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Transient Gestational Exposure to Hexavalent Chromium (CrVI) Adversely Affects Testicular Differentiation: A Study in Rat Model

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Abstract

Chromium (Cr), an essential trace element, turns into an endocrine disruptor and male reproductive toxicant when its concentration in drinking water exceeds the safe limit. Improper disposal of effluents from more than 50 industries that use Cr contaminate the environment, in addition to occupational exposure of the workers. Testis has come to stay as a target for the reproductive toxicity of hexavalent Cr (CrVI), whereas its impact on fetal testicular differentiation remains elusive. We tested the hypothesis "*In utero* exposure to CrVI may alter the level of specific proteins controlling differentiation of testicular cell types". Pregnant Wistar rats were exposed to drinking water containing 50, 100 and 200 ppm potassium dichromate (CrVI) during gestational days 14 to 21, covering the period of fetal differentiation of testicular cells. Testes were collected on postnatal day 1 and subjected to light microscopic histological studies and immunohistochemical detection of cell-specific proteins. Testis of neonatal rats with gestational exposure to high doses of CrVI showed shrunken and dispersed tubules with fewer gonocytes, extensive vacuolization of seminiferous cord accompanied by damaged epithelium, and shrunken Leydig cells present in large interstitial spaces and loose compaction of cells when compared coeval control group. Immunosignals of androgen and estrogen receptor β increased, whereas those of estrogen receptor α, follicle stimulating hormone receptor, anti-Mullerian hormone, P_{450} aromatase, inhibin, c-fos and c-jun decreased. Immunosignals of steroidogenic acute regulatory protein and CYP11A1 increased, whereas 3β -hydroxy steroid dehydrogenase and CYP17A1 proteins decreased, indicating compromised steroidogenic function. Our findings support the proposed hypothesis and we conclude that gestational exposure to CrVI disrupts specific hormones and hormone receptors that control fetal differentiation of testicular cells. The detrimental effect of gestational exposure to CrVI on functional differentiation of testicular cells may have a bearing on testicular function at adulthood.

Keywords: Gonocytes, Leydig Cell, Sertoli Cell, Steroidogenesis, Testis

Abbreviations

AR, androgen receptor; **3-βHSD**, 3β-hydroxy steroid dehydrogenase; **17-βHSD**, 17β-hydroxy-steroid dehydrogenase; **CrIII**, trivalent chromium; **CrVI**, hexavalent chromium; **CYP11A1**, cytochrome P450 family 11 subfamily A member 1; **CYP17A1**, cytochrome P450 family 17 subfamily A member 1; **E_2**, 17β estradiol; **ERα**, estrogen receptor α; **ERβ**, estrogen receptor β; **FSH**, follicle stimulating hormone; **FSHR**, FSH receptor; **GD**, gestational day; **LCs**, leydig cells; **LH**, luteinizing hormone; **LHR**, LH receptor; **PND**, postnatal day; **SCs**, sertoli cells; **StAR**, steroidogenic acute regulatory protein; **T**, testosterone; **USEPA**, united states environment protection agency.

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1. Introduction

Endocrine disrupting chemicals (EDCs) are major causative factors of male infertility, both in animals and humans[1]. Increasing incidences of hypospadias, cryptorchidism, testicular germ cell cancer, and declining sperm count and fertility rates worldwide due to EDCs have raised apprehensions about environmental influence on male reproductive health[2-4]. Chromium (Cr), an essential trace element[5-8] is also one among the top ten heavy metal pollutants of the environment[9] and endocrine disruptor[10,11]. Recent reports have shown that Cr pollution is one of the top six toxic threats to the world[12]. It is used in more than 50 industries across the world with applications including tanneries, wood processing, chrome plating, welding, wax, paint, steel manufacturing, alloy, cast iron, ammunition, etc[13,14]. Its exponential usage in various industries worldwide and improper disposal of the effluent contaminate the environment, which has deleterious effect on human health, including reproduction[15]. CrVI, released from industries, is deposited in the landfills and waterways, and affects millions of people exposed to the polluted sites, who drink Cr-containing water[16]. It has been envisaged that approximately 74 million people in 7000 communities who drink tap water polluted with Cr across the world are at risk[17]. The concentration of Cr in drinking water supplied in different parts of USA has been reported to be higher than the safe limit[17-21] approved by US EPA i.e. 0.1 mg/L (0.1ppm)[21]. In many parts of India also, Cr concentration in drinking water is higher than the recommended safe level,[16,22,23] particularly in Ranipet (Tamil Nadu State, India), a flourishing tanneries site, which has total Cr in the range of 3.1 to 246 ppm in drinking water wells, whereas in Kanpur, another hub of tanneries (Uttar Pradesh State, India), it is between 1.05 and 35.34 ppm[24].

Chromium has become a potential threat to human reproductive health[25]. Increased incidences of birth and developmental defects among children born to mothers living around tanneries, chrome and leather industries in the developing world may attest the adverse effect of Cr on reproduction[16]. Abortion by 12 weeks in pregnant women employed in a dichromate manufacturing factory in Russia has been reported[26]. Exposure to CrVI decreased sperm counts and increased sperm abnormalities by disrupting germ cell arrangement and spermatogenesis in rat and humans[27]. Welders who work in stainless steel industry have poor sperm quality when compared to others[28,29]. Subfertility in 673 welders from Danish population in the same geographical area was reported and the study lead to the conclusion that 79% of wives of welders had increased rate of delayed conception[30]. Poor semen quality, sperm abnormalities and infertility in workers exposed to CrVI in mild steel welding industry was attributed to developmental problems including cancer in children[31]. Altered sex ratio with reduced male births due to paternal exposure to the metal fumes containing Ni and Cr has been reported in workers in an Italian mint[32].

Experimental studies performed in animal models pointed out that excess Cr may be a male reproductive toxicant. Direct effect of Cr on mammalian testis was predicted with the finding of its accumulation in the interstitial tissues of rat[33]. This view got entrenched by the finding of uptake of CrVI by the testis and its persistence in the tissue for a prolonged period in the form of CrIII in rat and mouse[34]. Edema of interstitial tissues, congestion of blood vessels and complete absence of spermatocytes in the seminiferous tubules have been found in rabbits administered 0.7 mg CrVI/kg body weight CrVI through oral route for six weeks[36]. Administration of 2 or 3 mg CrVI/kg body weight for 69 days to adult rats decreased sperm count and motility, and disrupted spermatogenesis with decreased late stage spermatids and germ cell number at stage VII, and altered activities of testicular enzymes sorbitol dehydrogenase, lactate dehydrogenase, λ-glutamyl transpeptidase and glucose-6-phosphate dehydrogenase[37]. Intraperitoneal (i.p.) injection of CrVI (0.5 mg/kg body weight) for five days a week for eight weeks led to testicular atrophy and reduced sperm counts and motility in rats, which could be reversed after eight weeks of reversal period[38,39]. Injections (i.p.) of 2 mg CrVI/kg body weight/day to adult rats for 15 days resulted in ultrastructural changes in testis, which included leakage of SC tight junctions in seminiferous tubules, cytoplasmic vacuolization and degeneration of mitochondria in the seminiferous epithelium and disruption of mitochondrial sheaths in the tail and mid-piece of spermatids[40,41]. Enlarged intercellular spaces, tissue loosening and significant loss of gametes into the lumen of seminiferous tubules, decreased sperm motility and number, and increased sperm abnormality were observed in adult rats treated CrVI (1 or 2 mg/kg body weight) for fifteen consecutive days[42].

Studies performed in our laboratory[43,44] on a non-human primate (*Macaca radiata*) model entrenched the male reproductive toxicity of sub-lethal doses of CrVI

(50, 100, 200, 400 ppm) administered through drinking water for a chronic period of six months. Adult monkeys exposed to different doses of CrVI experienced decreased sperm counts and forward motility from the second month onwards leading to azoospermia by the sixth month[43,46]. Ultrastructural studies on these monkeys revealed the presence of multi-nucleate hypertrophied germ cells undergoing degeneration in the lumen of testis and epididymis[45], which was attributed to oxidative stress in these organs[46]. Azoospermia in CrVI-treated monkeys was associated with free radical toxicity due to decreased specific activities of antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), and the concentration of reduced glutathione in seminal plasma, which was reversed by supplementation of various doses of vitamin C (0.5, 1, 2 mg/L)[44]. The level of H_2O_2 in the seminal plasma/sperm of monkeys increased with increasing doses and duration of CrVI exposure[44]. While all these changes were reversed after 6 months of Cr-free exposure period, simultaneous supplementation of vitamin C prevented the development of Cr-induced oxidative stress[44]. Electron micrographs of testis of Cr-treated monkeys revealed premature release of spermatocytes and round spermatids into the lumen, which underwent degeneration beginning with hypertrophy; epididymis developed micro-canalization in an adaptive mechanism to avoid extravasation of sperm in monkeys exposed to Cr[45]. Abundance of basal cells, intraepithelial macrophages and lipofuscin (LF) material in these cell types of the epididymis increased in CrVI treated monkeys; the principal cells phagocytosed dead sperm from the lumen due to CrVI exposure and processed them partially into LF material[47].

It was further reported from our laboratory that swollen LCs with large vacuoles and lipid inclusions, swollen mitochondria with collapsed cristae and vacuoles in SER of prepubertal (PND30) and young adult (PND 60) rats born to mothers with gestational exposure to 50 ppm /100 ppm CrVI, whereas LCs in PND90 rats had normal organization[48]. Gestational or lactational exposure to CrVI led to temporal reversible changes in serum testosterone and estradiol titres in rats[48]. Recently, we reported atrophy of seminiferous tubules and interstitial edema with distorted tubular morphology and increased interstitial space in adult rats of mothers with gestational exposure to CrVI[10]. The above report also showed disruption of inter-SC tight junctions and SC-GC junctions leading to disruption of spermatogenesis and premature release of round spermatids into the lumen in sequel to decreased expression of *Ar* and *Fshr* and tight junction proteins occludin and claudin-11 in SCs.

An *in vitro* study has proved the adverse effect of Cr on differentiation and self-renewal mechanisms of spermatogonial stem cells (SSCs) and disruption of steroidogenic apparatus due to decreased expression of *Cyp11a1* and *3βHsd* and increased *Star* and *Cyp17a1* expression in TM3 LC lines challenged with various concentrations of CrVI; TM4 SC line challenged with CrVI showed decreased expression of tight junction signaling and cell receptor molecules like *Tjp1, Ocln, Vim*, and thus impaired secretory functions[49].

Testicular differentiation is a critical event during male sex development, beginning at GD14 with the appearance of seminiferous cords in rat[50] and is strictly regulated by proteins and hormones of testicular origin. However, the impact of excess CrVI on fetal testicular differentiation remains a grey area of research. Therefore, we hypothesized that *in utero* exposure to CrVI may affect the expression of specific proteins controlling differentiation of testicular cell types, which we put to test in this study.

2. Materials and Methods

2.1 Experimental Animals and Chemicals

The experiments in Wistar rats (*Rattus norvegicus*) were approved by the Institutional Animal Ethics Committee for studies on experimental animals (Ref: IAEC No. 03/013/08), which had a nominee of the Committee for Prevention of Cruelty and Safety to Experimental Animals (CPCSEA), Government of India, Ministry of Environment, Forests and Climate Change, New Delhi. The entire study was conducted in triplicate with rats in three separate batches. The female rats were divided into four groups: **Group 1, Control**: Pregnant rats which received regular normal drinking water. **Groups 2, 3 and 4 (CrVI-treated)**: Pregnant rats which received drinking water containing 50, 100 and 200 ppm $K_2Cr_2O_7$ respectively, from gestational day (GD) 14 to GD21, encompassing the critical window of fetal testicular differentiation[50]. These doses of Cr have been in practice in our laboratory for more than a decade based on dose-response studies performed in rat and monkey models[43,46,51]. Among the selected doses, 50 ppm is the minimum effective dose, 100 ppm is the half-maximal effective dose and 200 ppm is the maximum effective dose, which induced reproduc-

tive toxicity as assessed by serum hormones and spermatogenesis/sperm counts without any mortality in rats[10]. Doses lower than 50 ppm (12.5 and 25 ppm) did not produce any obvious change in reproductive physiology or hormonal profiles of experimental animals[43,46,51]. Since the objective of the study has been to test the reproductive toxicity of the metal, we did not test lower doses prescribed for 'No-observed adverse effect level (NOAEL)' for carcinogenic responses[52] in the present study. These doses are above the USEPA (2017) guideline for safe limit of Cr (0.1 ppm) in drinking water and comparable to Cr level in drinking water in many developing countries with Cr based industries[16,53]. Throughout the study, rats were fed standard rat pellet feed (Brooke Bond Lipton India Ltd.,Bengaluru) *ad libitum* and maintained under controlled temperature (25±1 °C) and photoperiod (12h Dark: 12h Light). The animals were dewormed with albendazole (10 mg/kg body weight, orally) one week before use. The estrous cyclicity of rats was checked by observing the vaginal smear, daily morning and evening under the microscope (Long and Evans, 1922). Adult male rats with proven fertility (stud) were dewormed and allowed to mate with the females (1:2) at late proestrous phase. Presence of vaginal plug in the morning confirmed successful mating and that day was considered as day '0' of pregnancy, and the conceptus was considered as one day old, 24 h later i.e., day 1 post-coitum (dpc) or gestaional day (GD 1). On PND1 the male pups were euthanized (3 pups in each group from three sets of animals i.e. a total of 9 pups in each group), testes were randomly dissected out and fixed in 4% para-formaldehyde solution for sectioning followed by immunohistochemical localization of various key proteins associated with testicular differentiation, whereas 10% neutral buffered formalin was used as fixative for histological analysis of testis.

2.2 Hematoxylin and Eosin Staining

Testes of newborn (PND1) rats were dissected and fixed in 10% neutral buffered formalin solution for 3 days. The tissues were embedded in paraffin and sectioned at 5 µm thickness, and prepared for Harris' hematoxylin and Eosin. The sections were mounted in Distyrene Plasticizer Xylene (DPX). The sections were examined in a Nikon Eclipse 80i microscope and photographs were obtained at 400x magnification using Network Internet System (NIS) -imaging and capturing software (Nikon Instruments, Tokyo, Japan).

2.3 Immunohistochemical Analysis

PND1 rat testes for immunohistochemistry were randomly selected after completion of treatment period from GD14 to GD21. Testes were fixed in 4% paraformaldehyde (Fisher Scientific, Mumbai, India) for 3 days, processed and embedded in paraffin at 58 ºC, sectioned at 5 µm thickness and stored at room temperature until processed for immunostaining. The sections were deparaffinized by using xylene and rehydrated with different dilutions of alcohol in their descending percentages, then treated with 3% H_2O_2 in methanol to suppress endogenous peroxidase activity. Antigen retrieval was performed by heating the sections in citrate buffer (1:10 dilution in deionized water, pH 6.0) (Sisco Research Laboratories, Mumbai, India). The avidin-biotin peroxidase method was used for immunostaining[54]. The sections were incubated with 1% normal bovine serum albumin in TBS for 45 min to reduce nonspecific staining. Thereafter, the sections were kept for incubation overnight at 4 ºC with specific primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The optimal working dilution of each antibody was determined by incubating sections with various concentrations of antibody ranging from (1:150 to 1:250 µL dilution). Following incubation with the primary antibody, the slides were washed in TBS (Tris Buffer Saline) and incubated with biotinylated secondary antibody anti-rabbit IgG or anti-goat IgG (1:300 µL) for 1 hour at room temperature, and then with avidin-biotin peroxidase (1:200 µL) (Vector Labs, Burlingame, CA) for 30 min. Further, the sections were exposed to 3, 3' diaminobenzidine tetra-hydrochloride (DAB) (Impact, Vector Labs, Burlingame, CA), counterstained with hematoxylin, and mounted with DPX (Sisco Research Laboratories, Mumbai, India).

The stained sections, after mounting, were observed in a Nikon Eclipse 80i microscope and photographed using NIS element software at 400x magnification. At least five 40x power fields were digitized for each sample with a NIS element software camera (Nikon Eclipse 80i microscope). The immunostained cells and the intensity of staining of each testicular subpopulation were considered to find the outcome in each group for analysis.

2.4 Statistical Analysis

All statistical analyses were performed using Prism (version 6.0; Graph Pad Software, San Diego, CA). For comparison of control and CrVI-treated (50 ppm, 100 ppm and 200 ppm) animals, one-way ANOVA followed by Newman Keuls post-test for multiple comparisons was used.

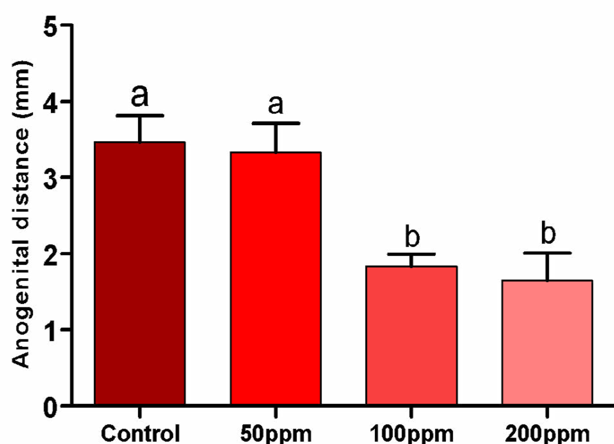

Figure 1. Impact of gestational exposure to CrVI on the AGD of neonatal rats. X-axis indicates doses of CrVI;the bar above each histogram denotes the SEM of five animals ; alphabets above each histogram indicate the statistical significance of difference between means;same alphabets in two means indicate no significance,whereas different alphabets indicate statistically significant difference at $p<0.05$ between the two means.

3. Results

3.1 Measurement of Anogenital Distance (AGD)

F_1 progeny of mothers exposed to drinking water containing 100 ppm and 200 ppm CrVI during GD14 to 21 had decreased AGD as compared to coeval control rats. The AGD of 50ppm CrVI treated male rats were comparable to the control (Figure 1).

3.2 Histological Changes

Testis of control rats showed normal arrangement of the spermatogenic cords with mononucleate gonocytes and spindle-shaped mesenchymal cells having normal distribution in interstitium (Plate 1A). F_1 progeny of mothers who consumed drinking water containing CrVI manifested various histopathological changes, which were exponential with increasing concentrations of the metal (Plate 1A-D). The F_1 progeny of mothers exposed to 50 ppm CrVI during gestational period showed unaltered histoarchitecture in general, except for loosening of tubular compaction and increased interstitial space (Plate 1B), whereas neonates of mothers exposed to 100 ppm CrVI showed shrunken tubules with damaged SCs with highly vacuolated cytoplasm and increased interstitial space (Plate 1C). The neonatal rats of mothers exposed to the highest concentration of (200ppm) CrVI exhibited marked disruption of spermatogenic cells with appearance of some multinucleate and vacuolated gonocytes (Plate 1D). The spermatogonial cells had increased heterochromatin in the 200 ppm CrVI treated neonatal rats.

3.3 Immunohistochemical Changes

AMH exhibited strong signal within the seminiferous tubule of control rat testis retrieved on PND1 (Plate 2A); consistent with the fact that SCs are its source, intense AMH signal was seen in and around these supportive cells. However, the signal intensity decreased in F_1 progeny of mothers exposed to 50 ppm, 100 ppm and 200 ppm CrVI during gestational period (Plate 2B,C,D)

Inhibin showed immunopositivity and strong signal of inhibin in seminiferous tubules (Plate 3A). Though the staining pattern in CrVI-treated pups bore resemblance to the testis of control rats, the signal intensity in 50 ppm, 100 ppm and 200 ppm CrVI-treated rats decreased significantly as compared to control (Plate 3B,C,D).

FSHR protein in F_1 neonatal rats showed strong immunostaining in control rats (Plate 4A), whereas a significant reduction in signal was observed in pups born to mothers who consumed drinking water containing CrVI during gestation (Plate 4B,C,D).

The LHR protein showed no immunopositive signal in the testis of F_1 neonatal rats of either control or CrVI treated group (Plate 5).

PND1 rat pups belonging to the control groups exhibited no conspicuous immunosignal of AR protein in the interstitium, peri-tubular myoid (PTM) cells and SCs of newborn control rat testis. Coeval rats born to mothers with gestational exposure to CrVI also showed very weak signal in the interstitium and PTM, especially in the group exposed to 200 ppm CrVI (Plate 6).

ERα protein exhibited strong signal in tubular compartment as well as in interstitial cells of control neonatal rat testis. On the other hand, F_1 progeny of mothers with gestational exposure to CrVI revealed decreased signal intensity in a dose-dependent manner, compared to coeval control in the order of control >50 ppm > 100 ppm > 200 ppm CrVI treated groups (Plate 7).

Neither control nor experimental rats with exposure to 50 ppm and 100 ppm CrVI showed any immunopositivity for ERβ. However, neonates born to mothers in 200 ppm group showed ERβ positivity in a few interstitial cells (Plate 8).

LCs of neonatal control rat testis showed immunopositivity of StAR protein, whereas F_1 progeny of mothers in 50 ppm, 100 ppm and 200 ppm CrVI treated groups exhibited increased signal intensity in SC cytoplasm along with LCs (Plate 9).

The control and F_1 neonates of mothers in 50 ppm CrVI treated group showed immunopositivity of CYP11A1 protein in LCs, and the intensity of the signal in both groups was comparable, whereas the signal intensity increased in 100 ppm and 200 ppm CrVI treated groups, not only in interstitial LCs but also in intraluminal compartment containing SC component. The shift in enzyme signal was more pronounced in SCs than interstitial LCs in the neonatal rats of mothers in 200 ppm CrVI group (Plate 10).

The interstitial LCs of neonatal control rats showed immunopositivity for 3βHSD protein, whereas F_1 progeny of mothers exposed to CrVI during gestational period exhibited diminished signal compared to coeval control in a dose-dependent manner. The intensity of the signal was in the order: control > 50 ppm > 100 ppm > 200 ppm (Plate 11).

The immunopositivity of CYP17A1 protein exhibited strong signal in the interstitium of control testis, which decreased in CrVI-treated rats. While decreased CYP17A1 signal intensity was observed in 50 ppm CrVI treated group, testicular cells of newborn rats of mothers in 100ppm and 200ppm CrVI treated groups did not show any immunopositive signal for CYP17A1 protein (Plate 12).

Immunostaining for P_{450} aromatase protein showed positivity in LCs and a weak signal within the seminiferous tubules of neonatal control rats and 50 ppm CrVI treated group. On the other hand, neonates of mothers with gestational exposure to 100 or 200 ppm CrVI exhibited no or very weak signal of aromatase, compared to coeval control (Plate 13).

The immunohistochemical localization of c-fos showed intense staining in the tubular compartment as well as LCs of control neonatal rat testis. The F_1 progeny of mothers with gestational exposure to CrVI exhibited diminished intensity in a dose-dependent manner, in the order: control > 50 ppm > 100 ppm > 200 ppm (Plate 14).

c-Jun protein, showed immunopositivity in the tubular and interstitial compartments of control rat testis, the intensity of which decreased in F_1 neonatal rats of mothers with gestational exposure to CrVI (Plate 15).

4. Discussion

The histological pictures clearly attest interruption in the testicular differentiation process in newborn F_1 progeny rats of mothers who consumed drinking water containing CrVI at concentrations higher than the safe limit prescribed by US EPA[21]. The changes that appeared in the seminiferous cords of fetal testis suggest that CrVI exposure during the critical window of differentiation interferes with the normal development of testicular structure.

Another important finding of the present study is that gestational exposure to CrVI may alter the male phenotypical characters in the progeny. This could be deduced from shortened AGD, a biomarker for male phenotype, which is longer in males than in females attesting its androgenic control[55]. AGD is known to be shortened in male rats exposed to numerous endocrine disruptors during prenatal period[56]. Puberty acceleration, sex differentiation and secondary sexual characters in rodents are marked by the AGD[57] which is under the control of androgens[58]. Thus, reduced AGD has been considered as a proxy indicator of decreased testosterone level[59]. The reduction of AGD in male rats with gestational exposure to flutamide, an anti-androgen, was attributed to subnormal androgen status[56]. Therefore, decreased AGD observed in the present study in new-born (PND1) rats of mothers exposed to CrVI during gestational period suggests that the heavy metal treatment has modified the androgen-dependent male phenotype. Our earlier report has shown sub-normal testosterone level in adult F_1 progeny of mothers with gestational exposure to CrVI[10]. Presumably, the F_1 neonatal male progeny rats of the present study might also have been in a state of skewed androgen status during the critical window of fetal testicular differentiation, resulting in reduced AGD.

Sexual differentiation, particularly masculinization programming window, is driven by anti-Mullerian hormone (AMH) and androgens secreted by fetal testis[60,61]. The earliest event in the testis differentiation beginning at GD14 in rat is the appearance of seminiferous cords within the gonadal ridge where the first appeared SCs undergo differentiation[62,63]; these fetal SCs secrete the essential male factor AMH which facilitates masculinization of the fetus by degenerating Mullerian duct[60] and, thus, ensure the development of male reproductive system under the direction of androgens[61]. Fetal SCs secrete AMH until birth, even after regression of Mullerian ducts[64,65]. Therefore, subnormal level of AMH detected in

experimental rat pups of the present study may suggest that gestational exposure to CrVI might interfere with sex differentiation process. Studies performed in our laboratory have clearly shown the predominance of female pups born to mothers with gestational exposure to CrVI[66,67]. The manifestation of LC hyperplasia and infertility have been reported in *Amh* knockout mice[68] indicating the role of AMH in regulating fetal LC function[69]. In the fetal testis, both AMH and testosterone coexist in higher concentrations[70]. Moreover, AMH is an impeccable marker for SC differentiation as its expression sharply drops during the perinatal and prepubertal period[71]. The hormone dependence of masculinization renders this process susceptible to disruption by factors interfering with hormone production[72]. Taken together, the findings of the present study, such as decreased AMH and diminution of androgen status evident from reduced AGD, and steroidogenic proteins (3βHSD and CYP17A1) and hormone receptors (LHR and FSHR) suggest that CrVI might have evoked an adverse effect on the fetal masculinization process by affecting differentiation of testicular cell types.

Another peptide hormone secreted from SCs is inhibin, first detected on GD14.5 in fetal rat[73], which selectively suppresses FSH secretion[74]. Fetal FSH stimulates SC proliferation, and secretion of AMH and inhibin[75]. Based on diminished level of inhibin in the F_1 newborn rats of mothers exposed to CrVI during gestational period, one may anticipate a possible shoot-up in FSH secretion and action on the testis. However, decreased FSHR noticed in the testis of the progeny of mothers with gestational exposure to CrVI in the present study goes against this suggestion. Thus, gestational exposure to CrVI, particularly during the male programming window, might have altered sexual differentiation by altering hormonal status and, thus, testicular function in F_1 rats.

Immunohistochemical studies on gonadotropin receptors revealed that the testis of newborn (PND1) rats express both *Fshr* and *Lhr* with the former being predominant. FSH interacts through its specific receptor on SCs, the number of which begins to rise by GD 20 in rodents[76]. *Fshr* mRNA is first detected at GD14.5 in SCs, and responds to FSH from GD15.5 onwards in rats[77,78]. SCs divide during the fetal and neonatal periods and cease to proliferate by PND17–18 in mice.[79] The proliferation of SCs is FSH-dependent during late fetal life in rats as decapitation or treatment with anti-FSH of rat fetuses at GD18.5 led to a decreased proliferation of SCs at GD20.5 and GD21.5, which was rescued by injections of FSH[51,80]. FSHR -/- mice had reduced number and proliferation index of SCs at birth suggesting that fetal FSH promotes the proliferation of SCs[81]. The FSH dependent proliferation of SCs during late fetal life has also been validated in the rats[80,82]. Thus, FSH and its receptor play a crucial role in the proliferation and functional differentiation of fetal SCs. Therefore, decreased level of FSHR noticed in F_1 progeny of mothers with gestational exposure to CrVI may be expected to have adverse effect on testicular differentiation by affecting the proliferation and functional differentiation of SCs. Histological pictures of the testis and secretory proteins of SCs of these animals may attest this suggestion.

The c-Fos and c-Jun are members of the AP-1 family of transcription factors (TFs) that stimulate the expression of genes in rat SCs[83]. *Fshr* gene in rat, mouse and sheep contain a number of important regulators including AP-1 site in the core promoter region[84]. Therefore, diminution of Fshr protein detected in the testis of neonates with prenatal exposure to CrVI may be linked to reduced level of c-Fos and c-Jun proteins. The reduction in Fshr may be attributed to defective SC proliferation and function in F_1 progeny of mothers exposed to CrVI during pregnancy.

Lhr in fetal LCs is first detected on GD 16.5, and significant amounts of LH are not seen until testosterone begins to decrease towards the end of gestation[85,86]. In addition, the male reproductive tract of *Lhr* knockout mice was similar to control animals at birth, supporting LH-independent production of testosterone[87]. The differentiation of FLCs does not require stimulation by LH[88,89] and, thus, lack of LH or LHR may not perturb FLC function or fetal gonadal development[90,91]. Since LH does not play role in steroidogenesis during fetal period, the unaltered level of LHR in the neonates due to CrVI exposure in the present study may not be of any significance.

The FLCs contain most of the steroidogenic proteins/enzymes such as StAR, CYP11A1, 3βHSD III and CYP17A1 except 17βHSD III which is located in SCs and converts androstenedione to testosterone[92,93]. During normal fetal development, intra-testicular testosterone level increases steadily from GD15.5 onwards with a peak on GD19.5 and subsequently declines on PND1[94]. In LCs, the translocation of cholesterol to the inner mitochondrial membrane is mediated by the transduceosome complex, an assembly of proteins including a marker trans-membrane protein StAR[95,96], to facilitate the conversion of cholesterol to pregnenolone by enzyme CYP11A1[97,98]. The augmented immunosignal of StAR and CYP11A1 and an

opposite trend in other components of steroidogenesis such as 3βHSD, CYP17A1 and P_{450} aromatase in the LCs of newborn rats of mothers exposed to CrVI during pregnancy indicate enzyme/protein specific interference. This might jeopardize steroidogenic activity in neonates with gestational exposure to CrVI. In addition, it is interesting to notice the shift of enzyme signal to the intraluminal compartment of the testis in pups of mothers with gestational exposure to CrVI, which appears to be a devise to cope up with the cellular stress generated due to CrVI toxicity. The attendance of StAR and CYP11A1 immunosignal in other than LCs in rats exposed to CrVI is consistent with the findings that expression of these proteins in SCs is a protective stress response[99, 100].

3βHSD and CYP17A1 enzymes of steroidogenic machinery catalyze conversion of pregnenolone to progesterone and progesterone to androstenedione, respectively[101,102]. Reduced immunodetection of 3βHSD and CYP17A1 clearly points out that gestational exposure to CrVI might have led to subnormal production of steroids. In addition, even the available substrate (pregnenolone) also might not have been utilized for the synthesis of progesterone and androstenedione since CrVI treatment had decreased the level of both 3βHSD and CYP17A1. Moreover, enhanced levels of StAR and CYP11A1 seen in CrVI treated pups might have been an attempt to counterbalance the decreased levels of 3βHSD and CYP17A1. Thus, our findings clearly indicate interference of CrVI at each level of steroidogenic pathway.

Fetal and neonatal testicular cells possess P_{450} aromatase, ERα and ERβ[103-106], which are known to regulate differentiation of testicular cells during late gestational period, and inhibit the number and activity of somatic and germ cells[107], Fetal and neonatal testes are estrogen sensitive, as the inactivation of ERα increased steroidogenesis, whereas ERβ inactivation enhanced development of the germ cell lineage in the male[107]. ERα knockout mice (ERαKO) showed increased testosterone production by the fetal testes as early as GD13.5[108]. The activity of FLCs increased by inactivation of the ERα gene causing hypertrophy of these cells and augmented expression of genes transcribing steroidogenic enzymes[108]. ErβKO mice showed increased number of gonocytes after birth due to boosted proliferation and decreased apoptosis.[109,110] Therefore, diminution of P_{450} aromatase and ERα, and augmented ERβ (200 ppm CrVI) in the rats exposed to might have also adversely affected the somatic and germ cells population, and functionality of FLCs by perturbing the expression of steroidogenic protein/enzymes, and thus cellular differentiation of fetal testis. Data on AR suggest that CrVI exposure did not modify this nuclear hormone receptor in any significant manner.

5. Conclusion

Taken together, the findings on enzymatic / non-enzymatic proteins of the steroidogenic apparatus, along with hormone receptors and peptide hormones AMH and inhibin, support our hypothesis so as to conclude that exposure of pregnant mothers to drinking water containing high concentration of CrVI much above the permissible safe level may disrupt the fetal endocrines controlling differentiation of the testis and male phenotype.

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7. References

1. Skakkebaek NE, Rajpert-De Meyts E, Main KM. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. Hum Reprod. 2001; 16: 972-8. https://doi.org/10.1093/humrep/16.5.972 PMid:11331648
2. Sharpe RM, Skakkebaek NE. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 1993; 341: 1392-5 https://doi.org/10.1016/0140-6736(93)90953-E
3. Acerini CL, Hughes IA. Endocrine disrupting chemicals: a new and emerging public health problem? Arch Dis Child. 2006; 91: 633-41. https://doi.org/10.1136/adc.2005.088500 PMid:16861481 PMCid:PMC2083052
4. Nordkap L, Joensen UN, Jensen MB, Jorgensen N. Regional differences and temporal trends in male reproductive health disorders: Semen quality may be a sensitive marker of environmental exposures. Mol Cell Endocrinol. 2012; 355: 221-30. https://doi.org/10.1016/j.mce.2011.05.048 PMid:22138051
5. Anderson RA. Nutritional role of chromium. Sci Total Environ. 1981; 17: 13-29. https://doi.org/10.1016/0048-9697(81)90104-2

6. Anderson RA, Polansky MM. Dietary and metabolite effects on trivalent chromium retention and distribution in rats. Biol Trace Elem Res. 1995; 50: 97-108. https://doi.org/10.1007/BF02789412 PMid:8605085
7. Cefalu WT, Wang ZQ, Zhang XH, Baldor LC, Russell JC. Oral chromium picolinate improves carbohydrate and lipid metabolism and enhances skeletal muscle Glut-4 translocation in obese, hyperinsulinemic (JCR-LA corpulent) rats. J Nutr. 2002; 132: 1107-14. https://doi.org/10.1093/jn/132.6.1107 PMid:12042418
8. Piva A, Meola E, Gatta P, Biagi G, Castellani G, Mordenti A, Luchansky J, Silva S, Mordenti A. The effect of dietary supplementation with trivalent chromium on production performance of laying hens and the chromium content in the yolk. Anim Feed Sci Technol. 2003; 106: 149-63. https://doi.org/10.1016/S0377-8401(03)00006-3
9. Blacksmith Institute (New York), World's worst toxic pollution problem: The top ten of toxic twenty, Report, 2011, pp. 34-37.
10. Kumar MK, Aruldhas MM, Banu SL, Balaji S, Vengatesh G, Ganesh MK, Shobana N, Navin AK, Felicia MM, Sankar V, Stanley JA, Ilangovan R, Banu SK, Akbarsha MA. Male reproductive toxicity of CrVI: In-utero exposure to CrVI at the critical window of testis differentiation represses the expression of Sertoli cell tight junction proteins and hormone receptors in adult F1 progeny rats. Reprod Toxicol. 2017; 69: 84-98. https://doi.org/10.1016/j.reprotox.2017.02.007 PMid:28192182
11. Shobana N, Aruldhas MM, Tochhawng L, Loganathan A, Balaji S, Kumar MK, Banu LAS, Navin AK, Mayilvanan C, Ilangovan R, Balasubramanian K. Transient gestational exposure to drinking water containing excess hexavalent chromium modifies insulin signaling in liver and skeletal muscle of rat progeny. Chem Biol Interact. 2017; 277: 119-28. https://doi.org/10.1016/j.cbi.2017.09.003 PMid:28911802
12. Pure Earth and Green Cross (Switzerland). The World's Worst Pollution Problems series -"The New Top Six Toxic Threats: A Priority List for Remediation." 2015; 10th Report.
13. Stohs JS, Bagchi D, Hassoun E, Bagchi M. Oxidative mechanism in the toxicity of chromium and cadmium ions. J Environ Pathol Toxicol Oncol. 2001; 20:77-88. https://doi.org/10.1615/JEnvironPatholToxicolOncol.v20.i2.10 PMid:11394715
14. Costa M, Klein CB. Toxicity and carcinogenicity of chromium compounds in humans. Crit Rev Toxicol. 2006; 36:155-63. https://doi.org/10.1080/10408440500534032 PMid:16736941
15. Akpor OB, Ohiobor GO, Olaolu TD. Heavy metal pollutants in wastewater effluents: sources, effects and remediation. Adv Biosci Bioeng. 2014; 2: 37-43. https://doi.org/10.11648/j.abb.20140204.11
16. Blacksmith Institute. Polluted Places- India. Final report, 2007. January 2005- December 2007. Project implemented by Blacksmith Institute. Supported under Poverty and Environment Program (PEP), Asian Development Bank.
17. Sutton, R. Chromium-6 in U.S. Tap Water, In: Houlihan J, Sharp R, Bruzelius N, (Eds.), Environmental Working Group, Washington DC, 2010; pp. 1-23.
18. Salnikow K, Zhitkovich A. Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: nickel, arsenic, and chromium. Chem. Res. Toxicol. 2008; 21:28-44. https://doi.org/10.1021/tx700198a PMid:17970581 PMCid:PMC2602826
19. Hawthorne M. Toxic chromium found in Chicago drinking water. 2011. Chicago Tribune, Chicago.
20. Honeycutt ME. Hexavalent chromium in Texas drinking water. Toxicol Sci. 2011; 119:423-24; author reply 425. https://doi.org/10.1093/toxsci/kfq347 PMid:21081757
21. USEPA. Drinking water contaminants- standard and regulations, Washington DC, 2017 https://www.epa.gov/dwstandardsregulations.
22. CPCB (Central Pollution Control Board), Report on groundwater quality in Kanpur: status, sources and control measures: GWQS/8/1996-97. Central Pollution Control Board, India, 1997; 1:4-5.
23. Sharma P, Bihari V, Agarwal SK, Verma V, Kesavachandran CN, Pangtey BS, Mathur N, Singh KP, Srivastava M, Goel SK. Groundwater contaminated with hexavalent chromium [Cr (VI)]: a health survey and clinical examination of community inhabitants (Kanpur, India). PLoS One, 2012; 7: e47877 https://doi.org/10.1371/journal.pone.0047877 PMid:23112863 PMCid:PMC3480439
24. INSA (Indian National Science Academy, New Delhi). Hazardous metals and minerals pollution in India, 2011, August, A position paper.
25. Remy LL, Byers V, Clay T. Reproductive outcomes after non-occupational exposure to hexavalent chromium, Willits California, 1983-2014. Environ Health. 2017; 16:18. https://doi.org/10.1186/s12940-017-0222-8 PMid:28264679 PMCid:PMC5340004
26. Shmitova LA. Content of hexavalent chromium in the biological substrates of pregnant women and women in the immediate post-natal period engaged in the manufacture of chromium compounds. Gigiena Truda i Professional'nye Zabolevaniya, 1980; 2:33-35.
27. Li H, Chen X, Li S, Yao W, Li L, Shi , Wang L, Castranova V, Vallyathan V, Ernst E, Chen C. Effect of Cr(VI) exposure on sperm quality: human and animal studies. Ann Occup Hyg. 2001; 45:505-11. https://doi.org/10.1016/S0003-4878(01)00004-7
28. Mortensen J. Risk for reduced sperm quality among metal workers, with special reference to welders. Scand J Work

Env Health. 1988; 14: 27-30. https://doi.org/10.5271/sjweh.1954

29. Bonde JP. Semen quality and sex hormones among mild steel and stainless steel welders: a cross sectional study. Br J Ind Med 1990; 47: 508-514. https://doi.org/10.1136/oem.47.8.508 PMid:2118383 PMCid:PMC1035221
30. Bonde JP. Subfertility in relation to welding. A case referent study among male welders. Dan Med Bull. 1990; 37:105-08.
31. Bonde JP. The risk of male subfecundity attributable to welding of metals: studies of semen quality, infertility, adverse pregnancy outcome and childhood malignancy. Int J Androl. 1993;16:1-29. https://doi.org/10.1111/j.1365-2605.1993.tb01367.x PMid:8070939
32. Figa-Talamanca I, Petrelli G. Reduction in male births among workers exposed to metal fumes. Int J Epedimiol. 2000; 29:381-83. https://doi.org/10.1093/ije/29.2.381 PMid:10817140
33. Danielsson BR, Dencker L, Lindgren A, Tjalve H. Accumulation of toxic metals in male reproduction organs. Arch Toxicol. 1984; 7:177-80. https://doi.org/10.1007/978-3-642-69132-4_26
34. Sipowicz MA, Anderson LM, Utermahlen WE Jr, Issaq HJ and Kasprzak KS. Uptake and tissue distribution of chromium (III) in mice after a single intraperitoneal or subcutaneous administration. Toxicol Lett. 1997; 93:9-14. https://doi.org/10.1016/S0378-4274(97)00064-7
35. Sutherland JE, Zhitkovich A, Kluz T, Costa M. Rats retain chromium in tissues following chronic ingestion of drinking water containing hexavalent chromium. Biol Trace Elem Res. 2000; 74:41-53. https://doi.org/10.1385/BTER:74:1:41
36. Behari J, Chandra SV, Tandon SK. Comparative toxicity of trivalent and hexavalent chromium to rabbits: III. Biochemical and histological changes in testicular tissue. Acta Biol Med Ger. 1978; 37:463-68.
37. Saxena DK, Murthy RC, Lal B, Srivastava RS, Chandra SV. Effect of hexavalent chromium on testicular maturation in the rat. ReprodToxicol. 1990; 4:223-28. https://doi.org/10.1016/0890-6238(90)90062-Z
38. Ernst E. Testicular toxicity following short-term exposure to tri- and hexavalent chromium: an experimental study in the rat. Toxicol Lett. 1990; 51:269-75. https://doi.org/10.1016/0378-4274(90)90069-X
39. Ernst E, Bonde JP. Sex hormones and epididymal sperm parameters in rats following sub-chronic treatment with hexavalent chromium. Hum Exp Toxicol. 1992; 11:255-58. https://doi.org/10.1177/096032719201100403 PMid:1354972
40. Murthy RC, Saxena DK, Gupta SK, Chandra SV. Ultrastructural observations in testicular tissue of chromium-treated rats. Reprod Toxicol. 1991a; 5:443-47. https://doi.org/10.1016/0890-6238(91)90008-4
41. Muthy RC, Saxena DK, Gupta SK, Chandra SV. Lead induced ultrastructural changes in the testis of rats. Exp Pathol. 1991b; 42:95-100. https://doi.org/10.1016/S0232-1513(11)80054-X
42. Marouani N, Tebourbi O, Mahjoub S, Yacoubi MT, Sakly M, Benkhalifa M, Rhouma KB. Effects of hexavalent chromium on reproductive functions of male adult rats. Reprod Biol. 2012; 12:119-33. https://doi.org/10.1016/S1642-431X(12)60081-3
43. Aruldhas MM, Govindarajulu P, Hasan GC. Effect of chronic chromium exposure on sperm maturation and male fertility. In: Final Technical Report of major research project sponsored by Council of Scientific and Industrial Research, Government of India, New Delhi. 2000; (no. 60(0022)/EMR II/97).
44. Subramanian S, Rajendiran G, Sekhar P, Gowri C, Govindarajulu P, Aruldhas MM. Reproductive toxicity of chromium in adult bonnet monkeys (Macaca radiata Geoffrey). Reversible oxidative stress in the semen. Toxicol Appl Pharmacol. 2006; 215:237-49. https://doi.org/10.1016/j.taap.2006.03.004 PMid:16678873
45. Aruldhas MM, Subramanian S, Sekhar P, Hasan GC, Govindarajulu P, Akbarsha MA. Microcanalization in the epididymis to overcome ductal obstruction caused by chronic exposure to chromium - a study in the mature bonnet monkey (Macaca radiata Geoffroy). Reproduction. 2004; 128:127-37. https://doi.org/10.1530/rep.1.00067 PMid:15232070
46. Aruldhas MM, Subramanian S, Sekar P, Vengatesh G, Chandrahasan G, Govindarajulu P, Akbarsha MA. Chronic chromium exposure-induced changes in testicular histoarchitecture are associated with oxidative stress: study in a non-human primate (Macaca radiata Geoffroy). Hum Reprod. 2005; 20:2801-13 https://doi.org/10.1093/humrep/dei148 PMid:15980013
47. Aruldhas MM, Subramanian S, Sekhar P, Vengatesh G, Govindarajulu P and Akbarsha MA. In vivo spermatotoxic effect of chromium as reflected in the epididymal epithelial principal cells, basal cells, and intraepithelial macrophages of a nonhuman primate (Macaca radiata Geoffroy). Fertil Steril. 2006; 86:1097-105. https://doi.org/10.1016/j.fertnstert.2006.03.025 PMid:16949592
48. Sekar P, Vengatesh G, Kumar MK, Balaji S, Akbarsha MA, Aruldhas MM. Impact of gestational and lactational exposure to hexavalent chromium on steroidogenic compartment of post-natal rat testis, J Endocrinol Reprod. 2011; 15:15-26.
49. Das J, Kang MH, Kim E, Kwon DN, Choi YJ, Kim JH. Hexavalent chromium induces apoptosis in male somatic and spermatogonial stem cells via redox imbalance. Sci Rep. 2015; 5:13921. https://doi.org/10.1038/srep13921 PMid:26355036 PMCid:PMC4564811

50. Magre S, Jost A. Sertoli cells and testicular differentiation in the rat fetus. J Electron Microsc Tech. 1991; 19:172-88. https://doi.org/10.1002/jemt.1060190205 PMid:1748901
51. Subramanian S. Reproductive toxicity of chromium in adult male rats: An endocrine and biochemical study. Ph.D. Thesis, University of Madras, Chennai, India, 2001.
52. Sun H, Brocato J, Costa M. Oral chromium exposure and toxicity. Curr Environ Health Rep. 2015; 2: 295-303 https://doi.org/10.1007/s40572-015-0054-z PMid:26231506 PMCid:PMC4522702
53. Johnson T. India's threatened water supplies, Council of Foreign Relations, 2007, Times of India.
54. Sar M, Welsch F. Differential expression of estrogen receptor-beta and estrogen receptor-alpha in the rat ovary. Endocrinology, 1999; 140:963-71. https://doi.org/10.1210/endo.140.2.6533 PMid:9927330
55. Hsieh MH, Breyer BN, Eisenberg ML, Baskin LS. Associations among hypospadias, cryptorchidism, anogenital distance, and endocrine disruption. Curr Urol Rep. 2008; 9:137-42. https://doi.org/10.1007/s11934-008-0025-0 PMid:18419998
56. McIntyre BS, Barlow NJ, Foster PM. Androgen-mediated development in male rat offspring exposed to flutamide in utero: permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. Toxicol Sci. 2001; 62:236-49 https://doi.org/10.1093/toxsci/62.2.236 PMid:11452136
57. Cowley JJ, Pewtress RK. Ano-genital distance as a factor in determining puberty acceleration in mice. J Reprod Fertil. 1986; 78: 685-91, PubMed PMID: 3643279. https://doi.org/10.1530/jrf.0.0780685
58. Atkinson TG, Blecher SR. Aberrant anogenital distance in XXSxr ("sex reversed") pseudo male mice. J Zool Lond. 1994; 233:581-589. https://doi.org/10.1111/j.1469-7998.1994.tb05366.x
59. Fisher JS. Environmental anti-androgens and male reproductive health: focus on phthalates and testicular dysgenesis syndrome. Reproduction 2004; 127:305-15. https://doi.org/10.1530/rep.1.00025 PMid:15016950
60. Rey R, Lukas-Croisier C, Lasala C, Bedecarras P. AMH/MIS: what we know already about the gene, the protein and its regulation. Mol Cell Endocrinol. 2003; 211:21-31. https://doi.org/10.1016/j.mce.2003.09.007 PMid:14656472
61. Welsh M, Saunders PT, Fisken M, Scott HM, Hutchison GR, Smith LB, Sharpe RM. Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. J Clin Invest. 2008; 118(4):1479-90. https://doi.org/10.1172/JCI34241 PMid:18340380 PMCid:PMC2267017
62. Jost A, Magre S, Agelopoulou R. Early stages of testicular differentiation in the rat. Hum Gene, 1981; 58:59-63. https://doi.org/10.1007/BF00284150 PMid:7286994
63. Magre S, Jost A. Sertoli cells and testicular differentiation in the rat fetus. J Electron Microsc Tech. 1991; 19:172-88. https://doi.org/10.1002/jemt.1060190205 PMid:1748901
64. Koopman P, Munsterberg A, Capel B, Vivian N, Lovell-Badge R. Expression of a candidate sex- determining gene during mouse testis differentiation. Nature. 1990; 348:450-52. https://doi.org/10.1038/348450a0 PMid:2247150
65. Munsterberg A, Lovell-Badge R. Expression of the mouse anti-Müllerian hormone gene suggests a role in both male and female sex differentiation. Development. 1991; 113:613-24.
66. Vengatesh G, Kathiresh Kumar M, Sheerin Banu L, Aruldhas MM. Mechanism underlying infertility of male rats subjected to gestational exposure to CrVI. XXVIII National Symposium of the Society for Reproductive Biology and Comparative Endocrinology, New Delhi (India), 2010.
67. Kumar M, Balaji S, Navin AK, Ganesh MK, Aswini S, Chandra S, Shankar S, Akbarsha MA, Aruldhas MM. Gestational exposure to hexavalent Cr interferes with expression of specific genes involved in sex differentiation. XXXI National Symposium of the Society for Reproductive Biology and Comparative Endocrinology, Karnatak University, Dharwad, Karnataka (India), 2013.
68. Behringer RR, Finegold MJ, Cate RL. Müllerian inhibiting substance function during mammalian sexual development. Cell. 1994;79:415-25.https://doi.org/10.1016/0092-8674(94)90251-8
69. Rouiller-Fabre V, Carmona S, Merhi RA, Cate R, Habert R, Vigier B. Effect of anti-Müllerian hormone on Sertoli and Leydig cell function in fetal and immature rats. Endocrinology. 1998; 139:1213-20. https://doi.org/10.1210/en.139.3.1213 PMid:9492056
70. Rey RA, Musse M, Venara M, Chemes HE. Ontogeny of the androgen receptor expression in the fetal and postnatal testis: its relevance on Sertoli cell maturation and the onset of adult spermatogenesis. Microsc Res Tech. 2009; 72:787-95.
71. Al-Attar L, Noel K, Dutertre M, Belville C, Forest MG, Burgoyne PS, Josso N, Rey R. Hormonal and cellular regulation of Sertoli cell anti-Mullerian hormone production in the postnatal mouse. J Clin Invest. 1997; 100: 1335-43.
72. Sharpe RM, McKinnell C, Kivlin C, Fisher JS. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. Reproduction. 2003; 125:769-84. https://doi.org/10.1530/reprod/125.6.769 PMid:12773099
73. Majdic G, McNeilly AS, Sharpe RM, Evans LR, Groome NP, Saunders PT. Testicular expression of inhibin and activin subunits and follistatin in the rat and human fetus and neonate and during postnatal development in the rat.

Endocrinology. 1997; 138:2136-47. https://doi.org/10.1210/en.138.5.2136 PMid:9112414

74. Vale W, Rivier C, Hsueh A. Chemical and biological characterization of the inhibin family of protein hormones. Recent Prog Horm Res. 1988; 44:1-34.
75. Grinspon RP, Rey R. Anti-Mullerian hormone and Sertoli cell function in paediatric male hypogonadism. Horm Re. Paedter. 2010; 73:81-92. https://doi.org/10.1159/000277140 PMid:20190544
76. Warren DW, Huhtaniemi IT, Tapanainen J, Dufau ML, Catt KJ. Ontogeny of gonadotropin receptors in the fetal and neonatal rat testis. Endocrinology. 1984; 114:470-476. https://doi.org/10.1210/endo-114-2-470 PMid:6317355
77. Rannikki AS, Zhang FP, Huhtaniemi IT. Ontogeny of follicle-stimulating hormone receptor gene expression in the rat testis and ovary. Mol Cell Endocrinol. 1995; 107:199-208. https://doi.org/10.1016/0303-7207(94)03444-X
78. Lecerf L, Rouiller-Fabre V, Levacher C, Gautier C, Saez JM, et al. Stimulatory effect of follicle-stimulating hormone on basal and luteinizing hormone-stimulated testosterone secretions by the fetal rat testis in vitro. Endocrinology. 1993; 133:2313-18. https://doi.org/10.1210/en.133.5.2313 PMid:8404683
79. Vergouwen RP, Huiskamp R, Bas RJ, Roepers-Gajadien HL, Davids JA, de Rooij GD. Postnatal development of testicular cell populations in mice. J Reprod Fertil. 1993; 99:479-85.
80. Orth JM. The role of follicle-stimulating hormone in controlling Sertoli cell proliferation in testes of fetal rats. Endocrinology. 1984; 115:1248-55. https://doi.org/10.1210/endo-115-4-1248 PMid:6090096
81. Migrenne S, Moreau E, Pakarinen P, Dierich A, Merlet J, Habert R, Racine C. Mouse testis development and function are differently regulated by follicle-stimulating hormone receptors signaling during fetal and prepubertal Life. PLoS ONE. 2012; 7:e53257. https://doi.org/10.1371/journal.pone.0053257 PMid:23300903 PMCid:PMC3531970
82. Migrenne S, Racine C, Guillou F, Habert R. Pituitary hormones inhibit the function and differentiation of fetal Sertoli cells. Endocrinology. 2003; 144:2617-22. https://doi.org/10.1210/en.2002-0011 PMid:12746325
83. Hall SH, Joseph DR, French FS, Conti M. Follicle-stimulating hormone induces transient expression of the protooncogene c-fos in primary Sertoli cell cultures. Mol Endocrinol. 1988; 2:55-61. https://doi.org/10.1210/mend-2-1-55 PMid:3135483
84. Heckert LL, Daggett MA, Chen J. Multiple promoter elements contribute to activity of the follicle- stimulating hormone receptor (FSHR) gene in testicular Sertoli cells. Mol Endocrinol. 1998; 12:1499 -512. https://doi.org/10.1210/me.12.10.1499 PMid:9773974
85. El-Gehani, F, Zhang FP, Pakarinen P, Rannikko A, Huhtaniemi I. Gonadotropin-independent regulation of steroidogenesis in the fetal rat testis. Biol Reprod. 1998; 58:116-23. https://doi.org/10.1095/biolreprod58.1.116 PMid:9472931
86. Zhang FP, Hamalainen T, Kaipia A, Pakarinen P, Huhtaniemi I. Ontogeny of luteinizing hormone receptor gene expression in the rat testis. Endocrinology. 1994; 134:2206-13. https://doi.org/10.1210/en.134.5.2206 PMid:8156923
87. Zhang, FP, Poutanen M, Wilbertz J, Huhtaniemi I. Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LHRKO) mice. Mol Endocrinol. 2001; 15:172- 183. https://doi.org/10.1210/me.15.1.172 PMid:11145748
88. Saez JM. Leydig cells: endocrine, paracrine, and autocrine regulation. Endocr Rev. 1994; 15:574-626. https://doi.org/10.1210/edrv-15-5-574 PMid:7843069
89. Zhang FP, Pakarainen T, Zhu F, Poutanen M, Huhtaniemi I. Molecular characterization of postnatal development of testicular steroidogenesis in luteinizing hormone receptor knockout mice. Endocrinology. 2004; 145:1453-63.
90. Kendall SK, Samuelson LC, Saunders TL, Wood RI, Camper SA. Targeted disruption of the pituitary glycoprotein hormone alpha-subunit produces hypogonadal and hypothyroid mice. Genes Dev. 1995; 9:2007-19.
91. O'Shaughnessy PJ, Baker P, Sohnius U, Haavisto AM, Charlton HM, Huhtaniemi I. Fetal development of Leydig cell activity in the mouse is independent of pituitary gonadotroph function. Endocrinology. 1998; 139:1141-46.
92. O'Shaughnessy PJ, Baker PJ, Heikkila M, Vainio S, McMahon AP. Localization of 17-beta-hydroxysteroid dehydrogenase/17-ketosteroid reductase isoform expression in the developing mouse testis- androstenedione is the major androgen secreted by fetal/neonatal Leydig cells. Endocrinology. 2000; 141:2631-37. https://doi.org/10.1210/en.141.7.2631 PMid:10875268
93. Shima Y, Miyabayashi K, Haraguchi S, Arakiwa T, Otake H, Baba TT, Matsuzaki S, Shishido Y, Ariyama H, Tachibana T. Contribution of Leydig and Sertoli cells to testosterone production in mouse fetal testis. Mol Endocrinol. 2013; 27:63-73. https://doi.org/10.1097/HNP.0b013e318280f738 PMid:23399706
94. Adamsson A, Salonen V, Pranko J, Toppari J. Effects of maternal exposure to di-isononyl phthalate (DINP) and 1, 1-dichloro-2, 2-bis (p-chlorophenyl) ethylene (p,p'-DDE) on steroidogenesis in the fetal rat testis and adrenal gland. Reprod Toxicol. 2009; 28:66-74. https://doi.org/10.1016/j.reprotox.2009.03.002 PMid:19490997
95. Stocco DM, Clark BJ. Regulation of the acute production of steroids in steroidogenic cells. Endocr Rev. 1996; 17:221-44.
96. Rone MB, Midzak AS, Issop L, Rammouz G, Jagannathan S, Fan J, Ye X, Blonder J, Venestra T, Papadopoulos V. Identification of a dynamic mitochondrial protein complex driving cholesterol import, trafficking, and metabolism

to steroid hormones. Mol Endocrinol. 2012; 26:1868-82. https://doi.org/10.1210/me.2012-1159 PMid:22973050 PMCid:PMC5416962

97. Payne AH, Hales DB. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr Rev. 2004; 25:947-70. https://doi.org/10.1210/er.2003-0030 PMid:15583024
98. Morohashi KI, Omura T. Ad4BP/SF-1, a transcription factor essential for the transcription of steroidogenic cytochrome P450 genes and for the establishment of the reproductive function. FASEB J. 1996; 10:1569-77. https://doi.org/10.1096/fasebj.10.14.9002548 PMid:9002548
99. Gregory CW, DePhilip RM. Detection of steroidogenic acute regulatory protein (StAR) in mitochondria of cultured rat Sertoli cells incubated with follicle-stimulating hormone. Biol Reprod. 1998; 58:470-74. https://doi.org/10.1095/biolreprod58.2.470 PMid:9475403
100. Ishikawa T, Hwang K, Lazzarino D, Morris PL. Sertoli cell expression of steroidogenic acute regulatory protein-related lipid transfer 1 and 5 domain-containing proteins and sterol regulatory element binding protein-1 are interleukin-1 beta regulated by activation of c-Jun N-terminal kinase and cyclooxygenase-2 and cytokine induction. Endocrinology. 2005; 146:5100-11. https://doi.org/10.1210/en.2005-0567 PMid:16123165
101. Chen H, Hardy MP, Zirkin BR. Age-related decreases in Leydig cell testosterone production are not restored by exposure to LH in vitro. Endocrinology. 2002; 143:1637-42. https://doi.org/10.1210/en.143.5.1637 PMid:11956144
102. Song L, Tang X, Kong Y, Ma H, Zou S. The expression of serum steroid sex hormones and steroidogenic enzymes following intraperitoneal administration of dehydroepiandrosterone (DHEA) in male rats Steroids. 2010; 75:213-18. https://doi.org/10.1016/j.steroids.2009.11.007 PMid:19961867
103. Van Pelt A, De Rooij D, Van der Burg B, Van der Saag P, Gustafsson J, Kuiper G. Ontogeny of estrogen receptor-beta expression in rat testis. Endocrinology.1999; 140: 478-483. https://doi.org/10.1210/en.140.1.478 PMid:9886860
104. Jefferson W, Couse J, Banks E, Korach K, Newbold R. Expression of estrogen receptor b is developmentally regulated in reproductive tissues of male and female mice. Biol Reprod. 2000; 62: 310-317. https://doi.org/10.1095/biolreprod62.2.310 PMid:10642567
105. O'Donnell L, Robertson KM, Jones ME, Simpson ER. Estrogen and spermatogenesis. Endocr Rev. 2001; 22:289-318.
106. Greco TL, Payne AH. Ontogeny of expression of the genes for steroidogenic enzymes P450 side-chain cleavage, 3 beta-hydroxysteroid dehydrogenase, P450 17 alpha-hydroxylase/C17-20 lyase, and P450 aromatase in fetal mouse gonads. Endocrinology. 1994; 135:262-68. https://doi.org/10.1210/en.135.1.262 PMid:8013361
107. Delbès G, Levacher C, Habert R. Estrogen effects on fetal and neonatal testicular development. Reproduction. 2006; 132:527-38. https://doi.org/10.1530/rep.1.01231 PMid:17008464
108. Delbes G, Levacher C, Duquenne C, Racine C, Pakarinen P, Habert R. Endogenous estrogens inhibit mouse fetal Leydig cell development via estrogen receptor alpha. Endocrinology. 2005; 146:2454-61. https://doi.org/10.1210/en.2004-1540 PMid:15661855
109. Delbes G, Levacher C, Pairault C, Racine C, Duquenne C, Krust A. Estrogen receptor beta-mediated inhibition of male germ cell line development in mice by endogenous estrogens during perinatal life. Endocrinology, 2004; 145:3395-403. https://doi.org/10.1210/en.2003-1479 PMid:15044378
110. Korach, KS, Couse J, Curtis S, Washburn T, Lindzey J, Kimbro K, Eddy E, Migliaccio S, Snedeker S, Lubahn D, Schomberg D, Smith E. Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes. Rec Prog Horm Res.1996; 51:929-35.

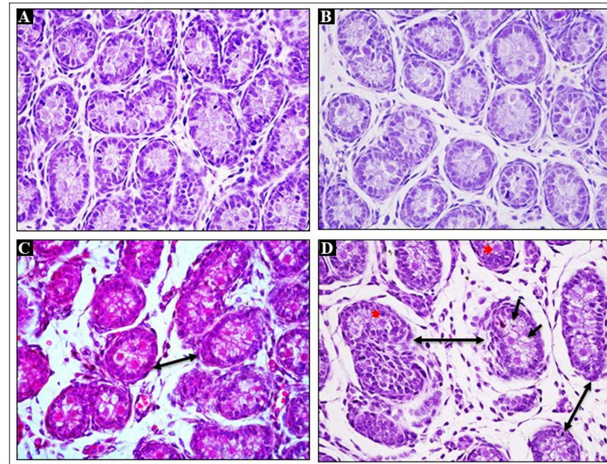
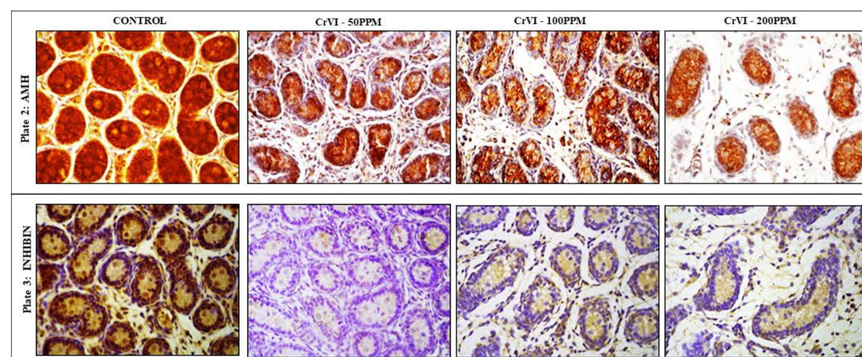
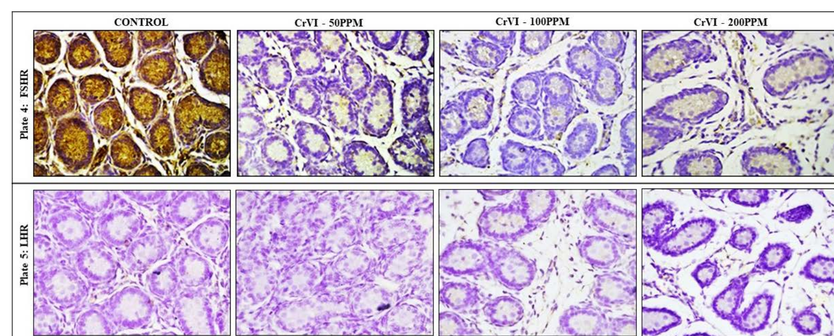


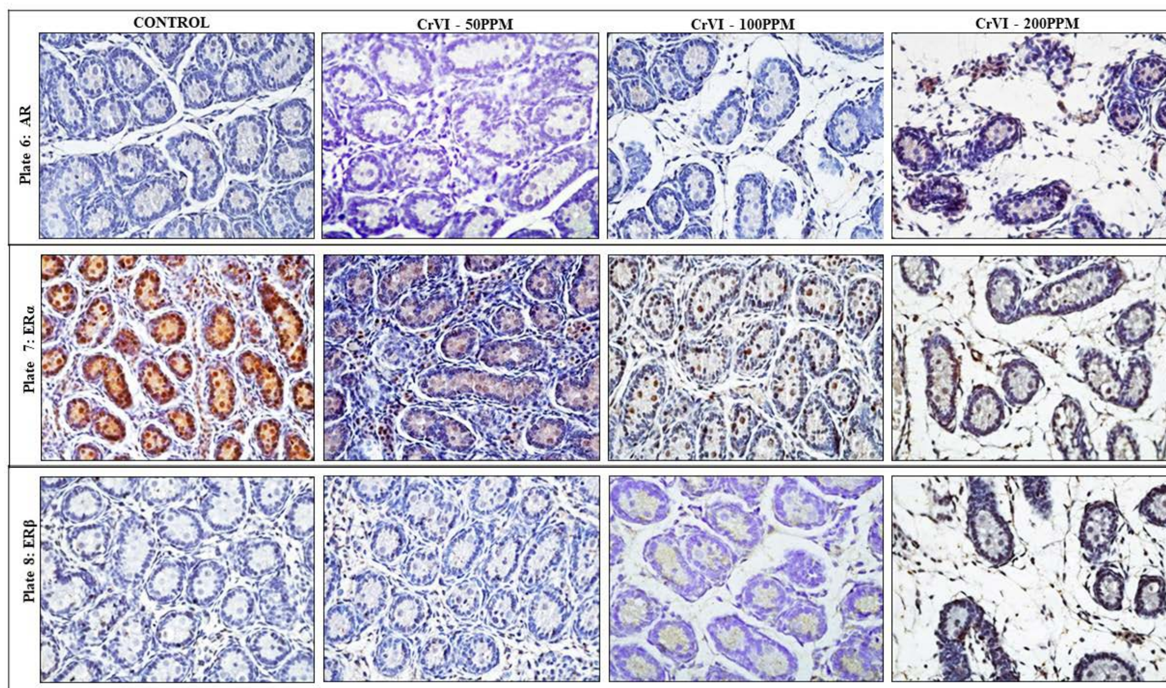
Plate 1. Testicular histology at PND1. (A) Testis of new born control rats showing seminiferous tubules with gonocytes surrounded by SCs around the periphery, and LCs present in the interstitial spaces. Testis of CrVI-exposed pups shows lack of compaction, and constricted tubules with large interstitial spaces (double-headed arrow). (B) Gestationally exposed to CrVI (50 ppm). Little if any change is indicated. (C & D) Testis of pups which had exposure to the higher doses of CrVI (100 & 200 ppm) exhibit reduced number of gonocytes. Gonocytes are located centrally in the seminiferous tubules due to vacuolization of SC cytoplasm (black single-headed arrow). Multinucleate gonocytes (red-forked arrow) are also visible in testes of pups whose mothers had exposure to 200 ppm CrVI. Hematoxylin and Eosin (H&E) staining. Original magnification: 400x.



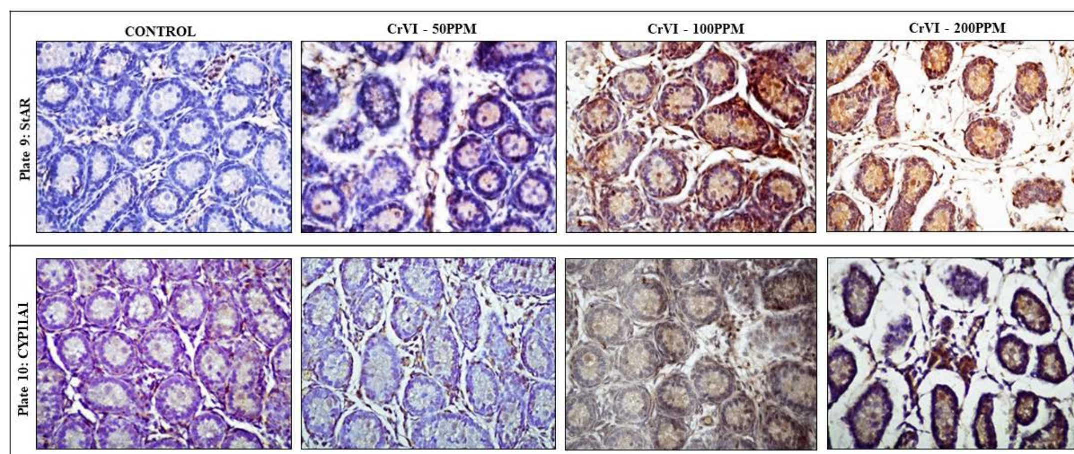
Plates 2-3. Immunohistochemical localization of AMH (Plate 2) and inhibin (Plate 3) proteins in testis of PND1 rats. The AMH protein shows strong immunostaining within the tubule of control rat testis **(A)** as compared to all groups treated with CrVI. The F_1 progeny of mothers with exposure to the high dose (200 ppm) CrVI show atrophied and constricted tubules (Plate 2). Inhibin protein shows strong immunopositivity in seminiferous tubules of control rat testis (A), whereas decreased intensity in all the treatment groups (B, C, D) as compared to control (Plate 3). Original magnification: 400x.



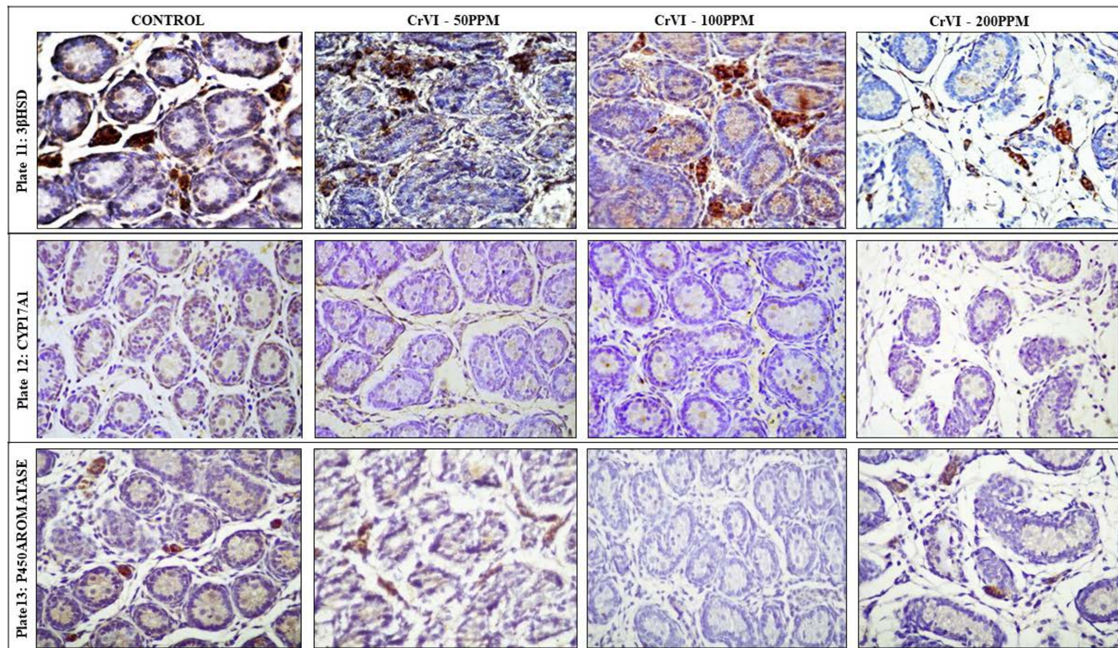
Plates 4-5. Immunostaining of FSHR protein shows intense signal in the SCs of control neonate testis (A) and very weak/ no immunostaining in the testis of F_1 progeny rats of mothers with transient gestational exposure to CrVI (B, C, D) (Plate 4). LHR protein shows no immunosignal in LCs of either control or F_1 neonates of mothers exposed to 50 ppm, 100 ppm and 200 ppm CrVI (Plate 5). Original magnification: 400x.



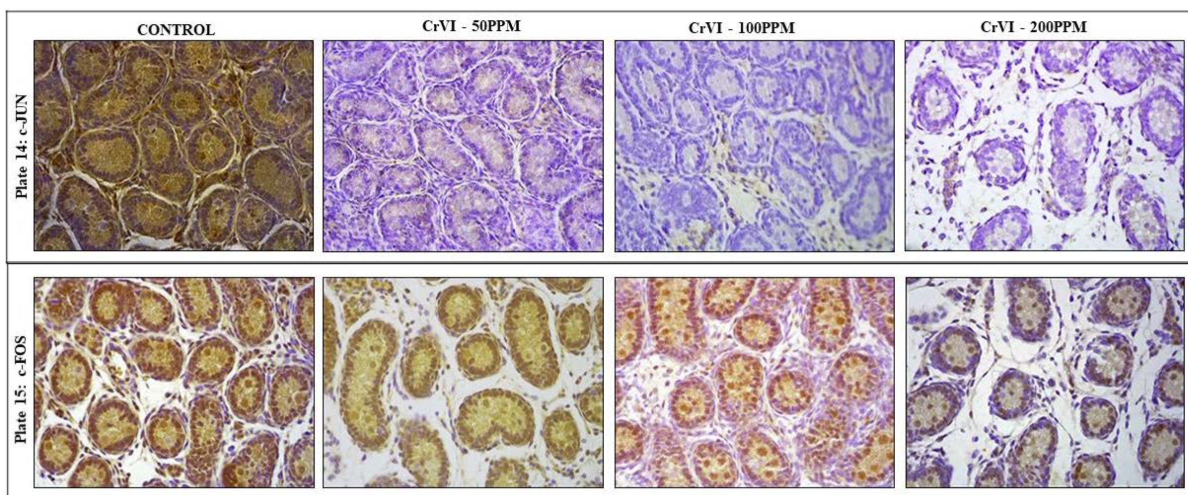
Plates 6-8. Neonate testis at PND1 immunostained for AR, ERα and ERβ. The AR signal shows intense staining in the interstitium, peri-tubular myoid (PTM) cells and weak/no staining in the SCs of neonatal testis of 200 ppm CrVI-treated rats as compared to coeval control, 50 ppm and 100 ppm CrVI-treated testis (Plate 6). The ERα immunostaining shows its presence in the tubular compartment as well as interstitial LCs in the testis of control rats. The changes in the immunohistochemical staining show a progressive decrease in a dose-dependent manner in CrVI treated pups (Plate 7). ERβ shows immunopositivity in testis, particularly in LCs, of F_1 progeny of mothers exposed to 200 ppm CrVI only (D) (Plate 8). Original magnification: 400x.



Plates 9-10. Immunohistochemical detection of steroidogenic protein StAR and CYP11A1 enzyme. The immunopositive signal of StAR protein appears in the LCs of control rats, whereas all CrVI treated neonates exhibit higher intensity as compared to coeval control. There is also a shift in the enzyme signal into the intra-luminal compartment containing SCs with mild staining in 50 ppm and maximally in 100 ppm and 200 ppm CrVI treated group (Plate 9). CYP11A1 shows immunopositivity in LCs of control and 50 ppm CrVI treated groups and are comparable; 100 ppm and 200 ppm CrVI treated groups exhibit increased signal intensity with shift in the enzyme signal into the intra-luminal compartment of seminiferous tubule (Plate 10). Original magnification: 400x.



Plates 11-13. Immunohistochemical detection of steroidogenic enzymes 3βHSD, CYP11A1 and P_{450} aromatase. Intense signal of 3βHSD appears in LCs of control rats (A) and progressively diminishes in intensity in pups with gestational exposure to 50 ppm, 100 ppm and 200 ppm CrVI (B,C,D) compared to control (Plate 11). The steroidogenic enzyme CYP17A1 shows immunostaining in the interstitium as well as in tubular compartments of control testis (A), marked decrease in signal intensity of 50 ppm CrVI treated rats (B) and no visible detection in F_1 progeny of mothers with gestational exposure to 100 ppm (C) and 200 ppm (D) CrVI (Plate 12). P_{450} aromatase protein shows strong intensity within seminiferous tubules and LCs of neonatal control rats and 50 ppm CrVI treated group; no signal is seen in the seminiferous tubules and LCs of 100ppm CrVI group, whereas 200 ppm treated group shows decreased intensity of aromatase in LCs only (D) compared to control (Plate 13). Original magnification: 400x.



Plates 14-15. Immunohistochemical detection of c-Fos and c-Jun proteins. c-Fos shows strong immunopositivity in the tubular and interstitial compartments of control rat (PND1) testis; however, testis of F_1 neonates with gestational exposure to 50 ppm, 100 ppm and 200 ppm CrVI shows progressively diminished signal (Photomicrograph 13). Strong c-Jun signal intensity appears in the tubular as well as interstitial compartments of control rat testis, and no signal in F_1 neonatal rats of 50 ppm, 100 ppm and 200 ppm CrVI exposure groups. (Plate 15). Original magnification: 400x.