Impact of gestational and lactational exposure to hexavalent chromium on steroidogenic compartment of post-natal rat testis

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Summary

Reproductive and embryonic toxicity of hexavalent chromium (CrVI) is known, and adult testis is one of its vulnerable targets. However, it is not known if gestational and lactational exposure to excess Cr affects development and functions of Leydig cells during postnatal life. It is hypothesized that gestational/lactational exposure to CrVI may affect Leydig cell development and differentiation and its functions during postnatal life extending into adulthood. Pregnant [gestational days 9 to 21] and lactating [postnatal days (PND) 1 to 21] rats were exposed to 50ppm and 100ppm CrVI ($K_2Cr_2O_7$) through drinking water, and testis was collected on PND 30, 60, 90 and 120, and subjected to light and transmission electron microscopic analysis. Serum testosterone and estradiol were determined adopting RIA. Histological evaluation of testes revealed hypertrophy and vacuolation of Leydig cells of CrVI-exposed rats; transmission electron micrographs (TEM) showed lipid accumulation, swollen mitochondria and disorganized smooth endoplasmic reticulum. Lactational exposure to CrVI led to decrease in the number of mitochondria and collapse of mitochondrial cristae. In general, the changes were obvious in PND 30 rats, and became less pronounced by PND 60 to become normal by PND 90. Serum testosterone and estradiol levels showed a general trend of opposite response to CrVI exposure. Gestational exposure to CrVI caused increase in testosterone level in prepuberal rats, but the trend was reversed by PND 60, and by PND 120 its level was more than in coeval controls. A similar trend was noticed in rats which had lactational exposure to CrVI but for a consistent increase in both steroids in PND 30 and PND 60 old rats which were exposed to 50ppm CrVI. By PND 90, testosterone remained elevated or normal, but by PND 120 its level was increased due to lactational exposure to CrVI. On the contrary, serum estradiol in these rats was low by PND 90 and became normal by PND 120. The findings partially support the hypothesis proposed and it is concluded that the fetal type Leydig cells are the major targets for the toxic effects of CrVI exposure during gestational and lactational periods where in lactational exposure may have a persistent effect leading to increased testosterone: estradiol ratio. Nevertheless, the effects of CrVI on testosterone and estradiol are reversible, as the adult type Leydig cells are unaffected.

***Key words*:** CrVI, estradiol, Leydig cells, Sertoli cells, testicular toxicity, testosterone

Introduction

Hexavalent chromium (CrVI) is a heavy metal contaminant accessing man through occupational as well as environmental routes. Human exposure to Cr originates at more than 50 industries including stainless steel and chrome-plating industries, refractories, tanneries, and soap and ammunition factories (Nriagu, 1988; Shankar et al., 2005). These industries release their waste materials in the effluent into water bodies, air or open landfills, and environmental contamination with the metal is associated with several health hazards ranging from dermatitis, lung and skin cancer, nasal perforation and infertility (De Flora and Wetterhahn, 1987; Ishikawa et al., 1994; De Flora et al., 1997) among non-industrial populations who reside in close proximity to large sites of chromate disposal and get exposed to contaminated drinking water or air (Pellerin and Booker, 2000; Zhitkovich, 2002; Costa, 2003). The routes of entry include cutaneous, nasal and oral. Inhalation and dermal contact are the important routes of occupational exposure to chromium (Hertel, 1986). Non-occupational Cr exposure occurs via ingestion of chromium present in food, water and soil (Langard, 1982; Pedersen, 1982; Agency for Toxic Substances and Disease Registry, ATSDR, 2008).

The optimum functioning of the hypothalamo-hypophysial - gonadal axis determines normal fertility of the individual. Any perturbation in one segment of this delicate axis leads to infertility or abnormal fetal development, if spermatozoa carrying genetic defect(s) fertilize normal mature ova (Haris et al., 2011). In the

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United States, approximately 15% of couples experience some difficulties when trying to conceive and in roughly 50% of these couples, male factor is partially responsible for failure to conceive (Jarrow and Zirkin, 2005; Poch and Sigman, 2010). No identifiable cause of their abnormal semen could be found in about 25% of men evaluated and, hence, the diagnosis of idiopathic male factor infertility is probably due to exposure(s) to environmental/ occupational xenobiotics that act as reproductive endocrine disruptors. Reproductive endocrine disruptors can alter the hypothalamic, pituitary and/or testicular hormones that regulate spermatogenesis (Jarow and Zirkin, 2005).

Chromium occurs in the environment primarily in two valence states, trivalent chromium (CrIII) and hexavalent chromium (CrVI) (De Flora et al., 1977). CrIII is essential for normal glucose, protein and fat metabolism and is, thus, an essential dietary element, but excessive intake of CrIII may lead to toxicity. It is known that CrVI is 1000 times more toxic than Cr III (Zhang and Jin, 2006). CrVI detoxification leads to increased levels of CrIII. At physiological pH, CrVI exists as a chromate oxy-anion and can readily cross cell membranes through the sulfate anion transport system (O'Brien et al., 2003). Once internalized, CrVI is rapidly reduced to the ultimate intracellularly stable oxidation state, CrIII. During this reduction process, intermediate high-valent oxidation states of Cr, *i.e.,* CrIV and CrV, are formed along with free radicals both *in vivo* and *in vitro* (Connett and Wetterhan, 1985; Harris and Shi, 2003; Pulido and Parrish, 2003; Leonard et al*.,* 2004; Valko et al., 2005).

One of the major areas of concern in terms of human health due to Cr exposure is the reproductive toxicity. The early epidemiological studies on human exposure to welding fumes containing CrVI showed decreased sperm count and motility, and increased number of abnormal sperm (Bonde, 1990 a, b, 1993; Bonde et al*.,* 1990). However, according to Bonde and Ernest (1992), low level exposure to CrVI in welding industries may not be a major hazard for human fertility. Nevertheless, other studies (Li et al*.,* 2001; Danadevi et al., 2003) have reported male reproductive toxic effects of Cr fumes. Most of these epidemiological studies were based on data from men exposed to welding fumes containing Cr in the form of fumes, predominantly through nasal route. On the other hand, in leather and soap industries Cr is in dissolved form and the workers are vulnerable to exposure through skin. Further, effluents from these industries in most instances are let into the water bodies, in which case Cr can become an environmental pollutant, making access through cutaneous and oral routes.

Experiments performed in animal models and cell lines have shown that excess CrVI can result in deterioration of male reproductive health (Zahid et al., 1990; Ernst and Bonde, 1992; Subramanian, 2000; Chowdhuri et al., 2001; Pereira et al., 2002, 2004, 2005; Aruldhas et al., 2004, 2005, 2006; Subramanian et al*.,* 2006). Whether it be occupational exposure, as observed in the epidemiological studies, or oral / subcutaneous exposure, as in the animal models, studies were mostly limited to reproductive outcomes / or semen parameters in adult men / animals. It is an established fact that several potential toxicants can bring about serious damage to male reproductive health if the exposure is during *in utero* or neonatal period (Veeramachaneni et al., 2001). This is because differentiation of the bi-potential gonad into testis and subsequent differentiation of its cell types towards establishment of the functional testis would be in jeopardy in the context of exposure to toxicants such as dioxins, plasticizers, pesticides, etc. (Guo et al., 2000; Damstra et al., 2002; Theobold et al., 2003; Hauser et al., 2005; Hutt et al., 2008). 3β-hydroxysteroid dehydrogenase (3β HSD) and 17β-hydroxysteroid dehydrogenase (17βHSD) are the key regulatory enzymes of testicular steroidogenesis (Jana et al., 2006). FSH is required for maintenance of quantitatively normal spermatogenesis in pubertal rats (Russell et al., 1987; Huhtaniemi and Aittomaki, 1998). Testosterone is needed for initiation and maintenance of the spermatogenic process and inhibition of germ cell apoptosis (Singh and Handelsman, 1995).

In the light of the published literature on the impact of Cr on the functional testis in terms of spermatogenesis, and the reports that chromium can induce disturbance of embryonic and fetal development in treated animals (ATSDR, 2000) it is hypothesized that gestational / lactational exposure to CrVI can produce serious consequences in Leydig cell development, differentiation and its functions during postnatal life. In this paper, we report that gestational and / or neonatal exposure to chromium potentially affects the fetal type Leydig cells (FLCs) as well as secretion of sex steroids.

Materials and Methods

The experimental protocol

The experimental protocol of the present study on male albino rats of Wistar strain (*Rattus norvegicus*) was approved by the Institutional Animal Ethics Committee (IAEC) constituted under the auspices of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India.

The following groups of animals were used in the present study. *Group I*: Control male rats of postnatal days (PND) 30 (Group 1a), 60 (Group 1b), 90 (Group 1c) and 120 (Group 1d) given drinking water free from CrVI. *Group II*: PND 30 (Group 2a), 60 (Group 2b), 90 (Group 2c) and 120 (Group 2d) old male rats with gestational exposure to 50ppm CrVI from ED-9 to birth. *Group III*: PND 30 (Group 3a), 60 (Group 3b), 90 (Group 3c) and 120 (Group 3d) old male rats with gestational exposure to 100ppm CrVI from embryonic day (ED) 9 to birth. *Group IV*: PND 30 (Group 4a), 60 (Group 4b), 90 (Group 4c) and 120 (Group 4d) male rats with lactational exposure to 50ppm CrVI from PND 1 to PND 21 (weaning). *Group V*: PND 30 (Group 5a), 60 (Group 5b), 90 (Group 5c) and 120 (Group 5d) male rats with lactational exposure to 100ppm CrVI from PND 1 to PND 21.

Experimental rats were provided with drinking water containing CrVI (potassium dichromate, $K_2Cr_2O_7$) at concentrations 50 and 100ppm *ad libitum*, during gestational (Groups II and III) from ED 9 to birth and lactational (Groups IV and V) from PND 1 to PND 21, respectively. $K_2Cr_2O_7$ is highly soluble in water and used in many industries. The concentrations of chromium were selected on the basis of a dose -response study conducted earlier from our laboratory (Aruldhas et al., 2005). At the end of the experimental period, the testes of control and experimental rats were removed surgically and used for light and transmission electron microscopic analysis (TEM) as explained elsewhere (Aruldhas et al*.,* 2005); the blood was collected and serum separated and used for hormone assays.

Tissue processing for light- and transmission electron microscopy (TEM)

Tissues mount for TEM were not subjected to perfusion fixation since samples from the same rats were also used for light microscopic study as well. The entire right testis was immersion-fixed in 2.5% glutaraldehyde in cacodylate buffer (Hess and Moore, 1993) immediately after removal. Thin slices of testis were again fixed in the same fixative to ensure proper fixation. The tissues were post-fixed in 1% osmium tetroxide and embedded in thin viscosity resin (Spurr's mix; Sigma, St Louis, MO, USA). Semithin sections (1μm) were obtained in an ultramicrotome (Reichert Jung, Vienna, Austria) and stained in toluidine blue O (TBO) for light microscopic observation. Ultrathin sections were cut with an ultramicrotome (Leica Microsystems, Nussloch, GmbH Nussloch, Germany), stained with uranyl acetate and lead citrate, and observed in a Phillips 201-c (Amsterdam, Holland) transmission electron microscope. Image analysis and processing were done using Axiovision image analysis software (Carl Zeiss, Jena, Germany).

Radioimmunoassay of serum testosterone and estradiol

Serum levels of testosterone and estradiol were estimated according to the method of Sufi et al. (1986) adopting liquid phase radioimmunoassay using [3H]-testosterone and [3H]-estradiol, respectively, and using specific antibodies as explained in our previous papers (Banu et al., 2002). The percentage binding of the antigen to the antibody was 36% for testosterone and 37–40% for estradiol. Minimum detectable levels of testosterone and estradiol were 0.3ng and 0.1pg per tube, respectively. Testosterone and estradiol concentrations were expressed as ng/ml and pg/ml serum, respectively.

Statistics

Data were subjected to one-way analysis of variance, and whenever the F value was significant, the data were analyzed by Duncan's multiple comparison test to find the within-group significance at the $P<0.05$ level. The values are depicted as mean ± SEM.

Results

Histopathological changes in Leydig cells

Testis of control rats

The organization of cells in the interstitium of control rats on PND 30, 60, 90 and 120 revealed the Leydig cells in their characteristic topography and organization i.e., clustering around the blood capillaries (Fig.1 a-g).

Testis of chromium-treated rats

Rats on PND 30 which were born to mothers exposed to 50ppm CrV1 during gestation (Group IIa) indicated swollen Leydig cells (Fig. 2a). Large vacuoles containing cell debris amidst smooth endoplasmic reticulum were also observed (Fig. 2b). The progeny of mothers exposed to 100ppm CrVI (Group IIb) revealed disorganized Leydig cells (Fig. 2c). TEM showed abundant lipid inclusions, swollen mitochondria with collapsed cristae and vacuoles in the smooth endoplasmic reticulum (Fig. 2d, 2e). The Leydig cells of the progeny of rats belonging to Group II b (mothers exposed to 50ppm CrVI during gestation) at PND 60 showed minor pathological changes such as appearance of large vacuoles containing cell debris (Fig. 2f). Leydig cells of PND 90 old progeny of mothers with (Group II a) gestational exposure to 50ppm CrV1 had normal organization in the light micrographs (Fig. 2g). However, TEM showed the Leydig cells with disrupted mitochondria and the macrophages revealed phagocytosed cell debris (Fig. 2h). In the testis of progeny of 100ppm CrVI-exposed mothers,

Fig.1a

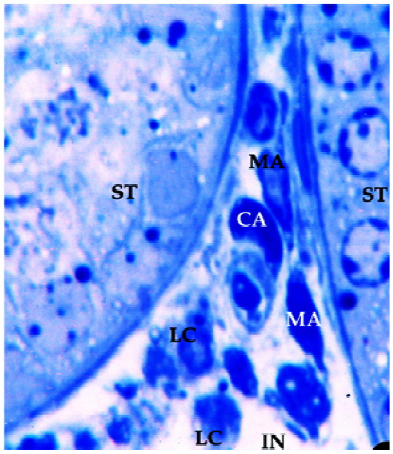


Fig.1b

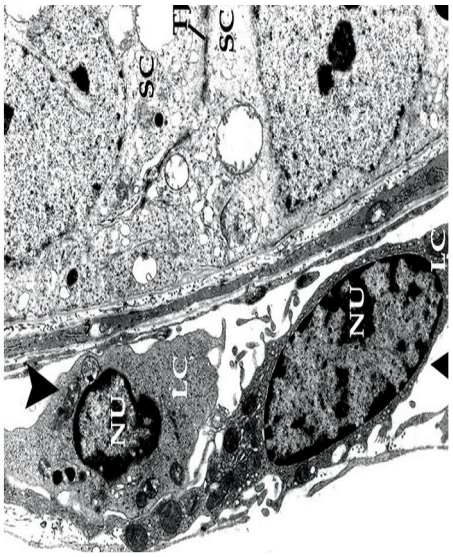


Fig.1c

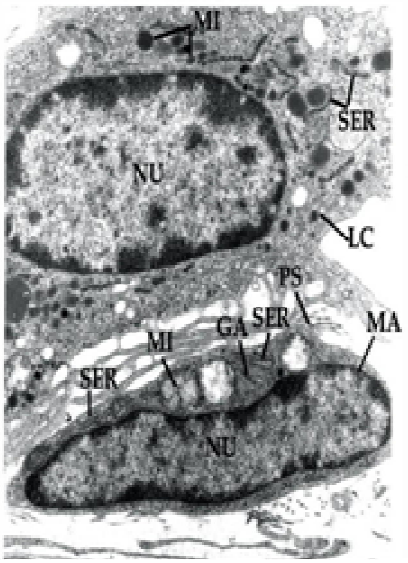


Fig.1a-1c. Organization of cells in the interstitium of control rats on PND 30. 1a: Light micrograph showing the interstitium (semithin; TBO; x 1,000). 1b: Low power TEM of Leydig cell (arrowheads) x 4,500). 1c: TEM of Leydig cell and macrophage (x 10,000). Other legends, as in Fig. 1d & c.

Fig.1d

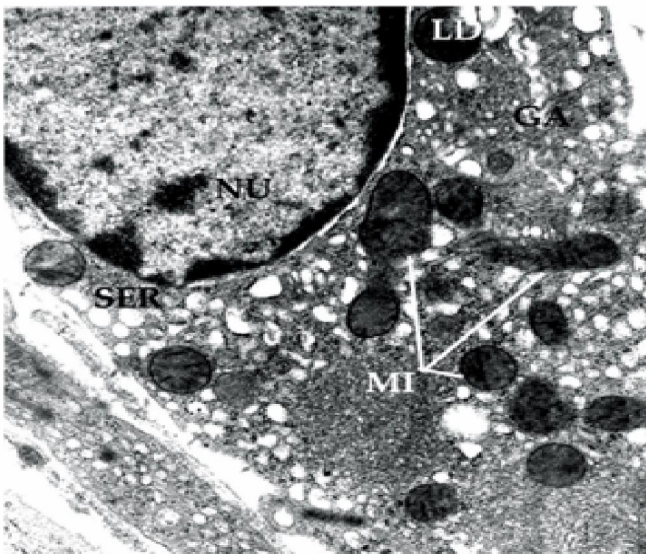


Fig. 1d. TEM. A portion of Leydig cell (x 20,000). CA, capillary; GA, Golgi apparatus; IN, interstitium; LC, Leydig cells; LD, lipid droplet; MA, macrophage; MI, mitochondria; NU, nucleus; PS, pseudopodial protuberances; SC, Sertoli cells; SER, smooth endoplasmic reticulum; ST, seminiferous tubules; TJ, tight junctions between Sertoli cells.

Fig.1e

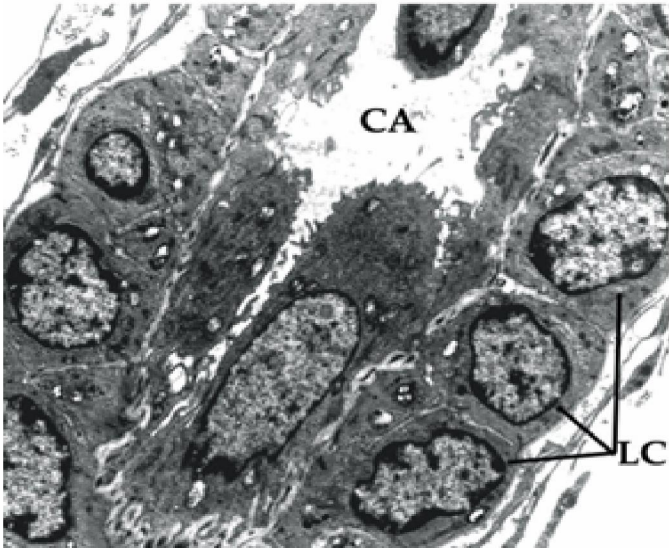


Fig.1e. TEM showing indications of establishment of adult type Leydig cells from peritubular cells in the testis of control pups on PND 30. Crowding of newly established adult-type Leydig cells around a capillary is seen (x 9,000). CA, capillary; LC, Leydig cells.

Fig.1f

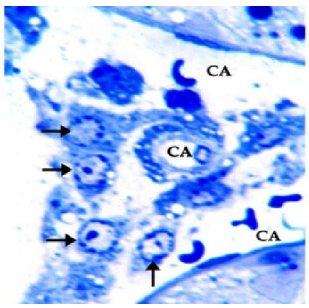


Fig. 1f. LM of seminiferous tubules and interstitium of control rat, PND 60. Interstitium shows normal Leydig cells (arrows) around the capillaries (semithin; TBO; x 1,000). CA, capillary.

Fig.1g

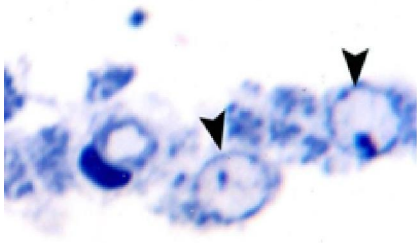


Fig. 1g. LM of seminiferous epithelium/Leydig cells of control rats on PND 90. Leydig cells with normal structure (arrowheads) (semithin; TBO; x 1,000).

Fig.2a

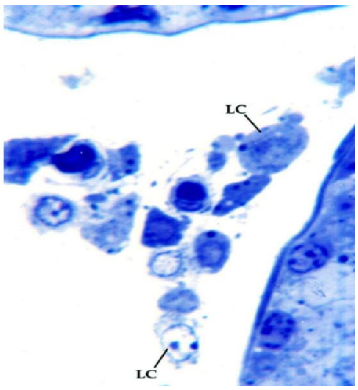


Fig.2b

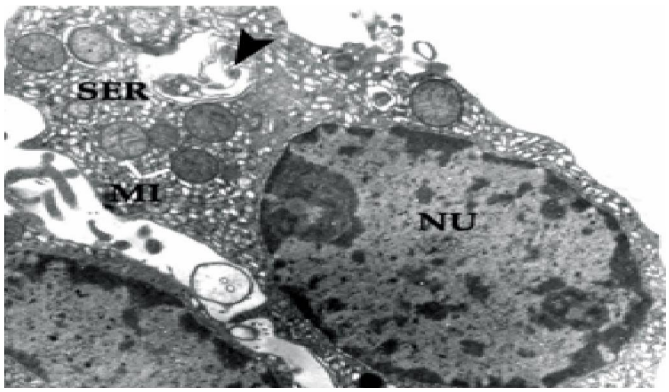


Fig. 2a, 2b. LM and TEM of Leydig cells of PND 30 progeny of mothers exposed to CrVI at 100 ppm concentration during gestation. 2a: LM showing hypotrophied Leydig cells (LC) (semithin; TBO; x 1,000). 2b: TEM showing a large vacuolar space containing cell debris (arrowheads) amidst smooth endoplasmic reticulum (SER) (x 7,000). LC, Leydig cells; MI, mitochondria; NU, nucleus; SER, smooth endoplasmic reticulum.

Fig.2c

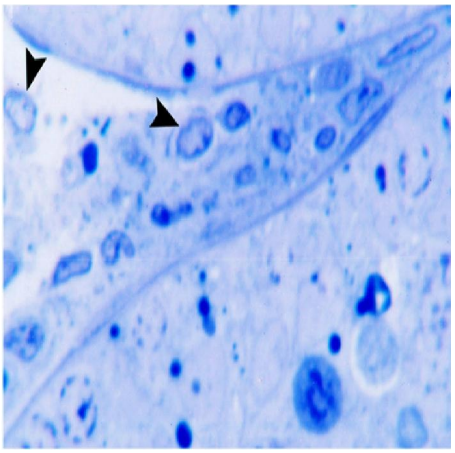


Fig.2d

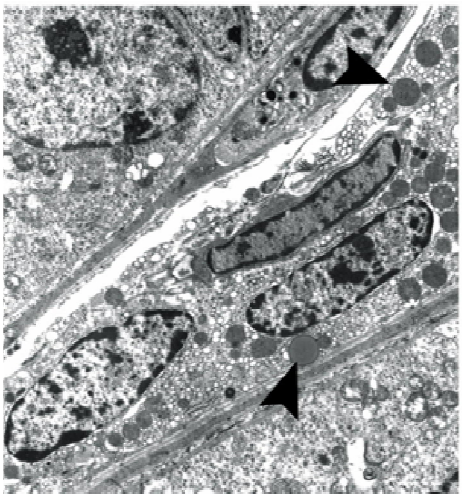


Fig.2e

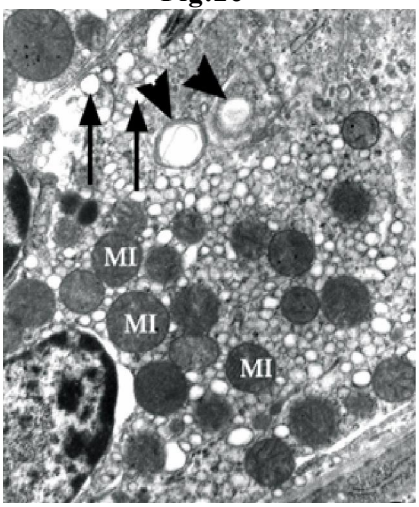


Fig. 2c, d, e. LM and TEM of a portion of interstitium of PND 30 progeny of mothers exposed to CrVI at 100 ppm concentration during gestation. 2c: LM showing disorganized Leydig cells (arrowheads) (semithin; TBO; x 1,000). 2d: TEM showing Leydig cells with dense accumulation of lipid inclusions (arrowheads) (x 4,500). 2e: TEM showing swollen mitochondria which have become highly electron-dense and vacuoles within the endoplasmic reticulum (arrows). In some of the mitochondria (arrowheads) the cristae are collapsed (x 20,000). MI, mitochondria.

Fig.2f **Fig.2g** **Fig.2h**

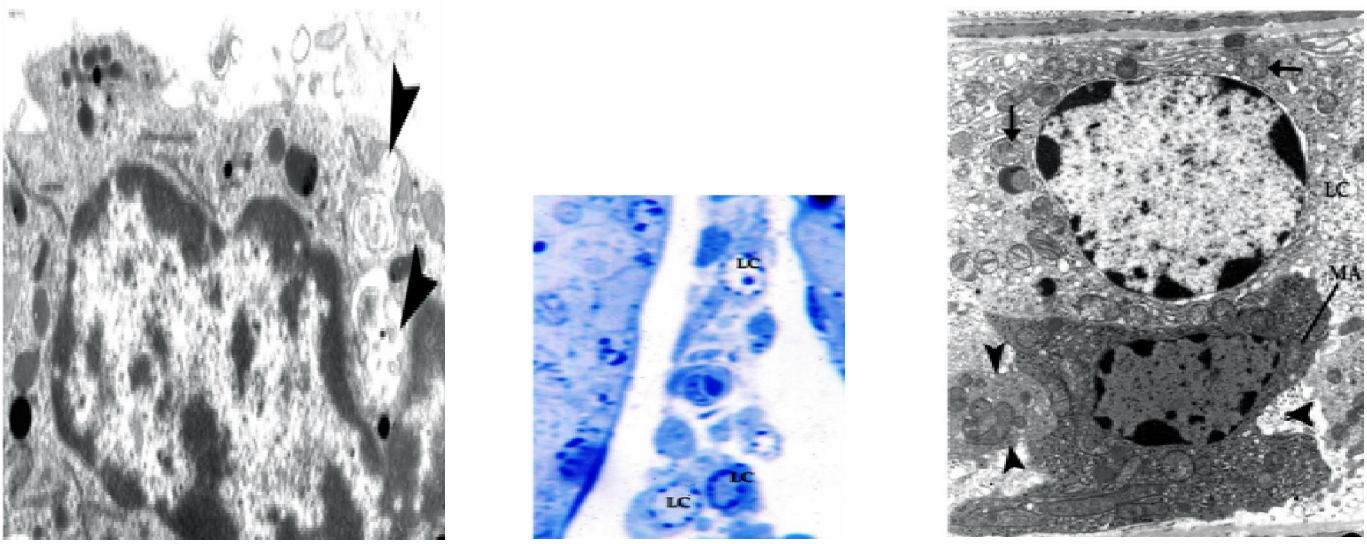


Fig. 2f. TEM of Leydig cell of PND 60 progeny of mothers exposed to CrVI at 50 ppm concentration during gestation. The Leydig cell reveals minor pathological change as seen in the large vacuoles (arrowheads) containing cell debris (x 10,000).

Fig. 2g. LM of Leydig cells of testis of PND 90 pogeny of mothers exposed to CrVI at 50 ppm concentration during gestation, showing normal Leydig cells (LC) (semithin; TBO; x 1,000).

Fig. 2h. TEM showing a Leydig cell (LC) with disrupted mitochondria (arrows) and a macrophage (MA) phagocytosing cell debris (arrowheads) (x 7,000).

Fig.2i **Fig.2j** **Fig.2k**

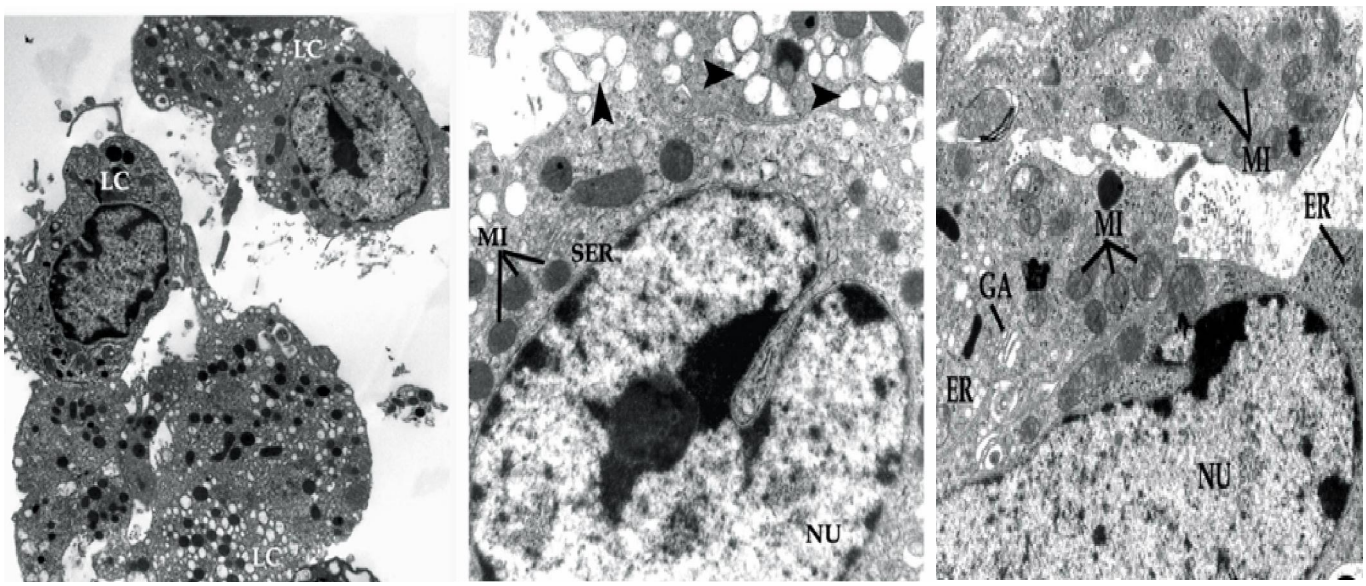


Fig. 2i, 2j. TEM of Leydig cells of testis of PND 90 progeny of mothers exposed to CrVI at 100 ppm concentration. 2j: A portion of Leydig cell on the top of figure 2i magnified, showing the vacuoles (arrowheads). (2i, x 3,000; 2j, x 20,000). GA, Golgi apparatus; LC, Leydig cell; MI, mitochondria; NU, nucleus; SER, smooth endoplasmic reticulum.

Fig. 2k. TEM of Leydig cells. GA, Golgi apparatus; MI, mitochondria; NU, nucleus; SER, smooth endoplasmic reticulum; ER, rough endoplasmic reticulum.

Fig.2l **Fig.2m** **Fig.2n**

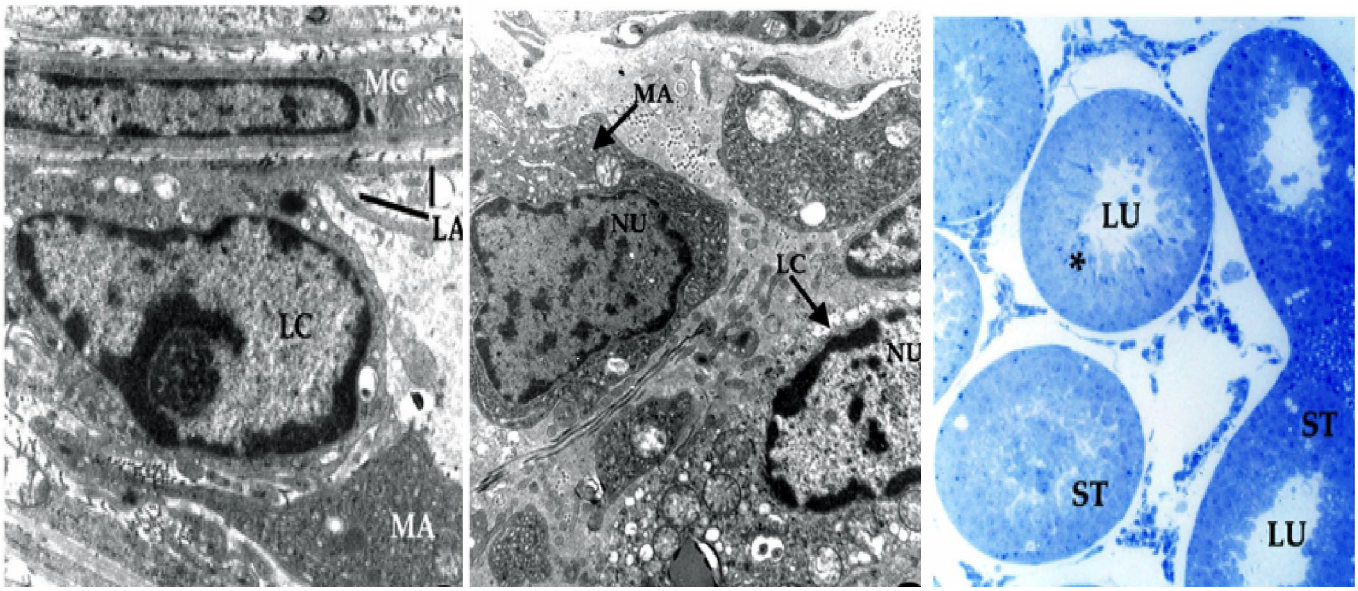


Fig. 2*l*. TEM of testis of PND 30 rat with lactational exposure to CrVI at 50 ppm concentration. A nascent adult type Leydig cell poor in content of mitochondria. A portion of a macrophage is also seen. (x 15,000). LA, lamina; LC, Leydig cell; MA, macrophage; MC, myoid cell; SC, Sertoli cell.

Fig. 2m. TEM of Leydig cells and a macrophage of PND 30 rat pups with lactational exposure to CrVI at 100 ppm concentration. (x 5000).

Fig. 2n. LM of testis of PND 60 rat with lactational exposure to Cr VI at 50ppm concentration. In the tubule marked with asterisk fully formed spermatics are adherent to the epithelium. LC, Leydig cell; MA, macrophage; NU, nucleus, ST, seminiferous tubules (TBO; x 100).

Fig.2o **Fig.2p** **Fig.2q**

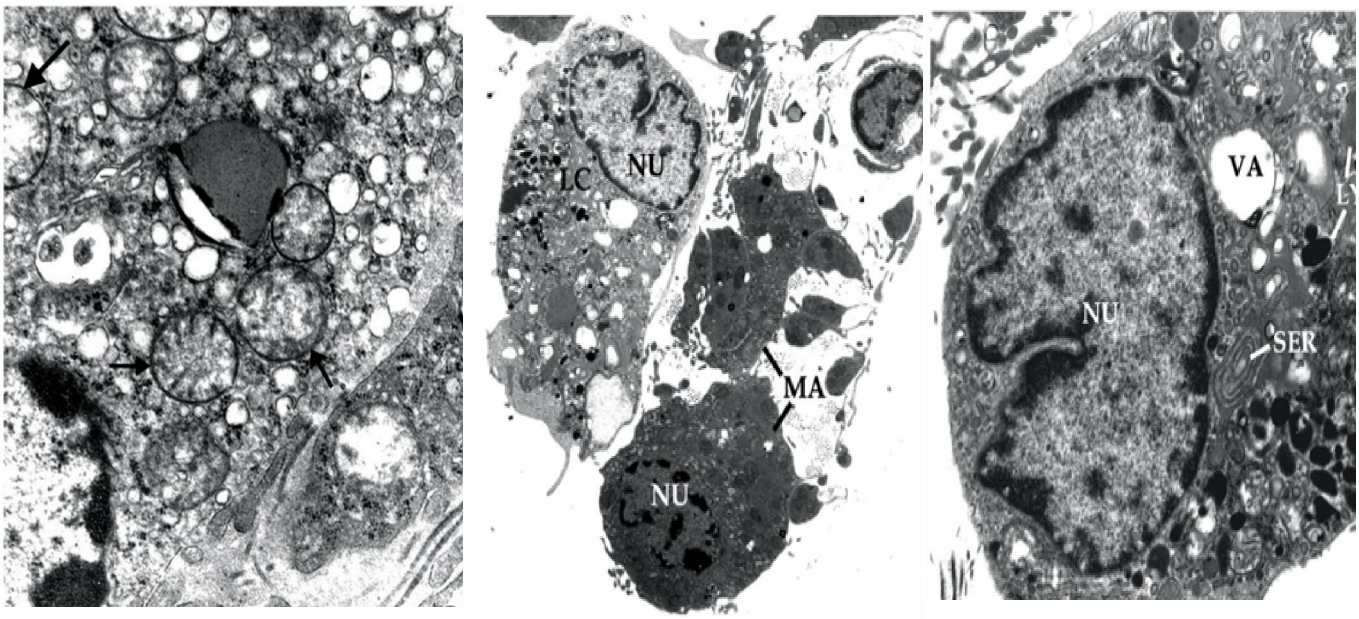


Fig. 2o, 2p, 2q. TEM of testis of PND 90 rat with lactational exposure to CrVI at 100 ppm concentration. 2o : A portion of Legdig cell with lipid inclusion and swollen mitochondria with disrupted cristae (x 15000). 2p : Leydig cell and macrophage (x 3,000). 2q : A Leydig cell magnified (x 10,000). LC, Leydig cell; LY, lysosome; MA, macrophage; NU, nucleus; SER, smooth endoplasmic reticulum; VA, vacuole.

vacuolated Leydig cells were predominant (Fig. 2i, 2j). The Leydig cells appeared perfectly normal in PND 120 old rats with gestational exposure to100 ppm CrVI during embryonic life (Fig. 2k).

Testis of PND 30 old rats which were lactationally exposed to 50ppm CrVI (group IV) had nascent adult type Leydig cells which had fewer mitochondria (Fig. 2l). The testis of coeval rats with 100ppm CrVI exposure (Group V) showed Leydig cells with collapsed mitochondrial cristae (Fig. 2m, 2n). In PND 60 old rats exposed to CrVI at 50ppm concentration during lactational period, the Leydig cells contained abundant lipid inclusions (Fig. 2o), whereas PND 90 old rats exposed to 100 ppm CrVI during lactational period revealed large vacuoles in the Leydig cells (Fig. 2p, 2q). However, PND 120 old rats with lactational exposure to CrVI at 100 ppm concentration, the Leydig cells were normal.

Hormone analysis

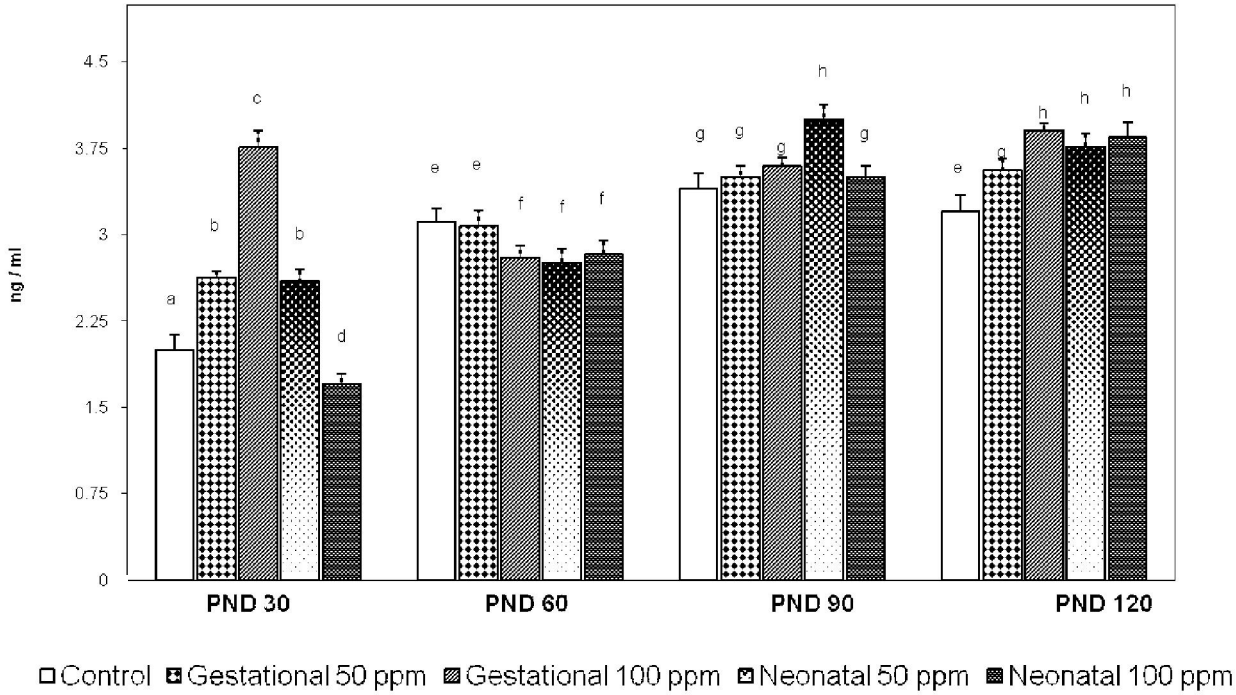
Testosterone (Fig. 3): In control rats, serum testosterone level increased significantly between PND 30 and PND 60, and it remained as plateau until PND 120. In PND 30 old rats, which experienced gestational exposure to CrVI, there was a significant increase in serum testosterone, and the magnitude of increase was higher in rats exposed to 100ppm CrVI than 50ppm. Lactational exposure to CrVI produced a dose-dependent differential effect on serum testosterone in PND 30 old pups, while 50 ppm CrVI exposure increased serum testosterone level, 100 ppm CrVI caused a marginal but significant decrease in the same. By PND 60, rats subjected to CrVI treatment recorded a marginal but statistically significant decrease in serum testosterone level except in the group that had gestational exposure to 50 ppm CrVI in which the level of testosterone was comparable to coeval controls. By PND 90, the experimental rats belonging to all treatment groups recorded normal serum testosterone levels except in rats with 50ppm CrVI exposure during lactational period, which showed an elevated level. By PND 120, all groups of experimental rats showed a significant increase in serum testosterone when compared with coeval control rats.

Estradiol (Fig. 4)*:* In the control rats, there was a sharp increase in the serum estradiol level by PND 60, which decreased thereafter and by PND 120, the level was comparable to PND 30 control rats. Gestational and lactational exposure to CrVI had a differential effect on serum estradiol titer. On PND 30, rats with gestational exposure of CrVI showed decreased serum estradiol level while lactational exposure to CrVI resulted in increased serum estradiol. By PND 60, serum estradiol level increased in rats exposed to CrVI during gestational as well as lactational periods. In PND 90 old rats, the level of serum estradiol was comparable between controls and gestationally CrVI exposed groups but a significant decrease in serum estradiol level was recorded in rats which had lactational exposure to CrVI. By PND 120 serum estradiol levels in all groups of CrVI treated rats remained normal when compared to the controls.

Discussion

The present study reveals that gestational *(in utero)* as well lactational exposure to chromium would affect the circulating levels of testosterone and estradiol, in prepuberal and puberal age with concomitant histopathological and ultra-structural changes in the Leydig cells. CrVI exposure during the two different windows (gestational and lactational exposure) induced changes in testosterone vary slightly. These effects of CrVI are partly or fully nullified from PND 90 onwards. These differences may be related to the two types of Leydig cells, fetal (FLCs) and adult (ALCs) types. In the progeny of mothers who had gestational exposure to CrVI, serum testosterone level increased during prepuberal peroid, whereas estradiol level decreased. This trend was not noticed with increase of postnatal days towards puberty, of course with slight difference between the groups exposed to different doses of CrVI. This suggests that CrVI exposure affects the conversion of testosterone into estradiol, perhaps due to interference with aromatase activity. Lactational exposure to CrVI produced almost an opposite trend where-in the testosterone level decreased (100 ppm, PND 30), whereas serum estradiol level increased. This is to be viewed in the perspective of the target Leydig cells. In the gestationally exposed group the target Leydig cells are FLCs because adult ALCs start appearing from PND 26 to PND 28 onwards (Ariyaratne and Mendis-Handagama, 2000). Thus, the gestational exposure (ED 9 to birth) to CrVI could impact only the FLCs. This can not impact the ALCs since LC start proliferating during postnatal life by PND 26-28 (the time by which FLCs undergo degeneration) and start differentiating as ALCs much later. The cells start expressing 11βHSD activity only when they transform from newly formed ALC into immature ALCs. Only mature ALCs acquire all necessary structures for steroid production and positive responsiveness to circulating LH (Shan and Hardy, 1992). ALCs gain more steroidogenic enzyme activities like 17βHSD, which catalyse the testosterone biosynthesis, with the gradual increase in smooth endoplasmic reticulum (Mendis-Handagama and Ariyaratne, 2001). Therefore,

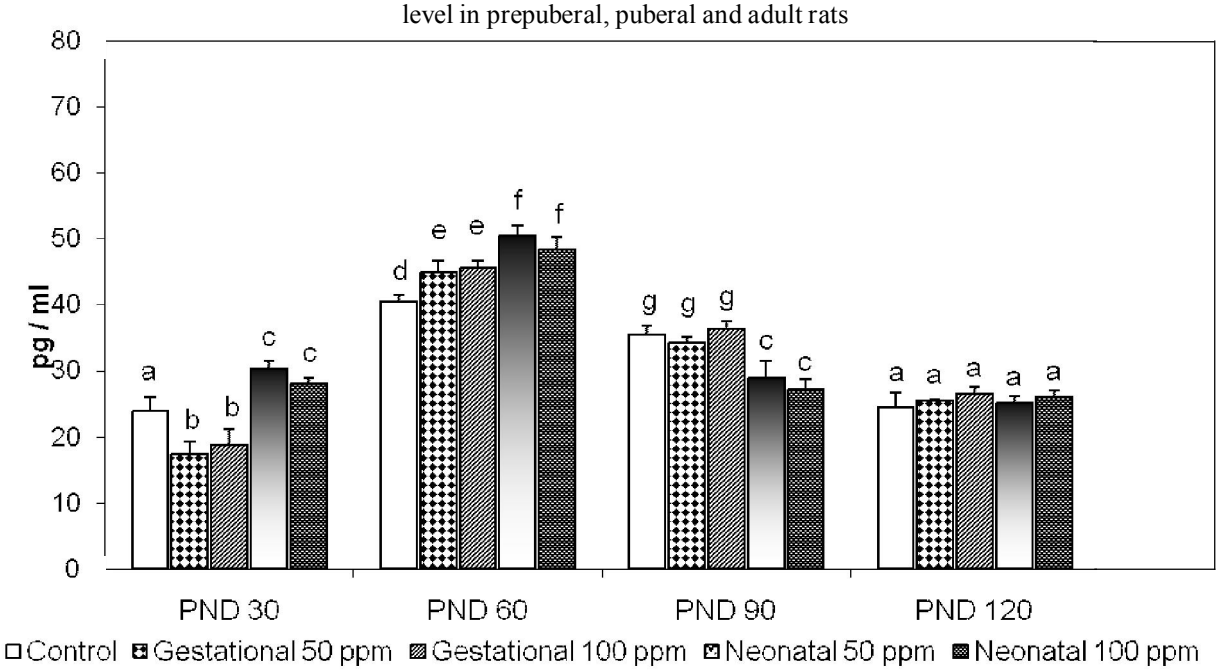
Fig: 3. Effect of gestational and neonatal exposure to chromium on serum testosterone level in prepuberal, puberal and adult rats



Gestational exposure: Pregnant rats were exposed to 50 or 100 ppm CrVI in drinking water from day 9 pc to 21 pc
Neonatal exposure : Lactating mothers were exposed to 50 or 100 ppm CrVI in drinking water from day 1 pp to 21 pp

Each bar represents the mean and the vertical line above denotes SEM. n=6. Histograms with same alphabets denote statistically insignificant difference between the respective means, while those with different alphabets indicate statistically significant difference between those means at *p< 0.05* level.

Fig. 4. Effect of gestation and neonatal exposure to chromium on serum estradiol level in prepuberal, puberal and adult rats



Gestational exposure: Pregnant rats were exposed to 50 or 100 ppm CrVI in drinking water from day 9 pc to 21 pc
Neonatal exposure : Lactating mothers were exposed to 50 or 100 ppm CrVI in drinking water from day 1 pp to 21 pp

Each bar represents the mean and the vertical line above denotes SEM. n=6. Histograms with same alphabets denote statistically insignificant difference between the respective means, while those with different alphabets indicate statistically significant difference between those means at *p< 0.05* level.

the gestational exposure to CrVI affects only the FLCs and may have no impact on the ALC.

Lactational exposure to CrVI was only up to PND 21. Here again the experimental protocol adopted has targeted only the FLCs and not the differentiated ALCs. Thus, in as much as CrVI and other toxicants can potentially affect Leydig cell structure and function, there is an inherent difference in that any impact in the FLCs vanishes when these cells disappear. Perhaps, if the pups are exposed to CrVI during the critical window of differentiation of ALCs, as was evident in the case of the fungicide iprodione (Blystone et al., 2007), it might produce a long-lasting or sustained impact, extending into the adult life. In fact, we have observed significant decrease in testosterone in rats with protracted exposure to CrVI from PND1to PND 90 or 120 (Unpublished data).

Thus, this study leads to the conclusion that exposure of rats to CrVI *in utero* or from PND 1 to PND 21 can impact only the FLCs, producing alteration in circulating steroid hormone levels transiently, since ALCs start differentiating only from PND 26-28 onwards. Our results in respect of testosterone level in rats with gestational exposure to CrVI and assessed on PND 30 are opposite to the observation of Akingbemi et al. (2001) who tested rats with DHEP. Never-the-less, these authors found that the effects were not apparent at PND 90. This could be further substantiated if an experiment is designed to test CrVI toxicity during the critical window of differentiation and maturation of ALC. Study in this line is in progress to find if the change in the FLC and the levels of testosterone and estradiol affect the testis and accessory reproductive organs.

Acknowledgments

This study was carried out with financial support from University Grants Commission (UPE, SAP & ASIST programs) and the Department of Science and Technology (FIST). P. Sekhar thanks the University Grants Commission, New Delhi, for the faculty Improvement Program award.

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