https://doi.org/10.48047/AFJBS.6.13.2024.6440-6455

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Study of lead induced immunomodulation and pro-inflamation in testicular macrophages leading to infertility in male -An in vitro study.

[1] Shamim Ahmed Shamim Khan Barbhuiya, [2] Ujjwal Barbhuiya, [3] Mahuya Sengupta, [4] Mahuya Sengupta,

1Department of Zoology, M.H.C.M Science College, Algapur, Hailakandi,2Department of Zoology, M.H.C.M Science College, Algapur, Hailakandi,3Professor, Deparatment of Biotechnology, Assam University, Silchar.

Corresponding author: Shamim Ahmed Shamim Khan Barbhuiya

shamimbiotech2009@gmail.com

Volume 6, Issue 13, Aug 2024

Received: 15 June 2024

Accepted: 25 July 2024

Published: 15 Aug 2024

doi: 10.48047/AFJBS.6.13.2024.6440-6455

ABSTRACT: Reproductive abnormalities are common in human population who were exposure of harmful substances such as lead. In the present work, the harmful effects of lead on the immunomodulation of testicular macrophages were experimentally investigated. Macrophages constitute the innate immune system, which is crucial for the host's defense mechanism. When testicular macrophages were exposed to lead (in vitro), there was a significant change in their morphology (16.2 ± 0.44%) between the control group and the lead poisoning group (82.48 ± 1.2%). The modified morphology thus affects the phagocytic index of testicular macrophages, making them ineffective in elimination of invasive microorganisms. Myeloperoxidase, an important enzyme produced by macrophages which are responsible for the oxidative elimination of invasive microorganisms, is also observed to be inhibited in lead exposed testicular macrophages (12.7 ± 0.2 µM) to (78.6 ± 1.4 µM). Macrophages from lead intoxicated testes significantly reduce nitric oxide release, a key cellular signaling enzyme released in response to external stimuli. This in turn affects the pro-inflammatory cytokine TNF-α and IL-6, which are important in maintaining the testicular immune response. These results indicate that lead significantly affects testicular macrophage shape, innate immune responses, enzyme release and proinflammatory response leading to reproductive failure in male Swiss albino mice.

Keywords: Lead, Testicular macrophages, Immunomodulation, Pro-inflamation.

INTRODUCTION:

The immune system provides protection against infectious organisms and other invaders. A microbe community of countless species both infects the host's body and lives within that host as commensal organisms. The innate immune system constitutes the host's first line of defense against infection and therefore plays an important role in the early recognition of invading pathogens and the subsequent triggering of an inflammatory response (Medzhitov and Janeway Jr, 2000). Cells and proteins in the damaged tissue detect the infection and release soluble mediators called cytokines, which interact with other cells and trigger an innate immune response. The general effect of the innate immune response is to induce an inflammatory state in the infected tissue.

However, the immune system deviates from its routine functions in immune-responsive organs such as the testes, eyes and brain. Unlike the latter two, testicular macrophages actively maintain immune privileges in the testicular environment (Chakraborty et al., 2014). It protects young developing spermatozoa against autoimmune attacks of cellular immunity and weakens the immune functions of testicular macrophages. Immune regulatory functions against invading microorganisms have also been transferred to testicular macrophages, which are an integral part of the innate immune system (Krause and Naz, 2009).

Exposure of animals to lead and its derivatives in daily life is unavoidable due to its widespread use and application. Lead is widely used in industry because of its malleability, corrosion resistance and low melting point. Although lead is one of the most useful metals, it is also one of the most toxic ones (Shotyk and Le Roux, 2005). The role of macrophages in heavy metal-induced immunotoxicological effects has been previously reported (Bishayi et al., 2004). In vivo studies have shown that lead is an immunotoxic agent that suppresses humoral immunity (Koller and Kovacic, 1974; Luster et al., 1978) and increases host susceptibility to bacterial infections (Hemphil et al., 1971; Lawrence, 1981).

In short, it adversely affects almost every body system. The reproductive effects of lead exposure are widespread (Patrick, 2006), affecting almost all aspects of reproduction (Zheng et al., 2003). Lead induces decreased sperm count, motility and increased morphological abnormalities in animals (Hsu *et al*., 1997; Hsu *et al*., 1998). Lead is known to impair male

reproductive function; however, the mechanisms mediating the side effects have not been clearly elucidated (Thoreux-Manlay et al., 1995).

The current study aims to evaluate an in vitro the changes in the morphology and modulation in innate immune responses, altered in enzyme release and inflammatory response in murine testicular macrophages due to lead exposure, leading to loss of testicular immunoprivilege and subsequent reproductive failure in male Swiss albino mice.

MATERIAL AND METHODOLOGY:

1.1 Reagents:

The reagents used were: RPMI 1640 (Gibco Life Technologies, Grand Island, NY), fetal calf serum (FCS) (SIGMA Aldrich), collagenase type IA, DNase I, Tosyl (Na-ptosyl-L-lysine chloromethyl ketone), Histopaque — 1077 (SIGMA St. Louis, MO). The other reagents used were also analytical grade.

1.2 Isolation of testicular macrophages:

Testicular macrophages were extracted using a slightly modified process of the Sikorski method (Sikorski et al., 1991). Cell-free culture supernatants were divided into two groups, one for control and one for lead acetate treatment, and lactate dehydrogenase (LDH) assay was performed.

1.3 Dose response study of lactate dehydrogenase release from testicular macrophages:

Cytotoxicity of the heavy metals was assessed by lactate dehydrogenase (LDH) assay. This test was performed to determine the amount of LDH released into the medium of dead cells as a result of lead cytotoxicity. Cell-free culture supernatants of testicular macrophages treated with lead (1, 10, 100 and 1000 ng/ml) were obtained and maximal lead cytotoxicity over time was determined along with LDH release at different exposure times. The cell-free supernatant was then used in various tests at the determined concentration and time period.

1.4 Preparation of bacteria (Staphylococcus aureus) for intracellular killing and phagocytosis assay:

To obtain mid-log phase bacteria, 10 mL of nutrient broth was combined with 100 µL of overnight culture and incubated for 2–5 h at 37 °C with orbital shaking. After washing in 10 mM

sodium phosphate buffer (pH 7.4), bacterial concentration was measured spectrophotometrically in A620 using the following formula: A620 0.2 = 5 x 107/ml (Yao et al., 1997).

1.5 Morphological Alteration:

Testicular macrophages were transferred to HBSS-BSA and fixed in 2.5% glutaraldehyde, centrifuged, and the pellet resuspended in HBSS. Glass slides were used to create smears, which were then stained with Giemsa and examined with an oil-immersion microscope. As a fraction of the total number of cells counted, each cell with an extensively smoothed surface was designated as polarized (Quet et al., 1995).

1.6 Phagocytosis:

Testicular macrophages were taken on glass slides. Non-adherent cells were washed away with DPBS. Sheep erythrocytes (sRBC) were added to glass slides with attached macrophages, incubated and washed with DPBS. Slides were fixed in methanol and stained with Giemsa and then viewed under an oil immersion microscope. Phagocytosis index was calculated based on sRBC (average no. sRBC macrophage × 100) (Czuprynski et al., 1984).

1.7 Myeloperoxidase (MPO) Release Assay:

The cell suspension was taken, stimulated with LPS and centrifuged. Supernatant was collected in separate micro-centrifuge tubes. Supernatant and cell lysate were then reacted with orthophenylenediamine (OPD) substrate and read spectrophotometer at 492 nm (Bos et al., 1990).

1.8 Nitric Oxide (NO) Release Assay:

Testicular macrophages were suspended in DPBS-BSA and stimulated with LPS. The cell-free supernatant was used in a nitric oxide release assay using the Griess reagent. Readings are taken with a UV spectrophotometer at 550 nm (Sasaki et al., 1998).

1.9 Cytokine Assay:

a) **TNF-α:**

Density gradient centrifugation was used to isolate testicular cells. Next, testicular macrophages were isolated by adhering to the polymer surface. Supernatants were collected after culturing 1 x 10^5 viable cells for 24 h in 0.2 ml RPMI 1640 medium supplemented with 5% FCS. Cells were

then spread in the microwells of flat 96-well microtiter plates. A sandwich ELISA was used to assess cytokine levels in culture supernatants. A TNF-α ELISA kit was used to assess cytokine levels. Biotinylated monoclonal secondary antibodies were used. The reaction was stopped with 3M H_2SO_4 and the optical density of each well was measured at 492 nm in a 96-well plate reader. Recombinant murine cytokines were used to generate standard curves. The lower density limit was 10 pg/ml (TNF-α).

b) **IL-6:**

Testicular cells were isolated by density gradient centrifugation. Testicular macrophages were then isolated by attaching the polymer to the surface. After cultivation of 1 x 10^5 viable cells for 24 h in 0.2 ml RPMI 1640 medium supplemented with 5% FCS, supernatants were obtained. Next, cells were distributed in flat 96-well microtiter plates using microwells. A kit from BD Biosciences was used to measure cytokine IL-6 levels in culture supernatants. Biotinylated monoclonal secondary antibodies were used. After stopping the reaction with 3M H_2SO_4, the optical density of each well was measured at 492 nm in a 96-well plate reader. Recombinant murine cytokines were used to generate standard curves. The lower limit of IL-6 density was 30 pg/ml.

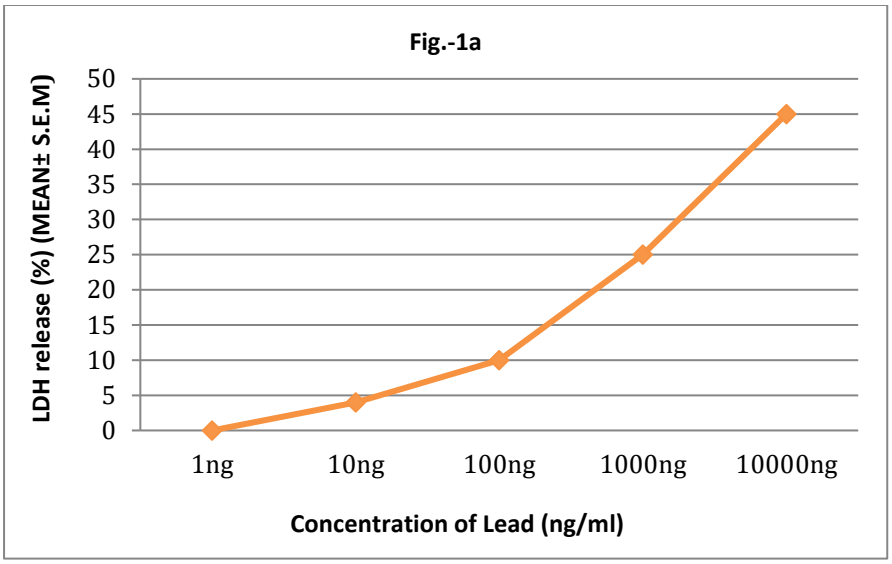
Statistical Analysis:

Data were expressed as mean ± standard deviation. Data were analyzed by Student's t test (two samples assuming unequal variances) to determine significant changes compared to control values. The level of significance was set at $P < 0.05 = P^*$ and $P < 0.001 = P^{**}$.

RESULTS

2.1 Dose response study in the release of lactate dehydrogenase (LDH) release from testicular macrophages exposed to lead:

Testicular macrophage toxicity is measured using dose-response assays of cell survival and cell membrane integrity as determined by cellular LDH release. In testicular macrophage lead poisoning, a concentration of 100 ng/ml was found to have the lowest cytotoxicity (LDH release) (Figure 1a) and an exposure time of 30 minutes (Figure 1b). Cell viability in control and lead-treated group was checked by trypan blue dye exclusion technique in testicular macrophages and more than 90% cells were found to be viable at 100 ng/ml.



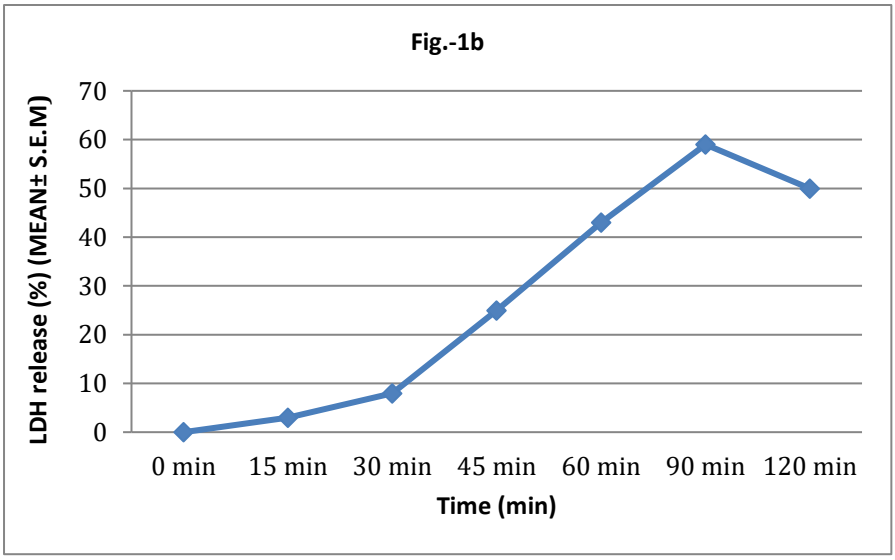


Figure 1: Percenatge of LDH release from lead intoxicated testicular macrophages isolated from Swiss albino mice (*In-vitro*). Figure 1a: In different concentrations. Figure 1b: with respect to time.

2.2 Effects of lead on morphological changes in testicular macrophages:

Macrophage morphology plays an important role in immunological function. To assess the effect of lead on testicular macrophages, a study was conducted on the morphological changes of macrophages. A significant abnormality in the morphology of testicular macrophages with lead intoxication was observed from 16.2 ± 0.44% to 82.48 ± 1.2% (Figure 2; P**).

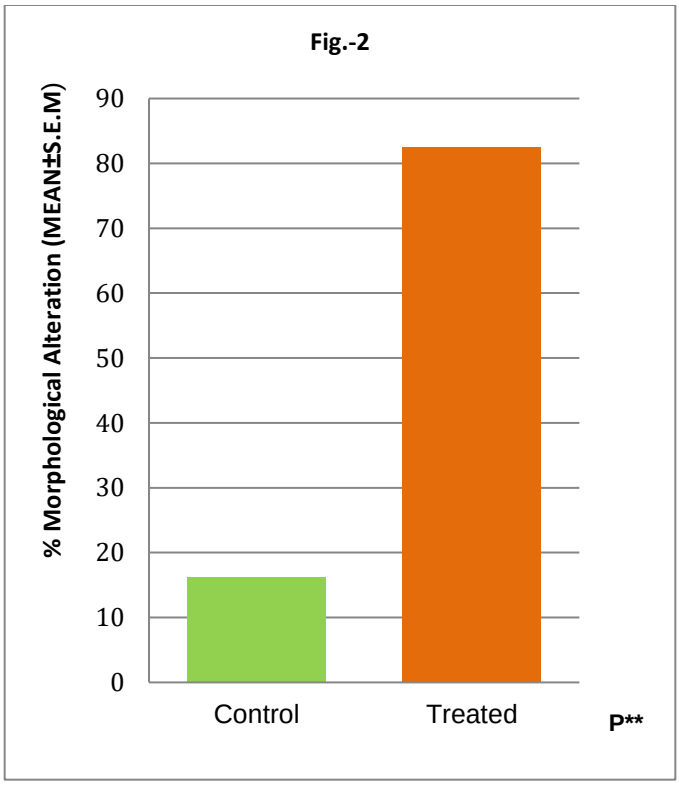


Figure 2: Study of effect of lead on the morphology of testicular macrophages isolated from adult male Swiss albino mice *(In-vitro).*

2.3 Effect of lead on the phagocytic capacity of testicular macrophages isolated from lead intoxicated Swiss albino mice:

To determine whether changes in phagocytic capacity due to lead poisoning are due to altered morphology of testicular macrophages, phagocytosis of heat killed S. aureus by macrophages was determined. The result shows a significant decrease in the phagocytic index from the control value from 29666.6 ± 1332.33 to 6333.36 ± 33.31 after lead poisoning (Figure 3; P**).

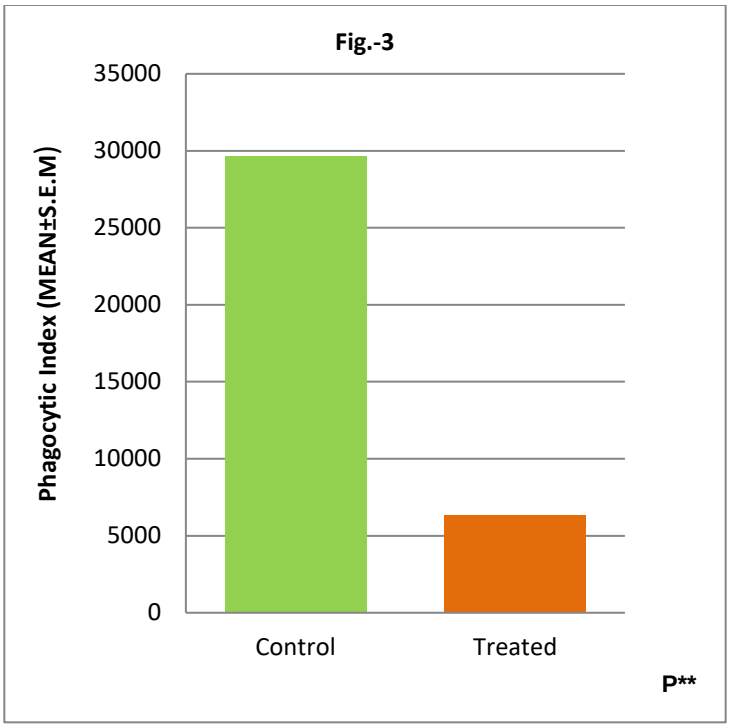


Figure 3: Study of effect of lead on phagocytic capacity of testicular macrophages isolated from adult male Swiss albino mice (*In-vitro).*

2.4 Effect of lead on myeloperoxidase (MPO) release from testicular macrophages isolated from lead-intoxicated Swiss albino mice:

Macrophage activation by bacterial cell wall lipopolysaccharide (LPS) initiates expression of high levels of the myeloperoxidase (MPO) enzyme. MPO reduces the amount of free radicals in our system. MPO release assay was performed to evaluate the effect of lead exposure on the release of MPO enzyme following LPS stimulation. A significant decrease in released MPO (µM) was observed. The value of MPO released by LPS stimulation of the control group was 78.6 ± 1.4 µM and 12.7 ± 0.2 µM in the lead treated group (Figure 4; P**).

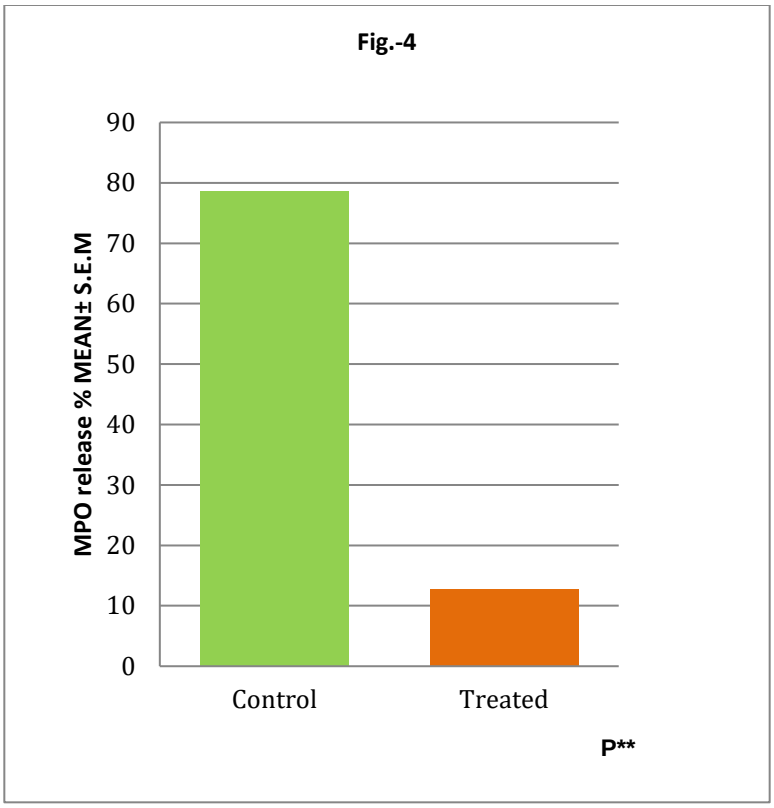


Figure 4: Study of effect of lead on myeloperoxidase enzyme release from testicular macrophages in adult male Swiss albino mice *(In-vitro).*

2.5 Effect of lead on nitric oxide (NO) release from testicular macrophages isolated from lead-intoxicated Swiss albino mice:

When macrophages are activated by bacterial LPS, they begin to express high levels of nitric oxide synthase, which oxidizes larginine to produce citrulline and nitric oxide (NO). Nitric oxide plays an important role in the destruction of the microbial pathogen within the macrophage itself and also in the formation of microbicides such as peroxynitrite and hypochlorites. The result shows that lead significantly decrease in NO release from the control value from 9.2 ± 0.45 to 2.6 ± 1.8 with lead treatment (Figure 5; P**).

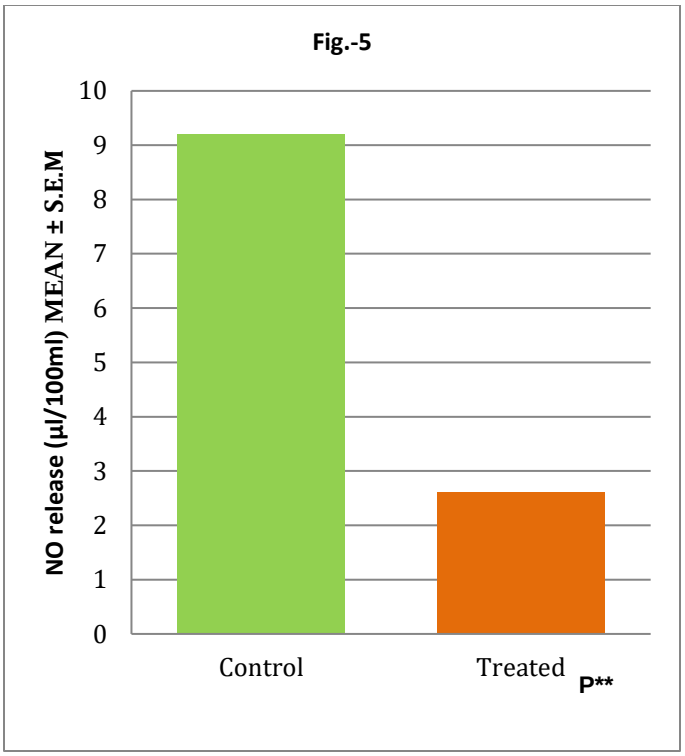
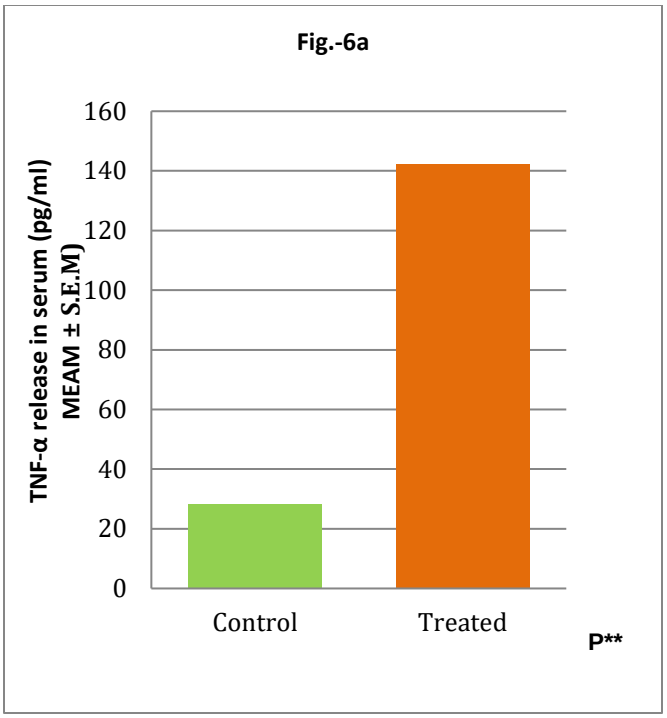


Figure 5: Study of effect of lead on nitric acid release in testicular macrophages isolated from adult male Swiss albino mice (*In-vitro*).

2.6 Effects of lead on the release of pro-inflammatory cytokines from testicular macrophages:

a. TNF-α: Testicular macrophages are possible source of pro-inflammatory cytokines. TNF-α is a multifunctional cytokine that affects not only the pro-inflammatory response but also immune regulation. The present study shows that lead (Pb) intoxication elevate TNF-α levels from 28.22 ± 1.2 to 142.41 ± 1.25 (Figure 6a; P**) results in inflammation and consequently leads to immunosuppression.

b. IL-6: The elevated level of TNF-α indicating multiple targets and sites of action of lead acetate, probably at the receptor level which as a result also increase the IL-6 level from 44.8 ± 8.4 pg/ml to 220.48 ± 18.6 pg/ml (Figure 6b; P**) after lead treatment followed by immune dysfunction. The inflammatory as well as the functional loss of immune surveillance may well be attributed to oxidative stress induced changes from an increased TNF-α titer. Thus the present study shows a decrease in NO and MPO release in lead induced testicular macrophages.



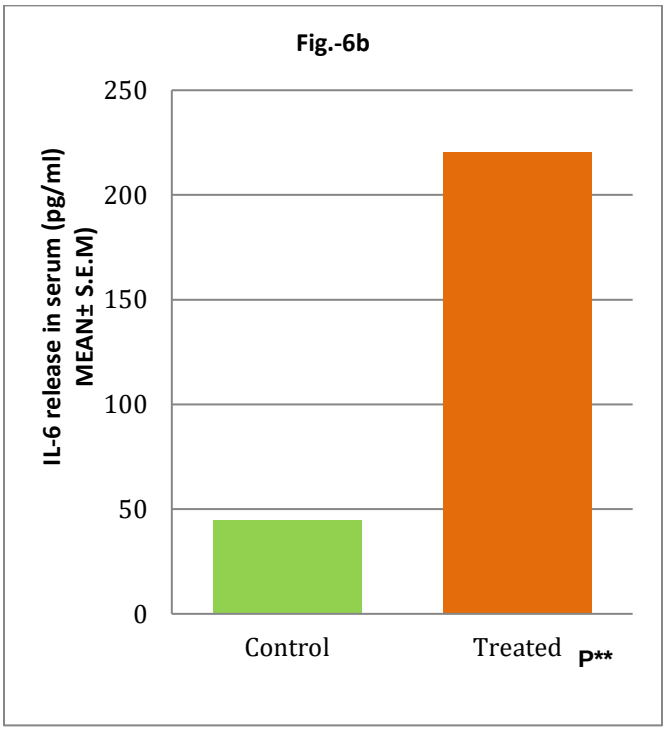


Figure 6: *In-vitro* study of effect of lead on release of Pro-inflammatory cytokines–(a) TNF-α in testicular macrophages isolated from adult male Swiss albino mice and (b) IL-6 in testicular macrophages isolated from adult male Swiss albino mice.

DISCUSSION:

The risks of reproductive abnormalities in male due to metals exposure is one of the fastest growing issues in the field of toxicology today. Sexual dysfunction is common in populations exposed to toxic substances such as heavy metals. Lead is a heavy metal that can be toxic if ingested and inhaled by humans and animals. This causes various destructive effects (Gennart et al., 1992). In humans, high concentrations of lead cause many serious diseases and organ dysfunctions (McGregor and Mason, 1990). The killing mechanism of macrophages is closely related to the ability of macrophages to adhere to a foreign body. Macrophages attach to the foreign particle via dendritic extensions. However, macrophages with an intensely rough surface or a spherical shape can be considered altered macrophages, because after antigen exposure, macrophages must have an optimal dendritic surface morphology. Deviation from the normal shape of macrophages can be the cause of dysfunction of testicular macrophages. Lead poisoning can cause changes in the expression of cell adhesion molecules, chemokine receptors etc. on the surface of macrophages, which further changes the shape and orientation of macrophages, indicating a decrease in the functional efficiency of macrophages.

Cell function studies, such as phagocytosis, have been performed to investigate the immunomodulatory effects of lead poisoning. Exposure of the organism to bacterial infection leads to the activation of several host defense mechanisms, such as phagocytosis. Contact between the pathogen and the phagocytic cell is necessary for the initiation of phagocytosis. This study shows that the lead poisoning group has a decreased phagocyte index compared to the control group, suggesting that lead somehow damages the integrity of the macrophage membrane so that the pseudo-pods are unable to attach to the foreign particle to form a functionally viable phagosome or efficient phagocytosis of invading microorganisms.

A possible mechanism for the increased survival of S. aureus in lead poisoned macrophages is likely to be the inactivation of both oxygen-dependent and oxygen-independent macrophage killing mechanisms. To understand the mechanism, two important parameters were analyzed: the release of myeloperoxidase and nitric oxide from macrophages in both control and treated groups. Upon activation, macrophages release a number of antimicrobial and cytotoxic substances that are responsible for the intracellular destruction of phagocytosed microorganisms. Activation of macrophages by bacterial cell wall lipopolysaccharide (LPS) begins to release large amounts of myeloperoxidase (MPO) enzyme. MPO also reduces the amount of free

radicals in our system. It was observed that the release of myeloperoxidase in the lead-treated group was significantly reduced in the lead poisoning group compared to the control group. Thus, this suggests that lead inhibits the release of myeloperoxidase, which further reduces immune capacity and increases oxidative stress in testicular macrophages.

Nitric oxide (NO) is an important molecule in activated rodent macrophages and has multiple effects modulating inflammation and playing a central role in the regulation of immune responses. Nitric oxide production in mammalian macrophages is strongly increased after infection. Thus, when macrophages are activated by bacterial cell wall lipopolysaccharide, they begin to express high levels of nitric oxide synthase, which oxidizes L-arginine to citrulline and nitric oxide. Nitric oxide itself has a strong antimicrobial effect and plays an important role in the destruction of phagocytosed microorganisms in macrophages. Nitric oxide also plays a role in cell signaling, which is responsible for coordinating and strengthening the immune system during infection. It is also observed that nitric oxide inhibits expression of numerous cytokines, hence, a fall in NO is critical for the development of inflammatory processes involving cytokines like TNF-α. Significant decrease in nitric oxide release was observed in lead treated testicular macrophage compared to control group suggesting further immunomodulatory effects involving over expression of TNF-α which promotes inflammation and thus impeding with cell signaling processes. (Barbhuiya and Sengupta, 2015).

Tumor necrosis factor alpha (TNF-α) is a multifunctional cytokine with effects not limited to pro-inflammatory response but also immunoregulatory responses and apoptosis (McGregor and Mason, 1990). The study revealed that lead exposure raised the levels of tumor necrosis factor alpha (TNF-α), leading to inflammation, which in turn resulted in testicular immunosuppression and compromised testicular immunoprivilege.

Elevated levels of TNF-α and inflammation leading to immunosuppression, indicating multiple targets and sites of action of lead acetate, probably at the receptor level, leading to elevated IL-6 levels after lead treatment, which may disrupt the blood-testis barrier followed by immune dysfunction and subsequent infertility in male.

Exposure of male mice to lead acetate altered testicular macrophage morphology and phagocytic counts, an index of testicular macrophages, indicating that the lead-treated groups were more susceptible to infection due to their inability to phagocytize effectively and making unable to eliminate invading microorganisms. The results of this study strongly suggest that

exposure to the heavy metal lead in the form of lead acetate affected heavily testicular macrophages both morphologically and functionally.

CONCLUSION:

The present experimental study showed that lead toxicity is clearly manifested in testicular macrophages, which in turn complicates the reproductive milieu of rodents, which may be particularly important in heavy metal induced loss of testicular immunoprivilege and subsequently lead to infertility in male.

CONFLICT OF INTEREST: There is no conflict of interest.

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