Toxic Effects of Lead Nitrate Pb(NO₃)₂ on Testis in the Catfish Heteropneustes fossilis

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ABSTRACT
Background: Pollution of the environment through the release of various chemical substances is one of the major concerns of modern society. The impact of heavy metals as well as other pollutants on aquatic biota can be evaluated by toxicity tests which are used to detect and evaluate the potential toxicological effects of chemicals on aquatic organisms. Fish is often considered the “sentinel” organism for the health of the water environment because they are capable of inhabiting practically all zones of the aquatic habitat and have a great commercial and recreational value.

Objective: In this study effects of heavy metal toxicity in the reproductive system of male catfish was investigated.

Materials and Methods: Sexually matured male catfish (Heteropneustes fossilis) were exposed to a sublethal concentration of Pb(NO₃)₂ at 1 mg L⁻¹ (1/5th of LC₅₀) for 3, 7 and 14 days and Gonado-Somatic Index (GSI), testicular morphology, biochemical parameters (protein, glucose, DNA, RNA) and reproductive hormone levels (testosterone and estradiol-17β) were evaluated. Preliminary series of short term static toxicity tests were run to determine LC₅₀ (96 h) of lead in H. fossilis which was 5 mg L⁻¹. Results: The results showed that there was a significant decrease in the GSI, total protein, glucose, fructose, DNA and RNA, estradiol-17β and testosterone levels in a duration dependent manner. The SDS-PAGE analysis of proteins showed the induction of a protein metallothionein, presumed to be an indicator of stress induction due to the lead exposure. Histological changes revealed successive degenerative changes in the spermatogonia, clumping of spermatogonial cells, rupture of interlobular tissue at 3 and 7 days. Exposure at 14 day revealed increased discontinuous interlobular connective tissue, dead sperms, empty seminiferous tubules and atrophy of spermatocytes. Conclusion: Thus, the results suggest that Pb(NO₃)₂ exposure causes suppression of catfish spermatogenesis and the data may serve as a reference in future toxicity assessments.

Keywords: Catfish, testicular toxicity, heavy metal, biochemical parameters, steroid hormones, histology, electrophoresis

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INTRODUCTION

During the last many decades pollution of water bodies has increased several folds, affecting adversely the life of aquatic organisms. Fish inhabits practically all zones of the aquatic habitat and play an important role in monitoring aquatic pollution as they respond with great sensitivity to changes in their environment. Fish chronically exposed to low levels of environmental pollutants like heavy metals (Pb, Cd, Hg etc.) may eventually suffer from reproductive dysfunction. Pb is used in the production of batteries, cables, pigments and chemical additives and in petrol products. The main sources of environmental Pb pollution are from foundries and mining industries, refineries, waste disposal and Pb-recycling industries. The impact of metals as well as other pollutants on aquatic biota can be evaluated by toxicity tests which are used to detect and evaluate the potential toxicological effects of chemicals on aquatic organisms.

The mechanisms by which contaminants can affect fish reproduction are several, like sex determination, sexual differentiation and reversal of sex, alteration of the synthesis or metabolism of reproductive hormones or interferences with egg formation process and damage of the reproductive tissues. During the last couple of decades, increases in several pathological disorders in the human male reproductive system, including cryptorchidism falling sperm counts, decreased semen quality and testicular cancer, have been observed.
Abnormalities in the male reproductive system of fish, reptiles, and mammals have also been reported. Recently, it has been suggested that the increasing incidence of male reproductive abnormalities may be the result of environmental pollution by man-made chemicals that have estrogenic effects. Such reproductive abnormalities not only impair the individual but may also threaten population as a whole. In some of the fish, retarded testicular growth was also observed.

Lead (Pb) is a toxic metal that induces a broad range of physiological, morphological, biochemical and neurological dysfunctions in human, wildlife, aquatic animals including fish. Pb has been one of the most important heavy metals with widespread applications for many centuries because of its broad industrial usage in the manufacture of batteries, fuel additives, pipes, pigments, solders, shielding, etc. Pb is a common occupational and environmental hazard throughout the world. Pb adversely affects the nervous, hematopoietic, endocrine, renal and reproductive systems and its toxicity remains a matter of public health concern. Reproductive consequences of Pb exposure are wide spread affecting almost all aspects of reproduction. Much of the inorganic Pb is bound to oxides, sulfides, hydroxides and carbonates and also may be bound to organic ligands in sediments, soil water or in water column. Depending upon pH, temperature and available ligand concentration, the solubility of Pb in water may vary. Lead loses solubility rapidly above pH 6.5. Weibe et al. noted that Pb causes reduced steroidogenesis. Thomas showed that Pb-exposed females of the Atlantic croaker (Micropogonias undulatus) displayed lower estrogen level. The male reproductive system of the catfish Heteropneustes fossilis is characterized by the presence of testes and one pair of seminal vesicle which open to the common sperm duct. In mammals, the epididymis and accessory sex organs secrete variety of chemical substances like proteins, fructose, hexosamines, sialic acid etc., into the lumen which facilitate various functions such as capacitation, nutrition and transport of spermatozoa.

The objective of the present study was to investigate toxicity effects of Pb on the testes of the catfish (Heteropneustes fossilis). Various parameters such as %GSI, total protein, glucose, fructose, DNA, RNA, testosterone, estradiol-17β hormone levels and histological changes were monitored through the period of exposure.

**MATERIALS AND METHODS**

**Collection and acclimatization of fish:** Adult male catfish *Heteropneustes fossilis* (30-40 g) were purchased from a local fish market during early spermatogenic phase (March) of the annual reproductive cycle. The fish were acclimatized in laboratory for 48 h under natural photoperiod and temperature (131: 11D, 28±2°C). They were fed with minced goat liver *ad libitum* till the day of the experiment. The experiments were performed in accordance with the guidelines of Banaras Hindu University for experimentation in animals and all care was taken to prevent cruelty of any kind. After acclimatization, fish were maintained for LC_{50} and exposure experiments. The tissues were kept at -80°C, till processed for steroid extraction, protein estimation and other biochemical parameters.

**Chemicals and reagents:** Pb(NO₃)₂ (98.9% purity, Vetac, Brazil) was purchased from Glaxo India Ltd. (Mumbai), India. Heparin was purchased from a local medical store, Estradiol-17β and testosterone ELISA kits (Diameter, Italy) were purchased from local suppliers. Diethylether, methanol and other chemicals were of analytical/HPLC grade and purchased from E-Merck, Mumbai, India.

**Estimation of LC_{50}:** For the estimation of LC_{50} for Pb(NO₃)₂, the protocol of Secretaria Estatal Do Meio Ambiente, SEMA was followed. Acclimatized male catfish (30-40 g) were divided into 5 groups of 10 fish each and kept in 10 L aquaria. Each group was exposed to the following nominal lead concentrations: 0 (control), 0.05, 1.0, 5.0 and 10 mg L⁻¹. Lead was added to the water as Pb(NO₃)₂. The fish were maintained for 96 h. Mortality and abnormal behavioural responses were recorded every 12 h during the 96 h exposure. Mortality of fish was recorded for each of the concentration during the 96 h exposure and used for the estimation of LC_{50} using the following equation:

\[ LC_{50} = \frac{LC_{10} \cdot \sum (Mean \, death \cdot Conc. \, Diff)}{No. \, of \, organisms \, per \, group} \]

The LC_{50} values for 96 h were estimated by the trimmed Spearman-Karber method. The LC_{50} value for male fish was 5 mg L⁻¹ and for further experiments 1/5th of LC_{50} (1 mg L⁻¹) dose was selected.
Experimental design: About 150 acclimatized fish were divided into various groups according to study parameters. Each parameter had its own respective control groups. Adult male fish were exposed to lead nitrate (1 mg L⁻¹) as decided from the LC₅₀ experiment for 3, 7 and 14 days. The fish were sacrificed after 3, 7 and 14 days of the treatment. Testes were sampled and fixed in Bouin's fixative for histology or stored at -20°C till processed for the assays of total protein, glucose, fructose, DNA, RNA, testosterone and estradiol-17β.

Parameters of the study
Gonado-Somatic Index (GSI): Five fish from each group were weighed and sacrificed by decapitation. Testes were removed and weighed. The weight of the testis was expressed in 100 g body weight. The GSI was calculated as:

\[ GSI (\%) = \frac{\text{Weight of testis (g)}}{\text{Weight of fish (g)}} \times 100 \]

Histology: The fixed testes were processed for paraffin embedding and 7 μm paraffin sections were taken in transverse plane and stained with Ehrlich's hematoxylin-eosin. At least 5 slides with 4 to 5 sections of the testis from different fish were examined under a Nikon microscope (Model CH3 of F100).

Protein estimation: Protein contents of the testes were determined by the method of Lowry et al. using bovine serum albumin as standard. The Optical Density (OD) of the protein samples was read at 750 nm against a reagent blank in a Systronics spectrophotometer.

Glucose estimation: Glucose content was measured with a standard O-toluidine method using a commercial glucose-reagent kit (New-Delhi, India) according to the manufacturer's protocol. Glucose test reagent consisting of 94% acetic acid and 6% o-toluidine. Testes were homogenized in 1 mL triple distilled water and centrifuged at 5000 rpm for 10 min. The supernatant was reacted with 3 mL of glucose reagent at 90°C for 10 min, cooled and optical density was recorded at 630 nm against the blank in a UV-VIS spectrophotometer. The concentration was determined from a standard curve using glucose (given with the kit) as a standard and expressed in µg mg⁻¹ testis weight.

Fructose assay: Testis (30-40 mg) was homogenized in 4 mL of 80% ethanol and centrifuged at 4000 rpm for 20 min. The supernatant was used for the fructose assay by the method of Mann. Optical density of the brown colour was recorded at 490 nm against the blank in a UV-VIS spectrophotometer. The concentration was determined from standard curve and expressed in µg mg⁻¹ tissue weight.

DNA and RNA estimation: About 10% homogenate of 250 mg testis was prepared in tris-HCl buffer on ice. Acid soluble free phosphates and lipid moieties were removed by 10% perchloric acid and ethanol-ether (3:1), respectively. The final precipitate was dissolved in 5% trichloroacetic acid (TCA), centrifuged at 5000 rpm for 20 min and supernatant was used for nucleic acid estimation. The DNA content was measured with freshly prepared diphenylamine reagent and the blue colour developed was measured at 600 nm. Standard curves were drawn using different known concentrations of calf thymus DNA. The RNA content was estimated with freshly prepared orcinol reagent and the greenish-blue colour developed was measured at 660 nm. Standard curves were drawn with yeast RNA.

Protein electrophoresis: The SDS-PAGE was performed according to Laemmli using 5 and 12% acrylamide in the stacking and resolving gel, respectively. Protein was extracted from the testis tissue from different experiments. The separation was carried out with a vertical slab gel apparatus. Electrophoresis was run at a constant voltage of 80 V till the bromophenol blue marker dye was at above 1 cm from the bottom of the resolving gel. After the electrophoresis, the gel was stained with 0.2% Coomassie brilliant blue R-250 in 50% methanol and 7% acetic acid, followed by destaining in 10% methanol and 7% acetic acid until the background became perfectly white. Molecular weights of proteins were approximately estimated by comparing the relative mobilities of marker proteins.

Steroid extraction: The testicular tissues from different experimental fish were thawed and homogenized separately in 4 volumes of cold PBS (0.02 M phosphate buffered saline solution; pH 7.4) with an ultrasonic homogenizer (XL-2000 Microson, Misonix, USA) 0°C for 5-10 sec. The homogenates were centrifuged at 5000 g for 20 min at 4°C and extracted with 3 volumes of diethyl ether, three times. The ether phase was collected, pooled, evaporated and dried under nitrogen gas, then stored at -20°C.

Testosterone assay: The assay was carried out using a Diametra ELISA kit according to the manufacturer's
protocol for testosterone. Briefly, 50 µL each of testosterone (T) standard (0, 0.1, 0.5, 2.0, 6.0 and 20.0 ng mL⁻¹) and reconstituted samples were transferred to the anti-testosterone immunoglobulin g (IgG)-coated plate. The immunoreaction was started by adding 50 µL of testosterone- HRP conjugate solution into each well, followed by incubation at 37°C for 1 h on a plate shaker at 200 rpm. The content from each plate was removed and washed with 300 µL of distilled water, 3 times. Water was completely drained out from each well. Next, 100 µL tetramethylbenzidine (TMB) substrate was dispensed into each well and incubated at room temperature for 15-20 min in dark on a plate shaker. Color development was stopped by adding 50µL of stop solution (1 M sulphuric acid). Absorbance was taken at 450 nM using a Multiscan microplate reader (Thermo Electron Corporation, USA).

**Estradiol-17β assay:** E2 was assayed using a Diametric ELISA kit according to the manufacturer’s instructions. Briefly, the samples were reconstituted and 50 µL sample and E2 standard (0, 20, 100, 500, 800 and 3200 pg mL⁻¹) each was pipetted into the anti-E2 IgG G-coated plate wells. The immunoreaction was started by adding 100 µL of E2-HRP conjugate solution to each well, followed by incubation at 37°C for 1 h on a plate shaker at 200 rpm. The content from each plate was removed and washed with 300 µL of wash buffer, 3 times. Wash buffer was completely drained out from each well. Next 150 µL TMB substrate was dispensed into each well and incubated at room temperature for 10-15 min on a plate shaker. Colour development was stopped by adding 50 µL of stop solution (1 M sulphuric acid). Absorbance was taken at 450 nM using a Multiscan microplate reader (Thermo Electron Corporation, USA).

**Statistical analysis:** Data were expressed as Mean±SEM and checked for homogeneity and normality. Further, the data were analyzed by one way ANOVA (p<0.001), followed by Newman-Keuls’ test (p<0.05) for statistical significance.

**RESULTS**

**Effect of lead on gonadosomatic index:** The exposure of the fish to lead caused an overall significant effect on the gonadosomatic index (Fig. 1, p<0.001, one way ANOVA). The GSI showed significant decrease in a duration-dependent manner. Compared to the control group, there was an insignificant decrease on day 3 and a significant decrease on day 7 and 14 (Newman-Keuls’ test; p<0.05).

![Graph showing the effect of lead on gonadosomatic index](image)

**Fig. 1:** Effects of lead nitrate on testicular gonadosomatic index (GSI %) in the catfish *Heteropneustes fossilis* (Mean±SEM, n = 5) after 3, 7 and 14 days of exposure in the preparatory phase of the reproductive cycle. Data were analysed by one way ANOVA (p<0.001) followed by Newman-Keuls’ test (p<0.05). Groups with different numbers show significant difference from the control group.

**Histological changes:** In the control testis, high spermatogenic activity was noticed with the seminiferous tubules containing a prominently active epithelium and the lumen filled with spermatids and sperm (Fig. 2a). The Pb exposure led to the inhibition of spermatogenic activity (Fig. 2b-d) and induced degenerative changes. The epithelium was thin fibrotic and interrupted without germ cells, the spermatids were pycnotic and clustered to form masses.

**Effects of lead on protein, glucose and fructose:** There was an overall significant variation in total testicular protein levels (Fig. 3, p<0.001, one way ANOVA). The total protein level increased in significantly on day 3 and decreased significantly after 7 and 14 days (Newman-Keuls’ test, p<0.05). Similarly, testicular glucose and fructose levels decreased duration-dependently after 7 and 14 days of lead exposure with no significant effect on day 3.

**Effects of lead on DNA and RNA:** There was an overall significant effect on testicular DNA and RNA contents after lead exposure (Fig. 4, p<0.001, one way ANOVA). The DNA level increased significantly on day 3 but decreased on day 7 and 14 duration dependently (Newman-Keuls’ test, p<0.05). The RNA level did not change significantly on day 3 and 7 but decreased on day 14 (p<0.05).
Effects of lead on steroid hormone levels
**Testosterone (T) and estradiol-17β (E₂):** There was an overall significant effect on testicular T and E₂ level after lead exposure (Fig. 5; p<0.001, one way ANOVA). The T and E₂ levels did not change significantly on day 3. On day 7 and 14, a significant decrease was noticed on the steroid level (Newman-Keuls’ test; p<0.05).

**SDS-PAGE:** The SDS PAGE analysis of testis protein showed a prominent band corresponding to the metallothionein zone, as indicated by the marker (Fig. 6). The Pb exposure led to a high induction of the protein on day 3 and 7. On day 14, the protein staining was weak, like the control group.

**DISCUSSION**

In the present study, exposure of the catfish *Heteropneustes fossilis* to lead nitrate Pb(NO₃)₂ decreased testicular gonadosomatic index (GSI) and total protein, glucose, fructose, DNA, RNA, testosterone and E₂ contents. It also produced a severe impact on spermatogenesis and induction of metallothionein protein, a potential marker for stress under heavy metal toxicity. The effects on the testis were significantly correlated in a duration-dependent manner. The GSI is a morphological indicator of the functional status of the testis and increases during spermatogenesis and decreases after spermiation. The exposure to Pb caused a duration dependent decrease indicating structural and functional alterations of the testis.
Fig. 3: Effects of lead nitrate on total protein, glucose and fructose levels in the testes of catfish *Heteropneustes fossilis* (Mean±SEM, n = 5). Data were analysed by one way ANOVA (p<0.001) followed by Newman-Keuls' test (p<0.05). Numbers (1-2) denote comparison of protein levels, letters (a-c) show comparison of glucose levels and (p-r) shows comparison of fructose levels. Groups with same numbers/letters are not significantly different.

Fig. 4: Effects of lead nitrate on DNA and RNA content in the testes of catfish *Heteropneustes fossilis* (Mean±SEM, n = 5). Data were analysed by one way ANOVA (p<0.001) followed by Newman-Keuls' test (p<0.05). Numbers (1-3) denote comparison of RNA levels, letters (a-d) show comparison of DNA levels. Groups with same numbers/letters are not significantly different.

The histological analysis of the testis after exposure to Pb at a concentration of 1 mg L⁻¹ reveals that the germinal epithelium of the seminiferous tubules has been severely affected. Spermatogonial cells are not seen, unlike in the control testes, the epithelium is populated

Fig. 5: Effects of lead nitrate on testosterone (T) and estradiol-17β (E₂) levels in the testes of catfish *Heteropneustes fossilis* (Mean±SEM, n = 5). Data were analysed by one way ANOVA (p<0.001) followed by Newman-Keuls' test (p<0.05). Letters (a-b) denote comparison of testosterone levels, numbers (1-2) show comparison of estradiol-17β levels. Groups with same numbers/letters are not significantly different.

Fig. 6: SDS-PAGE gel image showing testicular protein profile in the catfish *H. fossilis* exposed to lead nitrate. M: Molecular marker C: Control, 3: After 3 day, 7: After 7 day, 14: After 14 day. Note a strong induction of a protein of MW ~16 KD in the metallothionein zone of the gel.
with degenerated germ cells and fibroblasts, giving an attenuated epithelial morphology. Spermatids and sperm appeared pycnotic and formed pycnotic masses, enlarged lumen was observed on day 14. The interstitial Leydig cells were pycnotic and blood capillaries were lacking. The loss of spermatogonia, spermatids and spermatozoa probably occurred because of the inability of these epithelial cells to maintain them. This was evident from the impaired steroidogenesis, the level of testosterone and E2 decreased in a time-dependent manner. Hg has been reported to inhibit the conversion of cholesterol to pregnenolone, resulting in decreased synthesis of corticosterone in the adrenal of rats. Inhibition of 3β-hydroxy-Δ5-steroid dehydrogenase (3β-HSD) activity has been reported in other stereogenic tissues, such as the testis of Hg-treated rats and the testis of Hg-treated catfish. Cadmium has been reported to affect testicular steroid metabolism in brook trout and in rainbow trout. The interstitial Leydig cells which are stereogenic in the testes of several teleosts, including the catfish, showed histological signs of progressive degenerative changes, such as hyperactivity and pycnosis. These cellular alterations reflect adversely on the stereogenic potential of the cells. The enzyme, 3β-HSD which catalyses the conversion of pregnenolone to progesterone was inhibited by Hg. The resulting impaired steroidogenic activity might have led to the arrest of spermatogenic activity. In the present study, impairment of steroidogenesis by Pb might be due to a direct action of Pb on steroid biosynthesis or indirectly via hormonal feedback. In the catfish, pituitary gonadotropic activity was demonstrated to be inhibited by Hg treatment.

There was a significant decrease in testicular glucose and fructose contents in a duration-dependent manner. Monosaccharides have been reported as energy substrates in the gonads of a number of teleosts. In males of several fishes, inter and intra-specific seasonal variability in the concentrations of monosaccharides in the testes has been reported. In male catfish, glucose or fructose shows significant seasonal variations, fructose being the abundant sugar in the reproductive phase, as in most mammals. The significant decrease in glucose and fructose levels in the fish exposed for 7 and 14 days indicates that lead may cause hypoglycemia adversely affecting testicular functions. The percentage depletion of the glucose level at 14 days was more than 50%. Haux et al. and Haux and Larsson in a series of experiments have observed a significant and persistent hypoglycemia in lead-exposed Salmo gairdneri. Helmy et al. also noted hypoglycemia in the fish Liza macrolepis exposed to lead nitrate. The hypoglycemic response exhibited by Pb-intoxicated fish might be due to the Pb induced morphological and functional changes in renal tubule cells of the kidney along with reduced gluconeogenesis. Such effects are common in lead-exposed mammals. Pb can also affect glucose metabolism as shown by Salmeron-Flores et al., who reported increased blood glucose concentration in Sarotherodon aureus in response to lead exposure. A strong hyperglycemic response was also observed in fish exposed to other metals, such as cadmium and copper. Thus, Pb exhibits mixed response with respect to species, season and duration. Jha and Pandey have reported a significant decline in total carbohydrates of intestine, liver and gonads of Pb-exposed Channa punctatus and suggest the utilization of total carbohydrates to meet enhanced energy requirements due to the toxic stress.

In the present study, Pb had duration-dependent inhibitory effects on total protein of testis. The level of total protein in testis after Pb(NO3)2 treatment shows a highly significant decrease in the 7 and 14 day-treated groups, in comparison to control. Jana and Bandyopadhyaya have also noted a fall in total protein level of several tissues after exposing Channa punctatus to Pb. Jha similarly reported a decline in protein content of tissues after exposure of C. punctatus to Pb(NO3)2 for 30 days. Several heavy metals have been reported to stimulate interrenal activity and plasma corticosteroid and glucose levels in fish. Heavy metal exposure had impact on metabolic effects which include hyperglycemia, depletion of tissue glycogen reserves, catabolism of muscle protein and altered blood levels of protein, cholesterol and free fatty acids.

Metallothionein (MT) is a low-molecular weight (~2-16 kDa) cysteine-rich protein which has a high affinity for metals and plays important roles in the protection against metal toxicity. In addition to the detoxification of toxic metal such as cadmium (Cd) and mercury (Hg), MT is involved in the maintenance of homeostasis of essential trace elements such as Zinc (Zn) and Copper (Cu) and in the scavenging of free radicals. MTs have received much attention as potential biomarkers to monitor heavy metal pollution of aquatic ecosystems that are sensitive to pollutants especially to high amounts of heavy metals. However little or no information is available concerning MT's induction by Pb compounds on testis in teleosts. The MT gene expression is induced by many factors, including metal ions such as Cd, Zn, Cu and Hg, alkylating agents, UV irradiation and other chemical and physical stressors. In the present study, the SDS-PAGE
of testicular protein showed an increase in ~16 kDa protein expression on day 3, 7 of the Pb exposure and a decrease in the protein expression on day 14. The protein band corresponding to MW ~16 kDa may be a very weak MT protein, judging from the pattern of expression. The MT induction increased time-dependently up to day 7, indicating the impact of Pb exposure. At day 14, the impact was less and therefore, weak expression, as in the control fish.

Thus in the present study, it is suggested that Pb might have interfered with several steps in testicular steroid biosynthesis to lower the level of testosterone and estradiol and also severely affected metabolic biochemical substances related to testis functions. The histological observations indicate that spermatogenesis was impaired at a late stage, adversely affecting the transformation of spermatids into functional spermaresozoa.

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