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Impact of Hexavalent chromium on the Testicular lipogenic and lipolytic Enzymes in Albino rats

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Abstract
Chromium constitutes one of the heavy metals that are largely disposed to the environment through tannery wastes and from metallurgic, refractory and chemical industries. In this compounds denoted chromium (hexavalent compounds). In the present study, an attempt is made to know the chromium-induced cytotoxicity on the testis of pubertal albino rats. Since the reduction of chromium inside the cells, brings about various biochemical modifications, chromium-induced cytotoxicity on testis can be studied well by treating rats with chromium-mixed water. The general metabolism and steroidogenic processes of the testis, an attempt made to know the impact of chromium on these testicular lipogenic enzymes.The direct effects of chromium-induced cytotoxicity on the testicular architecture, lipogenic processes of the testis and serum hormonal profiles in these pubertal rats in a dose-dependent manner. Due to these deleterious effects of chromium-induced cytotoxicity, the spermatogenic and steroidogenic processes in the testis may get impaired, leading to infertility.

Keywords: Hexavalent chromium, chromium-induced cytotoxicity, albino rats

1. Introduction

1.1. Description of male reproductive system:

Male reproductive system comprises a pair of testes, epididymides and accessory sex glands. Testes are encapsulated ovoid organs consisting of seminiferous tubules separated by interstitial tissue. Testes has two main functions: production of spermatozoa, which transmit male's gene to embryo and male sex hormone testosterone, which plays an important role in maintaining spermatogenesis, accessory sex organs and secondary sexual characters.

Seminal vesicles are paired, bag-shaped glands and the internal surface consists of intricate system of folds to form irregular diverticula. Seminal vesicles secrete a viscous fluid, which is expelled along with sperm. It contains several essential nutrients which are required by the sperm for their fertilizing ability.

Ventral prostate is a bilobed structure situated ventral to urethra. It has numerous small ducts through which the secretions are discharged directly into the urethra. The secretions are rich in nutrients and serve as lubricant for the semen (Setchell ***et.al.,*** 1994).

1.2. Spermatogenesis:

Spermatogenesis is the process of gradual transformation of germ cells into spermatozoa over an extended period of time within the boundaries of the seminiferous tubules of testis. This process involves cellular proliferation by repeated mitotic divisions, duplication of chromosomes, genetic recombination through crossover and reduction division by meiosis to produce haploid spermatids and terminal differentiation of spermatids into spermatozoa (de Kretser ***et.al.,*** 2000). Seminiferous tubules contain a large number of germinal epithelial cells called spermatogonia, located in two to three layers along the outer border of the tubular epithelium and continually proliferate to replenish themselves. At the start of spermatogenesis, diploid spermatogonia proliferate producing three sub-populations of cells with markedly different destinies. One sub-population of spermatogonia are presumably identical to their progenitors and continue to function as stem cells and the majority of spermatogonia have been shown to enter a differentiative pathway to become spermatozoa (de Kretser ***et.al.,*** 2000). The stem spermatogonia (type A) are located immediately adjacent to the basement membrane of the germinal epithelium. Type A spermatogonia exhibiting fine pale-staining nuclear chromatin and type B with coarse granules or more heavily stained chromatin associated with nuclear membrane and nucleolus (Clermont, 1972). Type A spermatogonia, enter a cycle and produce a chain of aligned undifferentiated spermatogonia,

which differentiate into type A_1 spermatogonia. These cells undergo a sequence of six cell cycles and mitotic divisions resulting in the formation of A_2, A_3, A_4 intermediates and finally into slightly more differentiated cells, the type B spermatogonia. After several divisions these cells give rise to very large primary spermatocytes (Steinberger and Steinberger, 1975).

1.3. Effect of Chromium on Testicular function:

Chromium is an important heavy metal widely used in the metallurgic, refractory, chemical and tannery industries. (Barceloux 1999; Kumar ***et.al.,*** 2005) More than 170,000 tons of chromium wastes are discharged to the environment annually as a consequence of industrial and manufacturing activities (Gadd, 1993). Of the total chromium used in the processing of leather, 40% is retained in the sludge, disposal of which onto land and into water bodies has led to increased chromium levels reaching as high as 30,000 mg/Kg^{-13} (Kamaludeen ***et.al.,*** 2003).

The chromium (VI) Cr(VI) compounds are easily transported across the plasma membrane of cells through a non-selective anion channel that is normally utilized for uptake of physiologically relevant anions (Joiner ***et.al.,*** 1990), such as sulfate and phosphate, and immediately undergo reduction to chromium (III) (CrIII) (Dudek and Wetterhahn, 1994). This intracellular reduction of Cr(VI) to Cr (III) is responsible for producing both the cytotoxicity and genotoxicity associated with Cr(VI) compounds (Dudek and Watterhahn, 1994), inside the cells.

Cr(VI) has been shown to be carcinogen to both human and animals and cause DNA damages to different cells under both ***in vivo*** and ***in vitro*** systems (Bianchi ***et.al.,*** 1983; Levy and Venitt, 1986, see Stearns ***et.al.,*** 1994).

Afonne ***et.al.*** (2002) demonstrated chromium-induced testicular toxicity in mice. Exposure to chromium significantly reduces the sperm number, which indicates an interference with spermatogenesis. Therefore, in the present study, an attempt it made to know the direct of effect of chromium-induced toxicity on testis. Since testis is the major reproductive organ, the chromium-induced cytotoxicity may definitely affect the fertility of the male.

2. Materials and methods

90 days old healthy male albino rats of Wistar strain (Rattus norvegicus) with a body weight ranging from 110-130g were procured from the central animal facility, Dr. A.L.M. Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani, Chennai – 600 113 and were used in the present investigation. The rats were maintained in a well-ventilated laboratory condition, with 12 ± 1 hours light: 12 ± 1 hours dark, natural light schedule. They were supplied with standard rat pellet diet (Gold Mohur, Hindustan Lever Limited, Bangalore, India), supplemented with Bengal gram and carrots as their diet and clean drinking water ad libitum. Daily changes in the body weights of rats were recorded.

2.1. Experiment Groups:

The animals were divided into three groups, each consisted of the following.

Group I: (Control)

This group consisted of five rats, which served as controls. Each rat was given orally, 1 ml of vehicle (double distilled water) alone, daily for 7 days.

Group II: (Experimental – I)

This group consisted of five rats which served as experimental-I. To each rat of this group, 1 ml of Potassium dichromate solution ($K_2Cr_2O_7$, M.W: 294.18) containing a dichromate solution dosage of 10 mg/kg body weight of rat dissolved in double distilled water was given orally, daily for 7 days.

Group III: (Experimental – II)

This group consisted of five rats which served as experimental-II. To each rat of this group, 1 ml of potassium dichromate solution ($K_2Cr_2O_7$, M.W: 94.18) containing a dosage of 20 mg/kg body weight of rat dissolved in double distilled water was given orally, daily for 7 days.

Rats of both the control and experimental-I and II groups were given the vehicle/ potassium dichromate solution at a standard time (9.00 a.m). Daily changes in the body weight of both the control and experimental group rats were recorded.

After daily treatments with vehicle / potassium dichromate solution for one week, the animals were left untreated in the normal laboratory condition for a further period of five weeks duration to know the post effects of chromium toxicity. After the experimental period, both the control and experimental animals were sacrificed at a standard time which was uniformly followed for both control and experimental rats.

2.2. Chemicals and Reagents:

All the chemicals, reagents, substrates and enzymes used were of analytical grade (AR). Fine chemicals like substrates and enzymes were obtained from British Drug House (BDH), England, Loba chemie, Austria and sigma Chemical Company, U.S.A. other chemicals and reagents were obtained from British Drug House (India), Mumbai, Sisco Research Laboratory (SRL), Mumbai. Kochlight, USA, Glaxo Laboratories Limited, Mumbai and E.Merck, Germany.

Serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured by radioimmunoassay (RIA) using materials obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Hormone and Pituitary Program, Baltimore, Maryland, USA as gift.

Carrier-free iodine (^{125}I) (code I-3) for the radioiodination of hormones was supplied by Bhabha Atomic Research Centre (BARC), Mumbai, India. Serum estradiol and progesternone were assayed using the materials obtained from Diagnostic Products Corporation (DPC), Maryland, USA.

Only double distilled (glass) water was used for the preparation of reagents, until and otherwise specified.

2.3. Assay of serum Luteinizing hormone (LH): (NIDDK-r-LH-I-7)

Serum Luteinizing hormone was assayed according to the method of Lin ***et.al.*** (1988).

Reagents:

LH tracer (NIDDK-r-LH-I-7; AFP-9404B), rat LH antiserum (rabbit) (NIDDK-anti-r-LH-S-10; AFP-571487) were suitably diluted as described for the prolactin assay. Rat LH reference preparation (NIDDK-r-LH-RP-3; AFP-7187B) was prepared with a concentration of 5ng LH / ml and serial dilutions from this solution were made with concentrations of 0.25, 1, 4, 16, 32 and 64ng / LH / ml.

2.3.1. Assay of serum testosterone:
Testosterone was assayed using materials obtained from BIO DATA, Serono Diagnostics, S.A., Switzerland.

Principle
The assay is based on the principle that non-labelled testosterone molecules complete against ^{125}I labeled testosterone for binding to a limited amount of anti-testosterone antibodies. Thus, antigen-antibody complexes are formed following the Law of mass action. When the reaction reaches equilibrium, the antigen-antibody complexes are precipitated by the addition of constant amount of precipitating agent, then separated by centrifugation and counted in LKB-Wallace, RIA programmed microprocessor based automatic gamma counter. Percentage of radioactivity in antigen-antibody complexes and unlabelled testosterone are inversely proportional (Furuyama ***et.al.,*** 1970; Vermeulen, 1976).

Reagents
1. Buffer (0.05M phosphate buffer and 0.1% sodium azide, pH 7.6)
2. Reference testosterone preparation (Hypo Lab) dissolved in distilled water.
3. I^{125}-testosterone (1.5 μCi), dissolved in 12.5ml distilled water and mixed gently.
4. Lyophilized anti-testosterone rabbit serum (Hypo Lab) dissolved in distilled water and mixed gently.
5. 20% Polyethylene glycol (PEG) solution.

2.4. Biochemical Analysis:
2.4.1. Preparation of Tissue homogenates:
100mg of Testicular tissue samples were minced with scissors into small pieces and 5ml of 0.25M phosphate buffer (pH 7.2) was added and homogenized for 5 minutes in a Teflon homogenizer under ice-cold condition. All the subsequent steps in the fractionation procedure were carried out at 0°C to 4°C.

2.4.2. Centrifugation:
The homogenized tissue extracts were centrifuged at 900xg for 10 minutes, to remove the cell debris and unbroken cells in the suspension. The entire supernatant was carefully decanted into another tube and the sediment was discarded. The supernatant was again centrifuged at 5000 rpm for 20 minutes and the resulting supernatant was considered as enzyme extract.

2.4.3. Estimation of Protein:
The protein content of the testicular tissue samples were estimated by the method of Lowry ***et.al.*** (1951).

Principle:
The amino acids containing phenolic hydroxyl group viz., tyrosine and tryptophan reacts with Folin-Ciocalteu reagent to give blue colour due to reduction of phosphosmolybdate; the intensity of the colour is proportional to the concentration of proteins.

Reagents:
1. Reagent - A: 2% sodium carbonate in 0.1 N sodium hydroxide.
2. Reagent - B: 0.5% copper sulphate in 1.35% sodium potassium tartarate (prepared just before use)
3. Reagent - C Alkaline copper reagent: This was prepared just before use by mixing 50m1 of reagent A with 1.0 ml of reagent B.
4. Folin-Ciocalteu reagent (1N): Commercially available Folin_Ciocalteu phenol reagent (2N) (SRL) was diluted to 1N with distilled water.
5. Standard: A Standard solution of bovine serum albumin (BSA) containing 250 $\mu g / ml$ was prepared in 0.1N sodium hydroxide.

2.5. Statistical Methods:
The data were analyzed statistically using Student's 't' test and expressed as Mean ± standard Error of Mean (S.E.M) and was calculated as follows (Ostle, 1966).

$$S.E.M = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n(n-1)}}$$

Where x = individual observation
n = number of observations

't' value was calculated by the following formula and compared by the table value of 5% and 1% levels of significance.

$$t = \frac{x_1 - x_2}{S\sqrt{1/n_1 + 1/n_2}}$$

$$S = \frac{\sqrt{\sum x_1^2 \frac{(\sum x_1)^2}{n_1} + \sum x_2^2 - \frac{(\sum x_2)^2}{n_2}}}{n_1 + n_2 - 2}$$

Where

n_1 and n_2 denote the number of observed values in the two classes being compared (Ostle, 1966).
$p<0.00l$ to $p<0.05$ considered as significant data and $p>0.05$ as non-significant data.

3. Results & Discussion
3.1. Impact of Chromium (hexavalent)-induced toxicity on body weight and Testicular wet weights in pubertal rats:
The impact of the different doses of chromium-treatment for 7 days and subsequent withdrawal effect for 5 weeks on the body weight and the total wet weights of testis in pubertal rats in depicted in Tables and Figures.
When compared to their age-matched control pubertal rats, chromium treatment (10 mg/kg body weight; Experimental – I) for 7 days and subsequent withdrawal for 5 weeks has brought out a significant increase ($p < 0.01$) in their body weight.
This increase in bodyweight has increased to a further significant level ($p < 0.001$) in Experimental – II (20 mg/kg bodyweight / 7 days) animals when compared to their age-matched controls (**Table 1) and** (**Fig.1)**
The wet weights of testis in both low dose (Experimental – I) and high dose (Experimental – II) chromium-treated rats failed to elicit any significant alteration in both the groups when compared to their age-matched control pubertal rats (**Table 1) and** (**Fig.2)**

Table 1: Impact of chromium (hexavalent; $K_2Cr_2O_7$)-induced toxicity on body weight and testicular wet weights in pubertal albino rats

Parameters	Control	Experimental–I*	Experimental-II**
Body weight[@]	128.3±1.2	134.7±1.8[b]	152.9±2.3[c]
Testicular wetweight[$]	1.97±0.2	1.80±0.2	1.62±0.1

* $K_2Cr_2O_7$ – dosage (10mg/kg body weight/daily/7 days)

** $K_2Cr_2O_7$ – dosage (20mg/kg body weight/daily/7 days)
@ in grams
$mg/100g body weight
Each value is mean ± SEM of 5 animals
b P<0.01; c p<0.001;control Vs experimental

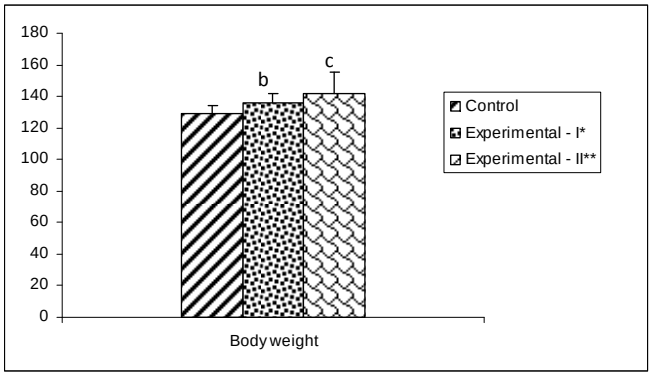


Fig 1. Impact of chromium (hexavalent; $K_2Cr_2O_7$)-induced toxicity on body weight in pubertal albino rats

* $K_2Cr_2O_7$ – dosage (10mg/kg body weight/daily/7 days)
** $K_2Cr_2O_7$ – dosage (20mg/kg body weight/daily/7 days)
@ in grams
$mg/100g body weight
Each value is mean ± SEM of 5 animals
b P<0.01; c p<0.001;control Vs experimental

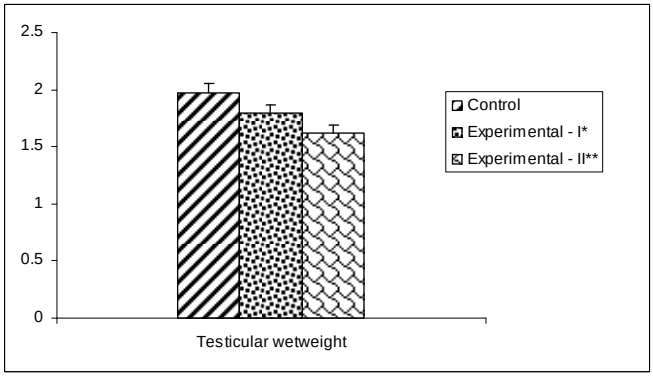
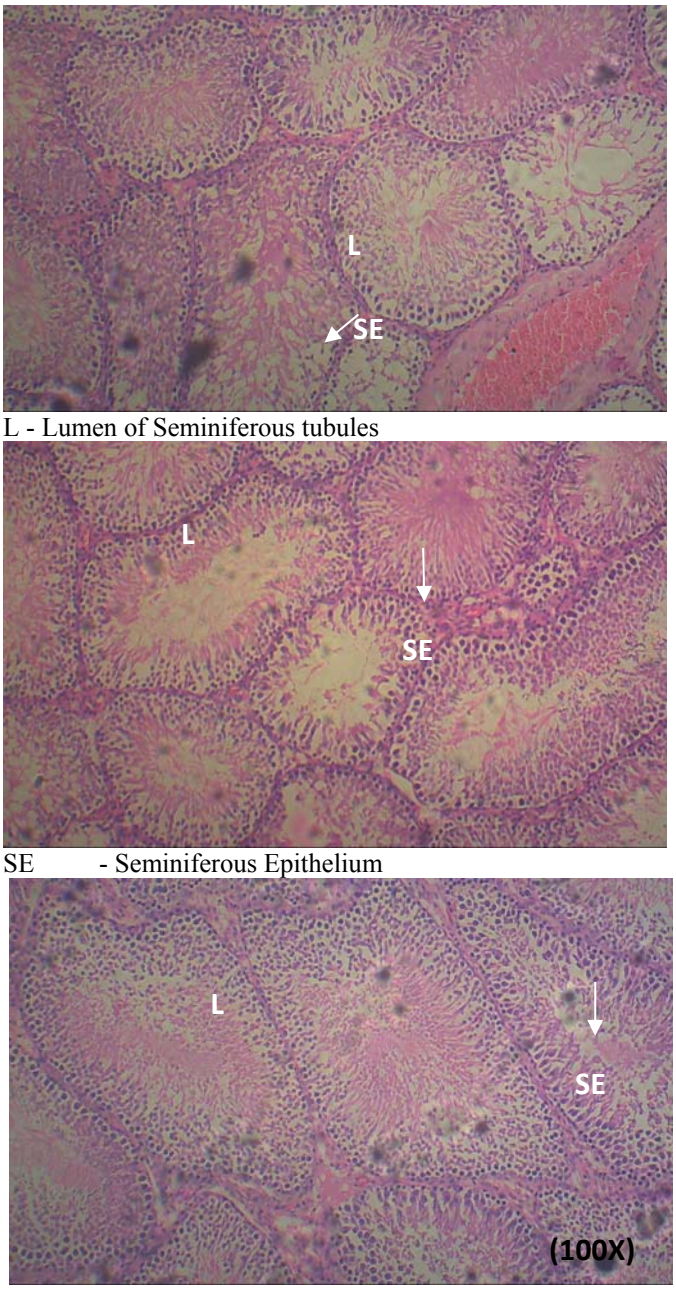


Fig 2. Impact of chromium (hexavalent; $K_2Cr_2O_7$)-induced toxicity on testicular wet weight in pubertal albino rats

* $K_2Cr_2O_7$ – dosage (10mg/kg body weight/daily/7 days)
** $K_2Cr_2O_7$ – dosage (20mg/kg body weight/daily/7 days)
@ in grams
$mg/100g body weight
Each value is mean ± SEM of 5 animals
NS Non-Significant; control Vs experimental

3.2. Testicular Histology (PLATE):

The histology of testis in both low dose (10 mg/kg.bodyweight) and high dose (20 mg/kg.bodyweight) chromium-treated pubertal rats are depicted in (**Fig. 3)**
The photomicrographs of testicular architecture in both control and experimental I and II groups were compared.
In the Experimental – I group of rats, chromium-treatment has indicated visible disruption in germ cell arrangements near the wall of seminiferous tubules and a decrease in the diameter of the seminiferous tubules.
In the Experimental – II group of rats, chromium-treatment has brought about the disruption of seminiferous epithelium along with the disruption in the attachment of sperm with the seminiferous epithelium. In these groups of rats, it was further observed the presence of intraepithelial vacuoles among the disrupted cells. Abnormalities affecting nearly all stages of germ cell development were seen in the seminiferous tubules, with narrowing up of the lumen of seminiferous tubules and necrotic mass of germ cells. The germ cells seem to be displaced towards the lumen of seminiferous tubules.



L - Lumen of Seminiferous tubules
SE - Seminiferous Epithelium

Fig 3. CHROMIUM (Hexavalent; $K_2Cr_2O_7$) treatment (Control, 10, 20mg/kg.body wt/daily/7 days) – (withdrawal for 5 weeks) - Histology of Testis of pubertal rats

3.3. Serum hormonal profiles in chromium-treated rats:

3.3.1. Gonadotropins:

Serum Luteinizing hormone (LH): (Table 2) and (Fig.4)
Chromium-treatment at low dose (10 mg/kg.bodyweight / 7 days) show increase in serum LH concentration ($p < 0.01$), when compared to the LH levels in the serum of control rats.
This level of increase in serum LH concentration is also reflected ($p < 0.05$) in the high dose (20 mg/kg.bodyweight / 7 days) chromium-treated rats when compared to their respective age-matched control pubertal rats.

3.3.2. Serum Testosterone (Table 2) and (Fig.5)

In contrast to the levels of gonadotropins, serum testosterone showed an opposite trend of decrease in both Experimental – I (low dose) ($p < 0.001$) and Experimental – II (high dose) ($p < 0.001$) chromium-treated pubertal male rats when compared to their respective age-matched control rats.

Table 2: Impact of chromium (hexavalent; $K_2Cr_2O_7$)-induced toxicity on serum hormonal profiles in pubertal male rats

Parameters	Control	Experimental – I*	Experimental - II**
Luteinizing hormone (LH)	0.45±0.05	0.67±0.02[b]	0.58±0.03[a]
Testosterone	1.52±0.09	0.57±0.08[c]	0.48±0.06[c]

* $K_2Cr_2O_7$ – dosage (10mg/kg body weight/daily/7 days)
** $K_2Cr_2O_7$ – dosage (20mg/kg body weight/daily/7 days)
The values are expressed as ng/ml of serum
Each value is mean ± SEM of 5 animals
[a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$; control Vs experimental

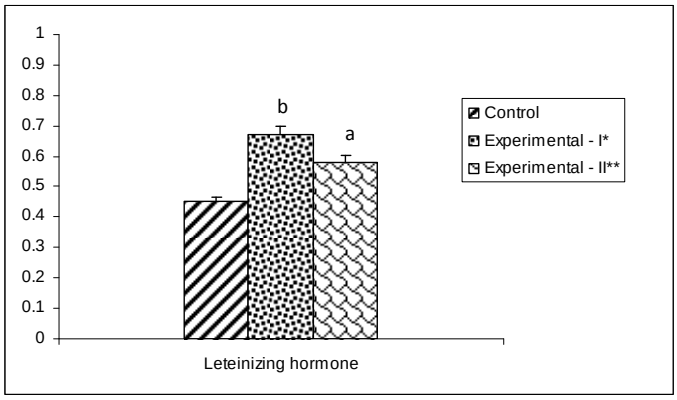


Fig 4. Impact of chromium (hexavalent; $K_2Cr_2O_7$)-induced toxicity on Luteinizing hormone in pubertal male rats

* $K_2Cr_2O_7$ – dosage (10mg/kg body weight/daily/7 days)
** $K_2Cr_2O_7$ – dosage (20mg/kg body weight/daily/7 days)
The values are expressed as ng/ml of serum
Each value is mean ± SEM of 5 animals
[a] $p < 0.05$; [b] $p < 0.01$; control Vs experimental

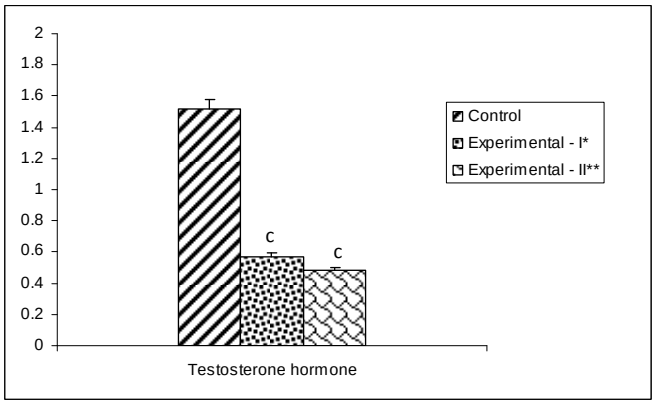


Fig 5. Impact of chromium (hexavalent; $K_2Cr_2O_7$)-induced toxicity on Testosterone hormone in pubertal male rats

* $K_2Cr_2O_7$ – dosage (10mg/kg body weight/daily/7 days)
** $K_2Cr_2O_7$ – dosage (20mg/kg body weight/daily/7 days)
The values are expressed as ng/ml of serum
Each value is mean ± SEM of 5 animals
[c] $p < 0.001$; control Vs experimental

3.4. Impact of chromium (hexavalent)-induced toxicity on the testicular histoarchitecture in pubertal albino rats:

Histological studies of low and high doses of chromium-treatments demonstrated the testicular structures of both control and chromium-treated pubertal rats. Control animals showed normal cellular arrangement in the seminiferous tubule. On the other hand, seminiferous tubular shrinkage was observed in both low and high doses of chromium-treated rat testes. Sloughing of germ cells from seminiferous epithelium was observed in the testis of high dose chromium-treated rats for 7 days. Degenerative changes in the testis with the disintegration of spermatocytes with above observations were also reported (Chandra ***et.al.,*** 2007). These degenerative changes due to chromium-treatment in the testis resulted in spermatogenic arrest with tubular necrosis and degenerating Leydig cells (Chandra ***et.al.,*** 2007).

In the present study also, the observed decreased levels of serum testosterone levels in both low and high doses of chromium-treated rats could have severely impaired the testicular histoarchitecture which has been clearly observed from the plates.

3.5. Impact of chromium (hexavalent)-induced toxicity on serum hormonal profiles in pubertal male albino rats:

Serum testosterone:

The decreased serum testosterone levels observed in both low and high doses of hexavalent chromium treated pubertal rats may be due to the diminished synthesis and secretion of the same.

The control of testosterone production is by the gonadotropin, LH, through the presence of specific receptors on the surface of Leydig cells (de Krester ***et.al.,*** 1971; see de Krester ***et.al.,*** 1995; see Multinger ***et.al.,*** 1996). The action of LH leads to a number of event that result in the provision of cholesterol substrate and in the stimulation of the side-chain cleavage enzyme to convert cholesterol to pregnenolone which is rapidly translated into a release of testosterone (see de Kretser ***et.al.,*** 1995). LH also enhances transcription of genes encoding the range of enzymes in the steroidogenic pathway (Waterman and Simpson, 1989).

Chowdhury (1995) have shown a decline of Leydig cell population with impaired steroidogenic machinery (Marouani ***et.al.,*** 2012) in rats treated to different doses of chromium. Due to this effect of reduction in Leydig cell population, the receptors of LH could also been down regulated, and Leydig cells being the sites of steroidogenic pathway, a reduction in the number of Leydig cells in chromium-treated rats could have been attributed to decreased testosterone levels in these rats.

4. Summary and Conclusions

The effect of low and high acute doses of hexavalent chromium induced cytotoxicity on testicular lipogenic enzymes in pubertal albino rats was studied in order to understand the biochemical mechanism underlying the actions of chromium-induced cytotoxicity on testis.

Potassium dichromate solution ($K_2Cr_2O_7$) at doses of 10mg/kg. body wt/7 days (low dose) and 20mg/kg. body wt/7 days (high dose) were administered to 90 days old pubertal rats by oral intubation. Control rats were given the vehicle (double distilled water) alone. After treatments, all the animals were left untreated for a further 5 weeks to know the withdrawal effect.

Daily changes in body weight were observed in both control and experimental animals. At the end experimental duration,

the wet weights of testis were noted. Serum was collected to study the serum hormonal profiles.

Both low and high doses of chromium-treatments to pubertal rats has brought about a significant elevation on their body weights, while the overall wet weights of testes were decreased in both low and high dose chromium-treated rats. Although the increased body weight in these chromium-treated rats could be attributed to the general metabolism, the decreased wet weights of testis could be due to the impairment of structural integrity of testis in these rats.

Serum LH were elevated, while the serum testosterone showed a trend of decreased level due to chromium-induced cytotoxicity.

The present data suggest the direct effects of chromium-induced cytotoxicity on the testicular architecture, lipogenic processes of the testis and serum hormonal profiles in these pubertal rats in a dose-dependent manner. Due to these deleterious effects of chromium-induced cytotoxicity, the spermatogenic and steroidogenic processes in the testis may get impaired, leading to infertility.

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