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EFFECT OF PROBIOTIC STRAIN *LACTOBACILLUS CASEI*STRAIN 17 AGAINST TOXICITYINDUCED BY CHROMIUM IN FEMALE REPRODUCTIVE SYSTEM OF RATS.

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ABSTRACT

The present study was aimed to investigate whether *Lactobacillus casei* strain17 could protect the chromium (Cr) VI-induced oxidative stress in female reproductive system of rats and to explore the underlying mechanism 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 *Lactobacillus casei*(Lc) strain 17 at the rate of 1 x 10^9 CFU/rat/day for 14 days. Group 4 was maintained as probiotic 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, antioxidant, 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 fertility impairment 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 markers in serum such as total protein were 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 *Lactobacillus casei*(Lc) strain 17 significantly ($p<0.05$) reversed the (Cr) VI induced changes.

KEY WORDS:Chromium, oxidative stress, α-tocopherol, female reproductive system.

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INTRODUCTION

Chromium is a transition element found in many compounds of Earth's crust[1] and ranks 21st in elemental abundance. Chromium provides also 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[5]. Chromium (VI) as reproductive toxicant is recently recognized and less studied[6].To check the chromium exposure into environment there are various treatment options, however, they are energy expensive and less successful due to their high running cost. In this context, biotransformation of Cr(VI) to Cr(III) by bacteria offers a viable, economically safe and sustainable alternative[7].Lactic acid bacteria (LAB) are ubiquitous in fermented and non-fermented foods and are common components of the human commensal microflora. They are a group of bacteria characterized by their ability to synthesize lactic acid and are widely used in food manufacturing for their beneficial technological properties and positive effects on the health. Lactic acid bacteria (LAB) have some probiotic functions, such as adjusting the balance of intestinal microflora, reducing serum cholesterol and revitalizing the immune system etc[8,9]. Many of their beneficial properties are related to their capacity to adhere or bind to different targets[10]. Heavy metals and aflatoxin B1 have been reported to passively bind to the bacterial surface by electrostatic and hydrophobic interactions[11,12]. The antioxidant effect of LAB has been reported only recently[13].The LAB could be comprised of about 20 genera. *Lactobacillus* is largest of these genera, comprising about 80 recognized species[14]. *Lactobacillus casei* has been reported to possess antioxidant activity and is able to decrease the risk of accumulation of reactive oxygen species (ROS) during the ingestion of food[15]. In this backdrop, the present work was designed to study the protective role of coated *Lactobacillus casei* strain 17 on the female reproductive system of rats exposed to chromium toxicity.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 & METHODS

i. Chemical Reagents

All the chemicals were of analytical grade and obtained from Qualigens Pvt. Ltd., Mumbai, India.

ii. Animals

Adult *Wistar* rats (24), aged about 60 days with average body weight of 140±10 g was 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).

iii. Experimental procedure

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 rats received $K_2Cr_2O_7$ (10 mg/ kg b.wt. subcutaneously single dose)along with coated *Lactobacillus casei*(Lc) strain 17 at the rate of 1 x 10^9 CFU/rat/day for 14 days. Group 4 rats received only coated *Lactobacillus casei*strain 17 at the rate of 1 x 10^9 CFU/rat/day for 14 days. The study was approved by Institutional Animal Ethics Committee.Potassium dichromate ($K_2Cr_2O_7$) was dissolved in sterile saline (Nacl 0.9%). Coated *Lactobacillus casei*strain 17 was dissolved in 1% Tween 80 and made into solution by adding double ionized distilled water and was given at the rate of 1 x 10^9 CFU/rat/day (10^9 CFU =0.01 gm).

iv. Collection and preparation of samples

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.

v. 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 (OECD Guidance Report Part 5, 2009)[16]. 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 prelabelled 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.

vi. Antioxidant markers

SOD was estimated by the method that involved inhibition of superoxide-dependent reduction of tetrazolium dye methyl thiazolyltetrazolium (MTT) to its formazan[17]. GSH was estimated based on a reaction of reduced glutathione with 5-5ditiobis-2-nitrobenzoic acid (DTNB)[18].

vii. Peroxidation markers

Malondialdehyde, the product of lipid peroxidation, was estimated by reaction with thiobarbituric acid as per the method prescribed by Balasubramanian*et al.*[19] 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[20].

viii. Sero-biochemical markers

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

ix. Total Protein

Total protein in the ovarian tissue was quantified as per Lowry *et al.*'s[21] method.

x. 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[22]. For transmission electron microscopy (TEM), the glutaraldehyde-fixed tissues were used. Specimen preparation, staining and the observations were done at the designated RUSKA Lab, SV Veterinary University, Hyderabad.

xi. 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 test and significance was set at $P < 0.05$.

RESULTS

i. Body weight gain

The average body weight gain was significantly ($p<0.05$) reduced in group 2 compared to control. But co-administration of *Lactobacillus casei*strain 17 with chromium exposure (group 3) showed a significant ($p<0.05$) increase in weights as compared to group 2 (Fig. 1).

ii. Occurrence of estrus cycle

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 *Lactobacillus casei*strain 17 along with chromium showed normal estrus cycle phases that were comparable to control (Table 1).

iii.Oxidative stress parameters

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 antioxidants such as SOD and reduced GSH were significantly ($p<0.05$) reduced compared with control. Co-administration of *Lactobacillus casei*strain 17 significantly ($p<0.05$) reversed the above values (Table 2).

iv.Functional markers

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 *Lactobacillus casei*strain 17 (Table 3).

v. Histology

Uterus of chromium-treated group showed atrophy of endometrial glands, fibrous tissue proliferation (Fig 2) and hyperplasia of uterine epithelium (Fig 3). Ovarian sections from group 2 revealed severe congestion, degeneration of follicles. In addition, cystic follicles were seenin large numbers (Fig 4). Ultrastructural changes like distorted nucleus, swollen and elongated mitochondria, altered epithelial size and shapewere also noticed in group 2 rats (Fig 5). Recovery from histological injury was observed in *Lactobacillus casei*strain 17co-administered rats, with mild fibrous tissue proliferation in uterus (Fig 6) and congestion in ovaries (Fig 7). Ultrastructurally changes like vesicular cytoplasm, distorted and dilated endoplasmic reticulum were noticed in ovaries of group 3 rats (Fig 8). In group 4, treatment with *Lactobacillus casei*strain 17alone, revealed normal architecture of uterus (Fig 9) and ovaries (Fig 10). Ultrastructurally no abnormal changes were seen in ovaries of group 4 rats (Fig 11).

Table 1
*Effect of Lactobacilluscaseis*train 17*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^{A}
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}	26.47±1.41^{A}	17.00±1.11^{B}	43.67±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).

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	9.01±0.26^{B}	65.22±2.04^{B}	0.48±0.02^{A}	0.40±0.03^{B}
4	10.11±0.34^{C}	68.16±3.6^{B}	0.37±0.02^{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^{c}	17.8±0.10^{a}	18.5±0.15^{a}	0.75±0.01^{a}	1.47±0.22^{A}
2	2.24±0.18^{a}	62.32±0.15^{c}	31.13±1.73^{c}	1.06±0.04^{b}	3.10±0.24^{B}
3	3.6±0.19^{b}	28.4±0.24^{b}	24.48±0.51^{b}	0.89±0.03^{c}	1.54±0.06^{A}
4	3.8±0.12^{c}	18.2±0.11^{a}	17.38±0.17^{a}	0.73±0.02^{a}	1.46±0.05^{A}

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

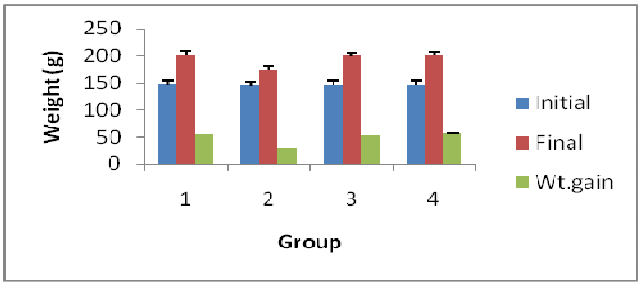

Figure 1
Effect of coated Lactobacillus casei on body weight gain in chromiumintoxicated rats

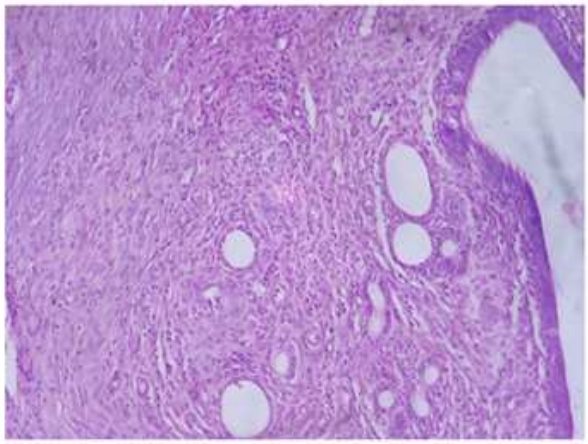

Figure 2
Photomicrograph of uterus showing marked atrophy of endometrial glands and fibrous tissue proliferation. H&E X 200 (Group 2).

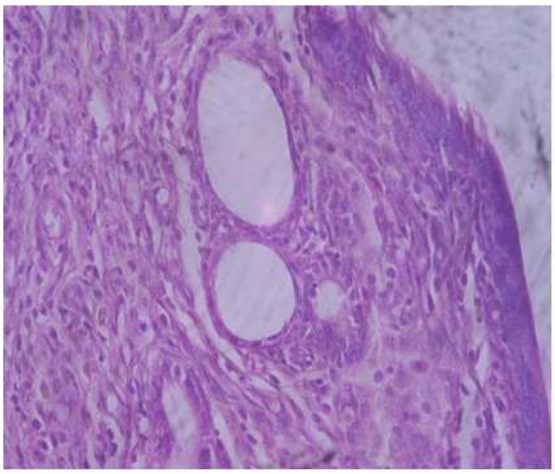

Figure 3
Photomicrograph of uterus showing hyperplasia of uterine epithelium. H&E X 400 (Group 2).

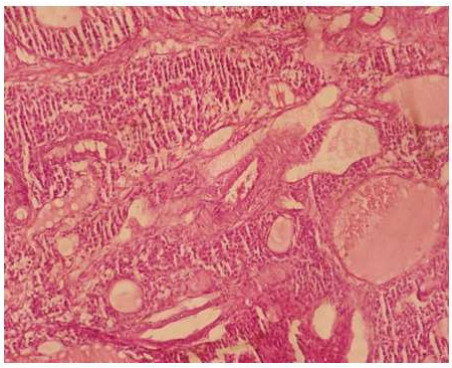

Figure 4
Photomicrograph of ovary showing congestion and cystic follicles.H&E X 200 (Group 2).

Figure 5
Photomicrograph of ovary of TEM (x5000) showing altered epithelial cell size and shape, distorted nucleus, swollen and elongated mitochondria, margination of chromatin (Group 2).

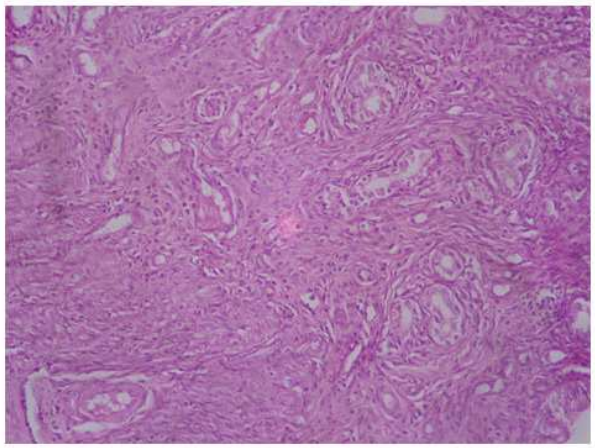

Figure 6
Photomicrograph of uterus showing mild fibrous tissue. H&E X 200 (Group 3).

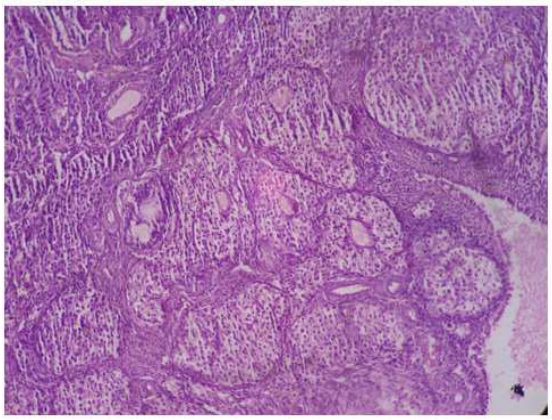

Figure 7
Photomicrograph of ovaries showing normal histoarchitecture.H&E X 200 (Group 3).

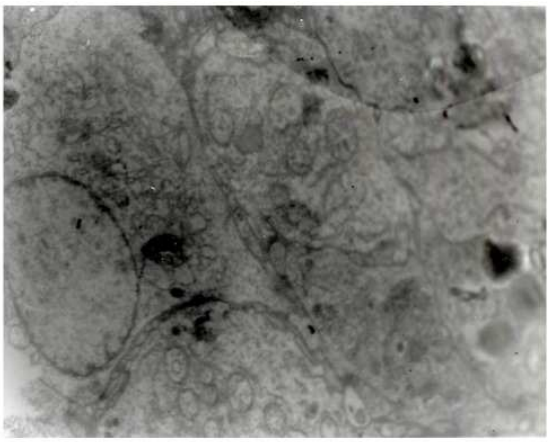

Figure 8
Photomicrograph of ovary of TEM (x5000) showing vesicular cytoplasm, distorted and dilated endoplasmic reticulum (Group 3).

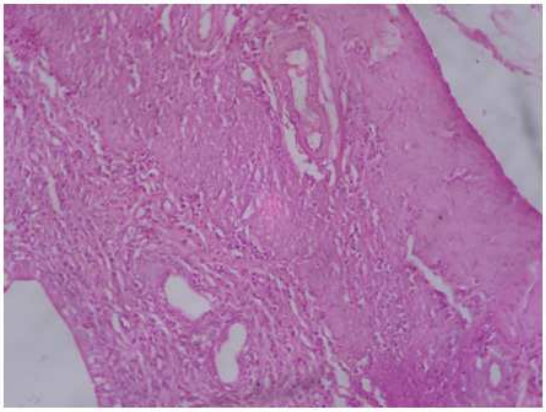

Figure 9
Photomicrograph of uterus showing normal histoarchitecture.H&E X 200 (Group 4).

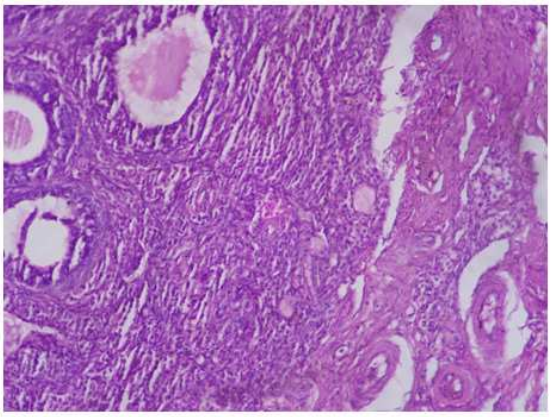

Figure 10
Photomicrograph of ovaries showing normal histoarchitecture.H&E X 200 (Group 4).

Figure 11
Photomicrograph of ovary of TEM (x5000) showing intact ovary and various stages of follicles(Group 4).

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 (DART IC) mentioned in 2007[6]. Due to their extensive use in industry, there is a need to investigate themulti-organ toxicity due to Cr (VI) and mitigative role of probiotic *Lactobacillus casei* strain 17.Previous studies showed that dichromate exposure increases the concentration of reactive oxygen species (ROS)[23], and provokes oxidative damage in hepatocytes[24], kidney[25], ovaries and uterus[26].Administration of Cr resulted in prolongation of diestrusphase. Estradiol is responsible for changes in the reproductive tract, mammary glands and for the regulation

of gonadotropins. The stages of estrus cycle and their inter conversions are mainly governed by the hormones viz., estrogens and progesterone[27]. Any change in these hormones would lead to changes in the cyclicity and impaired fertility. Hence the persistentdiestrus 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*[28].Steroid hormone synthesis is controlled by activity of several highly substrate selective cytochrome P_{450} 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[29]. 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.

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 seenin 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*[30]. 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 antioxidant 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 creatinineinthis study was suggestive of nephrotoxicity. The results of the present study are in agreement with earlier findings of reduction in the antioxidant markers with simultaneous increase in peroxidation markers and functional markers in rats under Cr influence[26].*Lactobacillus casei* is a well-known commercial probiotic strain. Biosorption, bioaccumulation and enzymatic reduction/oxidation are the processes by which the microorganisms interact with the toxic metals. The enzymatic reduction of Cr (VI) involves membrane bound chromate reductase under aerobic conditions and activity of which is enhanced by NADH or glutathione as enzyme co-factors[31]. In group 3 rats, simultaneous administration of Lc along with Cr significantly restored various biomarkers under study when compared to group 2. The favourable recovery in these biochemical profiles is further supported by the less severe histological changes in the liver and kidney. Recent studies have also shown that various strains of lactic acid bacteria exhibit antioxidative activity both*invivo* and *invitro*[32].In conclusion, the results of the study suggest that sodium alginate coated *Lactobacillus casei* possesses antioxidant property that was able to protect the liver and kidney from chromium-induced oxidative stress.

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