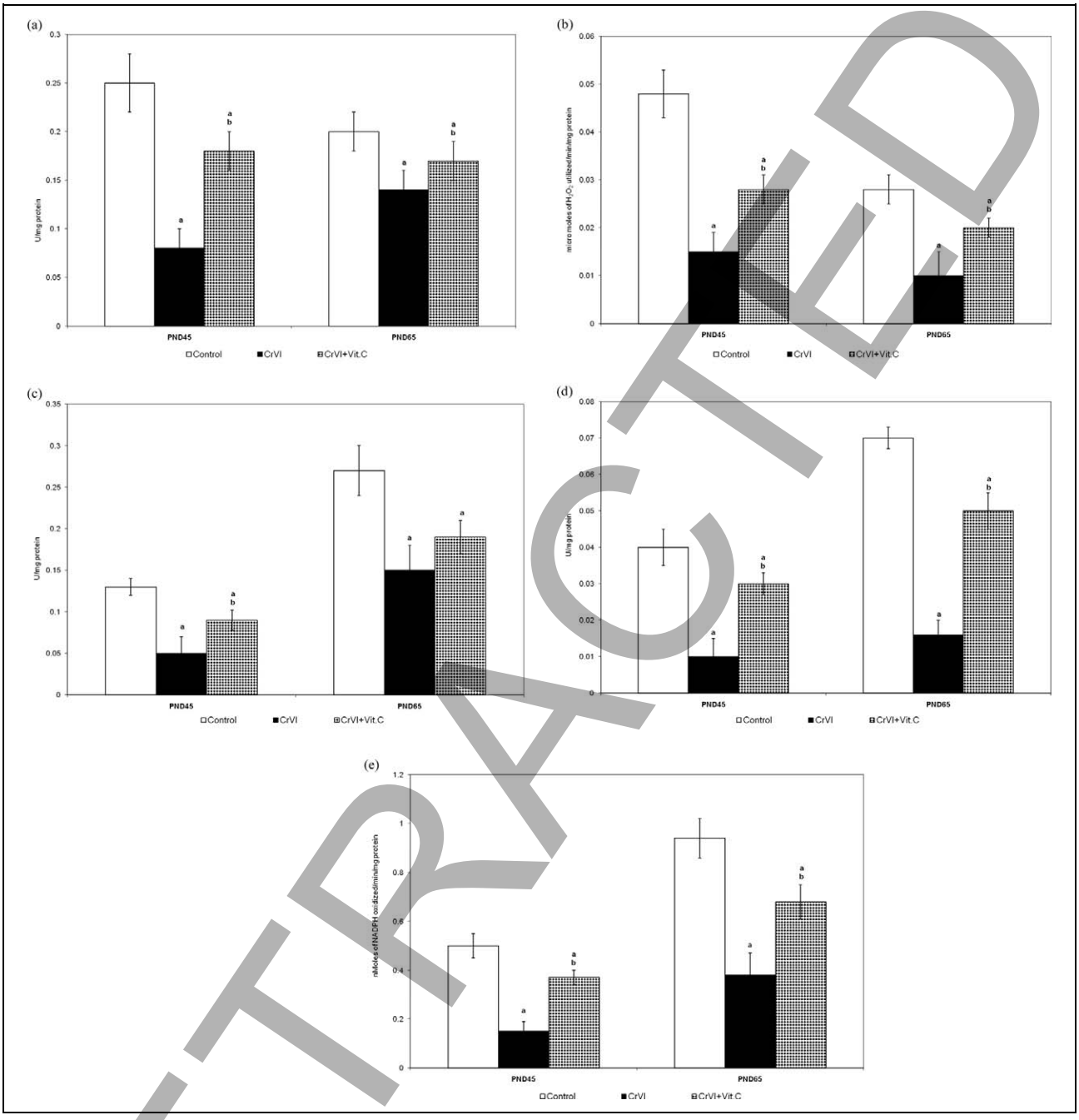

Figure 4. Effect of lactational exposure to hexavalent chromium (CrVI) on the specific activities of uterine superoxide dismutase (SOD; a), catalase (b), glutathione peroxidase (GPx; c), glutathione-S-transferase (GST; d) and glutathione reductase (GR; e). Each bar represents the mean and the vertical line above denotes the SEM of 24 female rats (from 6 mothers). See Materials and methods section for experimental details; statistical significance of difference among groups at $p < 0.05$; [a]control versus experiment; [b]CrVI versus CrVI + vitamin C.

body and uterus weight in both PND 45 and PND 65 rats. However, the toxic effects of CrVI on ovary was partially prevented by vitamin C. Earlier, we have reported the ovotoxic and uterotoxic nature of CrVI, where CrVI treatment resulted in permanent damage to ovarian cell population (Banu et al., 2008) with extended estrous cycle (Samuel et al., 2011b).

The involvement of oxidative stress was found in heavy metal toxicity (Susa et al., 1996). Cr-induced ROS can damage the cellular element in its target organs (Bagchi et al., 2001). CrVI produces excess

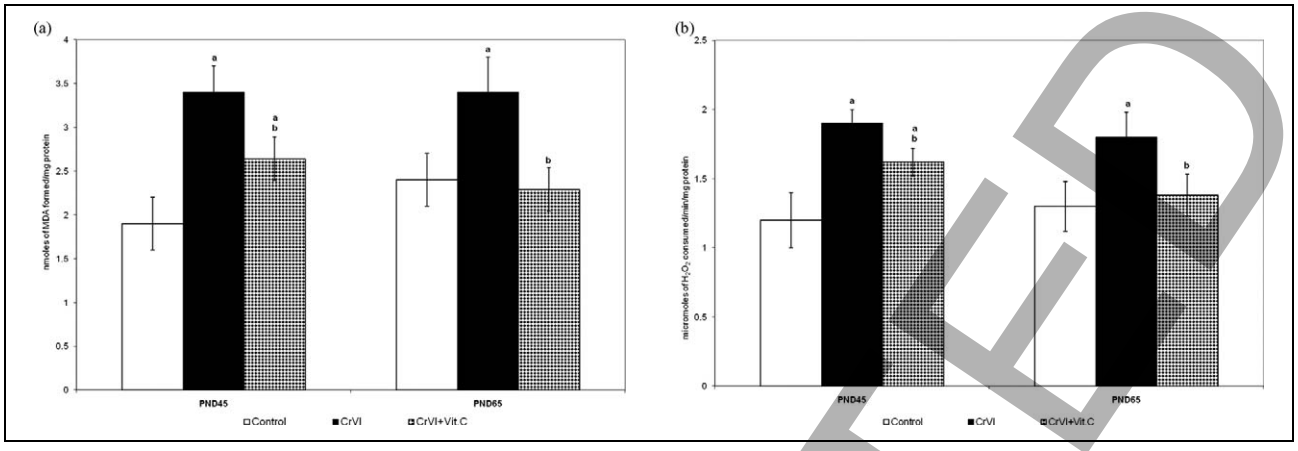

Figure 5. Effect of lactational exposure to hexavalent chromium (CrVI) on ovarian lipid peroxidation (LPO; a) and H_2O_2 (b). Each bar represents the mean and the vertical line above denotes the SEM of 24 female rats (from 6 mothers). See Materials and methods section for experimental details; statistical significance of difference among groups at $p < 0.05$; [a]control versus experiment; [b]CrVI versus CrVI + vitamin C.

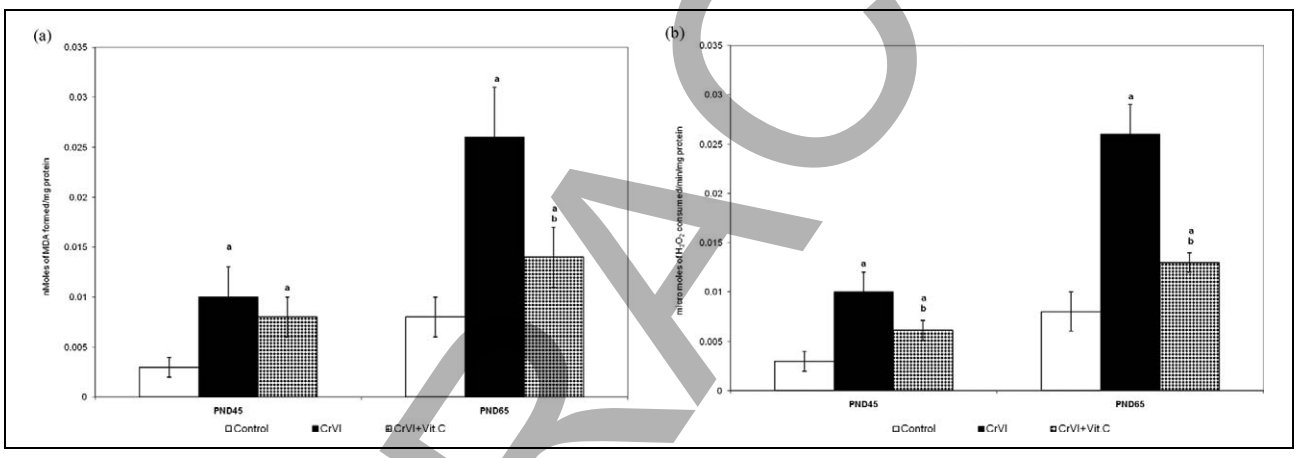

Figure 6. Effect of lactational exposure to hexavalent chromium (CrVI) on uterine lipid peroxidation (LPO; a) and H_2O_2 (b). Each bar represents the mean and the vertical line above denotes the SEM of 24 female rats (from 6 mothers). See Materials and methods section for experimental details; statistical significance of difference among groups at $p < 0.05$; [a]control versus experiment; [b]CrVI versus CrVI + vitamin C.

ROS including superoxide anion, singlet oxygen and hydroxyl radicals (Kawanishi et al., 1986). CrVI-exposure-induced impairment of the pituitary-ovarian axis in Teleost was reported (Mishra and Mohanty, 2010). CrVI-induced oxidative stress in mouse ovary (Rao et al., 2009) and rat uterus (Samuel et al., 2011b) were known. Results from the present investigation indicate the induction of oxidative stress in the ovary and uterus of rats exposed to CrVI through mother's milk, as there was a significant increase in the concentration of H_2O_2 and LPO with subnormal activity of the most of the antioxidant enzymes tested. As a transition metal, Cr can replace iron and copper in Fenton reaction and generate hydroxyl radical which is able to initiate LPO, leading to increased MDA concentration, one of the byproducts of LPO (Dey et al., 2003; Kalahasthi et al., 2006; Sawicka et al., 2010). The increase in H_2O_2 might induce the peroxidation of polyunsaturated fatty acids and lead to the formation of MDA. Higher concentration of MDA in serum of chrome-plating industry workers (Huang et al., 1999) and in mouse ovary exposed to CrVI (Rao et al., 2009) further highlights the involvement of oxidative stress in Cr-induced toxicity. Recently, we showed higher levels of MDA in rat uterus exposed to CrVI (Samuel et al., 2011b). Vitamin C treatment prevented the CrVI-exposure-induced oxidative stress and LPO in both ovary and uterus. Data from the present study revealed decreased levels of serum testosterone,

Table 3. Effect of lactational exposure to CrVI on circulating levels of steroid and pituitary hormones in developing rats (PNDs 45 and 65)

Hormone	Age	Control	CrVI	CrVI + Vit. C
Testosterone	PND 45	0.94 ± 0.07	0.45 ± 0.04[a]	0.89 ± 0.03[b]
	PND 65	1.07 ± 0.08	0.56 ± 0.05[a]	0.93 ± 0.07[b]
Estradiol	PND 45	43.8 ± 4.0	24.1 ± 2.3[a]	46.7 ± 4.5[b]
	PND 65	40.9 ± 3.5	27.3 ± 2.1[a]	38.9 ± 4.7[b]
Progesterone	PND 45	6.42 ± 0.45	3.70 ± 0.68[a]	5.53 ± 0.74[b]
	PND 65	9.01 ± 0.85	5.21 ± 0.68[a]	8.42 ± 0.82[b]
LH	PND 45	7.35 ± 0.85	9.34 ± 1.21[a]	7.90 ± 1.40
	PND 65	5.86 ± 0.55	7.49 ± 0.95	6.97 ± 0.77
FSH	PND 45	10.0 ± 1.12	16.47 ± 1.58[a]	11.6 ± 1.74[b]
	PND 65	9.30 ± 0.75	10.1 ± 1.28[a]	10.4 ± 1.84

CrVI: hexavalent chromium, FSH: follicle stimulating hormone, LH: luteinising hormone, PND: postnatal day, Vit. C: vitamin C.
Each value represents the mean and SEM of 24 female rats (from 6 mothers). See Materials and methods section for experimental details; statistical significance of difference among groups at $p < 0.05$.
[a]Control versus experiment.
[b]CrVI versus CrVI + vitamin C.

estradiol and progesterone. Estrogen deficiency was shown to be associated with oxidative stress (Muthusami et al., 2005). Thus, the impaired level of serum steroids observed in the present study might have led to increased level of MDA in ovary and uterus.

The levels of MDA were increased in both ovary and uterus of PND 45 and PND 65 rats exposed to CrVI through mother's milk. The observed increase in LPO was many folds higher in ovary than uterus. The increase in LPO may also be due to decreased activities of SOD and CAT, the free radical scavenging enzymes. The enzyme SOD catalyses dismutation of superoxide radical, leading to the formation of H_2O_2, which inturn was detoxified by the enzymes GPx and CAT (Rzeuski et al., 1998). The present study also showed decreased levels of SOD and CAT in both ovary and uterus of PND 45 and PND 65 rats exposed to CrVI and the decrease in SOD activity may result in more accumulation of O_2^-, which in turn may inhibit other antioxidant enzymes. The decreased activities of SOD and CAT in the present study were concomitant with our previous reports (Samuel et al., 2011b) and with that of Rao et al. (2009). CAT is the main scavenger of H_2O_2 at high concentration. Along with CAT, GPx is also involved in scavenging H_2O_2 and thereby protects cell membrane from LPO (Samanta et al., 1999).

The present study indicates that CrVI-exposed offspring exhibited delayed sexual maturation with extended estrous cycle when compared to control rats. The risk of damage to the ovarian follicle cell population from Cr depends mainly on the accessibility of Cr to the follicles. After entering the body by the oral route, CrVI reduced to CrIII by fluids in the digestive tract and then it can be sequestered by intestinal bacteria (Metze et al., 2005). CrVI that escapes reduction in digestive tract could also be reduced in the blood of the portal vein system and then in liver (De Flora et al., 1997). In spite of these protective mechanisms, CrVI can reach several other reproductive tissues, where it can be accumulated. In the present study, the post natal rats exposed to CrVI through mother's milk had increased levels of Cr in serum, ovary and uterus. Difference between ovary and uterine Cr accumulation could be due to distinctive properties of each tissue. Earlier, we have reported that higher accumulation of Cr reduced ovarian follicular cell population (Banu et al., 2008). In the present study, the observed oxidative stress and the Cr accumulation in target tissues might have delayed sexual maturation. CrVI is reported to arrest the cell cycle at G2/M phase along with decreased CAT levels in lung epithelial cells (Zhang et al., 2001). A similar mechanism might operate in the present study, which could have contributed to the decrease in ovarian follicle number through arrest in G2/M cell cycle in CrVI treated rats. Vitamin C treatment partially prevented the CrVI-exposure-induced accumulation of Cr in serum, ovary and uterus of PND 45 and PND 65 rats. The present study also showed decreased levels of sex steroid hormone levels. The decreased levels of steroid hormones observed in the present investigation might be due to the CrVI-induced decrease in the number of ovarian follicles and the associated oxidative stress.

Estradiol is not only associated with follicular maturation and oxidative stress but also regulates LH and FSH from the pituitary through positive and negative feedback on the hypothalamus and pituitary (Hoyer and Sipes, 1996). Earlier, we have reported the decreased expression of steroidogenic genes in ovarian cells exposed to CrVI (Banu et al., 2008). We have also reported the toxic effects of CrVI on developing rat ovary and uterus (Banu et al., 2008; Rodriguez et al., 2008; Samuel et al., 2011b). The CrVI exposure-induced impairment of steroidogenic mechanisms might have led to decreased synthesis of steroid hormones despite increase in the FSH levels. This may be the reason behind the delayed sexual maturation observed in the present investigation. The effects of LH and FSH are mediated by their respective receptors LHR and FSHR. Earlier, we have reported the CrVI reduced LHR and FSHR levels both *in vivo* (Samuel, 2001) and *in vitro* (Banu et al., 2008; Rodriguez et al., 2008). Collectively, these findings indicate the deleterious effects of CrVI on female reproductive system.

The protective effect of vitamin C on heavy metal toxicity is well documented via its detoxifying effects and free radical scavenging mechanisms (Banu et al., 2008; Kaderabkova et al., 1997; Subramanian et al., 2006; Suzuki, 1990). Earlier, we have reported the beneficial effect of vitamin C in CrVI-induced ovotoxicity (Banu et al., 2008). Recently, vitamin C has been shown to protect thyroid gland from toxic effects of CrVI (Qureshi and Mahmood, 2010). Vitamin C is an essential nutrient that functions as a nonenzymatic antioxidant in the cytosol. Once CrVI enters into the cells, it is rapidly reduced to CrIII by vitamin C, GSH and other antioxidants (Liu et al., 2005). During this reduction process, enormous amount of ROS is formed and are scavenged by antioxidants to protect cells from oxidative stress (Valko et al., 2005). In the present study, supplementation of vitamin C along with CrVI in rats exhibited protective effect in both ovary and uterus, as most of the parameters studied did not exhibit variation in comparison to control.

The present study reported the beneficial effects of vitamin C on ovarian and uterine toxicity induced by CrVI exposure through mother's milk. The present study design simulated the work environment where pregnant and lactating women were exposed to CrVI. The results from the present investigation revealed that lactational CrVI exposure affects the ovary and uterus and delayed sexual maturation of postnatal rats and the toxic effects were prevented by simultaneous supplementation of vitamin C. The present study reports the mitigative effects of vitamin C on CrVI-induced delay in ovarian and uterine antioxidant system and also on sexual maturation. Large-scale controlled studies are needed in order to support the beneficial effects of vitamin C in pregnant and lactating women who are all in potential danger of occupational CrVI exposure.

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Ameliorative effect of vitamin C on hexavalent chromium-induced delay in sexual maturation and oxidative stress in developing Wistar rat ovary and uterus

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Abstract

Hexavalent chromium (CrVI) is a highly toxic metal and major environmental pollutant and is extensively used in more than 50 industries. The major route of CrVI exposure for the general population is oral intake. Chromium is considered an important nutrient responsible for carbohydrate metabolism. However, excess CrVI exposure is associated with various pathological conditions including reproductive dysfunction. CrVI can traverse the placental barrier and cause wide range of abnormalities in fetal development. Cr is transported to offspring through mother's milk in lactating women exposed to CrVI. Therefore, the present study was carried out to determine the toxic effects of lactational CrVI exposure on ovary and uterus and the beneficial role of vitamin C in preventing/ameliorating the toxic effects of CrVI in developing female Wistar rats. Generation of oxidative stress is considered one of the plausible mechanisms behind Cr-induced cellular deteriorations. The present study evidenced a decrease in the specific activities of antioxidants, serum testosterone and progesterone and an increase in the levels of H_2O_2, lipid peroxidation (LPO) and follicle stimulating hormone in rats exposed to CrVI when compared to control. CrVI exposure also delayed the sexual maturation and extended the estrous cycle. Simultaneous administration of vitamin C significantly prevented the increase in LPO and enhanced the antioxidant status. These results suggest the protective effect of vitamin C against the CrVI exposure-induced toxicity and attest the significance of antioxidants in diet.

Keywords

Chromium, ovary, uterus, oxidative stress, sexual maturation, vitamin C

In recent years, contamination of the environment by chromium (Cr) has become a major area of concern. Cr exists in multiple oxidation states. The hexavalent chromium (CrVI) and trivalent chromium (CrIII) states are most important from a biological and an industrial standpoint. CrIII is an important nutrient for the human body; it enhances the action of insulin by increasing the number of insulin receptors, and enhances insulin binding and insulin sensitivity (Anderson, 2000). CrVI is an industrial contaminant of landfills and waterways (Cohen et al., 1993) and used on a large scale in more than 50 industries including metallurgical, electroplating, production

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of paints and pigments, tanning, wood preservation, pulp and paper production (Barceloux, 1999). The tanning industry is the large contributor of Cr pollution in water resources. In India about 2000–3200 tones of elemental Cr escape into the environment annually from the tanning industries, with a Cr concentration ranging between 2000 and 5000 mg L^{-1} in the effluent compared to the recommended permissible limit of 2 mg L^{-1} (Chandra et al., 1997). Sukinda (Orissa), Vapi (Gujarat) and Ranipet (Tamil Nadu) in India were listed as the world's top 10 worst polluted places because their drinking water is heavily contaminated with CrVI (The Blacksmith Institute, 2007). Excessive levels of Cr have been shown to produce various health hazards including reproductive dysfunction (Al-Hamood et al., 1998). The other health hazards caused by Cr include dermatitis bronchitis, lung cancer and nasal ulceration (Burrows, 1978; Halasova et al., 2009).

Occupational exposure to Cr is found among approximately half a million industrial workers in the United States and several millions worldwide; significant contamination with CrVI has been found in approximately 30% of the drinking water sources in California (Salnikow and Zhitkovich, 2008). The deposition of CrVI wastes in landfills and waterways by chromate industries affects millions of people who reside in the vicinity of dangerously polluted sites. Women working in dichromate manufacturing industries and living around Cr-contaminated areas encounter gynecological illness, postnatal hemorrhage and birth complications with high levels of Cr in blood and urine (Greene et al., 2010; Jendryczko et al., 1984; Shmitova, 1980; Zhang et al., 1992).

It is known that environmental chemicals can affect the function of female reproductive organs (Hoyer and Sipes, 1996). CrVI can traverse the feto-placental barrier and also transported to offspring through milk (Bailey et al., 2006; Banu et al., 2008; Junaid et al., 1996; Rodriguez et al., 2008; Samuel et al., 2011b). Earlier studies point out the deleterious effects of CrVI on the structure and function of ovary (Banu et al., 2008; Rao et al., 2009). CrVI exposure results in enhanced formation of reactive oxygen species (ROS) including super oxide anions, hydroxyl radical and nitric oxide, decreased cell viability, increased cellular genomic hepatic DNA fragmentation, enhanced intracellular oxidizing states, membrane damage, apoptotic and necrotic cell death (Bagchi et al., 2002). Previous findings demonstrated that Cr, administrated through drinking water led to abnormal gestational changes in mice and rats, with a decreased number of implantation sites (Junaid et al., 1996; Kamath et al., 1997). Pregnant mice exposed to CrVI through drinking water during gestation resulted in increased pre-implantation and post-implantation loss and decreased litter size (Trivedi et al., 1989). Delayed sexual maturation and reduced fertility were observed in female mice offspring treated with CrVI (Al-Hamood et al., 1998). Earlier, we have reported the ovotoxic nature of CrVI, where lactational CrVI exposure delayed follicular development and impaired ovarian steroidogenesis (Banu et al., 2008). Recently, we have reported the uterotoxic effects of CrVI, where the lactational exposure to CrVI are associated with delayed puberty and oxidative stress (Samuel et al., 2011b).

CrVI can escape from primary contact organs and blood erythrocytes and reach different organs (Barceloux, 1999; Dayan and Paine, 2001). CrVI is a well-known oxidizing agent (Bagchi et al., 1995). In biological systems, after entry into cells, CrVI is rapidly detoxified/reduced to CrIII by intracellular defensive reductant system that includes ascorbate, glutathione (GSH) and cysteine (Valko et al., 2005). Antioxidant compounds have been used to protect cells from various oxidative damages. Vitamin C is known to be an antistress and a powerful reducing agent and plays an important role in attenuating oxidative damage to the cellular membranes (Sugiyama, 1992). Vitamin C accounts for 80% of CrVI metabolism in target tissues such as lung, liver and kidney (Zhitkovich, 2005). In view of the existing background, the present study is aimed to test the hypothesis, 'Vitamin C prevents the lactational CrVI exposure-induced disruption of ovarian and uterine function in Wistar rats.'

Materials and methods

Animals and treatments

Pregnant Wistar rats (*Rattus norvegicus*), 90 days old, were maintained in plastic animal cages in a light controlled room (12-h day light) at 23–25°C and provided with standard rat pellet diet and clean drinking water *ad libitum*.

CrVI doses were selected based on our previous reports (Banu et al., 2008; Samuel et al., 2011b). Pregnant rats were divided into three groups. At birth, male pups were removed and the litters were culled to four female pups per mother rat. In cases where fewer than four female pups were delivered, additional pups were

fostered from other litters. (i) In the control group, the rats received regular drinking water from the day of parturition to day 21 postpartum. (ii) In CrVI-treated group, rats received CrVI (potassium dichromate, 200 mg/L) in drinking water from the day of parturition to day 21 postpartum. (iii) In CrVI + vitamin C group, rats received CrVI (200 mg/L) and vitamin C (ascorbate: 500 mg/L) simultaneously in drinking water from the day of parturition to day 21 postpartum.

During days 1–21 of postnatal life (postnatal day [PND] 1–21), the female pups were exposed to respective CrVI through their mother's milk. After weaning, on PND 45, female pups from 6 mother rats ($n = 24$; 4 pups/mother) from each group (control, CrVI-200 ppm, CrVI-200 ppm + vitamin C) were killed by decapitation; and their blood, ovary and uterus were collected. The remaining pups were maintained separately and fed with regular drinking water and diet. On PND 65, the rest of the rats ($n = 24$) were killed, and their blood, ovary and uterus were dissected, cleansed and used for biochemical assay. All the chemicals used in the present study were purchased from Sigma Chemical Company, St Louis, USA.

Tissue preparation for enzyme assays

Ovary and uterus were dissected out and washed in ice cold physiological saline repetitively, weighed accurately and homogenized in 0.1 mol/l Tris-HCl buffer, pH 7.4. The protein concentrations of the tissue homogenates were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. The detailed methodology for the antioxidant enzymes and ROS assays were described in our earlier article (Samuel et al., 2011b).

Antioxidant enzymes

SOD activity was estimated according to the method of Marklund and Marklund (1974). The enzyme activity was expressed as U/mg protein. Catalase (CAT) activity was quantified colorimetrically (Sinha, 1972) and expressed as mmoles of H_2O_2 utilized/min/mg protein. Glutathione peroxidase (GPx) activity was determined colorimetrically (Rotruck et al., 1973) and expressed as U/mg protein. Glutathione-*S*-transferase (GST) activity was determined by estimating the amount of enzyme that catalyzed the conjugation of a known amount of 1-chloro-2,4-dibenzene with GSH (Habig et al., 1974) and expressed as μmoles of GSH consumed/min/mg protein. Glutathione reductase (GR) activity was assayed colorimetrically (Staal et al., 1969) and expressed as nmoles of NADPH oxidized/min/mg protein.

Reactive oxygen species

Lipid peroxidation (LPO) was measured by the method of (Devasagayam and Tarachand, 1987). The results were expressed as nmoles of malondialdehyde (MDA) formed/mg protein. H_2O_2 production was assessed spectrophotometrically (Holland and Storey, 1981) and the content was expressed as μmoles of H_2O_2/mg protein.

Chromium estimation

Chromium level in ovary and uterus. Cr levels in ovary and uterus of control and CrVI-exposed rats were estimated as described earlier (Banu et al., 2008; Samuel et al., 2011b). To provide enough tissue for analysis, ovary and uterus in a dose group were pooled whenever needed. Fifty milligram of tissue was digested in 1 ml of concentrated HNO_3 and 1 ml of perchloric acid over a sand bath until the solution became pale yellow. If the color of the digest was brown, more HNO_3 and perchloric acid were added and the oxidation was repeated. The digest was made up to 1 ml with deionized water. Aliquots of this were used to estimate Cr, using an atomic absorption spectrometer (Perklin Elmer 2380), at 357.8 nm wavelength. The values are expressed as μg/g tissue.

Chromium level in serum. Serum Cr was directly quantified (Davidson and Secrest, 1972). Serum samples were diluted to the ratio of 1:10 with diluent containing 0.1% Triton and 0.1% HNO_3 in distilled deionized water. Aliquots of this were used to estimate the Cr content, using an atomic absorption spectrometer (Perklin Elmer 2380) at 357.8 nm wavelength. The values are expressed as μg/ml of serum.

Sexual maturation and estrous cycle. Vaginal opening was observed in rats from 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 (Banu et al., 2008; Samuel et al., 2011a, b).

Radioimmunoassay (RIA) of steroid hormones. Serum levels of estradiol, progesterone and testosterone

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

	Body weight (g)			Ovary weight (mg)			Uterus weight (mg)		
Age	Control	CrVI	CrVI + Vit. C	Control	CrVI	CrVI + Vit. C	Control	CrVI	CrVI + Vit. C
PND 45	69 ± 3.6	51 ± 3.8[a]	63 ± 2.7	54 ± 2.2	41 ± 1.9[a]	50 ± 2.4[b]	287 ± 12.4	256 ± 15.9[a]	266 ± 13.7
PND 65	98 ± 7.2	65 ± 5.3[a]	85 ± 5.9	62 ± 3.8	52 ± 4.7[a]	64 ± 5.2[b]	389 ± 20.2	328 ± 26.6[a]	354 ± 21.5

CrVI: hexavalent chromium, PND: postnatal day, Vit. C: vitamin C.
Each value represents the mean and SEM of 24 female rats (from 6 mothers). See Materials and methods section for experimental details; statistical significance of difference among groups at $p < 0.05$.
[a]Control versus experiment.
[b]CrVI versus CrVI + vitamin C.

were estimated by RIA as described earlier (Banu et al., 2008; Samuel et al., 2011a, b). 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 binding of the testosterone antibody was 36%, and the sensitivity of the assay was 0.3 pg testosterone/ml. Testosterone levels in serum are expressed as ng/ml. The maximum binding of progesterone antibody was 40% and the sensitivity of the assay was 0.3 pg/ml. Progesterone level is expressed as ng/ml.

Radioimmunoassay of peptide hormones. Serum levels of luteinising hormone (LH) and follicle stimulating hormone (FSH) were assayed by RIA as described earlier (Banu et al., 2008; Samuel et al., 2011b). The maximum binding of the LH antibody was 31.36% and the sensitivity of the assay was 0.2 ng/ml. The maximum binding of FSH antibody was 31% and the sensitivity of the assay was 0.2 ng/ml. LH and FSH levels were expressed as ng/ml.

Statistical analysis

Data were statistically analyzed using analysis of variance. When the F ratio was statistically significant, the data were subjected to Students Newman-Keul's test. Values were considered significant at $p < 0.05$.

Results

Lactational CrVI exposure leads to age-dependent changes in the specific activities of antioxidant enzymes, free radicals and hormones in postnatal rat ovary and uterus and simultaneous vitamin C treatment prevented the toxic effects of CrVI.

Body, ovary and uterus weight

Lactational CrVI exposure significantly decreased the body weight, ovary weight and uterus weight in both the age groups of CrVI-treated rats. Vitamin C treatment completely prevented the CrVI-induced decrease in body and uterus weight in PND 45 and PND 65 rats; however, vitamin C partially prevented the changes in ovary weight in both PND 45 and PND 65 rats (Table 1).

Chromium content in serum, ovary and uterus

In order to check, whether the decreased body, ovary and uterus weight is associated with the bioavailability of Cr, we have estimated the levels of Cr in serum, ovary and uterus of experimental offspring (Table 2). Cr content was significantly different between serum, ovary and uterus at basal conditions. Serum, ovary and uterus of Cr-exposed animals have higher Cr content than control animals. Vitamin C treatment prevented accumulation of Cr partially in PND 45 rats and completely in PND 65 rats.

Sexual maturation

Vaginal opening has been used as an index of sexual maturation. To evaluate the effect of CrVI on the sexual maturation, we have examined the vaginal opening every 24 h in the experimental rats from PND 22 onwards (Figure 1). CrVI exposure significantly delayed the sexual maturation in postnatal rats, while vitamin C restored the CrVI exposure-induced delay in sexual maturation.

Estrous cyclicity

In order to evaluate the effect of CrVI on the duration of estrous cycle, vaginal cytology was determined daily (Figure 2). CrVI exposure significantly extended the estrous cycle in diestrous stage when compared to

Table 2. Effect of lactational exposure to CrVI on the bioavailability of chromium in the serum, ovary and uterus in developing rats (PNDs 45 and 65)

	Serum level (μg/ml)			Ovary level (μg/g)			Uterus level (μg/g)		
Age	Control	CrVI	CrVI + Vit. C	Control	CrVI	CrVI + Vit. C	Control	CrVI	CrVI + Vit. C
PND 45	0.13 ± 0.02	0.35 ± 0.05[a]	0.29 ± 0.02[a]	0.38 ± 0.04	0.62 ± 0.07	0.43 ± 0.04[b]	0.33 ± 0.03	0.58 ± 0.04[a]	0.43 ± 0.04[a,b]
PND 65	0.11 ± 0.01	0.19 ± 0.01[a]	0.15 ± 0.02[a,b]	0.31 ± 0.04	0.59 ± 0.06[a]	0.33 ± 0.05[b]	0.32 ± 0.05	0.53 ± 0.05[a]	0.39 ± 0.03[b]

CrVI: hexavalent chromium, PND: postnatal day, Vit. C: vitamin C.
Each value represents the mean and SEM of 24 female rats (from 6 mothers). See Materials and methods section for experimental details; statistical significance of difference among groups at $p < 0.05$.
[a]Control versus experiment.
[b]CrVI versus CrVI + vitamin C.

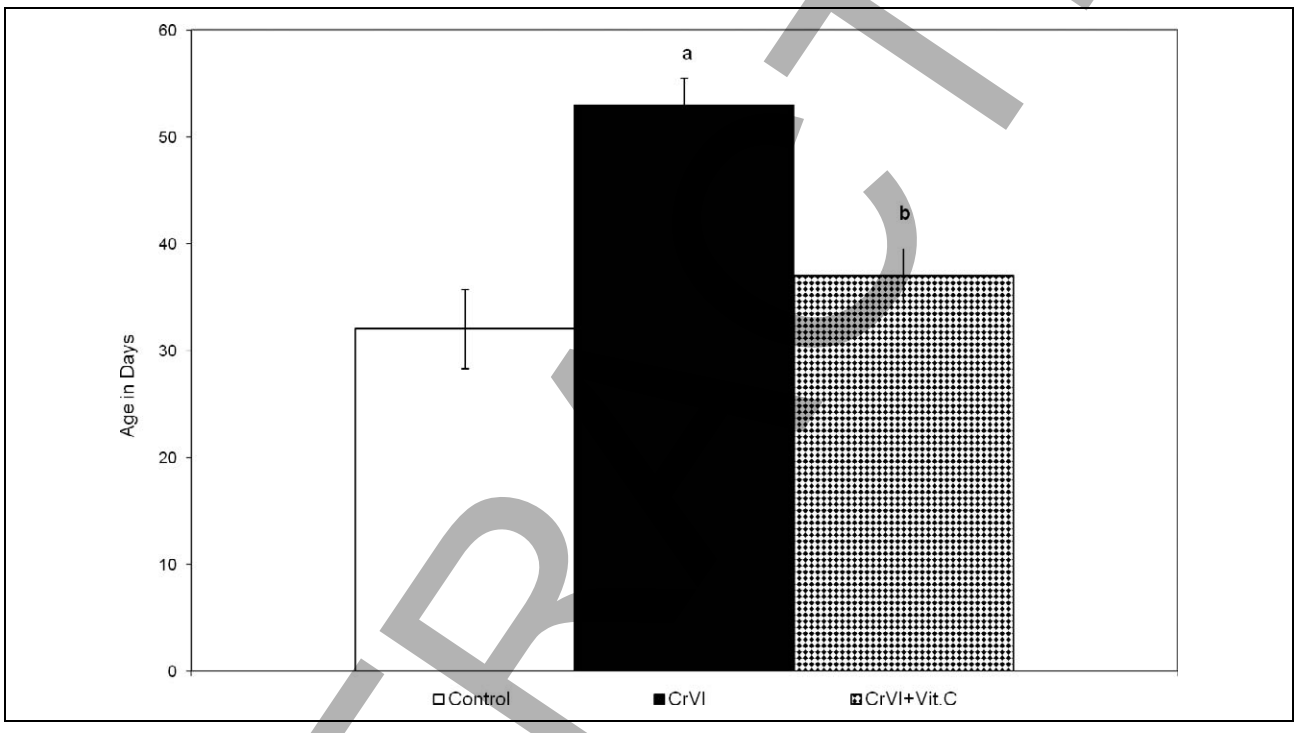

Figure 1. Effect of lactational exposure to hexavalent chromium (CrVI) on sexual maturation in developing rats (postnatal days [PNDs] 45 and 65). Each bar represents the mean and the vertical line above denotes the SEM of 24 female rats (from 6 mothers). See Materials and methods section for experimental details; statistical significance of difference among groups at $p < 0.05$; [a]control versus experiment; [b]CrVI versus CrVI + vitamin C.

control rats. CrVI exposure did not alter proestrous, estrous and metestrous phases of estrous cycle. Vitamin C prevented the extension of estrous cycle in CrVI-exposed rats.

Antioxidant enzymes

Effect of CrVI on the specific activities of different antioxidant enzymes in rat ovary and uterus are described in Figures 3(a–e) and 4 (a–e). CrVI exposure decreased the specific activities of SOD, CAT, GPx, GR and GST in the rat ovary and uterus of PND 45 and PND 65 rats. Vitamin C treatment partially prevented the CrVI exposure-induced decrease in ovarian SOD, CAT, GPx and GR levels in PND 45 rats, while it is completely restored in PND 65 rats. However, CrVI exposure-induced decrease in uterine antioxidants SOD, CAT, GPx, GST and GR were partially prevented by vitamin C treatment in PND 45 and PND 65 rats.

Lipid peroxidation and H_2O_2 concentration

Figures 5 (a, b) and 6 (a, b) represent the levels of MDA and H_2O_2 in control, CrVI with or without

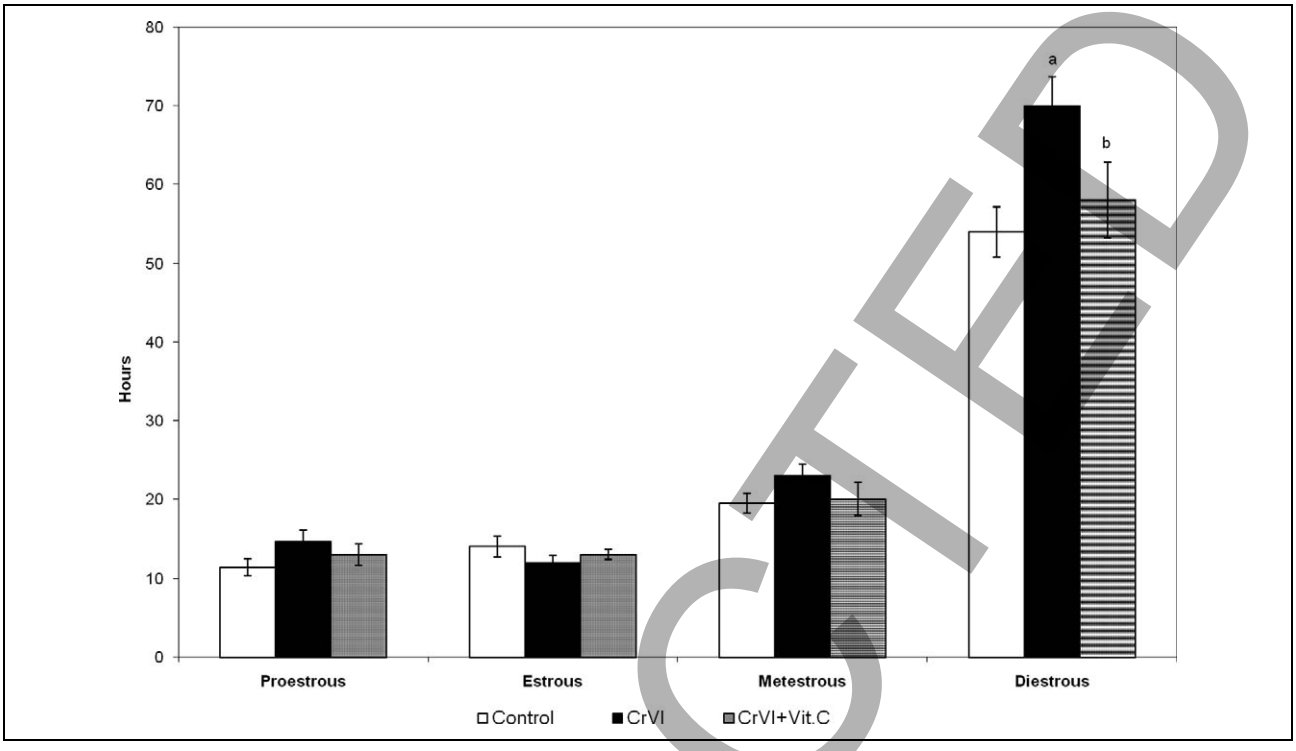

Figure 2. Effect of lactational exposure to hexavalent chromium (CrVI) on estrous cycle in developing rats (postnatal days [PNDs] 45 and 65). Each bar represents the mean and the vertical line above denotes the SEM of 24 female rats (from 6 mothers). See Materials and methods section for experimental details; statistical significance of difference among groups at $p < 0.05$; [a]control versus experiment; [b]CrVI versus CrVI + vitamin C.

vitamin C treated ovary and uterus of PND 45 and PND 65 rats. Increased concentrations of LPO and H_2O_2 generation were observed in CrVI-treated ovaries and uterus of PND 45 and PND 65 rats. However, simultaneous vitamin C treatment partially prevented the CrVI-exposure-induced increase in LPO and H_2O_2 levels in PND 45 and PND 65 uterus and PND 65 ovary, while it is completely prevented in PND 65 rat ovary.

Serum hormones

Table 3 represents circulating levels of steroid and pituitary hormones in postnatal rats exposed to CrVI and CrVI along with vitamin C. CrVI exposure decreased steroid hormones in both PND 45 and PND 65 rats, while vitamin C prevented the CrVI-exposure-induced decrease in steroid hormone levels. Pituitary hormones LH and FSH showed a varied pattern, FSH and LH significantly increased in PND 45 rats, while PND 65 rats did not show any significant difference between control and CrVI-exposed rats. Vitamin C treatment prevented the CrVI-exposure-induced increase in LH and FSH levels.

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. CrVI is the established endocrine disruptor reported to disturb the reproductive process in both males (Aruldhas et al., 2005) and females (Banu et al., 2008; Rao et al., 2009; Rodriguez et al., 2008; Samuel et al., 2011b). The purpose of this study was to investigate the toxic effects of CrVI in two major female reproductive organs, viz., ovary and uterus and to examine the possible beneficial role of vitamin C in preventing CrVI-induced female reproductive toxicity. In the present study, body and organ (ovary and uterus) weight were decreased significantly. This was consistent with our earlier reports (Samuel et al., 2011b) and also with others (Al-Hamood et al., 1998; Rao et al., 2009). The failure of postnatal rats exposed to CrVI through mother's milk to gain body weight may be due to decreased feed intake, malabsorption of nutrients from the gastrointestinal tract and impaired feed

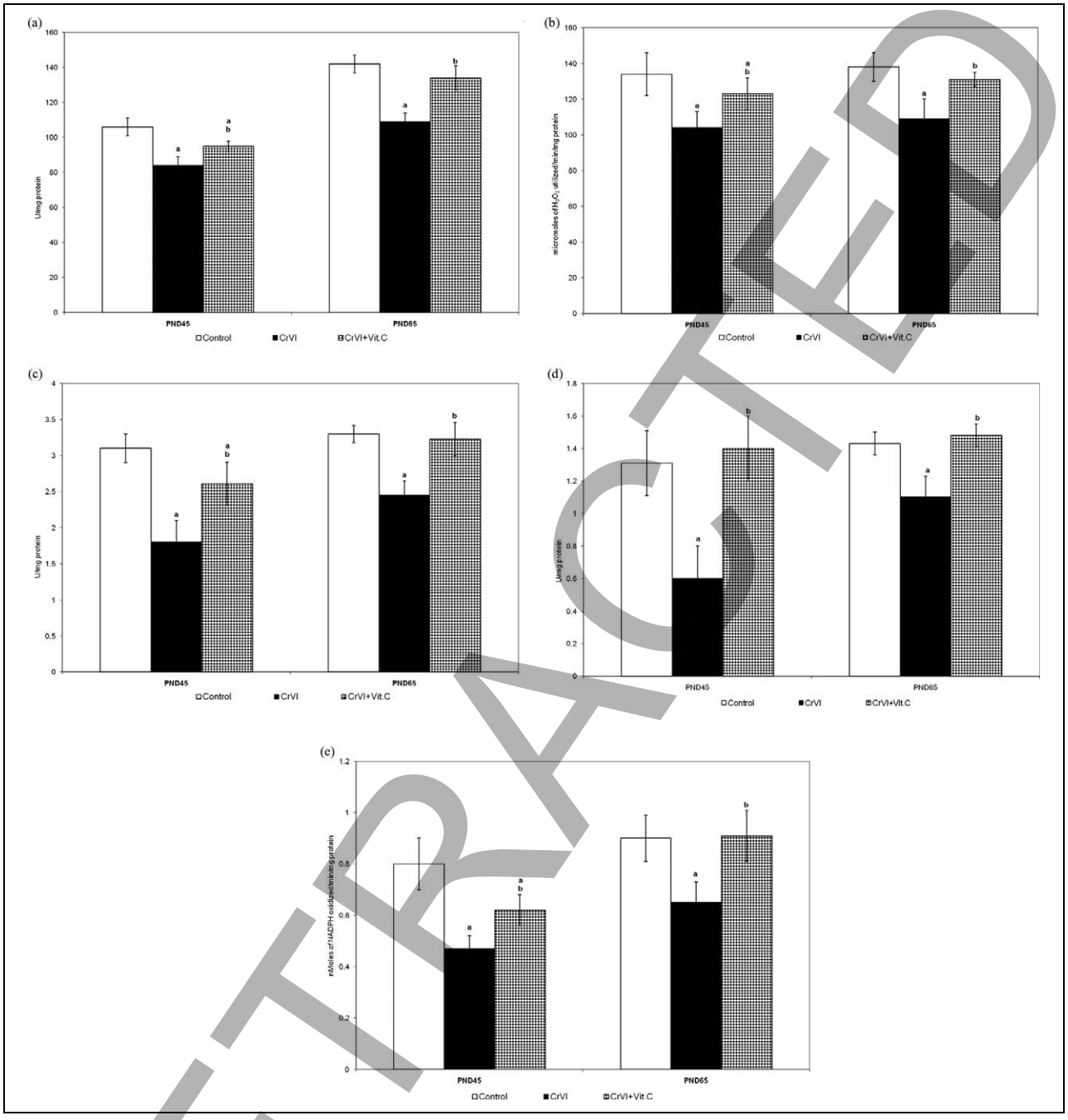

Figure 3. Effect of lactational exposure to hexavalent chromium (CrVI) on the specific activities of ovarian superoxide dismutase (SOD; a), catalase (b), glutathione peroxidase (GPx; c), glutathione-*S*-transferase (GST; d) and glutathione reductase (GR; e). Each bar represents the mean and the vertical line above denotes the SEM of 24 female rats (from 6 mothers). See Materials and methods section for experimental details; statistical significance of difference among groups at $p < 0.05$; [a]control versus experiment; [b]CrVI versus CrVI + vitamin C.

conversion efficiency (Ball and Chhabra, 1981). Cr was shown to be an essential micronutrient required for normal carbohydrate and lipid metabolism and acts as a cofactor for enzymes like cytochrome C, phosphoglucomutase and was involved in nucleic acid metabolism (Mertz, 1993). The observed decrease in body and organ (ovary and uterus) weight may be due to the influence of CrVI on the intermediary metabolism. Vitamin C treatment prevented the CrVI-exposure-induced reduction in

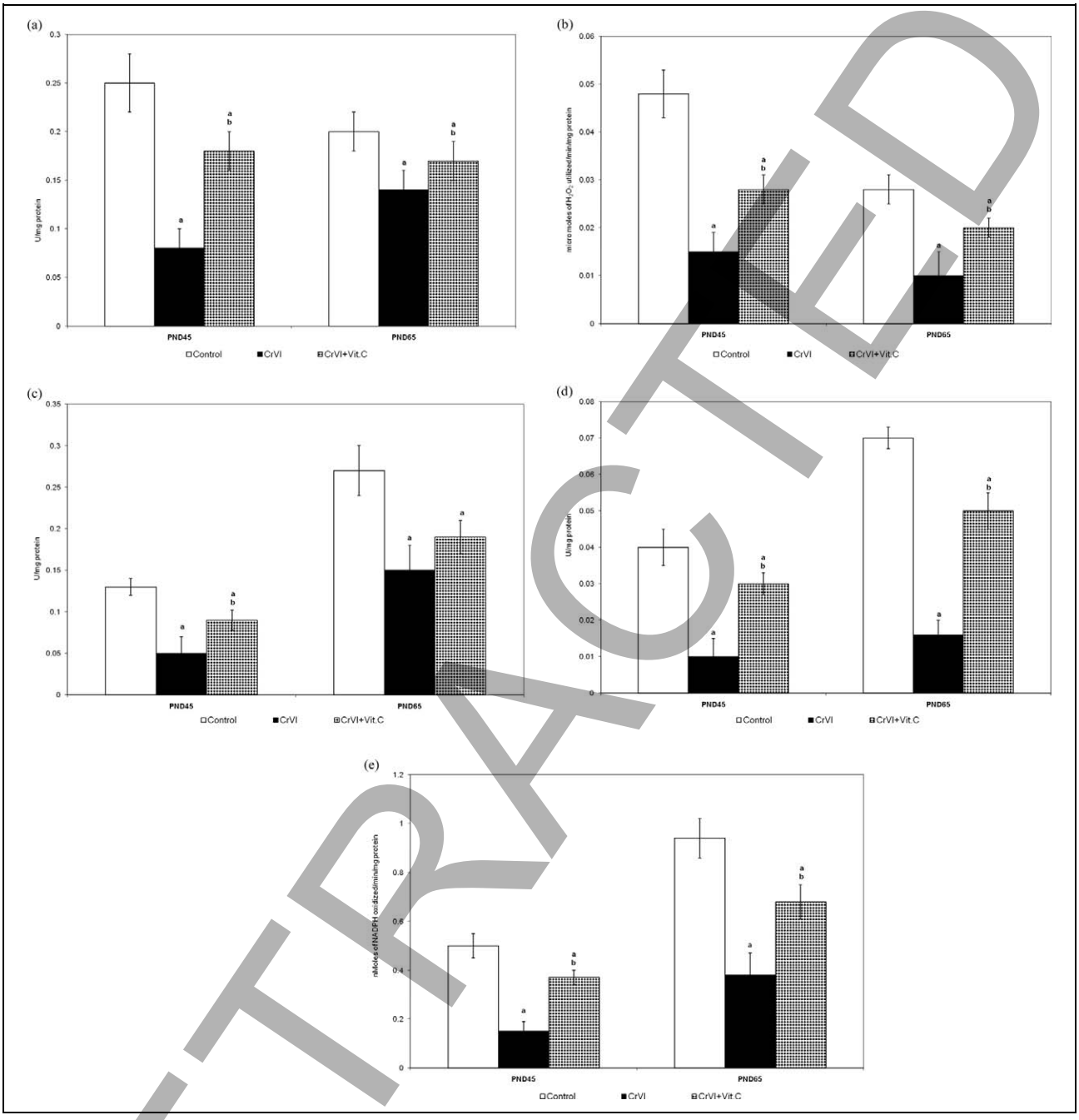

Figure 4. Effect of lactational exposure to hexavalent chromium (CrVI) on the specific activities of uterine superoxide dismutase (SOD; a), catalase (b), glutathione peroxidase (GPx; c), glutathione-S-transferase (GST; d) and glutathione reductase (GR; e). Each bar represents the mean and the vertical line above denotes the SEM of 24 female rats (from 6 mothers). See Materials and methods section for experimental details; statistical significance of difference among groups at $p < 0.05$; [a]control versus experiment; [b]CrVI versus CrVI + vitamin C.

body and uterus weight in both PND 45 and PND 65 rats. However, the toxic effects of CrVI on ovary was partially prevented by vitamin C. Earlier, we have reported the ovotoxic and uterotoxic nature of CrVI, where CrVI treatment resulted in permanent damage to ovarian cell population (Banu et al., 2008) with extended estrous cycle (Samuel et al., 2011b).

The involvement of oxidative stress was found in heavy metal toxicity (Susa et al., 1996). Cr-induced ROS can damage the cellular element in its target organs (Bagchi et al., 2001). CrVI produces excess

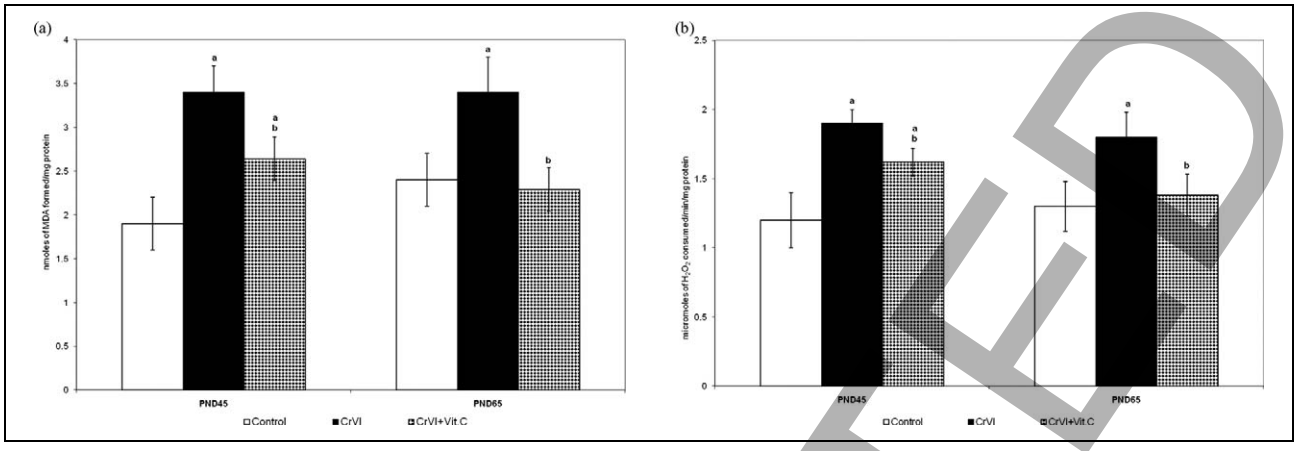

Figure 5. Effect of lactational exposure to hexavalent chromium (CrVI) on ovarian lipid peroxidation (LPO; a) and H_2O_2 (b). Each bar represents the mean and the vertical line above denotes the SEM of 24 female rats (from 6 mothers). See Materials and methods section for experimental details; statistical significance of difference among groups at $p < 0.05$; [a]control versus experiment; [b]CrVI versus CrVI + vitamin C.

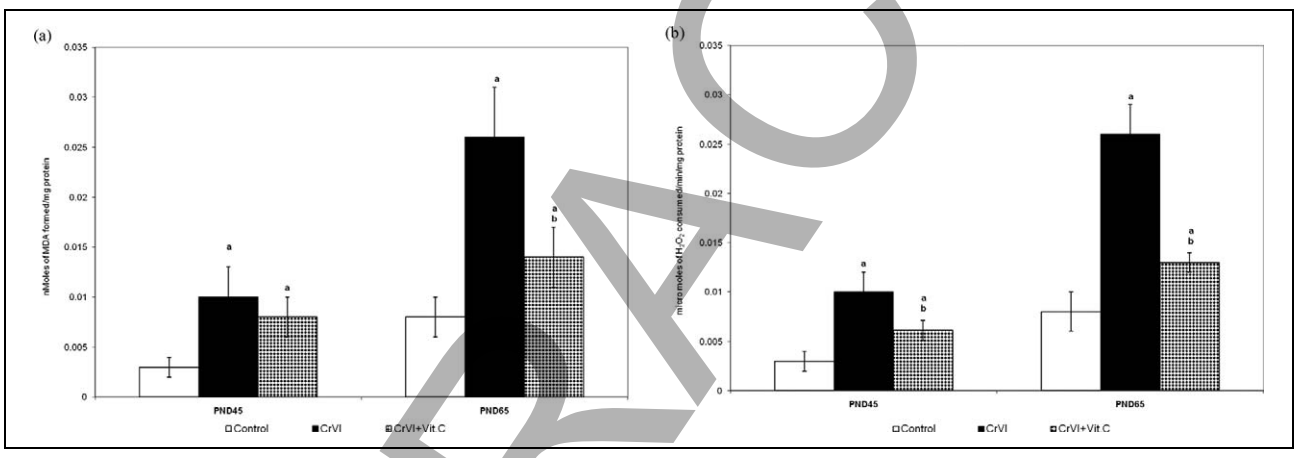

Figure 6. Effect of lactational exposure to hexavalent chromium (CrVI) on uterine lipid peroxidation (LPO; a) and H_2O_2 (b). Each bar represents the mean and the vertical line above denotes the SEM of 24 female rats (from 6 mothers). See Materials and methods section for experimental details; statistical significance of difference among groups at $p < 0.05$; [a]control versus experiment; [b]CrVI versus CrVI + vitamin C.

ROS including superoxide anion, singlet oxygen and hydroxyl radicals (Kawanishi et al., 1986). CrVI-exposure-induced impairment of the pituitary-ovarian axis in Teleost was reported (Mishra and Mohanty, 2010). CrVI-induced oxidative stress in mouse ovary (Rao et al., 2009) and rat uterus (Samuel et al., 2011b) were known. Results from the present investigation indicate the induction of oxidative stress in the ovary and uterus of rats exposed to CrVI through mother's milk, as there was a significant increase in the concentration of H_2O_2 and LPO with subnormal activity of the most of the antioxidant enzymes tested. As a transition metal, Cr can replace iron and copper in Fenton reaction and generate hydroxyl radical which is able to initiate LPO, leading to increased MDA concentration, one of the byproducts of LPO (Dey et al., 2003; Kalahasthi et al., 2006; Sawicka et al., 2010). The increase in H_2O_2 might induce the peroxidation of polyunsaturated fatty acids and lead to the formation of MDA. Higher concentration of MDA in serum of chrome-plating industry workers (Huang et al., 1999) and in mouse ovary exposed to CrVI (Rao et al., 2009) further highlights the involvement of oxidative stress in Cr-induced toxicity. Recently, we showed higher levels of MDA in rat uterus exposed to CrVI (Samuel et al., 2011b). Vitamin C treatment prevented the CrVI-exposure-induced oxidative stress and LPO in both ovary and uterus. Data from the present study revealed decreased levels of serum testosterone,

Table 3. Effect of lactational exposure to CrVI on circulating levels of steroid and pituitary hormones in developing rats (PNDs 45 and 65)

Hormone	Age	Control	CrVI	CrVI + Vit. C
Testosterone	PND 45	0.94 ± 0.07	0.45 ± 0.04[a]	0.89 ± 0.03[b]
	PND 65	1.07 ± 0.08	0.56 ± 0.05[a]	0.93 ± 0.07[b]
Estradiol	PND 45	43.8 ± 4.0	24.1 ± 2.3[a]	46.7 ± 4.5[b]
	PND 65	40.9 ± 3.5	27.3 ± 2.1[a]	38.9 ± 4.7[b]
Progesterone	PND 45	6.42 ± 0.45	3.70 ± 0.68[a]	5.53 ± 0.74[b]
	PND 65	9.01 ± 0.85	5.21 ± 0.68[a]	8.42 ± 0.82[b]
LH	PND 45	7.35 ± 0.85	9.34 ± 1.21[a]	7.90 ± 1.40
	PND 65	5.86 ± 0.55	7.49 ± 0.95	6.97 ± 0.77
FSH	PND 45	10.0 ± 1.12	16.47 ± 1.58[a]	11.6 ± 1.74[b]
	PND 65	9.30 ± 0.75	10.1 ± 1.28[a]	10.4 ± 1.84

CrVI: hexavalent chromium, FSH: follicle stimulating hormone, LH: luteinising hormone, PND: postnatal day, Vit. C: vitamin C.
Each value represents the mean and SEM of 24 female rats (from 6 mothers). See Materials and methods section for experimental details; statistical significance of difference among groups at $p < 0.05$.
[a]Control versus experiment.
[b]CrVI versus CrVI + vitamin C.

estradiol and progesterone. Estrogen deficiency was shown to be associated with oxidative stress (Muthusami et al., 2005). Thus, the impaired level of serum steroids observed in the present study might have led to increased level of MDA in ovary and uterus.

The levels of MDA were increased in both ovary and uterus of PND 45 and PND 65 rats exposed to CrVI through mother's milk. The observed increase in LPO was many folds higher in ovary than uterus. The increase in LPO may also be due to decreased activities of SOD and CAT, the free radical scavenging enzymes. The enzyme SOD catalyses dismutation of superoxide radical, leading to the formation of H_2O_2, which inturn was detoxified by the enzymes GPx and CAT (Rzeuski et al., 1998). The present study also showed decreased levels of SOD and CAT in both ovary and uterus of PND 45 and PND 65 rats exposed to CrVI and the decrease in SOD activity may result in more accumulation of O_2^-, which in turn may inhibit other antioxidant enzymes. The decreased activities of SOD and CAT in the present study were concomitant with our previous reports (Samuel et al., 2011b) and with that of Rao et al. (2009). CAT is the main scavenger of H_2O_2 at high concentration. Along with CAT, GPx is also involved in scavenging H_2O_2 and thereby protects cell membrane from LPO (Samanta et al., 1999).

The present study indicates that CrVI-exposed offspring exhibited delayed sexual maturation with extended estrous cycle when compared to control rats. The risk of damage to the ovarian follicle cell population from Cr depends mainly on the accessibility of Cr to the follicles. After entering the body by the oral route, CrVI reduced to CrIII by fluids in the digestive tract and then it can be sequestered by intestinal bacteria (Metze et al., 2005). CrVI that escapes reduction in digestive tract could also be reduced in the blood of the portal vein system and then in liver (De Flora et al., 1997). In spite of these protective mechanisms, CrVI can reach several other reproductive tissues, where it can be accumulated. In the present study, the post natal rats exposed to CrVI through mother's milk had increased levels of Cr in serum, ovary and uterus. Difference between ovary and uterine Cr accumulation could be due to distinctive properties of each tissue. Earlier, we have reported that higher accumulation of Cr reduced ovarian follicular cell population (Banu et al., 2008). In the present study, the observed oxidative stress and the Cr accumulation in target tissues might have delayed sexual maturation. CrVI is reported to arrest the cell cycle at G2/M phase along with decreased CAT levels in lung epithelial cells (Zhang et al., 2001). A similar mechanism might operate in the present study, which could have contributed to the decrease in ovarian follicle number through arrest in G2/M cell cycle in CrVI treated rats. Vitamin C treatment partially prevented the CrVI-exposure-induced accumulation of Cr in serum, ovary and uterus of PND 45 and PND 65 rats. The present study also showed decreased levels of sex steroid hormone levels. The decreased levels of steroid hormones observed in the present investigation might be due to the CrVI-induced decrease in the number of ovarian follicles and the associated oxidative stress.

Estradiol is not only associated with follicular maturation and oxidative stress but also regulates LH and FSH from the pituitary through positive and negative feedback on the hypothalamus and pituitary (Hoyer and Sipes, 1996). Earlier, we have reported the decreased expression of steroidogenic genes in ovarian cells exposed to CrVI (Banu et al., 2008). We have also reported the toxic effects of CrVI on developing rat ovary and uterus (Banu et al., 2008; Rodriguez et al., 2008; Samuel et al., 2011b). The CrVI exposure-induced impairment of steroidogenic mechanisms might have led to decreased synthesis of steroid hormones despite increase in the FSH levels. This may be the reason behind the delayed sexual maturation observed in the present investigation. The effects of LH and FSH are mediated by their respective receptors LHR and FSHR. Earlier, we have reported the CrVI reduced LHR and FSHR levels both *in vivo* (Samuel, 2001) and *in vitro* (Banu et al., 2008; Rodriguez et al., 2008). Collectively, these findings indicate the deleterious effects of CrVI on female reproductive system.

The protective effect of vitamin C on heavy metal toxicity is well documented via its detoxifying effects and free radical scavenging mechanisms (Banu et al., 2008; Kaderabkova et al., 1997; Subramanian et al., 2006; Suzuki, 1990). Earlier, we have reported the beneficial effect of vitamin C in CrVI-induced ovotoxicity (Banu et al., 2008). Recently, vitamin C has been shown to protect thyroid gland from toxic effects of CrVI (Qureshi and Mahmood, 2010). Vitamin C is an essential nutrient that functions as a nonenzymatic antioxidant in the cytosol. Once CrVI enters into the cells, it is rapidly reduced to CrIII by vitamin C, GSH and other antioxidants (Liu et al., 2005). During this reduction process, enormous amount of ROS is formed and are scavenged by antioxidants to protect cells from oxidative stress (Valko et al., 2005). In the present study, supplementation of vitamin C along with CrVI in rats exhibited protective effect in both ovary and uterus, as most of the parameters studied did not exhibit variation in comparison to control.

The present study reported the beneficial effects of vitamin C on ovarian and uterine toxicity induced by CrVI exposure through mother's milk. The present study design simulated the work environment where pregnant and lactating women were exposed to CrVI. The results from the present investigation revealed that lactational CrVI exposure affects the ovary and uterus and delayed sexual maturation of postnatal rats and the toxic effects were prevented by simultaneous supplementation of vitamin C. The present study reports the mitigative effects of vitamin C on CrVI-induced delay in ovarian and uterine antioxidant system and also on sexual maturation. Large-scale controlled studies are needed in order to support the beneficial effects of vitamin C in pregnant and lactating women who are all in potential danger of occupational CrVI exposure.

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Retraction notice

At the request of the Editor and the Publisher, the following article:

Samuel JB, Stanley JA, Vengatesh G, Princess RA, Muthusami S, Roopha DP, Suthagar E, Kumar KM, Sebastian MS, Aruldhas MM. (2012) Ameliorative effect of vitamin C on hexavalent chromium-induced delay in sexual maturation and oxidative stress in developing Wistar rat ovary and uterus, *Toxicology and Industrial Health* 28(8): 720-733. DOI: 10.1177/0748233711422728

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has been retracted from *Toxicology and Industrial Health* due to redundant publication by the same group of authors in another journal:

Samuel JB, Stanley JA, Roopha DP, Vengatesh G, Anbalagan J, Banu SK, Aruldhas MM. (2011) Lactational hexavalent chromium exposure-induced oxidative stress in rat uterus is associated with delayed puberty and impaired gonadotropin levels, *Human & Experimental Toxicology* 30(2):91-101. DOI: 10.1177/0960327110364638

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The two papers have used the same data and reporting with the *Toxicology and Industrial Health* manuscript inserting Vitamin C to differ the reporting. Additionally, the *Toxicology and Industrial Health* manuscript does not provide citations to original work by another author.