The Toxic Effects of Lead on Testicular Macrophage Immunomodualation and Sperm Cell Parameters in Mice

Shamim Ahmed S. K. Barbhuiya[1] and Mahuya Sengupta[1*]

[1]*Department of Biotechnology, Assam University, Silchar-788011, Assam, India.*

Authors' contributions

This work was jointly carried out by both the authors in Assam University. Author MS designed the experiment, arranged the required reagents/materials/analysis tools. Author SASKB performed the literature search, experiments and statistical analysis. Authors SASKB and MS wrote the manuscript. Both authors read and approved the final manuscript

Original Research Article	

ABSTRACT

Aims: In the present study the toxic effects of lead was investigated experimentally on the testicular macrophages and sperm cells isolated from testes of adult male mice to ascertain the extent of immunomodulation and reproductive dysfunctions (*in-vivo*).
Study Design: Experimental study.
Place and Duration of Study: Department of Biotechnology, Assam University, Silchar, Assam, India, between March 2013 and August 2014.
Methodology: Dose response study was carried out with an increasing concentration of lead acetate. Percent mortality was determined for these doses and plotted graphically against the respective doses. From the graphs, LD_{50} value was determined. To validate immunomodulation of testicular macrophages and reproductive dysfunction due to lead intoxication, mice were divided into two groups. One group is treated with lead acetate (10 mg/kg body weight) and the other group with isotonic saline solution for 15 days. The isolated testicular macrophages were used to study the phagocytic property, alteration of enzyme release, cytokine release assay and the sperm cell were used for studying the sperm parameters in both control and treated group.

Corresponding author: Email: senguptamahuya35@gmail.com, shamimbarbhuiya@ymail.com;

Results: From the study significant decrease in phagocytic index (25516.61±1352.69 to 5154.67±437.37), myeloperoxidase release (77.3±10.7µM to 23.6±4.9µM), nitric oxide release (9.2±1.13 to 4.7±1.69) and a concomitant rise in the pro-inflammatory cytokine TNF-α were observed. These leads to an increased oxidative stress and inflammatory damage in the testes and subsequently less sperm count (78±1.155 to 24.33±1.764), sperm motility and abnormal sperm morphology was documented.

Conclusion: Thus it could be concluded that the toxic potential of lead diminished the functional capacities of testicular macrophages, led to immunomodulation and inflammatory damage in testes and thus impede the sperm function parameters, which bear particular significance in heavy metal induced immune infertility in male.

1. INTRODUCTION

Today one of the fastest growing areas of research concern is the reproductive hazards from metal exposure in males. Sexual dysfunctions are common in the general population exposed to toxic elements like heavy metals. Lead is a pervasive and persistent environmental pollutant that can be detected in almost all phases of environment and biological systems. Exposure of animals to lead and its derivatives in day-to-day life are unavoidable due to its wide applications and usage [1]. Although lead has many uses industrially and commercially, it is also one of the most toxic ones [2]. The toxic effects of lead demand attention in particular because of its bio-accumulative characteristic associated with a slow turnover and a long biological half-life [3].

Lead serves no useful purpose in the human body and its presence in the body can lead to toxic effects. It is indicated that lead can cause neurological, hematological, gastrointestinal, respiratory, reproductive, circulatory and immunological pathologies [4]. The effect of lead on immunocompetent cell activity has been well studied [5]. Role of macrophages in heavy metal induced immunotoxicologic effect has been reported earlier [6]. Lead exposure has been found to cause alteration of hematopoietic as well as immune cell functions [7]. In short it adversely affects almost all the organ systems of the body. Adverse consequences of lead exposure on reproductive health are widespread [4], equally affecting reproductive, endocrine and immune parameters [8]. Lead intoxication results in the inhibition of testicular functions along with those of the secondary sexual glands like the prostrate, epididymis and seminal vesicle, altering their biochemical composition and affecting both steroidogenesis as well as gametogenesis [9].

Lead is known to encumber the male reproductive functions; however the mechanisms through which these adverse effects are mediated are yet to be clearly elucidated [10]. Testicular macrophages play the dual role of immunoregulation as well as maintenance of testicular immunoprivilege [11,12]. The current study attempts to evaluate the modulation of innate immune responses, altered enzyme release and pro-inflammatory responses due to lead intoxication in murine testicular macrophages that subsequently alters sperm functions in male Swiss albino mice.

2. MATERIALS AND METHODS

2.1 Animals

As per CPCSEA guidelines animal experiments were in accordance with the instructions for care and use provided by the institution at which the research was carried out. All experiments were conducted after obtaining ethical clearance from the Institutional Ethics Committee (IEC), Assam University. Male Swiss albino mice (average body wt 20 gm) were collected from Pasteur Institute, under the Department of Health and Family Welfare, Shillong, Government of Meghalaya. Adult male Swiss albino mice were divided into two groups (1) Control group and (2) Lead treated groups. The second group was given intra-peritoneal dose of lead acetate solution (10 mg/kg body weight) and the first group was given an isotonic saline solution (0.9% NaCl) daily for 15 days. The animals were kept in plastic cages in the animal house, Department of Biotechnology, Assam University. All the animals were kept under laboratory conditions (up to 3 mice per cage, 12 hours light-dark cycle, temperature 25±40ºC, humidity 80±10.0%). The animals were fed standard diet and had free access to water *ad libitum*. All experiments were conducted in triplicate.

2.2 Reagents

The reagents used to carry out the experiments were as follows: Collagenase Type IA, DNase I, Fetal calf serum (FCS) Tosyl (Na-p-tosyl-L-lysine chloromethyl ketone), (SIGMA-Aldrich); Histopaque-1077 (SIGMA-Aldrich); RPMI 1640 (Gibco Life Technologies, Grand Island, NY). All other reagents used in the experiments were of analytical grade.

2.3 Dose Response Study

To determine the LD_{50}, mice were administered with increasing concentrations of lead acetate (1, 5, 10, 20, 50, 100, 200, 300 and 400 mg/kg body weight) till 30 days period. Percent mortality was determined for these doses and plotted graphically against the respective doses. From the graphs, LD_{50} values were determined. Sub-lethal dose of lead acetate (5% of LD_{50}) concentration was standardized to study its toxic effect *in vivo*. These experiments were conducted after obtaining ethical clearance from the Institutional Ethics Committee (IEC), Assam University.

2.4 Testicular Macrophage Isolation

Testes were excised from mice and immediately placed in Alsever's solution. Single cell suspension was prepared and centrifuged. Cells were then allowed to adhere on plastic surface. The adherent cells were collected and Trypan Blue dye exclusion technique was used to test the cell viability [13]. Macrophages from both control and lead exposed mice were used for assays.

2.5 Preparation of Bacteria (*Staphylococcus aureus* MC524) for Phagocytosis Assay

100 µl of an overnight culture made in nutrient broth was added to 10 ml of nutrient broth and incubated for 2-5 h at 37ºC with orbital shaking to get bacteria in the mid logarithmic phase of growth. 10 mM sodium phosphate buffer (pH 7.4) was used to wash the bacteria. Concentration of the bacteria was estimated by spectrophotometry at A_{620} on the basis of the relationship: A_{620} 0.2 = 5×10^7/ ml.

2.6 Scanning Electron Microscopy of Testicular Tissue

JSM-6360 (Jeol) scanning electron microscope (SEM) was used to observe the testicular macrophage and sperm cell at Sophisticated Analytical Instrument Facility (SAIF), North-Eastern Hill University Campus, Meghalalya, India [14,15].

2.7 Phagocytosis

Testicular macrophage was taken on glass slides and allowed to adhere to them. DPBS was used to remove the non-adherent testicular macrophages. Glass slides containing adhered testicular macrophage were treated with heat-killed bacteria and incubated at 37ºC. Then the slides were washed with DPBS and air dried. Methanol was used to fix the slides which were then stained with Giemsa. Slides were next observed under an oil immersion microscope. Phagocytosis was calculated as an average number of SRBC per macrophage × 100 [16].

2.8 Myeloperoxidase (MPO) Enzyme Release Assay

Cells from different groups were taken and stimulated with LPS for 1 h after which they were centrifuged for 10 min. The supernatants and cell lysates were collected in separate tubes. Ortho phenylenediamine (OPD) was added to both supernatants and cell lysates and allowed to react. Readings was taken at 492 nm in a spectrophotometer [17].

2.9 Nitric Oxide (NO) Enzyme Release Assay

Respectively, 100 µl of macrophages was isolated from both treated and control group and suspended in DPBS-BSA. The cells were then stimulated with LPS and centrifuged. 100 µl Griess reagent was added to cell-free supernatant and incubates. Readings were taken in a UV spectrophotometer at 550 nm and compared to a sodium nitrate standard curve [18].

2.10 Pro-Inflammatory Cytokine Assay

Testicular macrophages were isolated by density gradient centrifugation. Isolated testicular macrophages were allowed to adhere to the plastic surface. Then 1 X 10^5 viable number of

cells in RPMI 1640 medium (0.2 ml) containing 5% FCS were distributed in flat 96 well microtitre plates. After 24 hrs of culture, supernatants were collected. Concentration of the cytokine in culture supernatants were measured by RayBio-mouse TNF-α ELISA kit. Biotinylated monoclonal secondary antibodies were used. 3 M H_2SO_4 was used to stop the reaction and optical density of each well was measured at 492 nm. All determinations were done in triplicate. Triplicate assays were done to get standard curves using recombinant mouse cytokines. Lower density limits were found to be 10 pg/ml (TNF-α).

2.11 Evaluation of Sperm Concentration and Motility

Sperm extraction: Two parts of cauda epidydimus were taken from male mouse, make small pieces and poured into microcentrifuge tubes containing 500 µl of warm Whittens-2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic media and incubated for 10-15 min. That allows the sperm to swim up. Concentration and motility of sperm was determined using a Neubauer-improved haemocytometer. The numbers of total and motile sperm were assessed twice per sample by counting sperm cells within the 25 group squares. In case of uneven distribution of the sperm cells within the counting chamber, two or more corners of the large squares were counted and the total amount of cells was divided by the number of analyzed fields. Sperm concentration was expressed as the number of spermatozoa per milliliter. Motility was counted using automated sperm counter and was expressed in percentage.

2.12 Sperm Morphology Assessment

After liquefaction, 10 µl of sperm supernatant was taken into a glass slide, made into a smear and allowed to air-dry at room temperature. The smear was stained with Giemsa and sperm morphology was observed under bright field microscope at a 1000x magnification and oil immersion microscope.

2.13 Statistical Analysis

Student's *t*-test (two-sample assuming unequal variances) was used to analyze the data, to determine the significant changes of treated value from control. The data were expressed as mean ± standard error of mean. Level of significance was set at P*= *P*<.05 and P**= *P*<.001.

3. RESULTS

3.1 Dose Response Study for *In vivo* Exposure to Lead Acetate

The LD_{50} values of lead acetate (*in vivo*) in mice were found to be 200 mg/kg body weight. It was found that 50% of the experimental population died at a concentration of about 200 mg/kg body weights and there was a significant decrease of survival according to the increment of dose (Fig. 1). Therefore, at 5% of the LD_{50}, a dose of 10 mg/kg body weight (sub-lethal) was chosen for further experiments to elaborate the underlying mechanisms. The volume of drug administered (ip) was 50 µl (concentration of lead acetate 0.2 mg/ml). The injections (ip) of both control groups and lead acetate treated group were given daily in the forenoon for a period of 15 days. No death was recorded among the control group.

3.2 Effect of Lead Acetate on Phagocytic Capacity of Testicular Macrophages Isolated from Lead Treated Male Albino Mice

Results of the present experiment showed a marked decrease in the phagocytic index from control 25516.61±1352.69 to 5154.67±437.37 after lead intoxication (Fig. 2; P**), indicating a drastic change in immunomodulation.

3.3 Effect of Lead Acetate Treatment on Myeloperoxidase Enzyme Release from Testicular Macrophages Isolated from Lead Treated Male Albino Mice

It was observed that, there was a significant decline in MPO release (%) after treatment with lead. MPO release in control group (77.3±10.7%) was much higher as compared to lead treated group (23.6±4.9µM) (Fig. 3; P**) indicating a rise in oxidative stress in the cellular level.

3.4 Effect of Lead Acetate Treatment on Nitric Oxide Enzyme Release from Testicular Macrophages Isolated from Lead Treated Male Albino Mice

Nitric oxide is an important enzyme, which kills the pathogens inside the macrophages. Our present study shows that, lead treated group showed a significant decrease in nitric oxide release (4.7±1.69 µM) as compared to control (9.2±1.13 µM) (Fig. 4; P*).
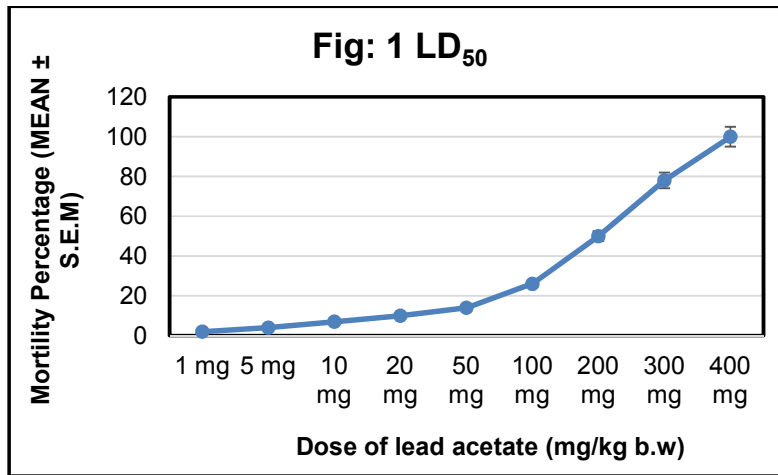
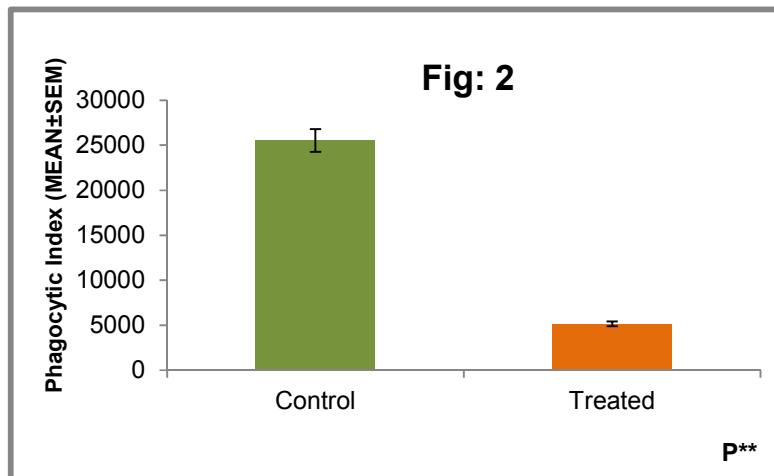**Fig. 1. Study of *in vivo* dose response study (LD$_{50}$) of lead acetate**

Fig. 2. Study of effect of lead on phagocytosis by testicular macrophages
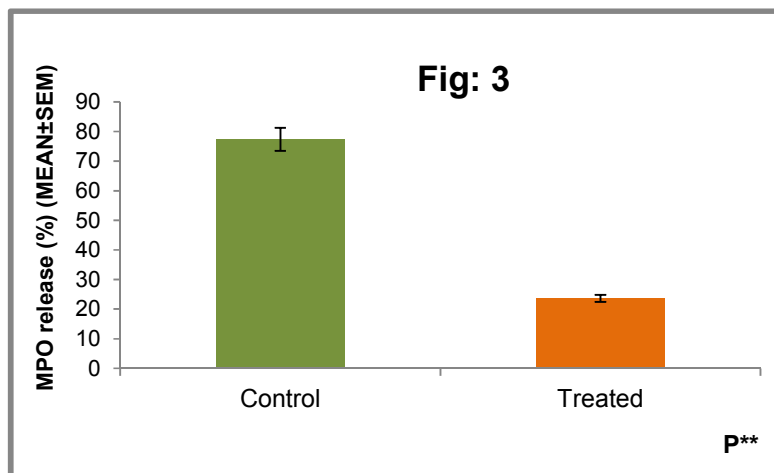

Fig. 3. Study of effect of lead acetate in the release of enzyme myeloperoxidase from lead treated testicular macrophages
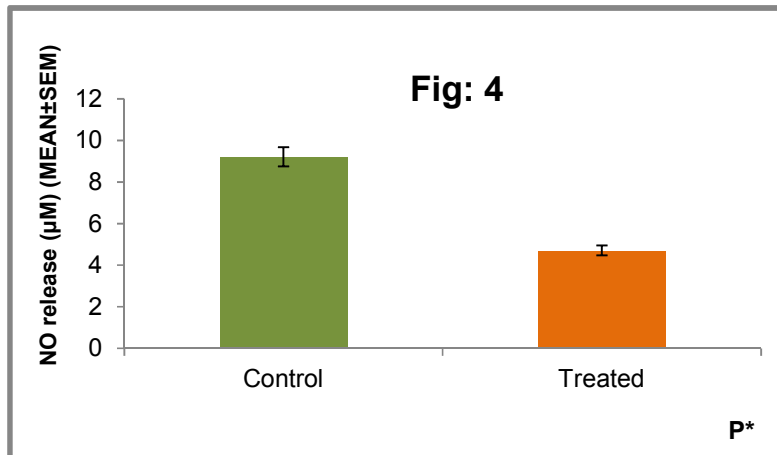
Fig. 4. Study of effect of lead acetate in the release of enzyme nitric oxide from lead treated testicular macrophages

3.5 Effect of Lead Acetate Treatment on Pro-Inflammatory Cytokine Release from Testicular Macrophages Isolated from Lead Treated Male Albino Mice

TNF- α is an important parameter which regulates the testicular homeostasis. The present study shows that lead intoxication elevated TNF-α levels from 152±1.37pg/ml to 312.33±1.17 pg/ml (Fig. 5; P**) resulting in inflammation and immunosuppression.

3.6 Effect of Lead Intoxication on Sperm Parameters

(i) Sperm count: From the present study it has been documented that lead had a drastic effect on number of sperms counted. Sperm count in lead intoxicated group (24.33±1.764 million/ml) significantly declined as compared to control group (78±1.155 million/ml) (Fig. 6a; P**).

(ii) Sperm motility: Sperm motility also declined in lead exposed group (23 ± 1.5523%) as compared to control group (68±0.8819%) (Fig. 6b; P*).

(iii) Sperm morphology: Sperm morphological alteration was found to be more prevailing in the lead treated group. In the present study more abnormal sperm cells are counted (76.33±0.8819%) in the lead exposed group as compared to control group (22±1.155%) (Fig. 6c; P**).
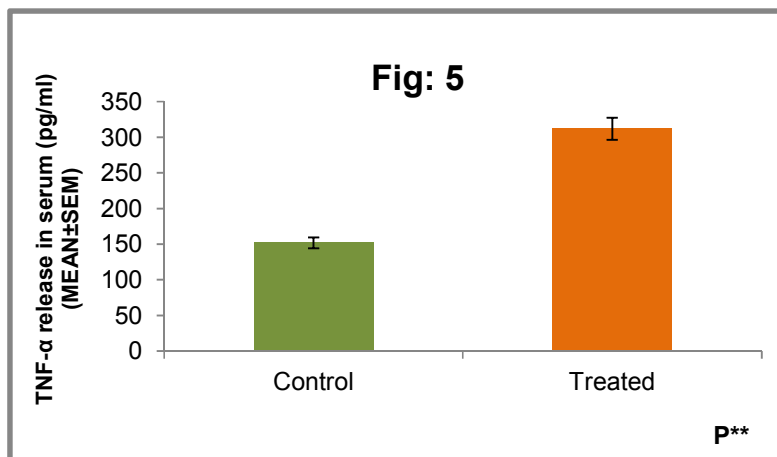

Fig. 5. Study of effect of lead acetate treatment in the release of pro-inflammatory cytokine (TNF-α) from lead treated testicular macrophages
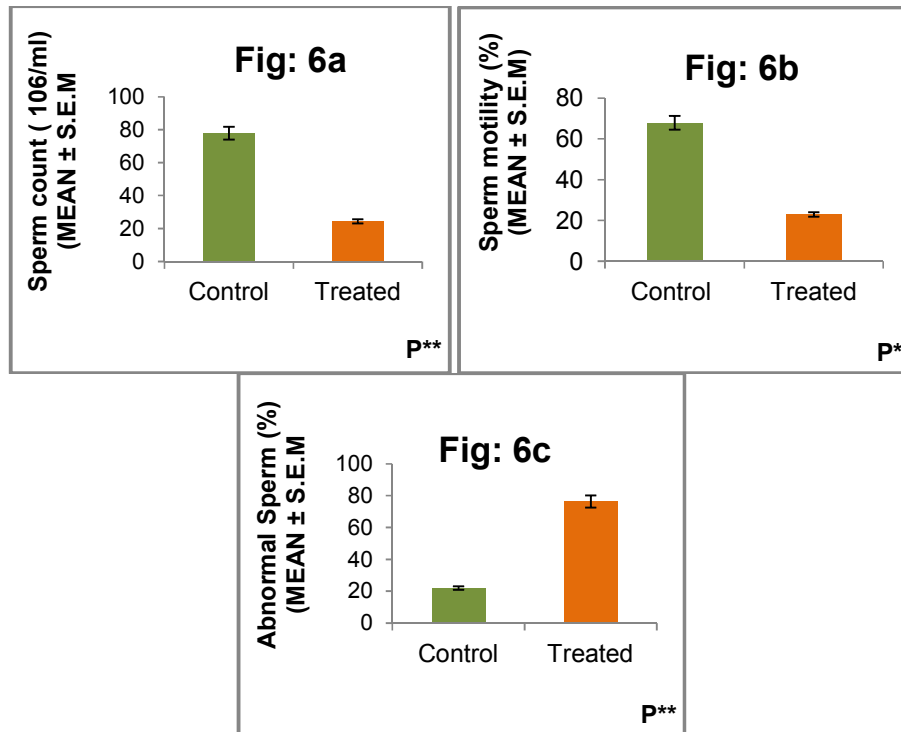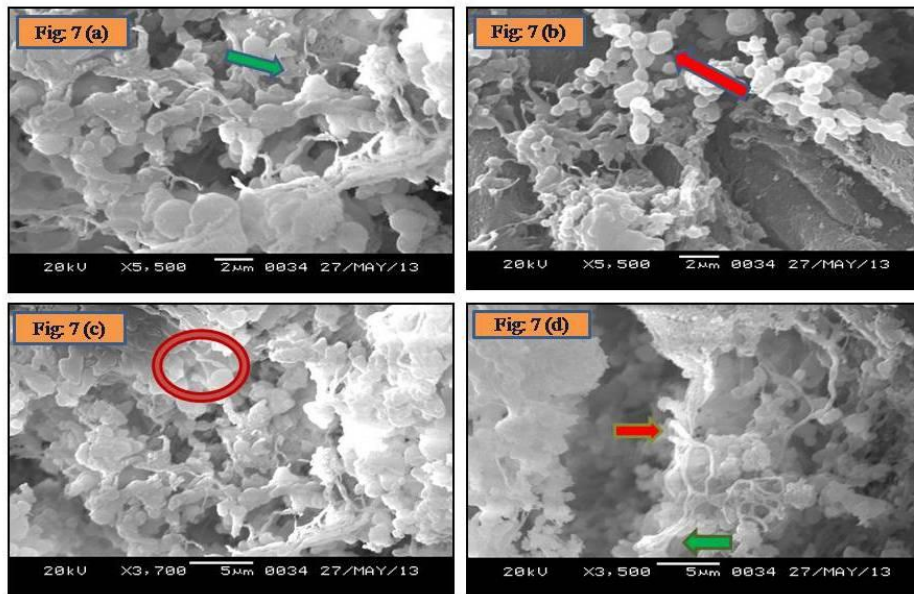
Fig. 6. Study of effect of lead on sperm parameters, 6(a) Sperm count, 6(b) Sperm motility, 6(c) Sperm morphology

JSM-6360 (Jeol) scanning electron micrograph

Fig. 7(a). Testicular macrophages with differentiated pseudopodia were scored as normal in control group. 7(b) Testicular macrophages with un-differentiated pseudopodia were scored as polarized in lead treated group. 7(c) Showing bent neck region, an indication of abnormal sperm morphology in lead exposed group. 7(d) Abnormal sperm morphology and sperm tangles in lead treated group

3.7 Effect of Lead Treatment on Morphology of Testicular Macrophages and Sperm Cells as Determined by Scanning Electron Microscopy (SEM)

Morphology is an important part of cell functioning. Any deviation in morphology leads to reduced functional status of the cells. Presence of spherical macrophage cells with smooth surface or highly polarized cells were an indication of morphologically altered or deformed cells. Lead exposure was found to alter testicular macrophage morphology significantly. Scanning electron micrographs of lead treated testes showed smoother periphery which rendered the differentiation of testicular macrophages and was deficient of pseudopods (dendritic extensions) as compared to the control (Fig. 7b above). Abnormal sperm cells were also more prominent in lead treated (Figs. 7c, 7d above).

4. DISCUSSION

Exposure of macrophages to heavy metal like lead is known to affect several functions of these cells, such as phagocytosis, motility, response to migration-inhibitory factor, antigen presentation etc. particularly there is a striking decreased capacity to kill intracellular pathogens. It is known that the microbicidal activity of macrophages depends on the production of highly reactive metabolites of oxygen [19], the synthesis of which is triggered during the phagocytic process.

To elucidate immunomodulatrory effect of toxicity due to lead intoxication, cell function study like phagocytosis was performed. Exposure of organism to bacterial infection results in the activation of variety of host defense mechanism such as phagocytosis. In phagocytosis the antigens adhere to the macrophage cell membrane induces membrane protrusions called pseudopodia. The pseudopodia extend around the attached material and fuse to form a phagosome, which then enters into the endocytic pathway of antigen processing and presentation. Lead intoxicated group shows a decrease in the phagocytic index as compared to control group which suggests that lead somehow damages the membrane integrity of the testicular macrophages, therefore pseudopodia can neither attach with the foreign particles properly to form phagosomes nor hold the effective phagocytosis of the invading microorganisms.

Not only are the exogenous factors responsible for failure of effective phagocytosis by the macrophages but also there are some endogenous factors that are intricately linked up with such failure such as inactivation of oxygen depending killing mechanism of macrophages. To establish the fact, two important parameters such as myeloperoxidase and nitric oxide release from macrophages isolated from both lead intoxicated and control group were studied.

Macrophages are key components of the innate immune response; on activation, they express a variety of antimicrobial and cytotoxic substance such as myeloperoxidase (MPO). MPO released into the phagosome reacts with H_2O_2 to generate hypochlorous acid (HOCl), a potent antibacterial substance [20]. MPO is not only a potent anti-microbicidal enzyme but it is also responsible for decreasing the free radical levels in our body system. It is observed that the myeloperoxidase release significantly decreased in lead intoxicated group when compared with control group. Thus it suggests that lead inhibits the release of myeloperoxidase in the physiological milieu which further decreases the immune competence and increase the oxidative stress in macrophages.

Nitric oxide (NO) is as an important effector molecule in activated rodent macrophages and exerts multiple modulating effects on inflammation and plays a key role in the regulation of immune responses. Production of nitric oxide in mammalian macrophages is strongly up-regulated following infection. Thus when macrophages are activated with bacterial cell wall lipopolysaccharide, they begin to express high level of nitric oxide synthase, which oxidizes L-arginine to yield citrulline and nitric oxide. NO itself has a potent antimicrobial effect and plays a significant role in killing of phagocytisized microorganism within macrophages. NO has also a role in cell signaling responsible for coordinating and boosting the immune system upon 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 suggesting further immunomodulatory effects involving over expression of TNF-α which promotes inflammation thus impeding with cell signaling processes.

In the testicular milieu pro-inflammatory cytokine TNF-*α* was significantly enhanced with lead exposure. Tumor necrosis factor alpha (TNF-α) is a multifunctional cytokine with effects not limited to pro-inflammatory response but also immunoregulatory responses and apoptosis [21]. Further tumor necrosis factor alpha (TNF-α) has an inhibitory role in gonadal functions, particularly in the steroidogenesis of Leydig cells. Elevated tumor necrosis factor alpha (TNF-α) level has been observed in human patients with critical illness, burns, and sepsis who experience depressed gonadal functions with low serum testosterone levels [22]. The present study documented that lead intoxication elevated tumor necrosis factor alpha (TNF-α) levels resulting in inflammation that consequently led to immunosuppression of the testis as well as compromise of testicular immunoprivilege [11]. In addition, a rise in tumor necrosis factor alpha (TNF-α) is also known to cause a vicious cycle of less testosterone production which is intricately linked up with sperm parameters and maturation in the testicular milieu and leads to functional loss of sperm cells which subsequently causes infertility in males.

5. CONCLUSION

The present experimental findings provided a well-documented idea that exposure to heavy metals like lead toxicity adversely reduces the functional capacity of testicular macrophages and sperm cell parameters in mice. Lead reduced the functional capacities of testicular macrophages, leading to immunomodulation (diminished MPO and NO levels with an augmented TNF titre) and inflammatory damage (as envisaged from the morphologic alterations) in the testicular milieu and was found to be associated with heavy metal induced subfertility in male mice.

CONSENT

It is not applicable.

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. Animal experiments were in accordance with the instructions for care and use provided by the institution at which the research was carried out.

All experiments were conducted after obtaining ethical clearance from the Institutional Ethics Committee (IEC), Assam University.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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