Original Article

Evaluation of protective action of α-tocopherol in chromium-induced oxidative stress in female reproductive system of rats

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Abstract

The present study was aimed to investigate whether α-tocopherol could protect the chromium (Cr) VI-induced oxidative stress in female reproductive system of rats and to explore the underlying mechanisms of the same. A total of 24 *Wistar* adult female rats were equally divided into four groups. Group 1 served as control, while groups 2 and 3 were administered $K_2Cr_2O_7$ (10 mg/kg b.wt. s.c. single dose). In addition to Cr, group 3 also received α-tocopherol @ 125 mg/kg daily by oral gavage for 14 days. Group 4 was maintained as α-tocopherol control (dose as above). Body weights were recorded at the beginning and at the end of experiment. Further, the rats were observed for occurrence of estrus cycle. At the end of 14 days, blood samples were drawn for sero-biochemical analysis. Subsequently, all the rats were sacrificed to collect uterus along with ovaries for assay of tissue peroxidation, anti-oxidant and functional markers, and histopathology. Administration of chromium (Cr) VI to rats revealed a significant ($P < 0.05$) accumulation of cholesterol and a prolonged diestrus phase leading to impaired fertility in rats. Administration of chromium (Cr) VI significantly ($P < 0.05$) reduced the antioxidant markers such as superoxide dismutase (SOD) and reduced glutathione (GSH), along with significant ($P < 0.05$) increase in peroxidation markers such as malondialdehyde and protein carbonyls in ovaries. The functional marker in serum such as total protein was decreased, whereas other functional markers *viz* alanine transaminase (ALT), blood urea nitrogen (BUN) and creatinine were increased. Prominent pathological changes were observed in the uterus and ovaries of Cr-treated group. Co-treatment with α-tocopherol significantly ($P < 0.05$) reversed the (Cr) VI induced changes.

Key words: α-tocopherol, chromium, female reproductive system, oxidative stress

INTRODUCTION

Chromium is a transition element found in many compounds of Earth's crust[1] and ranks 21st in elemental abundance. Chromium also comes from anthropogenic sources as: Chemical, metallurgical, refractory industry.[2] Chromium (Cr) is found in the environment in two valence states: Trivalent Cr (III) and hexavalent Cr (VI). Chromium (III) compounds have been reported to be less toxic than Cr (VI) compounds because latter can cross the cell membrane easily. Reduction of Cr (VI) to Cr (III) results in the formation of reactive oxygen species (ROS) that induce oxidative damage.[3] This, in turn, is responsible for various health hazards including cancers, dermatitis, damage to the liver and kidneys, infertility in both males and females, defects in embryo and developmental problems in young children.[4] Chromium exposure through drinking water has been shown to impair ovarian follicular maturation and differentiation.[1] Chromium (VI) as reproductive toxicant is recently recognized and less studied.[5]

The potential role of oxidative stress in injury associated with Cr^{6+} exposure suggests that anti-oxidant supplementation may mitigate chromate-induced toxicity.

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Vitamin E (α-tocopherol) is an important component in human diet and considered the most effective liposoluble anti-oxidant found in the biological system. It reacts with peroxy radicals 10,000-fold faster than do polyunsaturated lipids. Therefore, vitamin E is potentially useful as therapeutic agent in the treatment of several disorders associated with oxidative damage.[6] It might diminish lipid peroxidation (LPO) induced by heavy metals, including dichromate and protects the body's biological systems.[7] The first well known and the most established function of vitamin E is the regulation of reproductive functions in both male and female.[8] Because of the health problems induced by many environmental pollutants, much effort has been expended in evaluating the relative antioxidant potency of vitamin E.[9]

In light of the above data, the present study was undertaken to assess the effects of chromium on ovarian steroidogenesis and its possible protection by α-tocopherol.

MATERIALS AND METHODS

Chemicals

All the chemicals were of analytical grade and obtained from commercial sources.

Animals

Adult *Wistar* rats (24), aged about 60 days with average body weight of 140 ± 10 g were obtained from National Institute of Nutrition (NIN), Hyderabad. The animals kept in polypropylene cages were maintained under standard conditions prescribed by the committee for the purpose of control and supervision on experiments on animals (CPCSEA). The experimental protocol was approved by the Institutional Animal Ethics Committee (Approval No. I/7/2012).

Experimental design

A total of 24 rats were randomly divided into four groups with six rats in each. Group 1 was maintained as normal, while group 2 rats acted as Cr toxicity control. These rats were given Cr as $K_2Cr_2O_7$ dissolved in sterile saline (NaCl 0.9%) @ 10 mg/kg b.wt. as a single s.c. injection. Group 3 received Cr as above, but along with α-tocopherol, daily for 14 days by oral gavage. Group 4 was maintained as α-tocopherol control and was given α-tocopherol daily for 14 days by oral gavage. The study was approved by Institutional Animal Ethics Committee.

In this experiment, the dose of Cr to induce oxidative stress was based on a report by Biber *et al.*[10] The selected dose of α-tocopherol was as per Laura *et al.*[7] who stated that α-tocopherol at a dose of 125 mg/kg body wt. for 14 days effectively protected the kidney against Cr-induced alteration in lipid patterns.

Body weights were recorded at the beginning and at the end of experiment. Further, the rats were observed for occurrence of estrus cycle for 3 consecutive cycles. After completion of 14 days, the blood samples were collected from retro-orbital plexus of experimental rats for studying serum biochemical profile (ALT, BUN, creatinine and total protein). Then all the rats were euthanized. Uterus along with ovaries was collected immediately and ovaries were kept in ice cold phosphate buffer. A portion of the ovaries was homogenized with tissue homogenizer individually to make 10% homogenate to assay antioxidants, peroxidation and functional markers. Pieces of tissues from ovary and uterus were immediately kept in 10% of formalin fixative to study histological alterations, if any. Pieces of ovary were also kept in glutaraldehyde fixative to study subcellular alterations.

Occurrence of estrus cycle

The rats were observed for occurrence of estrus cycle every day in the morning between 9.00 AM and 10.00 AM by examination of cellular morphology of vagina by cotton swab smear technique.[11] The cotton wool tip was moistened slightly by dipping in saline. The rat was held around the thorax, ventral surface facing up. The tip of the swab stick was inserted carefully into the vagina to a depth of about 1 cm with a rotating action of swab and at an angle of 45° to animal body. The tip was rolled gently onto a clean pre labelled glass slide and the smears were examined under light microscope. Basing on the cell types, *viz* nucleated epithelial cells - Proestrus (PE), swollen cornified cells - Estrus (E), combination of nucleated epithelial cells, swollen cornified cells and leucocytes - Metestrus (ME), leucocytes-Diestrus (DE), each phase of estrus cycle was identified. The rats were examined for estrus cycle phase continuously for 3 consecutive cycles. The findings were tabulated as % of each estrus cycle phase continuously in 3 consecutive cycles.

Biochemical analysis

Antioxidant markers

SOD was estimated by the method that involved inhibition of superoxide-dependent reduction of tetrazolium dye methyl thiazolyl tetrazolium (MTT) to its formazan.[12] GSH was estimated based on a reaction of reduced glutathione with 5-5ditiobis-2-nitrobenzoic acid (DTNB).[13]

Peroxidation markers

Malondialdehyde, the product of lipid peroxidation, was estimated by reaction with thiobarbituric acid as per the method prescribed by Balasubramanian *et al.*[14] Protein carbonyls were estimated, based on the reaction of amino

carbonyls with 2, 4-dinitrophenyl hydrazine to form hydrazones, which can be detected spectrophotometrically at 372 nm.[15]

Sero-biochemical markers

Total protein, ALT, BUN and creatinine were estimated in serum by using the standard diagnostic kits.

Total Protein

Total protein in the ovarian tissue was quantified as per Lowry *et al.*'s[16] method.

Histology

For light microscopy examination, the formalin fixed tissues were dehydrated through ascending grades of alcohol, cleared in three changes of xylene, and were embedded in paraffin. Serial sections, each of 4-micron thickness, were cut and stained with H and E as per standard protocols.[17] For transmission electron microscopy (TEM), the glutaraldehyde-fixed tissues were used. Specimen preparation, staining and the observations were done at the designated RUSKA Lab, Hyderabad.

Statistical analysis

The data were subjected to statistical analysis by applying one way ANOVA using statistical package for social sciences (SPSS) version 12.0. Differences between means were tested using Duncan's multiple comparison tests and significance was set at $P < 0.05$.

RESULTS

The average body weight gain was significantly ($P < 0.05$) reduced in group 2 compared to control. But co-administration of α-tocopherol with chromium exposure (group 3) showed a significant ($P < 0.05$) increase in weights as compared to group 2 [Figure 1].

Vaginal smear examination of group I rats revealed normal cyclicity with 4 to 5 days of estrus cycle with appropriate duration of all four phases. Group II animals showed prolongation of diestrus phase with slight reduction of proestrus and metestrus phase. Co-administration of α-tocopherol along with chromium showed normal estrus cycle phases that were comparable to control [Table 1].

In Cr toxic group, the peroxidation markers such as malondialdehyde (MDA) and protein carbonyls in ovaries were significantly ($P < 0.05$) increased and the levels of anti-oxidants such as SOD and reduced GSH were significantly ($P < 0.05$) reduced compared with control. Co-administration of α-tocopherol significantly ($P < 0.05$) reversed the above values [Table 2].

The functional marker of ovaries *viz* total cholesterol was significantly ($P < 0.05$) increased when compared to control. The functional markers of liver in serum such as total protein were significantly ($P < 0.05$) decreased, while the ALT levels were significantly ($P < 0.05$) increased following Cr administration. Kidney functional markers such as serum creatinine and BUN were also significantly ($P < 0.05$) increased compared to those of control group. The above altered functional markers were significantly ($P < 0.05$) reversed with co-administration of α-tocopherol [Table 3].

Uterus of chromium-treated group showed atrophy of endometrial glands, fibrous tissue proliferation [Figure 2] and hyperplasia of uterine epithelium [Figure 3]. Ovarian sections from group 2 revealed severe congestion, degeneration of follicles. In addition, cystic follicles were seen in large numbers [Figure 4]. Ultrastructural changes like distorted nucleus, swollen and elongated mitochondria, altered epithelial size and shape were also noticed in group 2 rats [Figure 5]. Recovery from histological injury was observed in α-tocopherol co-administered rats, with mild cloudy swelling in uterus [Figure 6] and congestion in ovaries [Figure 7]. Ultrastructurally, no changes were noticed in group 3 rats [Figure 8]. In group 4, treatment

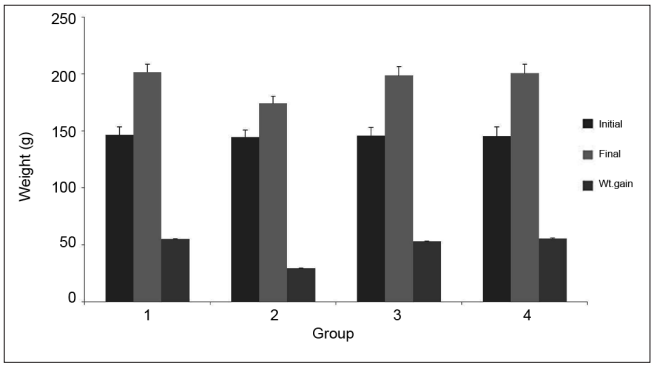

Figure 1: Photomicrograph of Mean weights of different groups of rats

Table 1: Effect of α-tocopherol on frequency of estrus cycle

Group	Estrus stages (% of cycle)			
	Proestrus	Estrus	Metestrus	Diestrus
1	12.99±1.02^{B}	27.67±1.41^{A}	17.67±0.99^{B}	41.67±1.74
2	9.33±1.42^{A}	26.0±1.42^{A}	15.00±0.89^{A}	49.67±2.17^{B}
3	12.86±1.02^{B}	27.47±1.41^{A}	17.00±1.11^{B}	42.67±1.74^{A}
4	12.67±0.89^{B}	27.00±0.89^{A}	18.00±1.47^{B}	42.33±1.47^{A}

Values are mean±SEM (n=6) One way ANOVA (SPSS), Means with different superscripts differ significantly (P<0.05)

with α-tocopherol alone, revealed normal architecture of uterus [Figure 9] and ovaries [Figures 10-11].

DISCUSSION

Hexavalent chromium is an important reproductive and developmental toxicant as Office of Environmental Health Hazard Assessment (OEHHA) and the Developmental and Reproductive Toxicant Identification Committee (DARTIC) mentioned in 2007.[5] Due to their extensive use in industry, there is a need to investigate the multi-organ toxicity due to Cr (VI) and mitigative role of vitamin E.

Previous studies showed that dichromate exposure increases the concentration of reactive oxygen species (ROS),[18] and provokes oxidative damage in hepatocytes,[19] kidney,[20] ovaries and uterus.[21]

Administration of Cr resulted in prolongation of diestrus phase. Estradiol is responsible for changes in the reproductive tract, mammary glands and for the regulation of gonadotropins. The stages of estrus cycle and their interconversions are mainly governed by the hormones viz., estrogens and progesterone.[22] Any change in these hormones would lead to changes in the cyclicity and impaired fertility. Hence, the persistent diestrus phase of the estrus cycle in the chromium treated rats could be correlated with decreased estradiol levels. These findings are in consistent with earlier report by Rao *et al.*[23]

Steroid hormone synthesis is controlled by activity of several highly substrate selective cytochrome P_{450}

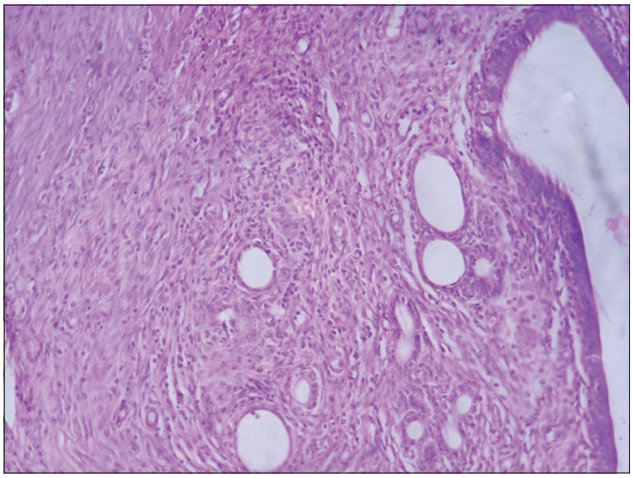

Figure 2: Photomicrograph of uterus showing marked atrophy of endometrial glands and fibrous tissue proliferation H and E ×200 (Group 2)

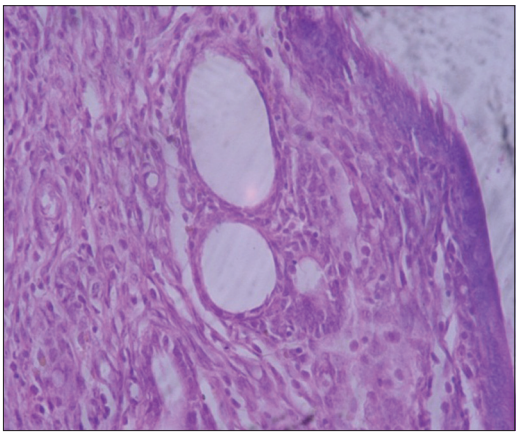

Figure 3: Photomicrograph of uterus showing hyperplasia of uterine epithelium. H and E ×400 (Group 2)

Table 2: Effect of α-tocopherol on antioxidant defenses and peroxidation biomarkers in ovarian homogenates

Group	Antioxidant markers		Peroxidation markers	
	SOD (Units/mg protein)	GSH(μM/mg protein)	TBARS (M of MDA/g of protein)	Protein carbonyls (nM/mg protein)
1	10.21±0.66^{C}	68.20±5.6^{B}	0.36±0.01^{A}	0.32±0.03^{A}
2	5.21±0.44^{A}	48.57±3.10^{A}	1.12±0.04^{B}	0.66±0.04^{C}
3	9.06±0.36^{B}	66.22±2.44^{B}	0.38±0.02^{A}	0.40±0.03^{B}
4	10.01±0.44^{C}	68.06±4.6^{B}	0.37±0.04^{A}	0.33±0.02^{A}

Values are mean±SEM (*n*=6) One way ANOVA (SPSS), Means with different superscripts differ significantly (*P*<0.05)

Table 3: Effect of α-tocopherol on functional markers of rats

Group	Functional markers				
	Liver		Kidney		Ovary
	Total protein (g/dl)	ALT (IU/L)	BUN (mg/dl)	Creatinine (mg/dl)	Cholesterol (mg/100 mg tissue)
1	3.9±0.14^{B}	17.8±0.10^{A}	18.5±0.15^{A}	0.75±0.01^{A}	1.47±0.22^{A}
2	2.2±0.18^{A}	62.3±0.15^{C}	31.1±1.73^{C}	1.06±0.04^{B}	3.10±0.24^{B}
3	3.8±0.19^{B}	20.4±0.24^{B}	19.98±0.81^{B}	0.76±0.03^{A}	1.50±0.06^{A}
4	3.9±0.12^{B}	17.2±0.11^{A}	18.58±0.22^{A}	0.74±0.02^{A}	1.48±0.05^{A}

Values are mean±SEM (*n*=6) One way ANOVA (SPSS), Means with different superscripts differ significantly (*P*<0.05)

enzymes and a number of steroid dehydrogenases and reductases. Interferences with steroid biosynthesis may result in impaired reproduction, alterations in development, sexual differentiation and growth.[24] The steroidogenic dehydrogenases are important regulatory enzymes necessary for the synthesis of steroid hormones. The exploration of these enzymes after chromium treatment results in blockage of steroidogenic pathway, which is evident by significant accumulation of cholesterol in ovaries of chromium treated rats.

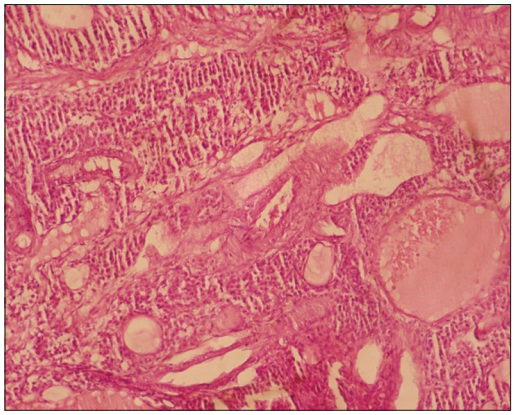

Figure 4: Photomicrograph of ovary showing congestion and cystic follicles. H and E ×200 (Group 2)

Figure 5: Photomicrograph of ovary of TEM (×5000) showing altered epithelial cell size and shape, distorted nucleus, swollen and elongated mitochondria, margination of chromatin (Group 2)

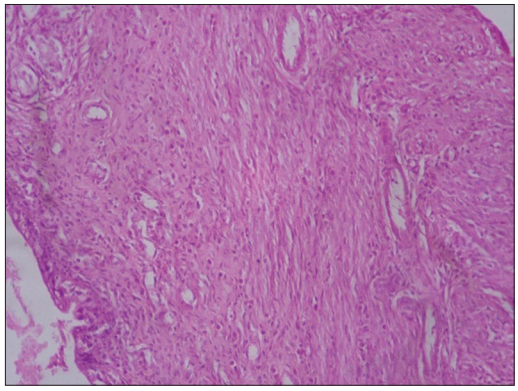

Figure 6: Photomicrograph of uterus showing mild cloudy swelling. H and E ×200 (Group 3)

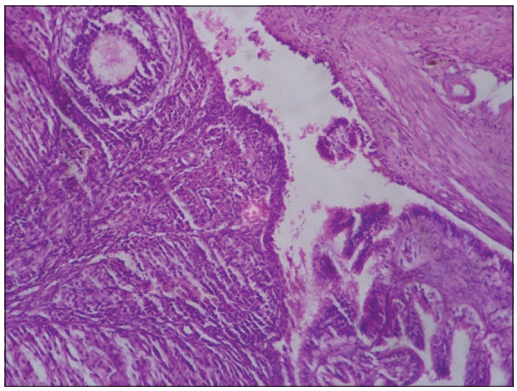

Figure 7: Photomicrograph of ovary showing mild congestion. H and E ×200 (Group 3)

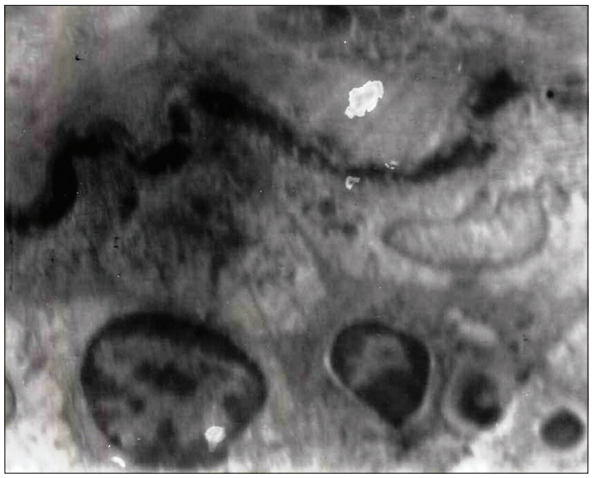

Figure 8: Photomicrograph of ovaries of TEM (×5000) showing various stages of follicle (Group 3)

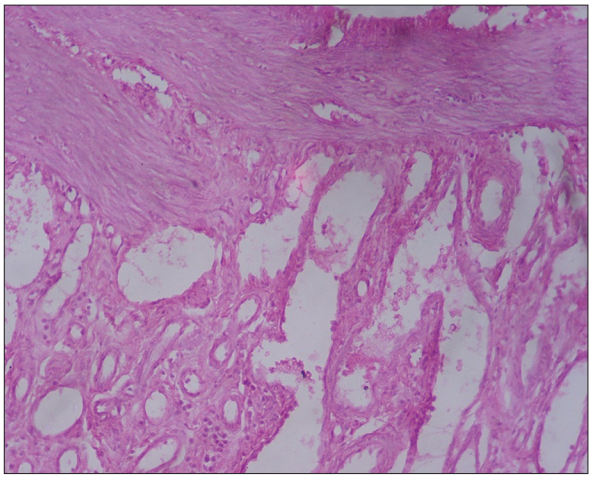

Figure 9: Photomicrograph of uterus showing normal histoarchitecture. H and E ×200 (Group 4)

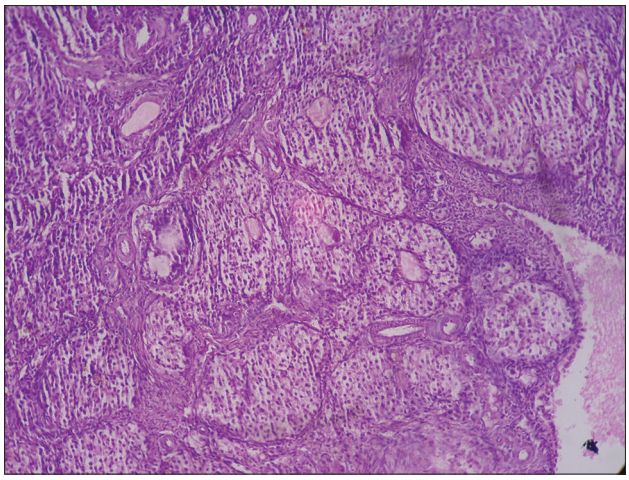

Figure 10: Photomicrograph of ovaries showing normal histoarchitecture. H and E ×200 (Group 4)

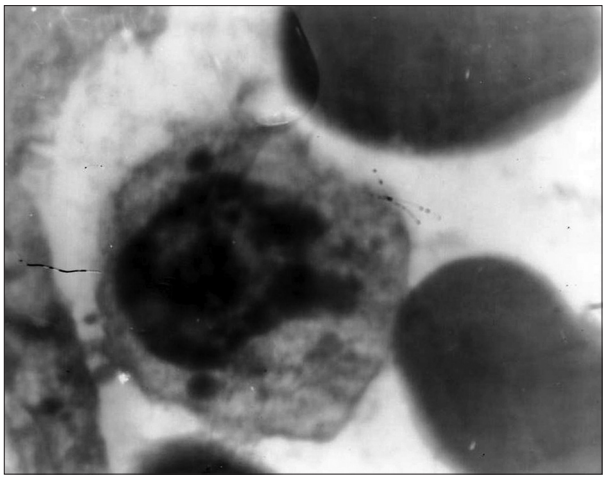

Figure 11: Photomicrograph of ovaries of TEM (×5000) showing normal follicles and basement membrane with adipose tissue (Group 4)

Administration of Cr resulted in oxidative stress in female reproductive system of rats that was reflected by altered histoarchitecture, with atrophy of endometrial glands in uterus, hyperplasia of uterine epithelium and fibrous tissue proliferation. Ovarian sections revealed severe congestion and degeneration of follicles. In addition, cystic follicles were seen in large numbers. Severe histological changes like follicular atresia, induced fibrosis and necrosis of primary and secondary follicles of Cr treated rats were earlier reported by Royce *et al*.[25] Cr induces free radical production by multiple mechanisms leading to peroxidation, which in the present study was evinced by significant increase in peroxidation markers such as MDA and protein carbonyls, and decrease in anti-oxidant markers such as SOD and GSH in ovaries. Peroxidative damage also occurred in liver and kidney, which resulted in reduced hepatic and kidney function, and was reflected by significant decrease in total protein with significant increase in ALT activity indicating hepatotoxicity. Significant increase in serum levels of BUN and creatinine in this study was suggestive of nephrotoxicity. The results of the present study are in agreement with earlier findings of reduction in the anti-oxidant markers with simultaneous increase in peroxidation markers and functional markers in rats under Cr influence.[21]

Vitamin E, a lipid soluble membrane localized anti-oxidant, protects cells and tissues from oxidative damage induced by a wide variety of free radical species. It functions as a chain breaking anti-oxidant that prevents the propagation of free radical reaction and preserves cell membranes by protecting against lipid peroxidation through reaction with lipid peroxyl radicals and conversion to a non-reactive tocopheroxyl radical.[26] In the present study, when vitamin E was supplemented along with chromium, a remarkable resurgence was observed in all the parameters. The results of the present study were in agreement with previous studies, where supplementation of vitamin E restored ovarian steroidogenic indices and cyclicity to normal in rats administered potassium dichromate.[23]

In conclusion, the study revealed that chromium exposure affected ovarian steroidogenesis and cyclicity along with histological alterations in ovaries and uterus, which affected the fertility potential of treated females, besides affecting hepatic and renal function. However, vitamin E supplementation along with chromium to rats, manifested significant protective effects.

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