Research Article

Loss of Immune Function and DNA Damage in Cadmium-Induced Altered Cytokine Milieu – an *In Vitro* Study

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ABSTRACT

The testis though recognized as an immuneprivileged organ, there are a number of immunocompetent cells like macrophages, neutrophils and monocytes within the testicular interstitial compartment which give evidence of testicular immune response. Among them, macrophage population is the largest one and is known to influence the function of the neighboring Leydig cells. Testicular macrophages are the largest population of immune cells in the rodent testes. Exposure to cadmium chloride *in-vitro* is known to inhibit the testicular macrophage functions. But the extent of effect or the mechanism thereof is not well elucidated. The present study examined the extent of immunomodulatory effect in murine testes after *in-vitro* exposure to cadmium. To elucidate the immunomodulatory effects of cadmium, cell function studies such as morphological alteration, phagocytosis, intracellular killing mechanism, DNA fragmentation, enzyme activity (nitric oxide, myeloperoxidase synthase) and proinflammatory cytokines TNF-α and IL-6 release assays were performed in testicular macrophages isolated from cadmium-treated and control group of adult male BALB/c mice. The *in vitro* study shows morphological alteration in cadmium treated (*in*-vitro) group along with DNA damage as compared to control. It also indicates an impairment of the microbial capacity due to suppressed phagocytosis and intracellular killing consorted to a reduction of inflammatory response. This is due to decrease of oxygen species both reactive nitrogen and in the phagocytic niche in the presence of cadmium ions. Thus it is observed that the enzyme release is also affected due to *in-vitro* cadmium treatment. The present work shows that treatment with cadmium is responsible for significant alteration in macrophage function.

Keywords: Cadmium, DNA fragmentation, Myeloperoxidase, TNF-α, IL-6, Phagocytosis.

INTRODUCTION

The immune system provides defense against infectious organisms and other invaders. Through a series of steps called the immune response, the immune system attacks organisms and substances that invade body systems and cause disease. The testis is known to be an immunopriviledged site largely due to the existence of the blood-testes-barrier (BTB). The concept of immunopriviledge in mammals includes two aspects of notions. Some tissues are predisposed to induce tolerance after being transplanted to an allogeneic recipient and some tissue readily accepts foreign cells without the induction of an immune rejection. The testes exhibit both the two aspects of immune privilege [1].

The effect of exposure to a toxin vary with the degree of exposure or 'dose'. One such toxin is the heavy metal, Cadmium (Cd). Its exposure has been associated with a wide range of toxic effects including effects on male reproductive physiology and immune system and alters the immune response which results in loss of sexual function in humans and animals [2].

For the testes this means safeguarding reproductive capability. Notwithstanding its immune privileged status, the testis is clearly capable of mounting normal inflammatory responses, as proven by its effective response to viral and bacterial infection. The mechanisms responsible for the testicular immune privilege are still far from being understood, but it is apparent that the identified factors involved are multiple and probably redundant. Overall, long regarded as a peculiar side issue of the functional testes, immune privilege is now established as part of the general scheme of male gamete formation and successful reproduction [3]. Further research in the area will not only help to improve diagnosis and treatment of immune infertility in males, but will also open new avenues in contraceptive development and transplantation medicine. The present study addresses the immunomodulatory properties of testicular macrophages functions, morphology and determination of alteration of enzyme release and pro-inflammatory response (TNF-α and IL-6) from murine

testicular macrophages due to cadmium chloride intoxicated male Swiss albino mice.

EXPERIMENTAL

Reagents

The following reagents were used: collagenase Type IA, DNase I, Tosyl (Na-p-tosyl-L-lysine chloromethyl ketone), Histopaque-1077 (SIGMA St. Louis, MO); RPMI 1640 (Gibco Life Technologies, Grand Island, NY), fetal calf serum (FCS) (SIGMA-Aldrich); All other reagents were of analytical grade.

Isolation of testicular macrophages

Testicular macrophages were isolated following a slightly modified procedure [4]. Cell free culture supernatants were divided into two group one for control and another for cadmium chloride treatment and lactate dehydrogenase (LDH) assay was performed.

Effect of cadmium on Lactate dehydrogenase release from testicular macrophages *in-vitro*

The cytotoxicity of any heavy metal was determined by the lactate dehydrogenase (LDH) assay. This assay was performed to measures the activity of LDH released into the medium from dead cell due to cytotoxicity of cadmium. Cell free culture supernatants from cadmium (1,10,100,1000 ng/ml) treated testicular macrophages were collected and the LDH release was measured with a different of exposure time interval and maximum cadmium cytotoxicity with the time interval was meseared. After that the cell free supernatant with the calculated concentration and time period were used for different assays.

Preparation of bacteria (*Staphylococcus aureus* MC524) for intracellular killing and phagocytosis assay

To obtain bacteria in the mid logarithmic phase 100 µl of an overnight culture made in nutrient broth was added to 10 ml of nutrient broth and incubated for 2-5h at 37^{o}C with orbital shaking. The bacteria was washed in 10 mM sodium phosphate buffer (pH 7.4) and their concentration was estimated by spectrophotometry at A_{620} on the basis of the relationship: A_{620} 0.2 = 5×10^{7}/ml [5].

Morphological alteration of macrophages

Cells were observed under oil immersion microscope. Any cell devoid of pseudopodia was scored as polarized and this was expressed as a percentage of the total number of cells counted [6].

DNA fragmentation assay

The DPA reaction was performed according to the method of [12]. Perchloric acid (0.5 M) was added to the pellets containing uncut DNA (resuspended in 200 µl of hypotonic lysis buffer) and to the other half of the supernatant containing DNA fragments. Then 2 volumes of a solution containing 0.088 M DPA, 98% (vol/vol) glacial acetic acid, 1.5% (vol/vol) sulfuric acid, and a 0.5% (vol/vol) concentration of 1.6% acetaldehyde solution were added. The samples were stored at 4°C for 48h. The colorimetric reaction was quantified spectrophotometrically at 575 nm. The percent fragmentation was calculated as the ratio of DNA in the supernatants to the total DNA [7].

Phagocytosis assay

Testicular macrophages from both control and cadmium chloride exposed groups were allowed to adhere separately on glass slides for one hour. Phagocytosis assay was performed with 10% SRBC and Phagocytic Index calculated [8].

Intracellular killing Assay

Bacteria were incubated with testicular macrophages. After various time intervals(15, 30 and 45 min), sample was treated with Gentamycin to kill extracellular adherent bacteria and viability of intracellular bacteria was determined [9].

Myeloperoxidase release assay

Cell suspension was taken, stimulated with LPS and centrifuged. The supernatant was collected in separate microcentrifuge tubes. Supernetant and cell lysate were allowed to react with OPD substrate and readings were taken at 492 nm in a spectrophotometer [10].

Nitric oxide release assay

Testicular macrophages were suspended in DPBS-BSA and were stimulated with LPS. The cell-free supernatant was used for nitric oxide release assay using Griess reagent. Readings are taken in a UV spectrophotometer at 550 nm [11].

Cytokine assay

Testicular cells were separated by density gradient centrifugation. Then testicular macrophages were obtained by adherence to plastic surface. A number of 1 X 10^{5} viable cells in 0.2 ml RPMI 1640 medium supplemented with 5% FCS were distributed in microwells in flat 96 well microtitre plates and, after 24 h culture, supernatants were collected. Cytokine concentrations in culture

supernatants were measured by sandwich ELISA estimating TNF-α using RayBio-mouse TNF-α ELISA kit and IL-6 using BD Biosciences kit. Biotinylated monoclonal secondary antibodies were used. The reaction was stopped with 3 M H_2SO_4 and the optical density of each well was measured in a 96-well plate reader at 492 nm. All determinations were done in triplicate. Standard curves were generated by recombinant mouse cytokines. Lower density limits were 30 pg/ml (IL-6) and 10 pg/ml (TNF-α).

Statistical analysis

The data was expressed as mean ± SD. A two tailed student's t-test was performed to estimate the difference in means and the level of significance thereof.All the experiments were done in triplicate. P* = P<.05, P**= P<.001, comparing with control.

RESULTS AND DISCUSSION

Effect of cadmium on Lactate dehydrogenase release from testicular macrophages *in-vitro*

When disease, injury or insult from toxic material damages tissues, cells release LDH. Since LDH is a fairly stable enzyme, LDH has been widely used to evaluate the presence of damage and toxicity of tissue and cells. Quantification of LDH has broad range of applications. So the cytotoxicity was determined by Lactate dehydrogenase (LDH) assay which measures the activity of LDH release into the medium from dead cells as a result of cytotoxicity. The toxicity was measured in dose-effect experiments with reference to cell survival and integrity of cell membrane as determined by LDH release. In *in vitro* condition it was found that 10 ng/ml concentration of cadmium had maximum cytotoxicity (LDH) (Figure 1a) with an exposure time of 30 min (Figure 1b). Viability of cell in control and cadmium treated group was assayed by trypan blue dye exclusion technique in vitro in testicular macrophages. It was also observed that at 10 ng/ml concentration more than 95% cells were found to be viable.

Effect of cadmium on the morphology of testicular macrophages

Cell specificity was determined by non-specific esterase staining. In order to demonstrate whether there was any change in the number of deformed cells, i.e., inactivated cells due to cadmium exposure, morphological alteration under light microscope was studied. It was observed that exposure to cadmium chloride leads to morphological changes in macrophages since more deformed cells were obtained in the cadmium chloride treated group (56.3 ± 1.96%) with respect to control (15.55 ± 2.2%)(Figure 2; P**). Our results demonstrate that alteration in testicular macrophage morphology in cadmium treated mice is significantly higher then that of control. Cadmium induced deviation in the normal shape of macrophages may be the cause of the reduced functional status of testicular macrophages.

Effect of cadmium on DNA damage of testicular macrophages

From the present study it is confirmed that morphological imbalance occurs in the testicular macrophages due to cadmium exposure. Further, to observe the extent of effect at the nuclear level it was important to study the DNA fragmentation occuring due to cadmium exposure. DNA fragmentation of macrophages was also reported in mice exposed to lead [12, 13]. In order to maintain the steady state levels of the various hematopoitic cells, cell division and differentiation is balanced by a process called programmed cell death. Cells undergoing this process often exhibit distinct morphological changes collectively referred to as apoptosis. This includes pronounced decrease in cell volume, membrane blebbing, condensation of the cytoplasm and chromatin and degradation or fragmentation of DNA[14]. To assess the effect of cadmium on apoptosis mechanism, the DNA fragmentation pattern of testicular macrophages isolated from the respective groups were studied. From the graph, it is clear that cadmium chloride induced the DNA fragmentation of testicular macrophages by six folds (from 9.25 ± 1.04% to 59.34 ± 0.39%) (Figure 3; P**). Such evidences may be due to a direct effect of heavy metal on the DNA structure, oxidative mechanisms [15] or indirectly due to another mechanism involving the activation of caspases in the process of cell death [16]. Thus, DNA fragmentation in the testicular macrophages isolated from cadmium-treated and control group of mice (*in vitro*) demonstrate that the DNA damage in testicular macrophages is much higher in cadmium treated group than in control group which leads to imbalance in the functioning of the macrophages.

Effect of cadmium on phagocytic capacity of cadmium intoxicated testicular macrophages

Macrophages are known to play an important role during bacterial infection and a huge amount of macrophages are accumulated

during various microbial infections. So, possibilities of the interaction of toxic metal cadmium with the cells cannot be precluded. Therefore, we sought to establish whether there is any alteration in the phagocytic capacity of testicular macrophages in cadmium chloride exposed mice with respect to that of control. It was observed that the phagocytic index in control group was (25333.33 ± 333.333) which was found to be (12333.333 ± 666.667) in cadmium chloride treated cells (Figure 4; P*). In phagocytosis the antigens adhere to the macrophage cell membrane. Adherence induces membrane protrusions, called pseudopodia, to extend around the attached material and fuse to form a phagosome, which then enters the endocytic processing pathway [17]. *In vitro* cadmium treated group shows decrease in the phagocytic index as compared to control group (Figure 4). This indicates that *in vitro* treatment of cadmium somehow damage the membrane as pseudopodia disintegrate as a result cannot attach with the foreign particle to form phagosomes and so could not kill the invading microorganism.

Effect of cadmium on killing capacity of testicular macrophages

Cadmium intoxicated mice has any effect in the killing capacity of testicular macrophages is the important objective for the study. *Staphylococcus aureus,* a virulent pathogen causes life threatening infection[18]. The results suggested that cadmium intoxication in control mice reduced the intra cellular killing capacity which suggest that cadmium treated mice are prone to infection and less effective in clearing invading pathogens as it were evident that testicular macrophages from cadmium exposed group could not able to kill the intracellular *Staphylococcus aureus* competently (Figure 5; P**). As it is evident that bacterial viability was more in *in-vitro* cadmium intoxicated group compared to control group, it can be suggested that *in-vitro* cadmium intoxicated group unable to kill the *Staphylococcus aureus* and so can be considered as immunocomromised and were more prone to infection, as they could not phagocytosed efficiently. As a result, these macrophages could not clear out the invading microorganisms, which lead to a diseased state upon bacterial invasion. Hence, *in- vitro* cadmium exposure may reduce the ingestion capacity of testicular macrophages, suggested that the cells were less potent to kill the ingested bacteria efficiently.

Effect of cadmium on Myeloperoxidase release of testicular macrophages

Macrophage ingest the invading microorganism in its vacuoles i.e., phagosomes and fuse with the enzyme containing granules and form phagolysosomes. Myeloperoxidase enters the phagolysosomes following such macrophage-microbe encounter, stimulates a respiratory burst response and helps in the oxidative killing and scavenging of the microbe. Activation of macrophages with bacterial cell wall lipopolysaccharide (LPS) begins to express high levels of myeloperoxidase (MPO) enzyme. MPO decreases the free radical level in our system. MPO release assay was performed to evaluate the effect of cadmium exposure on the release of this enzyme after LPS stimulation. Significant decrease in MPO released (µM) was observed. MPO released from the control group with LPS stimulation, showed a value of 47.8 ± 0.64 µM and 24.9 ± 2.3 µM in the cadmium treated group (Figure 6; P**). *In-vitro* study proves that there is inhibition of myeloperoxidase release which further decreases the immune competence of testicular macrophages.

Effect of cadmium on nitric oxide (NO) release of testicular macrophages

The effect of cadmium chloride *in-vitro* on nitric oxide release by the testicular macrophages was studied for further clarification on the role of cadmium in immunomodulation. The enzyme nitric oxide (NO) synthase is responsible for the generation of nitric oxide from L-arginine, which is an induced in activated macrophages. A number of antimicrobial and cytotoxic substances released by activated macrophages are responsible for the intracellular destruction of phagocytosed microorganism. When macrophages are activated with bacterial cell wall lipopolysaccharide, they begin to express high levels of nitric oxide synthase which oxidises L-arginine to yield citrulline and nitric oxide (NO). Nitric oxide itself has potent antimicrobial activity and it can also combine with superoxide anion to yield even more potent antimicrobial substance[17]. The effect of cadmium on NO release seeks to demonstrate the immuno-modulatory effect of cadmium. Significant decrease in NO released (µM) was observed in cadmium intoxicated group of mice. Result shows that cadmium causes a marked decrease in nitric oxide release from control 14 ± 0.58 to 6 ± 1.5 after cadmium treatment (Figure 7; P**). Significant decrease in nitric oxide release was observed in acute

cadmium exposure (*in-vitro*) compared to control. Nitric oxide synthase is an important effectors molecule involved in immune regulation and defense mechanism.

Effect of cadmium on pro-inflammatory cytokines release of testicular macrophages

It appears that immune privilege in the testis is maintained by a unique testicular environment that controls immune cell activity, inducing and maintaining peripheral tolerance and suppressing adaptive immunity in a tissue-localized manner [18, 19]. Interstitial testicular macrophages have been described a possible a source of TNFα. Normally, a rise in the TNFα causes heightened immune activity. However, contrary to this, we found that cadmium chloride caused a rise in TNFα levels (Figure 8a) and inflammation, leading to immunosuppression, thus indicating multiple targets and sites of action of cadmium chloride, probably at the receptor level which as a result decreases the IL-6 level (Figure 8b) after cadmium treatment followed by immune dysfunction. That cadmium chloride affects the cellular microenvironment and the signaling cross-talk within is evident from the functional status of the testicular macrophages. The inflammatory as well as the functional loss of immune surveillance may well be attributed to oxidative stress induced changes from an increased TNFα titer. The present study shows a decrease in NO and MPO in cadmium chloride exposed testicular macrophages despite a rise in TNF-α at levels leading to the conjecture that NO and MPO escape the phagocytic milieu of the testicular macrophages to increase oxidative stress and associated inflammatory damage in the testicular environment, including the developing sperm cells [20]. Cadmium chloride probably, alters the composition of macrophage population drastically and shifts the cytokine balance in favor of an inflammatory response with potential to overcome immune privilege.

CONCLUSION

The current *in-vitro* study demonstrates that *in-vitro* exposure of cadmium chloride result in altered structural as well as nuclear morphology followed by reduction of cell function study such as phagocytosis and intracellular killing which indicates that cadmium chloride treated groups are more prone to infection, as they cannot phagocytose efficiently and so cannot clear out the invading microorganism. Cadmium chloride intoxicated testicular macrophages were not able to kill the intracellular *Staphylococcus aureus* competently as compared to control. Further study shows the decrease in NO and MPO in cadmium chloride exposed testicular macrophages despite a rise in TNF-α at levels leading to the conjecture that NO and MPO escape the phagocytic milieu of the testicular macrophages to increase oxidative stress and associated inflammatory damage in the testicular environment.

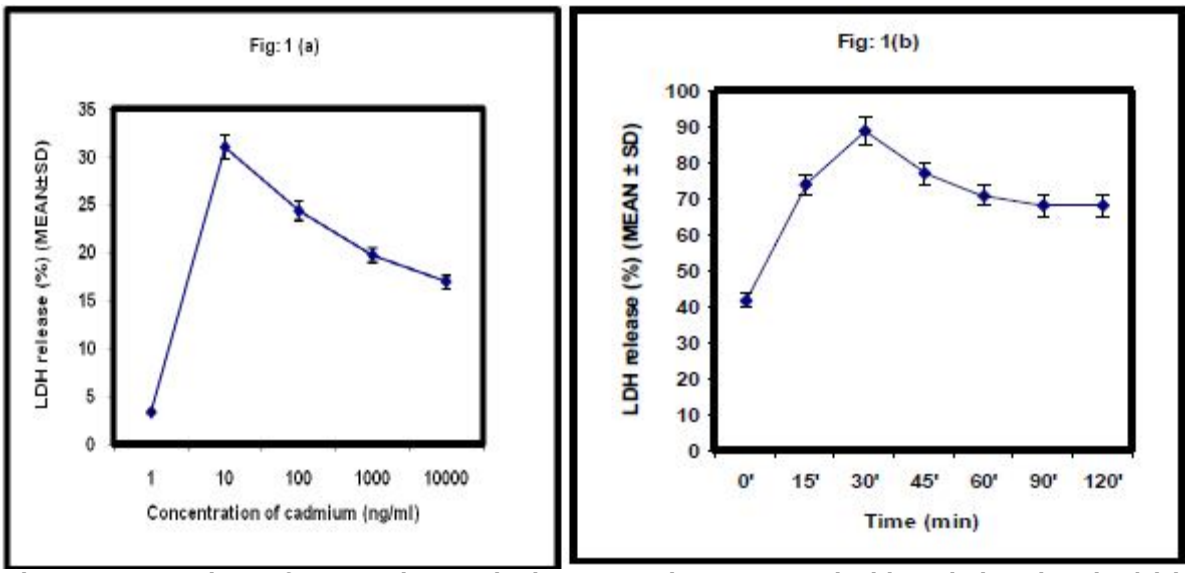

Fig. 1: % LDH release from murine testicular macrophages treated with cadmium (*In-vitro)* (a) in different concentration (b) with respect of time

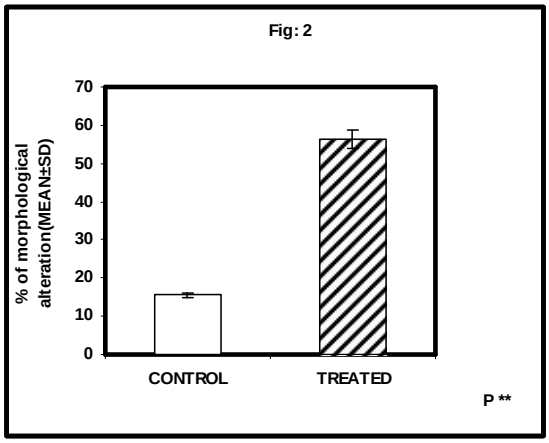

Fig. 2*: In-vitro* study of effect of cadmium on the morphology of testicular macrophages in adult male Balb/c mice

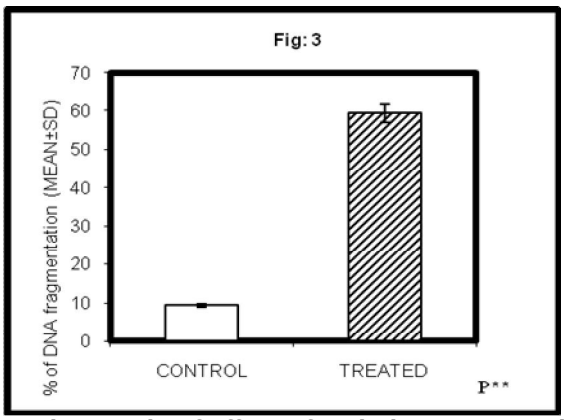

Fig. 3: *In-vitro* study of effect of cadmium on DNA damage of testicular macrophages in adult male Balb/c mice

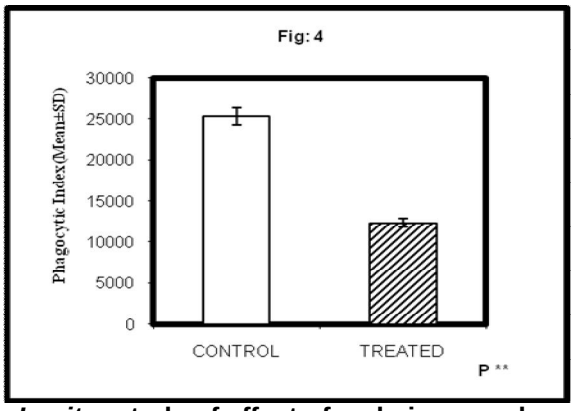

Fig. 4: *In-vitro* study of effect of cadmium on phagocytic capacity of testicular macrophages in adult male Balb/c mice

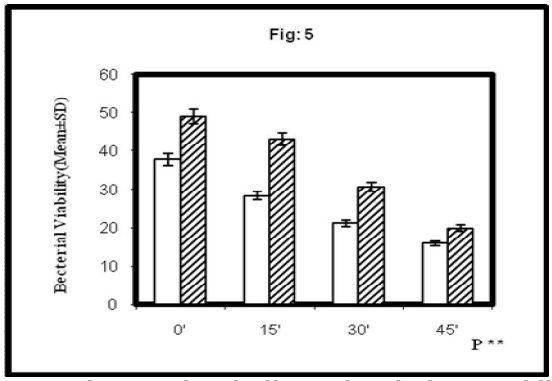

Fig. 5: *In-vitro* study of effect of cadmium on killing capacity of testicular macrophages isolated in adult male Balb/c mice

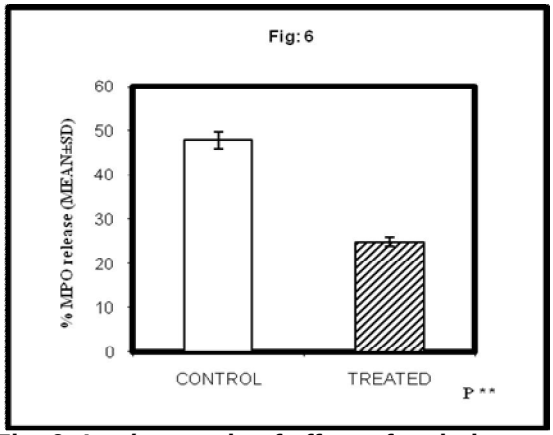

Fig. 6: *In-vitro* study of effect of cadmium on myeloperoxidase enzyme release in testicular macrophages in adult male Balb/c mice

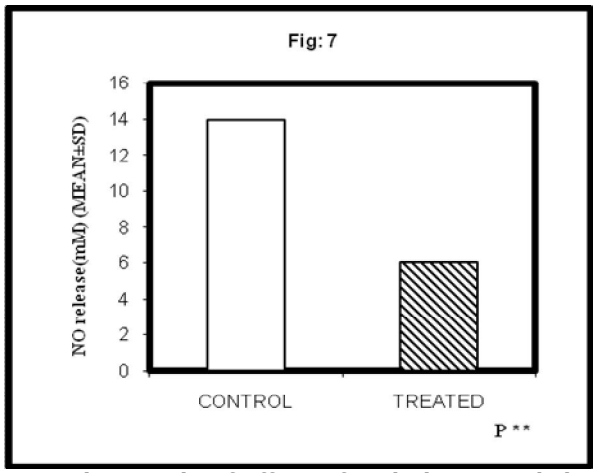

Fig. 7: *In-vitro* study of effect of cadmium on nitric acid release in testicular macrophages isolated in adult male Balb/c mice

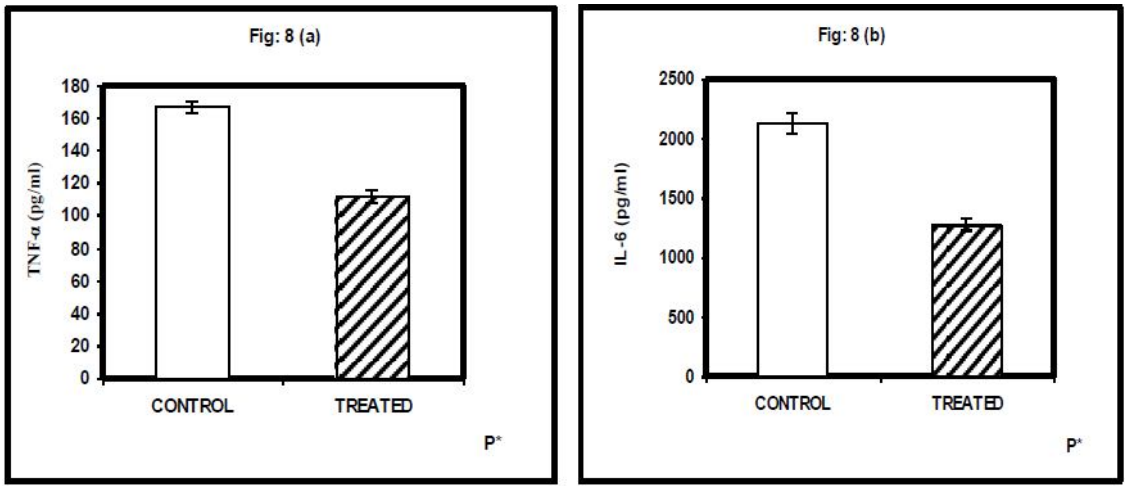

Fig. 8: *In-vitro* study of effect of cadmium on Cytokine release Pro-inflammatory – (a) TNF-α in testicular macrophages isolated in adult male Balb/c mice. (b) IL-6 in testicular macrophages isolated in adult male Balb/c mice

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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