Chapter-2

Dose-duration and toxicity study of chromium (VI) in male Albino rats

Contents

2.0 Introduction:

The toxicity of inorganic compounds has been of increasing interest in the area of environmental research, occupational health and clinical medicine. Metallic elements form a variety of inorganic salts and complexes. The most important binary and ternary compounds, both from technological and toxicological viewpoints are oxides and oxyanions. Oxidation state may be Changed either by non-enzymatic action (depending upon redox states inside cell or biological fluid) or through action of enzymes. The addition or removal of electrons from the metal atom will influence the chemical reactivity and capability of the metal to bind with tissue ligands for exerting biological effects.

The adverse effects of chromium compounds in human health were being defined (Baruthio, 1992). Chromium plays a contradictory role in nature. It is essential micronutrient element in low concentration but at higher concentration its toxicity appears. Thus, the chromium plays a dual biological role with its dual (trivalent and hexavalent) appearance in the environmental systems. On one hand, chromium (III) is regarded as essential micronutrient necessary for certain physiological functions, and requires a minimum daily intake. From this point of view the most extensively investigated effect of chromium (III) is its involvement in the metabolism of glucose, as a potentiating agent for insulin (WHO, 1990). Chromium has been prescribed as a dietary supplement in case of deficiency of food intake and of impaired glucose tolerance, in the form of glucose tolerance factor. On the other hand, overexposure to certain chromium (III) compounds is important from a toxicological view point specially its long-term effects. Most of the information on the chromium (VI)-induced carcinogenesis comes from the epidemiological studies

of exposed workers (ATSDR, TP-9/08, 1993). Hexavalent chromium compounds are considered too toxic to aquatic and terrestrial organisms. They are much more soluble in soil / water systems than trivalent chromium compounds (Losi et al, 1994). A high intake of hexavalent chromium has mutagenic (Chovatovicova et al, 1993) and carcinogenic effects (De Flora et al, 1990). In addition to toxicity to liver (Bagchi et al, 2002a) and renal toxicity has also been reported in workers and animals exposed to chromium (VI) (Hojo and Satomi, 1991). Chromium (VI) can easily enter inside cells passing through the membranes with the help of sulphate anion transport system (De Flora and Wetterhahn, 1989). Chromium (VI) is reduced intracellularly through "reactive intermediates" such as chromium (V), chromium (IV) to more stable form of chromium (III) by cellular reductants (Sugiyama et al, 1991). This reduction process produces huge amount of free radical species such as active oxygen radicals $O_2^{\bullet -}$ (Chrovaticova et al, 1993) which may be the prime cause of the mitochondrial oxidative stress leading to cell death (Orrenius et al, 2007). The present investigation was intended to study the dose and duration selection of hexavalent chromium in terms of their toxicity *in vivo* rat model setup.

2.1 Methodology

2.1.1 Chemicals and Reagents

Potassium dichromate salt and fine chemicals were purchased from Sigma Chemical Company, USA. All other chemicals and reagents were purchased from Sigma Chemical Company, USA; Sisco Research Laboratory Pvt Ltd. (SRL), Mumbai, India and were of analytical grade. The glass and plastic items were obtained from Borosil India Ltd

2.1.2 Maintenance of animals

Male albino rats, average weight between (80-100 g) were procured in laboratory. They were kept in well maintained cage and fed with a lab-prepared diet with water *ad libitum* as described (Dey et al, 2003). Laboratory acclimatized rats were grouped separately for serial experimental studies. The body weights of rats were taken daily at a fixed time of the day in treatment schedule.

2.1.3 Mode of treatment

Animals are divided into several groups and induced by intra peritoneal (*i.p.)* injection with $K_2Cr_2O_7$ for this study.

2.1.4 Experimental design

2.1.4.1 Rats were grouped for dose dependent study as follows:

a) Control Group-The rats were injected intraperitoneally (*i.p.*) with 0.9% NaCl solution for 28 days.

b) Chromium treated Group-There four doses of $K_2Cr_2O_7$ were used for dose dependent study, those are as follows:

Group I - 200 micrograms/ 100 grams of body weight / day

Group II - 400 micrograms/ 100 grams of body weight / day

Group III - 600 micrograms/ 100 grams of body weight / day

Group IV - 800 micrograms/ 100 grams of body weight / day

2.1.4.2 Rats were grouped for duration dependent study as follows:

a) **Control Group**

The rats were injected with 0.9% NaCl solution (*i.p.*).

b) **Chromium treated Group**

There are six durations of $K_2Cr_2O_7$ were used for duration dependent study, those are as follows:

Group I - 1st day (800 µg / 100 gm body weight /day)

Group II - 3rd day (800 µg /100 gm body weight /day)

Group III - 7th day (800 µg / 100 gm body weight / day)

Group IV -14th day (800 µg / 100 gm body weight/day)

Group V -21st day (800 µg / 100 gm body weight / day)

Group VI - 28th day (800 µg / 100 gm body weight / day)

2.1.4.3 Rats were grouped for toxicity study as follows:

a) **Control Group**

The rats were treated with 0.9% NaCl solution (*i.p.*) for 28 days.

b) **Chromium treated Group**

The rats were injected with $K_2Cr_2O_7$ (800 µg per 100 gram body weight per day for 28 days.

2.1.5 Collection of blood samples and preparation of serum

At the end of scheduled day of experiment, rats are kept fasting over-night and then sacrificed by cervical dislocation after proper anaesthesia. Then blood samples were collected from hepatic vein and immediately serum was separated out of clotted blood samples by centrifugation at 1500 × g for 15 min.

2.1.6 Collection of Tissues

The liver and lungs were dissected out of the body and collected separately into marked containers immediately after soaking the blood with blotting paper. The containers were kept at -20^0C**.**

2.1.7 Homogenization of tissues

Tissues were weighted and homogenized in glass homogenizer with ice cold 0.2 M PBS (pH 7.4). For different biochemical assays 5% of the homogenized tissues were used.

2. 1.8 Estimation of chromium

Tissues were mixed with an acid mixture containing, nitric acid, sulphuric acid and perchloric acid in a ratio of 6:1:1 over a regulated heater. The acid mixture after digestion was evaporated with occasional additions of triple distilled water. Thereafter final solution was obtained for chromium content estimation of particular tissues by Atomic Absorption Spectrophotometer.

2.1.9 Estimation of liver function enzymes

Alkaline phosphatase (ALP) was measured in serum, liver and lungs tissues according to Kind and King (1954). The activities of transaminases (AST & ALT) in

serum, liver and lungs tissues were estimated according to Reitman and Frankel (1957).

2.2 Oxidative stress parameters

2.2.1 Estimation of Malondialdehyde

MDA was estimated according to Ohkawa et al, (1979) using thiobarbituric acid. Tissues were used to estimate the MDA level at 530 nm wavelength with the help of UV spectrophotometer (Shimadzu, UV 1800). The lipid peroxidation level was noted as nmole/mg protein. Conjugated dienes were estimated as per the method described by Slater (1980).

2.2.2 Estimation of Superoxide dismutase activity

SOD activity variation in mitochondria of rat liver and lungs was determined from its ability to inhibit the auto-oxidation of pyrogallol according to Marklund and Marklund, (1974). The reaction mixture consisted of 50mM Tris (hydroxymethyl) aminomethane (pH 8.2), 1mM diethylen-etriaminepenta acetic acid, and 20–50 μL of isolated mitochondria of liver and lungs. The reaction was initiated by the addition of 0.2mM pyrogallol and the absorbance measured kinetically at 420 nm at 25°C for 3min. SOD activity was expressed in unit/min/mg of protein.

2.2.3 Estimation of Catalase activity

Catalase (CAT) activity was estimated in liver and lungs by the method of Luck (1963). CAT activity was calculated by using the molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$for H_2O_2. The activity of CAT was expressed in terms of m mol H_2O_2 consumed/min/mg protein.

2.2.4 Estimation of reduced Glutathione

Reduced glutathione (GSH) was measured from tissue homogenate as per the previously reported method (Dash et al, 2014). A standard curve was obtained with standard GSH. The levels of GSH were expressed as mg of GSH mg/protein.

2.2.5 Estimation of oxidized Glutathione

The oxidized glutathione level present in the tissue homogenate was measured after derevatization of GSH with 2-vinyl pyidine according to the modified method of (Mahapatra et al, 2009). The level of GSSG was calculated with standard GSSG curve. The levels of GSSG were expressed as mg of GSSG mg/protein.

2.2.6 Estimation of Glutathione Peroxidase

Glutathione peroxidase (GPx) activity was tested according to the method of Paglia and Valentine (1967). The reaction mixture was prepared with 50 mM potassium phosphate buffer (pH 7.0), 1 mM reduced glutathione, 1 U glutathione reductase, 0.2 mM NADPH, 1 mM sodium azide and1 mM EDTA. The sample was allowed to equilibrate for 5 min at 25◦C. The GPx activity was expressed in terms of nmol NADPH consumed/min/mg protein.

2.7 Estimation of Glutathione reductase

The activity of glutathione reductase (GR) was measured using the method of Miwa (1972). The tubes of enzyme assay were incubated at 37^0 8C, and contained 2.0 mL of 9 $mmol{\cdot}L^{-1}$ GSSG, 0.02 mL of 12 $mmol{\cdot}L^{-1}$ NADPH.Na4, 2.68 mL of 1/15 $mol{\cdot}L^{-1}$ phosphate buffer (pH 6.6), and 0.1 mL of cell lysate. The activity of enzyme

was determined by monitoring the decrease in absorbance at 340 nm. The molar extinction coefficient of 6.22×10^3 $mol{\cdot}L^{-1}{\cdot}cm^{-1}$ was used to determine GR activity.

2.2.8 Estimation of (Glutathione-S-Transferase) GST

Glutathione-S-transferase (GST) activity was estimated by the method of Gautam et al, (2012). The GST activity was expressed in terms of nmol NADPH consumed/min/mg protein.

2.3 Estimation of Protein

Protein was estimated according to Lowry et al, (1951) using bovine serum albumins as standard**.**

2.4 Statistical Analysis

All the parameters were repeated at least three times. The data were presented as mean ± SEM. By performing ANOVA test (using a statistical package, Origin 6.1, Northampton, MA 01060, USA), the means of control and treated group were compared by multiple comparison t-test having $P<0.05$ as a limit of significance. (Fisher and Yates, 1974).

2.5 Results

Activities of ALP (Fig-1), AST (Fig-2) and ALT (Fig-3) in serum are increased significantly in the different doses of chromium when it is compared with control group.

Figures 4, 5 & 6 show that the ALP, AST and ALT activities in liver and are significantly decreased following exposure to chromium at different doses.

Figures 7, 8 & 9 reveal that the ALP, AST and ALT activities of lungs are significantly decreased in the different doses of chromium-treated group when it was compared with the control group.

The data represented in Figures 10, 11 & 12 reveal the ALP, AST and ALT activities in serum for the duration dependent study in response to chromium. It has been found that the activities of ALP, AST and ALT in serum are increased significantly from the 7^{th} day of chromium treatment and became maximum on 28^{th} day treatment.

Figures 13, 14 & 15 show the ALP, AST and ALT activities in liver following exposure to chromium for duration dependent study. Results show that the activities of ALP, AST and ALT in liver are significantly decreased from the 7^{th} day of chromium treatment when it is compared with control group.

Figures 16, 17 & 18 reveal that the ALP, AST and ALT activities of lungs are significantly decreased following exposure to chromium for duration-dependent study. It has been found that the ALP, AST and ALT activities in lungs are significantly decreased from the 7^{th} day of chromium treatment.

Body weight changes during the 28 days period of chromium treatment are depicted in Figure-19. It was noted that the body weight decreased markedly after chromium administration in comparison with control. Chromium contents are also increased significantly after chromium treatment (Table-1).

MDA and conjugated dienes levels are significantly increased in liver and lungs in chromium exposed rats compared to control group (Figures 20 & 21). Significantly decreased catalase and SOD activities in both liver and lungs tissues are observed in

response to chromium when it has been compared with control (Figures 22 & 23). It has been found that GSH and GSSG level have decreased noticeably in both Liver and Lungs after exposure of hexavalent chromium (Figures 24 and 25). On the other hand, GPx, GR and GST activities in selected tissues are decreased significantly in challenge to chromium, comparing with control groups (Figures 26, 27, 28).

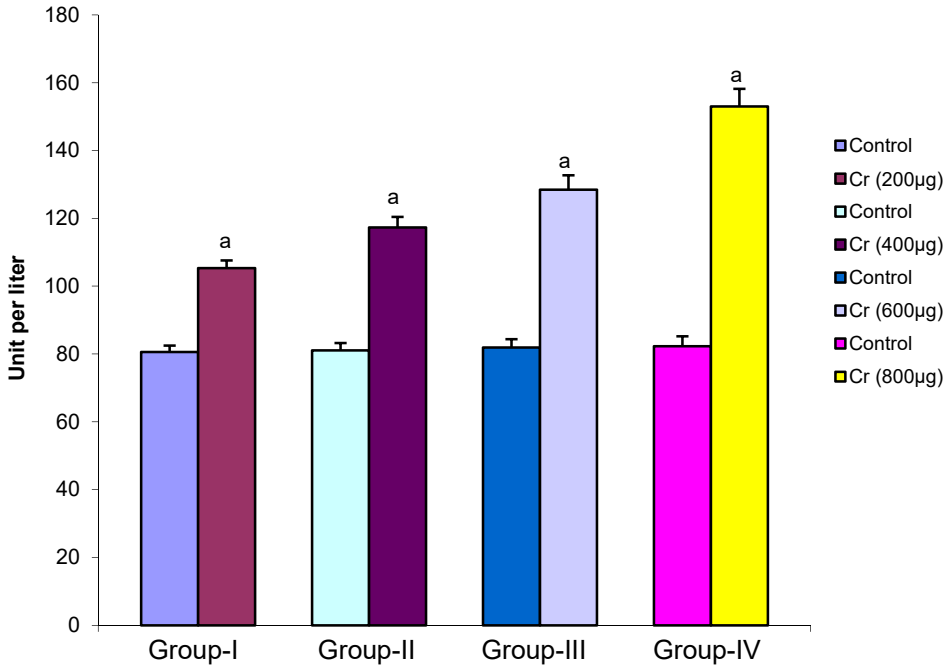

Figure 1: Changes in the serum ALP activity at different doses of chromium treated rats. [a] noted significant difference ($P<0.05$).

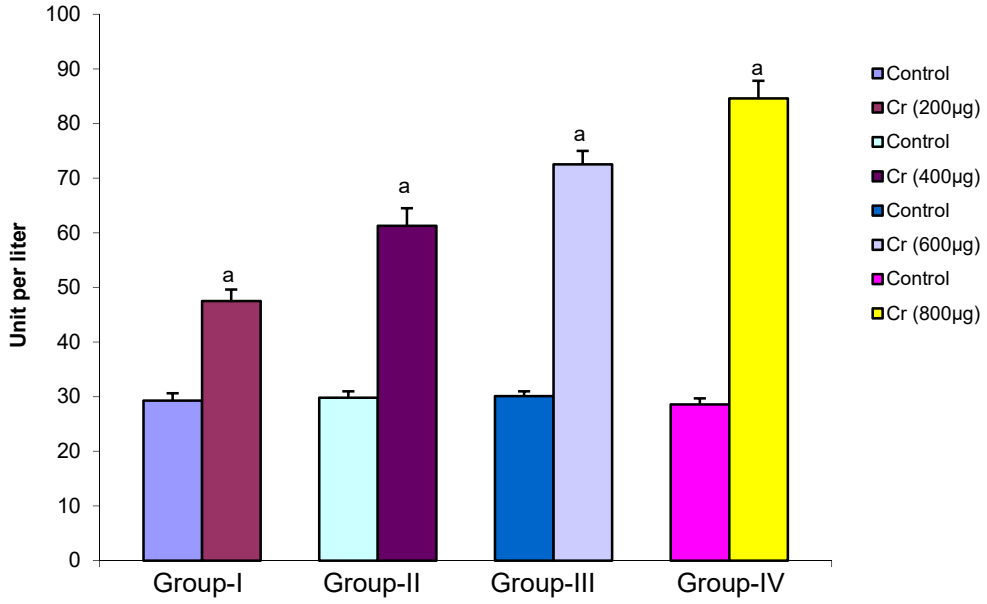

Figure 2: Changes in the serum AST activity at different doses of chromium treated rats. [a] noted significant difference (P<0.05).

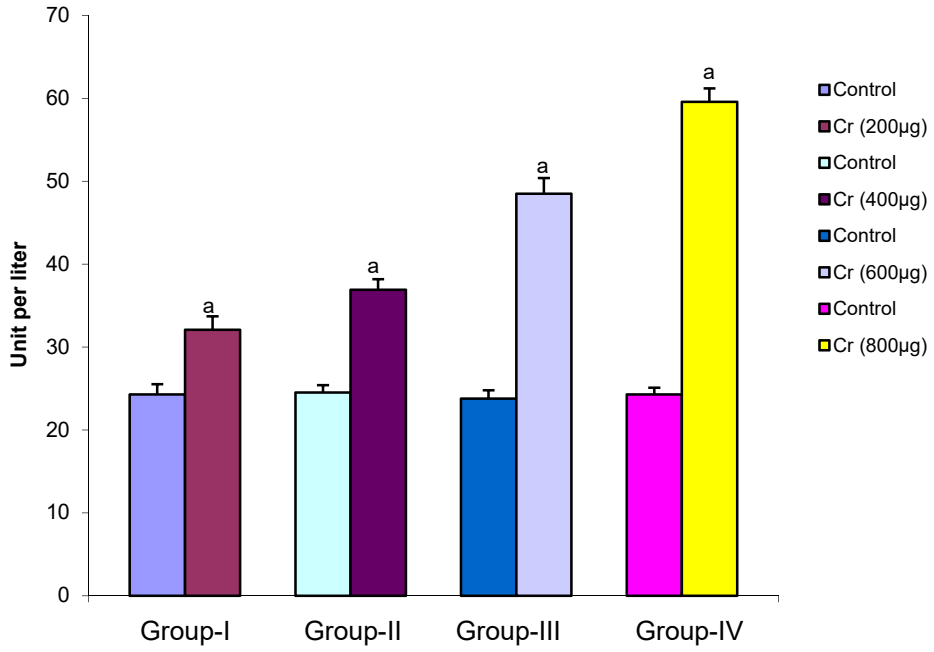

Figure 3: Changes in the serum ALT activity at different doses of chromium treated rats compared to control. [a] noted significant difference (P<0.05).

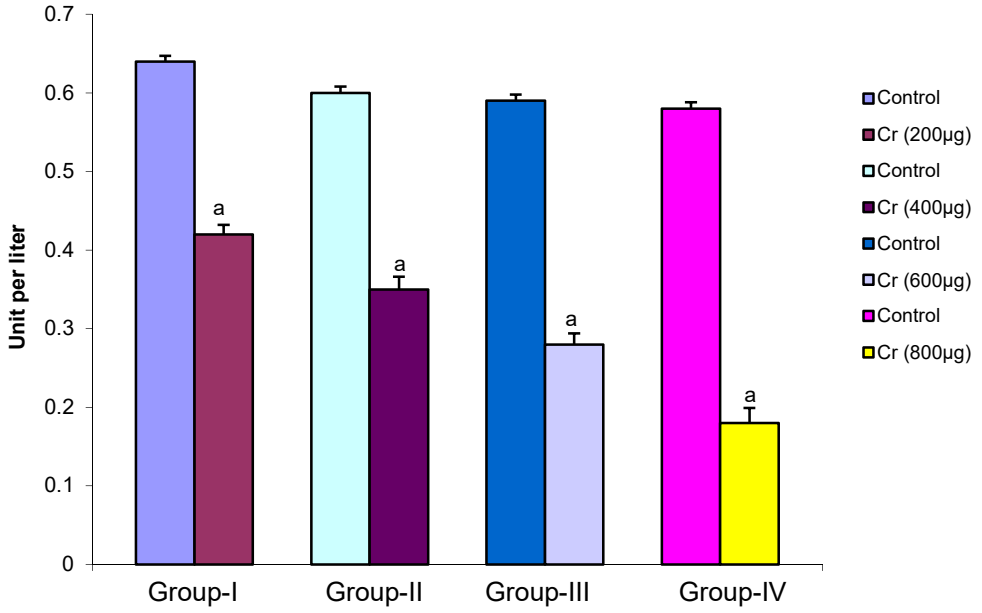

Figure 4: Change the ALP activity of liver at different doses in response to chromium. [a] noted significant difference (P<0.05).

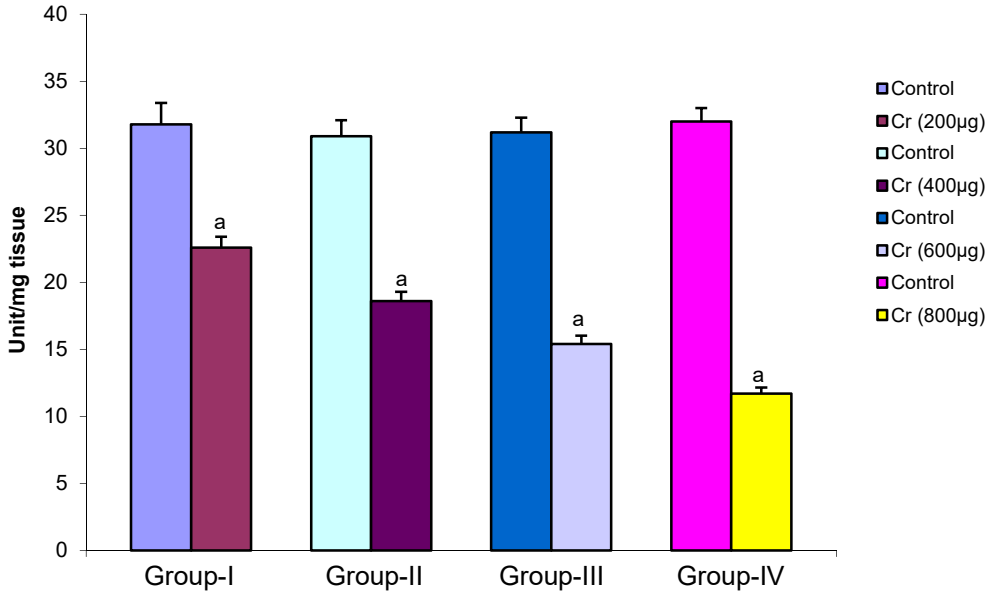

Figure 5: Changes in the AST activity of liver following exposure to chromium at different doses. [a] noted significant difference (P<0.05).

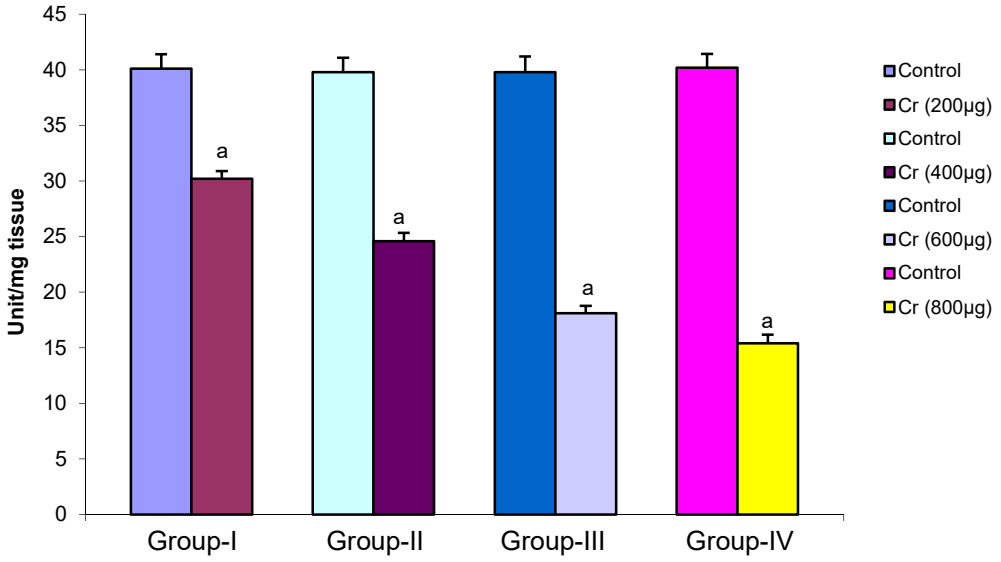

Figure 6: Change in the ALT activity of liver following exposure to chromium at different doses. [a] noted significant difference (P<0.05).

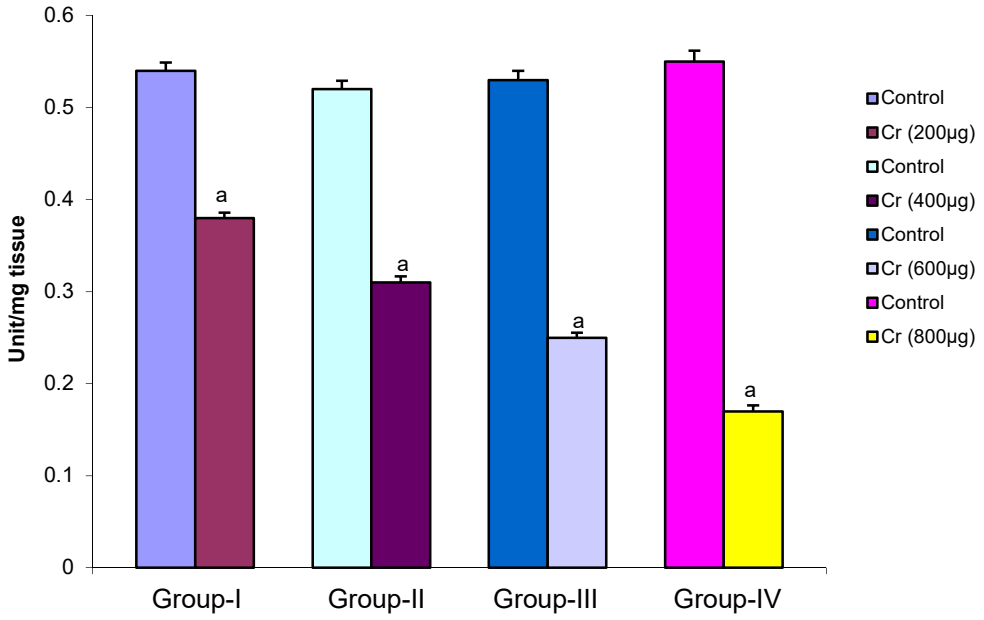

Figure 7: Change in the ALP activity of lungs in response to chromium at different doses. [a] noted significant difference (P<0.05).

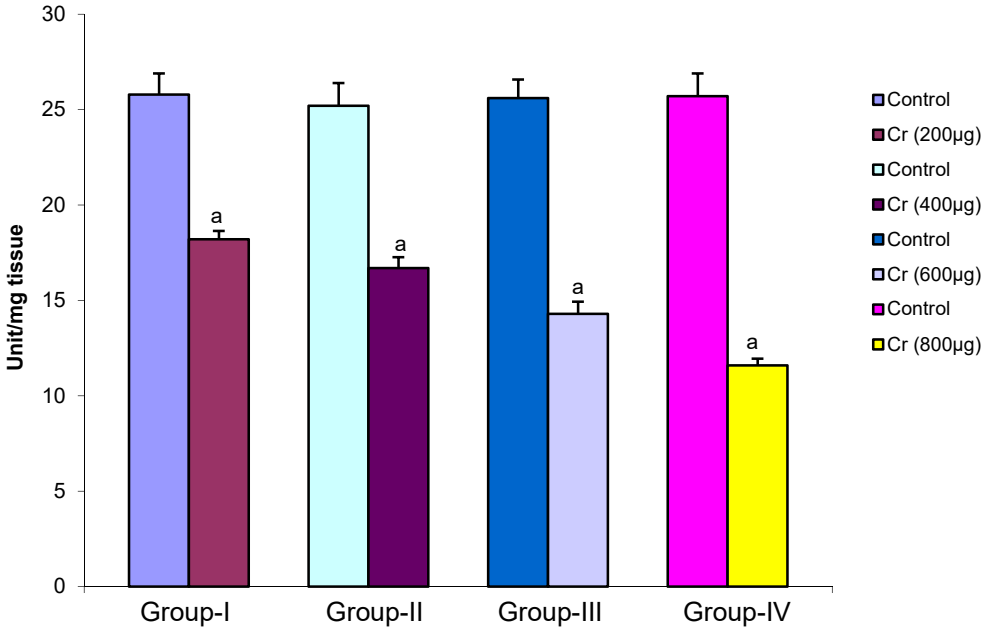

Figure 8: Change in the AST activity of lungs in response to chromium at different doses. [a] noted significant difference (P<0.05).

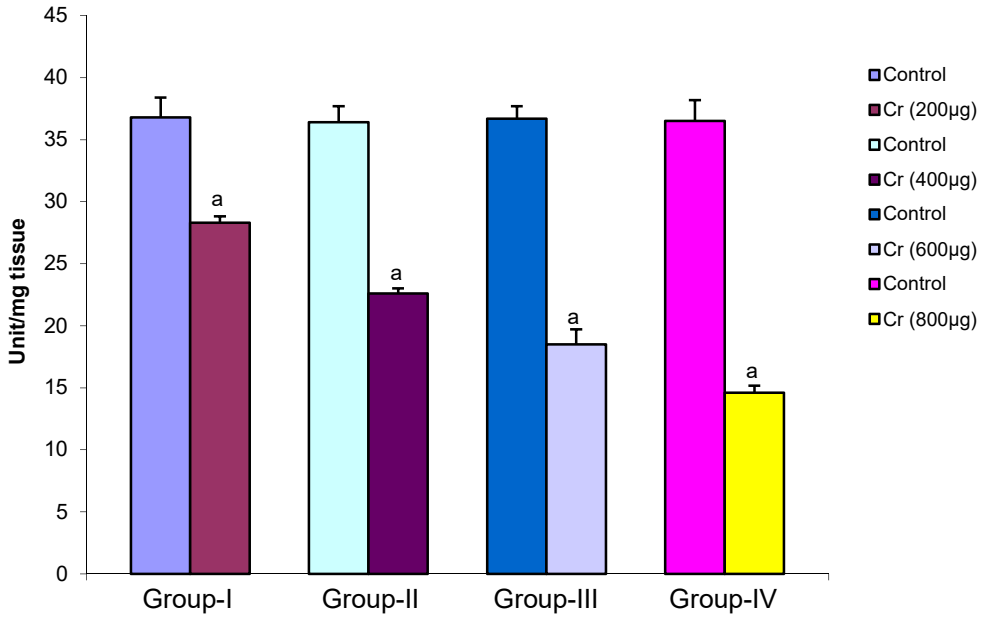

Figure 9: Change in the ALT activity of lungs after chromium treatment at different doses. [a] noted significant difference (P<0.05).

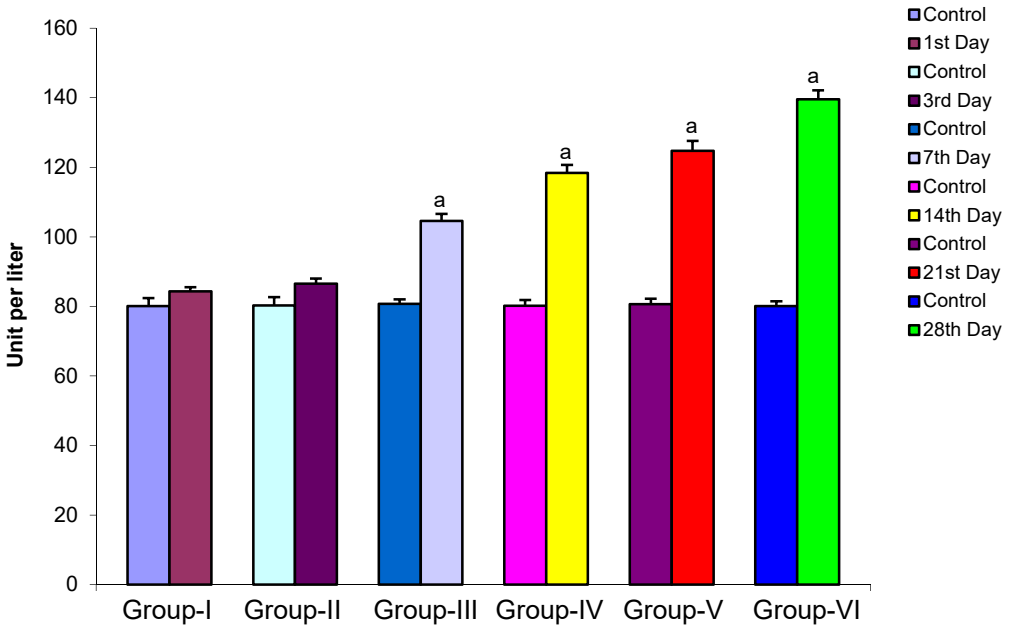

Figure 10: Changes in the serum ALP activity in duration dependent study of chromium treated rats. [a] noted significant difference (P<0.05).

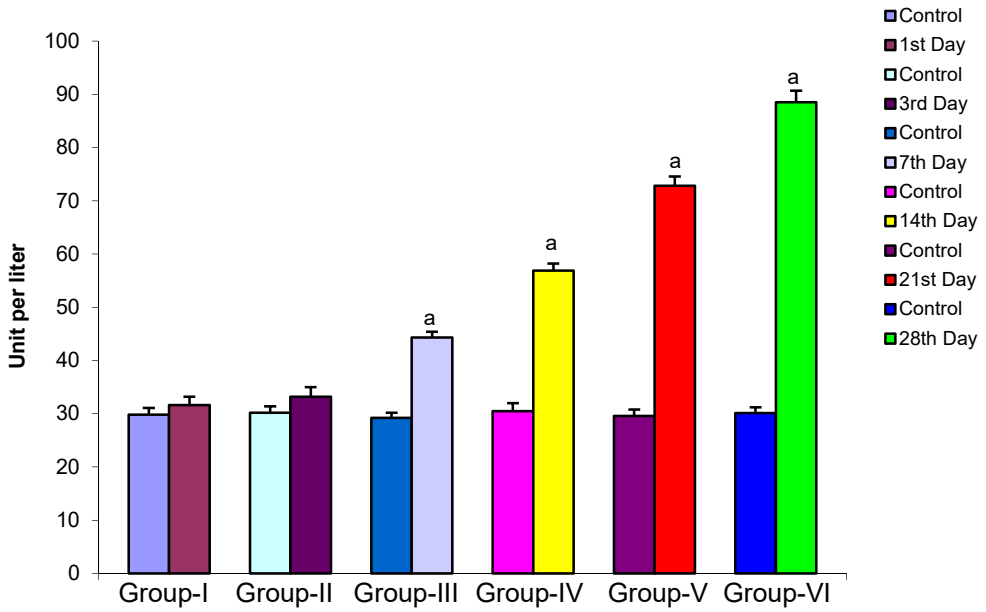

Figure 11: Changes in the serum AST activity in duration dependent study of chromium treated rats. [a] noted significant difference (P<0.05).

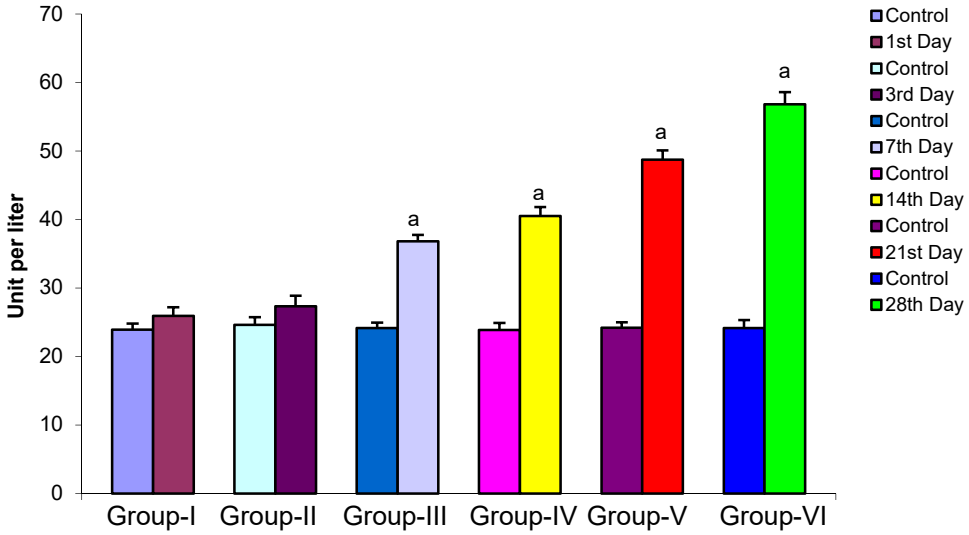

Figure 12: Changes in the serum ALT activity in duration dependent study of chromium treated rats. [a] noted significant difference (P<0.05).

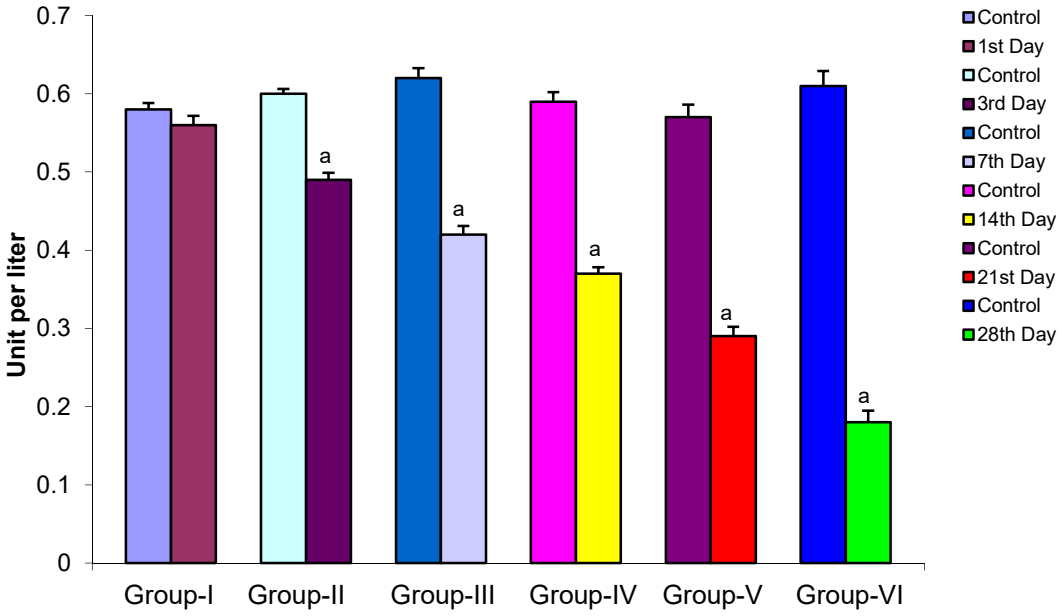

Figure 13: Change in the ALP activity of liver in response to chromium in duration dependent study. [a] noted significant difference (P<0.05).

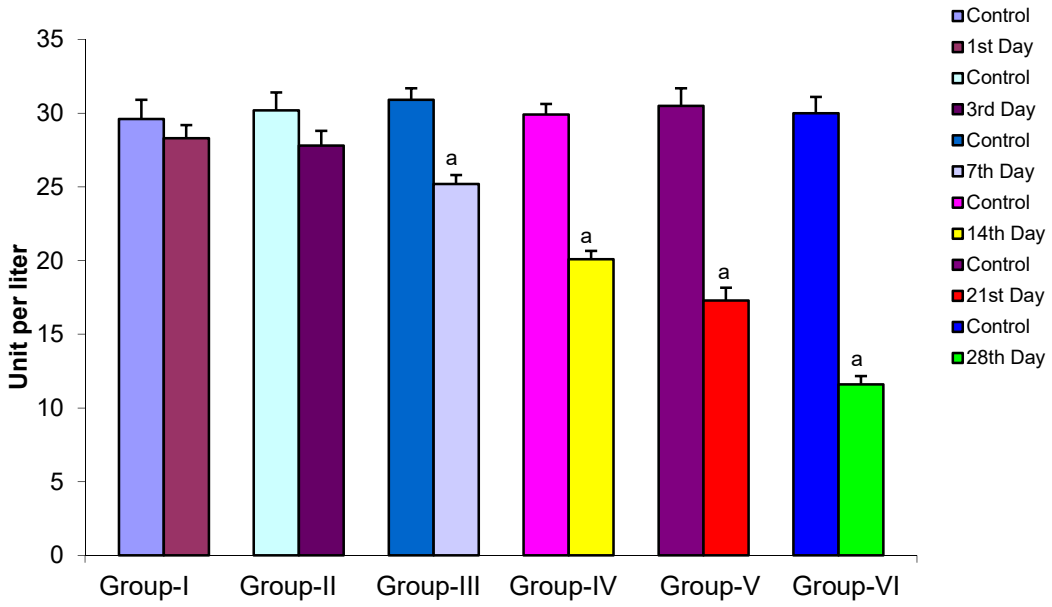

Figure 14: Change in the AST activity of liver in response to chromium in duration dependent study. [a] noted significant difference (P<0.05).

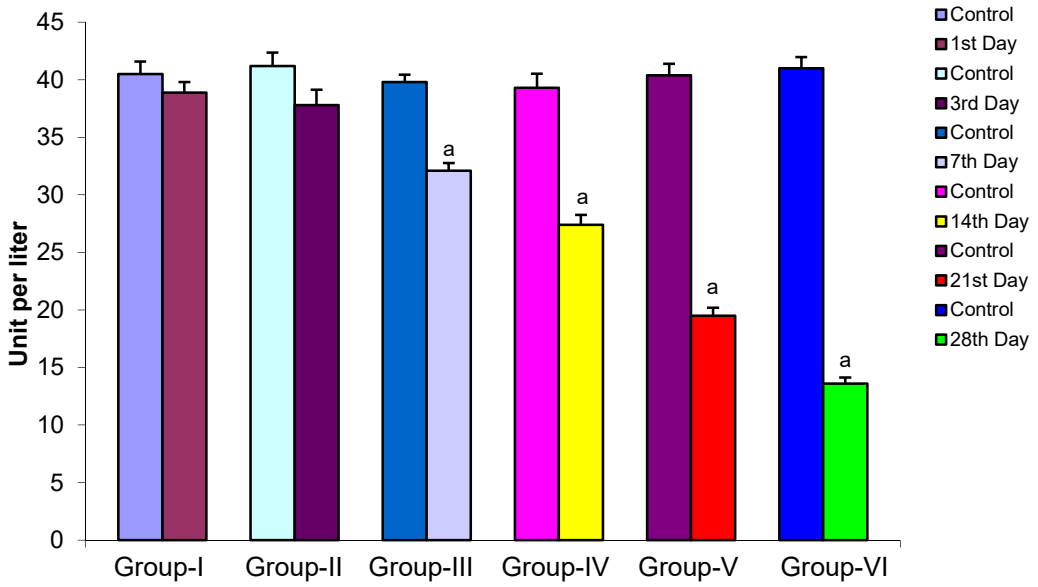

Figure 15: Change in the ALT activity of liver following exposure to chromium in duration dependent study. [a] noted significant difference (P<0.05).

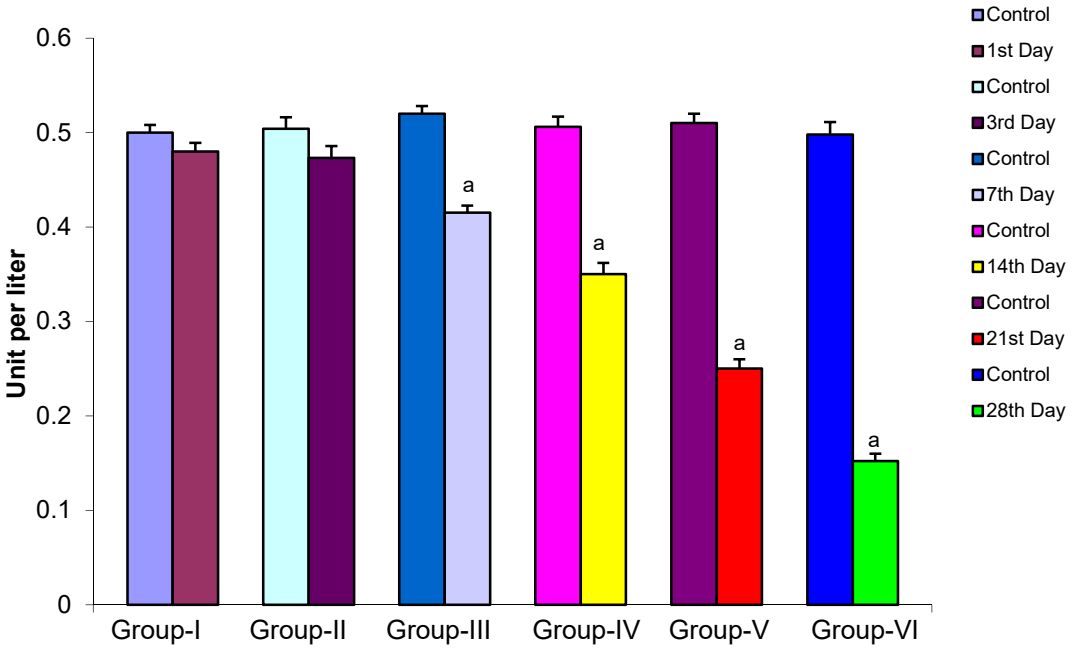

Figure 16: Change the ALP activity of lungs following exposure to chromium in duration dependent study. [a] noted significant difference (P<0.05).

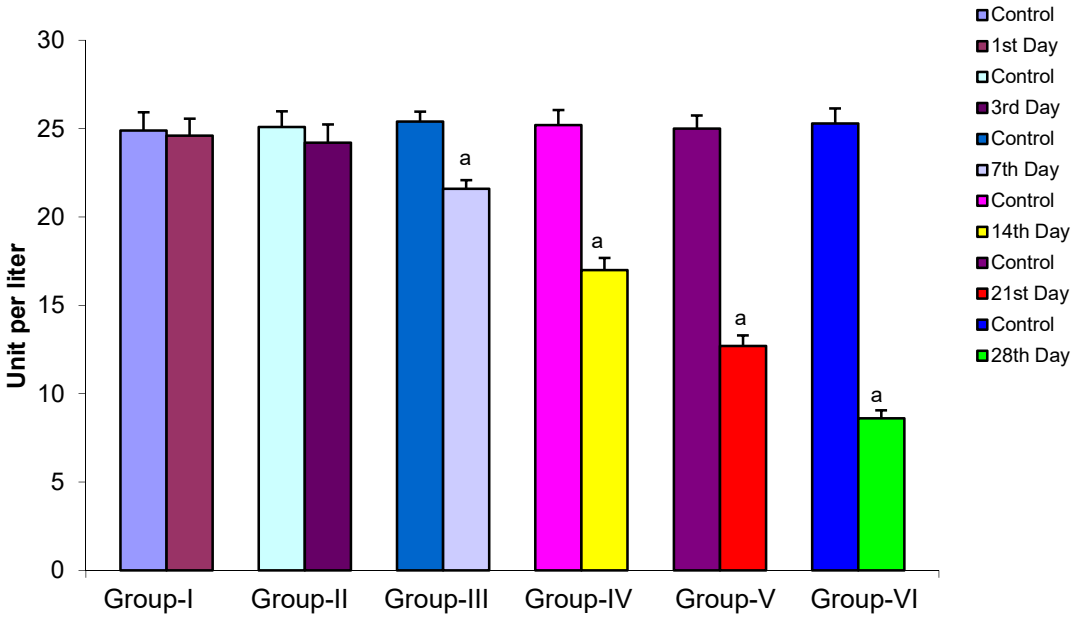

Figure 17: Changes of the AST activity of lungs following exposure to chromium in duration dependent study. [a] noted significant difference (P<0.05).

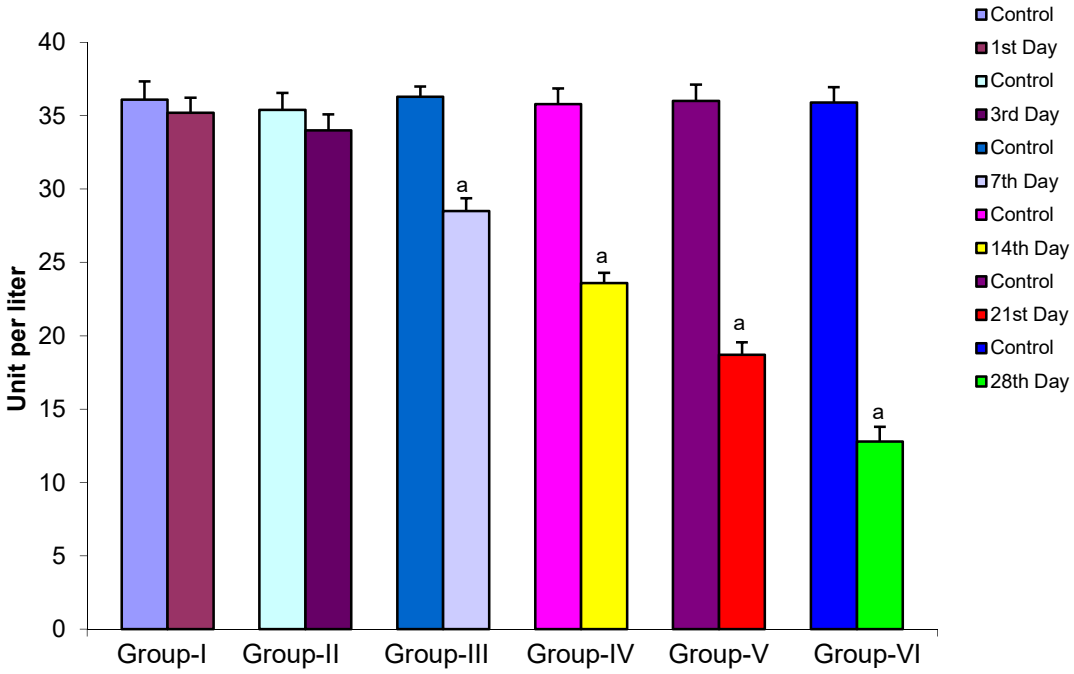

Figure 18: Changes in the ALT activity of lungs following exposure to chromium in duration dependent study. [a] noted significant difference (P<0.05).

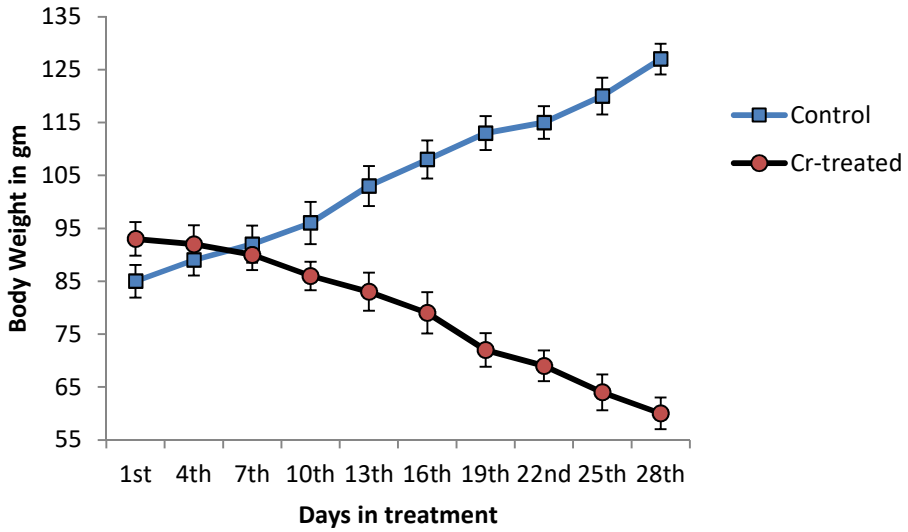

Figure 19: Changes the body weight of chromium treated rats.

Table 1: Mean organ weight and chromium content following Cr (VI) administration.

Tissues	Groups of animals	Organ weight (g/100g bw)	Chromium content (µg/g tissue)
Liver	**Control**	3.07 ± 0.10	0.34 ± 0.03
	Chromium treated	4.66 ± 0.22*	2.97 ± 0.14*
Lungs	**Control**	0.90 ±0.01	0.56 ± 0.04
	Chromium treated	0.88 ±0.01	3.74 ± 0.08*

* indicates significant differences (P<0.05).

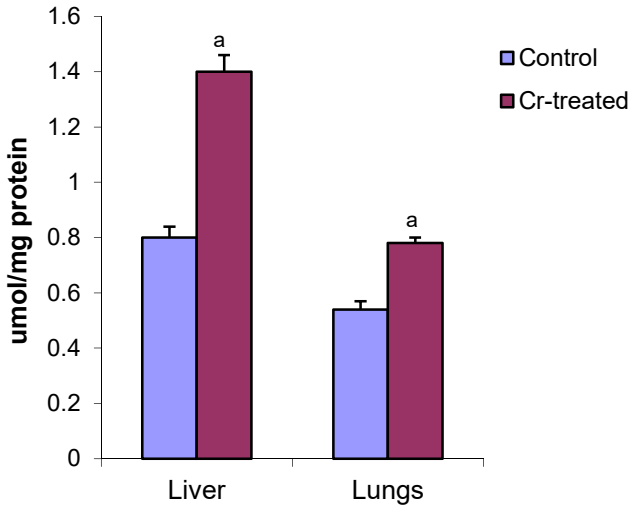

Figure 20: Changes in the MDA level of liver and lungs after chromium treatment when compared to control. [a] noted significant difference (P<0.05).

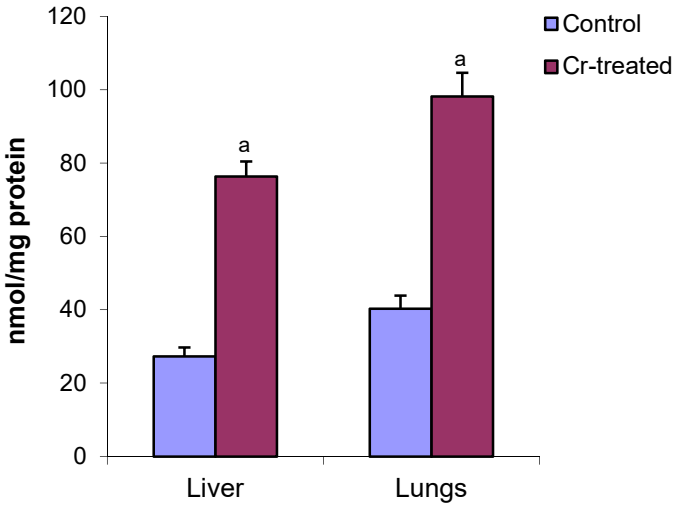

Figure 21: Changes in the Conjugated dienes level of liver and lungs in Cr (VI) treated rats compared to control. [a] noted significant difference (P<0.05).

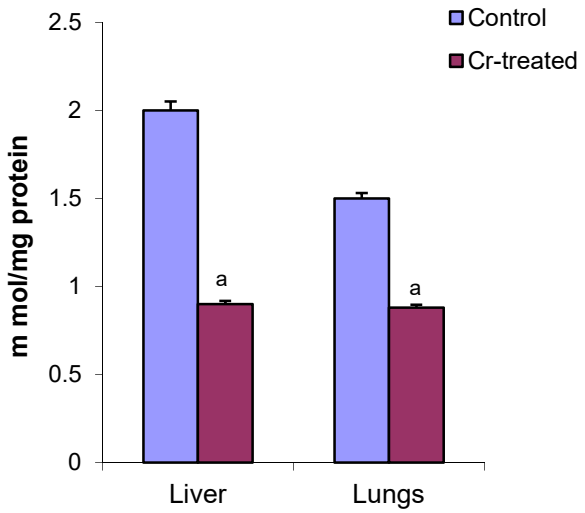

Figure 22: CAT activity using liver and lungs homogenates decreased much more than Cr (VI) treated group. [a] noted significant difference (P<0.05).

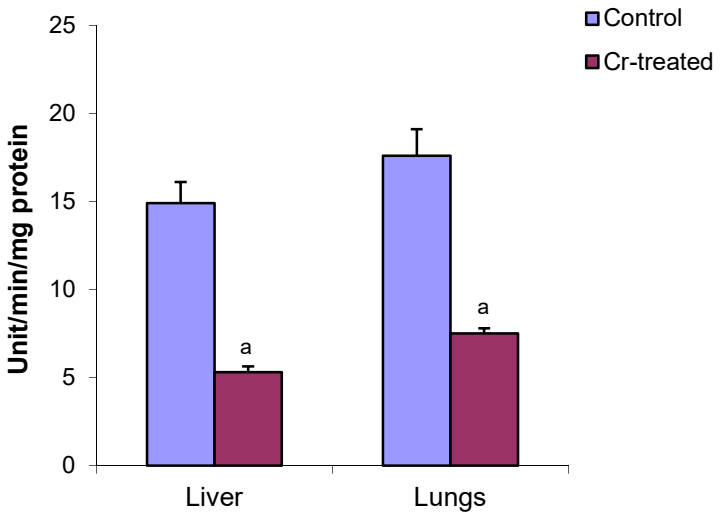

Figure 23: Measured SOD activity using liver and lungs homogenates decreased much more than Cr (VI) treated group. [a] noted significant difference ($P<0.05$).

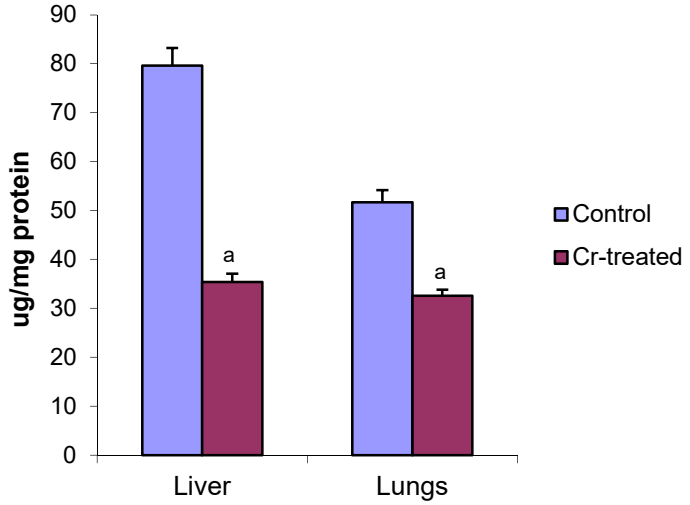

Figure 24: Changes in the GSH activities of liver and lungs in Cr (VI) treated rats compared to control. [a] noted significant difference ($P<0.05$).

Figure 25: Changes in the GSSG activities of liver and lungs in Cr (VI) treated rats compared to control. [a] noted significant difference ($P<0.05$).

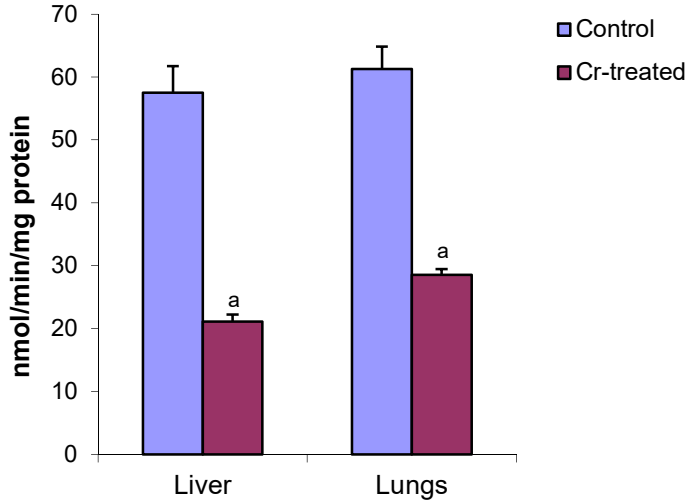

Figure 26: Changes in the GPx activity of liver and lungs in Cr (VI) treated rats compared to control. [a] noted significant difference ($P<0.05$).

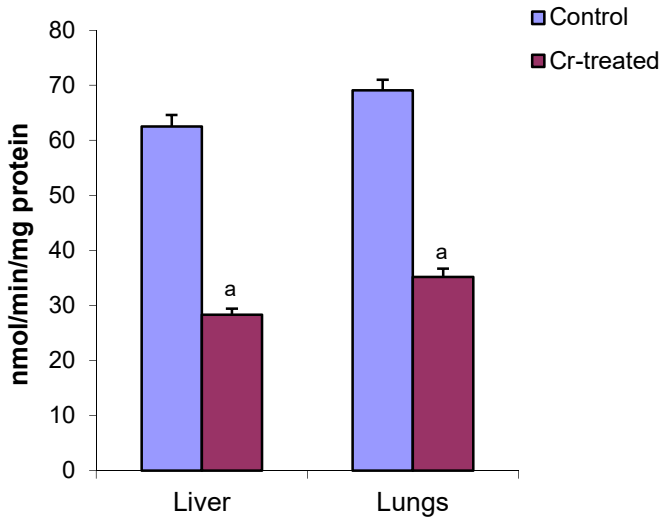

Figure 27: Changes in the GR activity of liver and lungs in Cr (VI) treated rats compared to control. [a] noted significant difference (P<0.05).

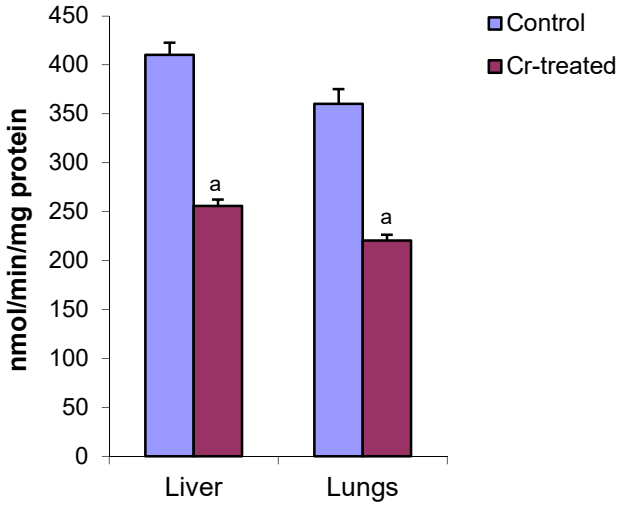

Figure 28: Changes in the GST activity of liver and lungs in Cr (VI) treated rats compared to control. [a] noted significant difference (P<0.05).

2.6 Discussion

The data in Figures 1, 2 & 3 represented marked elevation of serum ALP, AST and ALT activities in the different doses of chromium-treated group in comparison with control group. Most significant changes we have found in the group treated with chromium dose of 800 microgram per 100 gram body weight per day more than the control group.

We have observed that the ALP, AST and ALT activities in liver were significantly decreased following exposure to chromium at different doses (Figures 4, 5 & 6). It was revealed that the ALP, AST and ALT activities of lungs were decreased in significant amount in the different doses of chromium-treated group than the control group (Figure 7, 8 & 9).

The data represented in our study revealed that the ALP, AST and ALT activities in serum for the duration-dependent study in response to chromium (Figures 10, 11 & 12). It was found that the ALP, ALT and AST activities in serum were significantly increased from the 7^{th} day of chromium treatment to a maximum on the 28^{th} day of treatment.

Figures 13, 14 & 15 show the ALP, AST and ALT activities in liver following exposure to chromium for duration-dependent study. Results showed that the ALP, ALT and AST activities in liver were decreased significantly from the 7^{th} day of chromium treatment when it was compared with the control group.

Figures 16, 17 & 18 revealed that the ALP, AST and ALT activities of lungs were also significantly decreased following exposure to chromium for duration-dependent

study. It was found that the lungs ALP, ALT and AST activities were significantly decreased from the 7th day of chromium treatment.

From the above results it was found that the serum levels of ALP, ALT and AST were maximally elevated at the dose of 800 microgram per 100 gram of body weight per day on 28 days treatment of hexavalent chromium. On the other hand, it was also found that the ALP, AST and ALT activities have reduced in liver and lungs tissues at the dose of 800 microgram per 100 gram body weight per day on 28 days treatment of hexavalent chromium. The hexavalent and trivalent chromium inhibit succinic dehydrogenase, acid phosphatase and adenosine triphosphatase (Behari et al, 1978). Nehru and Kaushal (1993) reported marked increase in (ALP) alkaline phosphatase activity as a result of lead intoxication. Compounds of chromium and other heavy metals after chronic exposure have raised activities of many aminotransferases. Raised level of serum aminotransferases like AST activity was significantly higher in animals injected with lead and Chromium than zinc, cobalt and manganese, while higher serum ALT level were found in cobalt than zinc, chromium, and manganese (Awadallah and Hanna,1980). Dey et al, (2011) reviewed that ALT and AST activities are more in workers of tannery than the shoe factory workers.

The elevated levels of ALP, AST and ALT in serum and decreased activities in liver and lungs tissues of Cr-induced rats strongly indicate that continuous accumulation of Cr (VI) can damage the hepatic membrane probably due to reduction of antioxidant status.

Lysosomes containing ACP are primary responding cell organelles towards metal toxicity. ACP activity is decreased in response to chromium exposed tested organs

may be due to the direct effect of chromium on lysosomes, though it is considered the more stable portion of the cell (Susa et al, 1989). Trivalent Cr (III) has possible role in the cell membrane stabilization (Ueno et al, 1990). Therefore, the Cr (VI) produced from Cr (VI) curtails release of ACP, leading to the observed decrease in its activity. Like ACP, ALP activity also decreased remarkably in all the tissues. This observation seems to be in conformity with earlier research data (Dey et al, 1997). As ALP is a membrane-bound enzyme, significant damage of the plasma membrane due to chromium treatment, decrease in its activity are observed here. Very Similar findings in kidneys (Kumar and Rana, 1984) and liver (Anjum and Shakoori, 1997) of animals have been reported previously.

Body weight of chromium-treated rats has significantly decreased (Figure 19) and all the tested organs showed increased chromium contents (Table-1) as compared to that of the control group. Body weight changes probably resulted from direct effects of chromium but not due to reduced food intake. This reduced body weight did not match with the individual organ weights (Table-1). Only liver showed a major increase in its weight despite the increased chromium content, but lungs demonstrated unaltered weight in spite of increased chromium content (Table-1). These observations suggest that chromium has a different deleterious impact on tissues and organs.

Enhanced lipid peroxidation suggested by raised MDA and conjugated dienes in liver and lungs is a characteristic observation in chromium treated rats (Figures 20 & 21). Chromium is reduced into its metabolites mainly in liver and to a significant extent in lung. It plays a crucial role in the pathogenesis tissue toxicity (Rana and Kumar, 1984). Superoxide radicals are supposed to initiate primary events of Cr

(VI) induced cytotoxic tissue injury (Kawanishi et al, 1986; Shi and Dalal, 1990a). Cr (VI) increases hepatic mitochondrial and microsomal lipid peroxidation (Bagchi et al, 1995). Thus, the increased MDA level and conjugate dienes content in liver and lungs of chromium injected rats in the present study may be directly related to excessive generation of free radicals by chromium.

The host antioxidant defence and scavenging mechanism to avert cell damaging insult of Cr (VI) plays a crucial role in cell integrity (Lima and Savin, 2002). GSH protects the liver and lungs from oxidative injury against stress by detoxifying exogenous toxicants and quenching reactive oxygen species (ROS). Intracellular GSH content in isolated rat liver cells was reduced after Cr (VI) administration (Ueno et al, 1988). GSH is major intracellular antioxidants regulating the duration and extent of oxidative 'burst' (Abidi et al, 1999).

High level of intracellular GPx protects cells against oxidative injury utilizing GSH to catalyse the reduction of hydrogen peroxide (Ray and Husain, 2002). Thus, chromium mediated excess H_2O_2 and lipid peroxides are scavenged by GPx efficiently. The diminished activity of this enzyme activity reflects fluctuation in normal oxidative mechanisms after chromium uptake and metabolism. The GR enzyme actively induces the conversion of GSH from GSSG. GSH has other alternative metabolic functions independent of its antioxidant properties. Detoxification of xenobiotics is possible with active participation of GSH as substrate for the enzyme glutathione-S-transferase. Enzyme superoxide dismutase (SOD) catalyses the dismutation reaction to make highly reactive superoxide anion to and less reactive species O_2 and H_2O_2 (Okado-Matsumoto and Fridovich, 2001) and protects tissues against oxidative stress (Huang et al, 1997; Lin and Beal, 2006).

Hence, decreased SOD activity (Figure 23) expressed in the current study indicates a feedback inhibition or oxidative enzyme inactivation due to excess ROS production. Catalase, an antioxidant enzyme, plays an important preventive and protective role against the deleterious effects of lipid peroxidation (Pigeolot et al, 1990). The reduced CAT activity (Figure 22) is an evidence of increased of superoxide anion generation during the injection of chromium since superoxide anion is a strong inhibitor of catalase (Ashakumary and Vijayammal, 1996). Present study revealed that GSH and GSSG activities are decreased to a significant level (Figures 24 and 25). The study also showed that GPx, GR and G-S-T activities are diminished to great extent (Figures 26, 27, 28). Owing to excess utilization of CAT, SOD, GST, GSSG, GSH, GPx and GR enzymes during intracellular detoxification of chromium in liver and lungs, depletion of the above enzymes reflecting their lower levels are clearly noticed.

2.7 Conclusion

The current research indicated that the maximum toxicity of hexavalent chromium is seen at the dose of 800 microgram per 100 gram body weight per day and on 28th day treatment. Hence, above dose and duration of hexavalent chromium treatment is most tolerable dose -duration for survival of the test animals for the study of Cr (VI) toxicity. On the other hand, this initial research outputs also guide that Chromium treatment at the above dose and duration induces toxicity and oxidative stress in liver and lungs tissues.