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Irreversible Histopathological Modifications Induced by Iron Oxide Nanoparticles in the Fish, *Oreochromis mossambicus* (Peters, 1852)

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ABSTRACT: The aim of present study is to investigate if the histopathological alterations induced by iron oxide nanoparticles (Fe_3O_4NPs) in the fish *Oreochromis mossambicus* is reversible after the treatment withdrawal. Sublethal concentration (15 mg/L) of Fe_3O_4NPs was exposed to fish for short-term (96 h) and long-term (60 days) durations along with control group. Histopathological modifications in gill, liver and brain tissue were examined after the exposure periods. Gill tissue showed blebbing of gill epithelium, mucous deposition, vacuolization, hyperplasia of gill arches, aneurysm and loss of secondary lamella after short- term and long-term exposure of nanoparticles. Exposure to Fe_3O_4NPs in liver tissue resulted in segmentation of hepatocytes, vacuolization, spindle shaped nucleus, necrosis and aggregation of melanomacrophages, where the severity of lesions increased in time-dependent manner. Lesions of brain tissue included degeneration and severe loss of granular cells after 96 h followed by neurodegeneration and aggregation of gliosis after 60 days of nanoparticles treatment. Histomorphological modifications appeared in gill, liver and brain tissue remained unaltered after the treatment withdrawal period thereby indicates irreversible tissue damage. The present findings indicate the defensive mechanism of tissues to overcome nanoparticles induced stress and also represent an important parameter to study the ecotoxicological impact of nanoparticles on fish population.

Keywords: Fe_3O_4NPs, Gill, Liver, Brain, Histopathology, *Oreochromis mossambicus.*

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INTRODUCTION

Iron oxide exists in six different forms composed of Fe and O, they includes magnetite (Fe_3O_4), hematite (α-Fe_2O_3), β-Fe_2O_3, maghemite (γ-Fe_2O_3), ε-Fe_2O_3 and wustite (FeO) (Cornell and Schwertmann, 2003). Naturally nano-sized iron oxides are generated from environmental sources such as volcanoes, forest fire where they exist either as magnetite and maghemite crystalline structures. However, some nanoparticles are artificially engineered and extensively used in several biomedical applications including magnetic resonance imaging, gene and drug delivery, cell separation, protein immobilization etc (Salata *et al*., 2004; Sun *et al*., 2016). Iron oxide nanoparticles derived from magnetite and maghemite possess large applications as it is also used to remove metals from aqueous solutions and environmental remediation (Grover *et al*., 2012).

There is a growing concern regarding the increased exposure of nanoparticles to ecosystems and humans, associated to its large scale production. Nanoparticles are exposed to organisms by four possible routes namely air, water or food, dermal contact and through sediment deposition. The accidental spillage from the production site, effluents from wastewater treatment plants, during transportation and usage and rainwater runoff are the major ways of nanoparticles that reaches the aquatic ecosystems (Garner and Keller, 2014).When it reaches the aquatic organisms, nanoparticles overcome the tissue barrier or cross cellular membranes thereby causing dramatic changes in physiology, morphology and behaviour of the exposed animals. Iron oxide nanoparticles are highly biocompatible so that it can easily interact with biomolecules such as protein, lipids and nucleic acids (Gupta and Gupta, 2005). Such nano-biointeractions account for the development and application of engineered nanoparticles in biomedical purposes. Meanwhile, the interactions also pose negative impacts in different cell types when it damages the biomolecules (Singh *et al*., 2010).

The toxicity of iron oxide nanoparticles have been well studied in different cell lines and rodent models. Several review of literatures documented that the particle size, coating and surface charging of nanoparticles have crucial role on the intracellular uptake and its possible toxicity (Vladimir *et al*., 1999).

Fe_3O_4NPs at 1 mg/L concentration when exposed to Indian major carp for 96 h has been shown to cause alterations in hematological, biochemical, ionoregulatory and enzymological parameters (Saravanan *et al*., 2011). Iron oxide at 0.1 to 100 mg/L concentrations showed developmental toxicity in embryo of zebrafish causing mortality, hatching delay and malformation (Zhu *et al*., 2012). Recently, sublethal concentrations of iron oxide nanoparticles on exposure for 7 days have been shown to alter the hematological and biochemical parameters in the fish, *Labeo rohita* (Keerthika *et al*., 2017). Due to the continuous rise in the amount of nanoparticles exposed in the aquatic environment, the focus of the present study was to assess the histomorphological alteration induced by Fe_3O_4NPs in the vital tissues as gill, liver and brain of the fish, *Oreochromis mossambicus*. The study also critically evaluated the persistence of nanoparticles by the treatment withdrawal after long-term exposure of Fe_3O_4NPs in order to confirm the permanent impact of nanoparticles on the exposed organism.

MATERIALS AND METHODS

A. Test animal

Oreochromis mossambicus weighing 6±1.5 g and length 6.5±1cm were collected from local fish farm, Safa Aquarium, Kozhikode, Kerala (11º22'N, 75º85'E). Fish were acclimatized to the laboratory conditions prior to experiment by maintaining in glass tanks (40L) of dechlorinated water provided with good aeration and light. The physico-chemical features of the tap water were estimated as per APHA guidelines (1998). Standard water temperature (28±2°C), oxygen saturation of water (70 and 100 %) and pH (6.5 to 7.5) was maintained in a standard range throughout the experiment in both control and treatment groups.

B. Preparation of test solution

Fe_3O_4NPs (Cat. No. 637106) were obtained from Sigma Aldrich, Germany. The particles pre-characterized before the experiment was found pure, free from impurities and the size derived using Scherrer's formula was confirmed as 15.65 nm. The nanodispersion was prepared just before exposure by ultra-sonication at 100 kHz for 30 min using double distilled water and maintained as stock. The test concentration of nanoparticles was selected based on maximum dispersion in aqueous solution and agglomeration i.e., 150 mg/L, the concentration above which showed aggregation, agglomeration and high mortality. Thus one-tenth of the above concentration i.e., 15 mg/L was selected as sublethal concentration.

C. Grouping and toxicity testing

Fish were grouped into four groups as follows:
Group I: Control group (Toxicant-free)
Group II: Fe_3O_4NPs at 15mg/L for 96 h (short-term)
Group III: Fe_3O_4NPs at 15mg/L for 60 days (long-term)
Group IV: Fe_3O_4NPs (15mg/L for 60 days) followed by Toxicant-free medium (60 days)
At the end of each treatment periods, fishes were caught using small dip nets, avoiding stress to the animal and the gill, liver and brain tissues were dissected out for histological examinations.

D. Histology of tissues

Gill, liver and brain tissues were collected by sacrificing the fish and were fixed in 10% buffered formalin for 24 h. Dehydration of tissues were done in ascending grades of alcohol and cleared in xylene until they became translucent. Tissues were transferred to molten paraffin wax for 1 h to remove xylene completely and then impregnated with wax. Blocks were cut in a rotary microtome to prepare sections of thickness 4 to 6 microns. The sections were stained with hematoxylin and eosin and mounted in DPx. The structural alterations of gill, liver and brain tissues were observed under light microscope and were compared with the control tissue. Photomicrographs were taken using Canon shot camera fitted to the Carl Zeiss Axioscope 2 Plus Trinocular Research Microscope.

RESULTS AND DISCUSSION

Recently ecotoxicological studies pay great attention to investigate suitable methods to identify decline in the water quality and develop several preventive measures owing to the increase in aquatic pollution. Healthy fish population is essential to manage biotic integrity of aquatic ecosystems. Aquatic organisms are biologically sensitive to changes in the water quality which are reflected by alteration in behavioural, biochemical, physiological and histological parameters. Early detectable signs of damage at cellular level can be achieved by histological analysis as biomarker. Iron oxide nanoparticles induced histopathological alterations was observed in gill, liver and brain tissues of the fish *Oreochromis mossambicus*. Basic toxicology evaluates the toxicity of any chemicals depending on the concentration and duration of exposure. Duration of exposure is very important toxicological perspective as the consequences of nanoparticles depend on either exposure to minimum concentration for longer duration or higher concentration for shorter time period. The present study focused on both short-term and long-term exposure of nanoparticles at sublethal concentration because low threshold effect of toxicant is often useful to diagnose individual fitness in an ecosystem. The persistent effects of nanoparticles are further assessed by treatment withdrawal for 60 days which is important to predict if the fish recover from the exposed nanoparticles within the specific duration.

Gill tissue are extremely sensitive to the pollutants as it undergo physical modifications owing to the change in the environment, mainly because of the large surface area of gill epithelium and high rate of perfusion that makes easy entry of pollutants (Hinton, 1992). Gill tissue in toxicant free condition showed normal architecture with distinct gill epithelium, gill arches, primary and secondary lamellae (Fig. 1a). Exposure to Fe_3O_4NPs for 96 h resulted in mucous deposition, vacuolization, aneurysm, hyperplasia and absence of secondary lamellae (Fig. 1b). Fe_3O_4NPs treatment for 60 days showed blebbing of epithelium, aneurysm, hyperplasia and absence of secondary lamellae and the severity of damages were more prominent (Fig. 1c). Mucous deposition, epithelial edema and epithelial upliftment are the defensive mechanisms of the fish to avoid from contact with the toxicants (Shephard, 1982; Mallatt, 1985). Mucous produced from goblet cells form a discrete layer in between tissue and water, which prevents the entry of pollutants. Excess mucous deposition in the epithelial layer along with upliftment and edema are the symptoms of nanotoxicity as well as the response of gill tissue against the exposed nanoparticles. Regressive changes such as aneurysm, loss of secondary lamella and blebbing of epithelium are recognized as non-specific damages where the injury of pillar cells increase blood flow inside the lamellae leading to aneurysm (Johari *et al*., 2015).

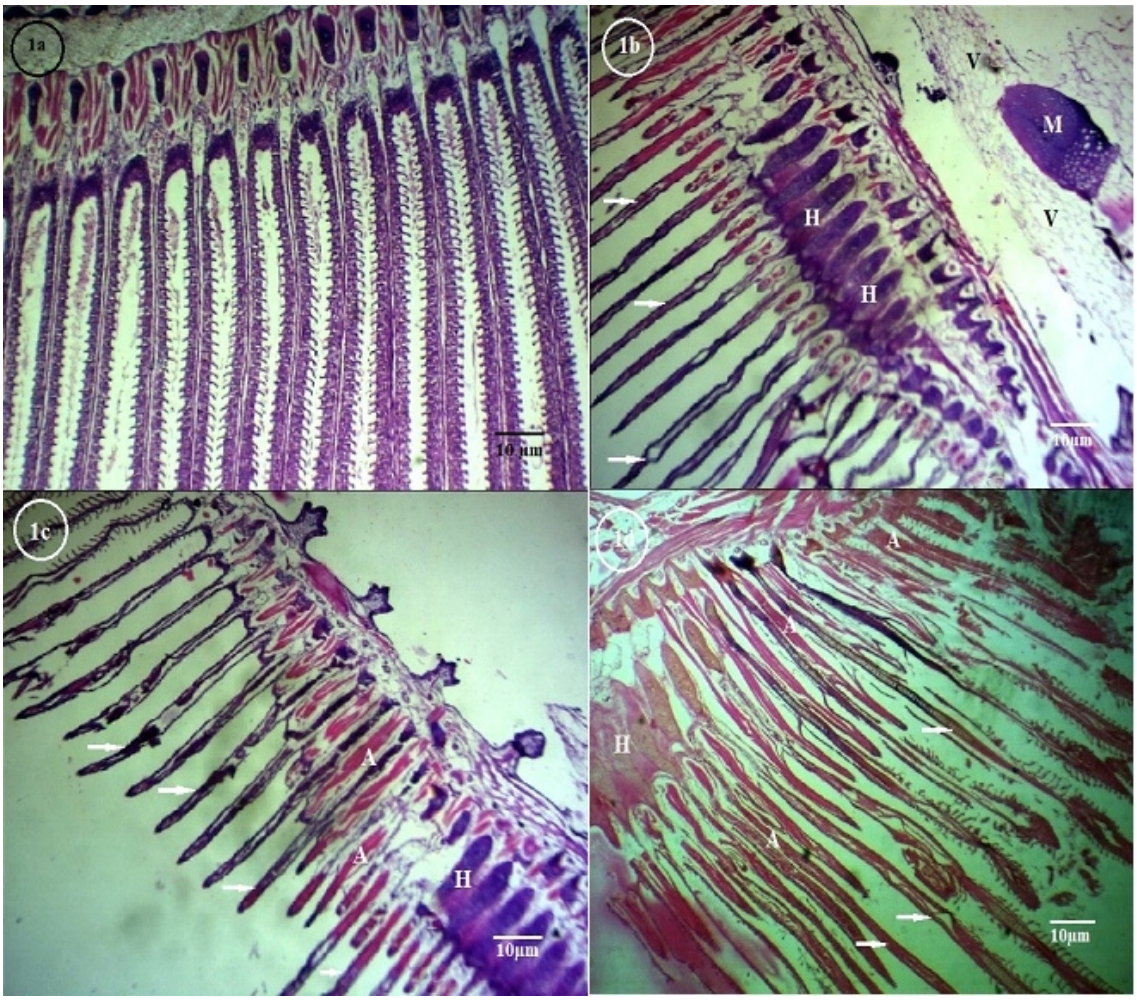

Fig. 1. Histomorphology of gill tissue exposed to Fe_3O_4NPs in *Oreochromis mossambicus*. 1a-Gill control; 1b: Fe_3O_4NPs at 15mg/L exposed for 96 h showing mucous deposition (M), vacuolization (V), hyperplasia (H), absence of secondary lamellae (→); 1c: Fe_3O_4NPs at 15mg/L exposed for 60 days showing blebbing, aneurysm (A), hyperplasia (H), absence of secondary lamellae (→); 1d: Treatment withdrawal showing aneurysm (A), hyperplasia (H), absence of secondary lamellae (→).

Most of the histopathological lesions of gill observed in the present study were in agreement with exposure to silver nanoparticles for 21 days in rainbow trout (Johari *et al*., 2015). The gill tissue of treatment reversal group retained the structural lesions similar to that of the treatment groups (Fig. 1d) thereby confirming irreversible tissue damage in *Oreochromis mossambicus*.

Control liver tissue showed normal histomorphology having hepatocytes with homogenous cytoplasm and a large central or subcentral spherical nucleus (Fig. 2a). Liver tissue exposed to Fe_3O_4NPs for short-term duration showed notable lesions such as segmentation of hepatocytes and spindle shaped nucleus (Fig. 2b), and when the treatment was increased for 60 days were observed with aggregation of melanomacrophages followed by severe necrosis (Fig. 2c). Melanomacrophages are aggregates of highly pigmented phagocytes involved in immune defenses and also for the phagocytosis of exogenous and endogenous indigestible materials (Herraez and Zapata, 1986). Fe_3O_4NPs induced necrosis is the direct effect of nanoparticles and aggregation of melanomacrophages in the hepatocytes represents the exogenous accumulation of toxic materials. In addition, melanomacrophage aggregation signifies hepatic degeneration that ultimately results in necrosis of the tissue which was evident after 60 days of nanoparticles exposure.

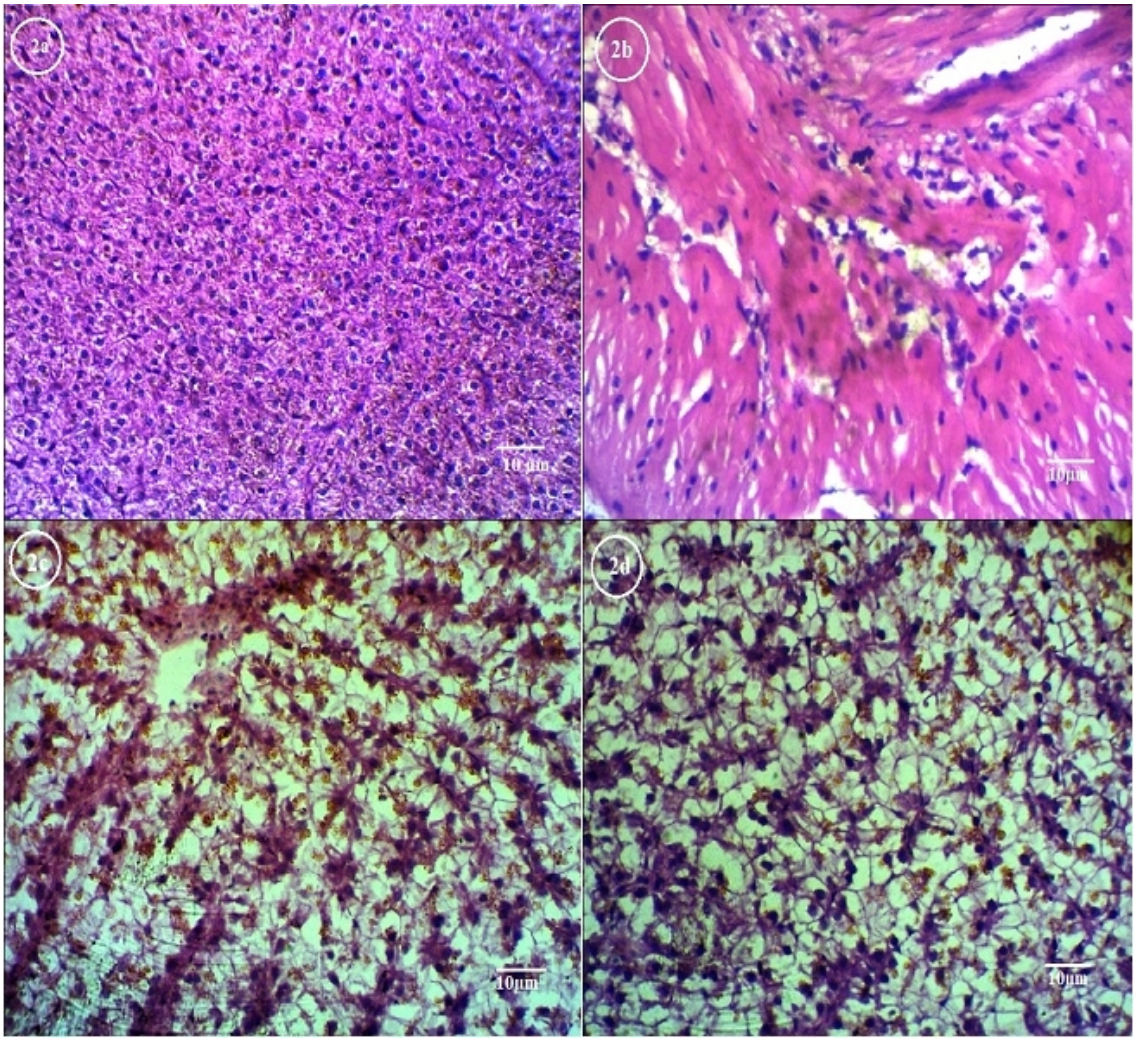

Fig. 2. Histomorphology of liver tissue exposed to Fe_3O_4NPs in *Oreochromis mossambicus*. 2a-Liver control; 2b: Fe_3O_4NPs at 15mg/L exposed for 96 h showing segmentation of hepatocytes and spindle shaped nucleus; 2c: Fe_3O_4NPs at 15mg/L exposed for 60 days showing severe necrosis and aggregation of melanomacrophages; 2d: Treatment withdrawal showing severe degenerated cytoplasm with deposition of melanomacrophages.

Similar hepatic lesions have been observed in the hepatocytes of *Tilapia zilli* exposed to three different concentrations of aluminium for 96 h (Hadi and Alwan, 2012). Degenerated cytoplasm with deposition of melanomacrophages retained even after the treatment withdrawal (Fig. 2d), which indicates failure of liver tissue to recover from the exposed nanoparticles.

Brain tissue from control group showed normal histological architect without any indication of lesions (Fig. 3a). Nanoparticles exposed fish brain showed degeneration of neurons, vacuole formation and severe loss of granular cells after 96 h of treatment (Fig. 3b). Neuronal degeneration after 60 days of Fe_3O_4NPs exposure was more prominent by means of more distinct changes as vacuolization, intracellular edema, congestion of neural cells and aggregation of gliosis (Fig. 3c). The impairment of brain tissue by the pathological lesions observed would result in alteration of physiological and behavioural functions of the fish. Similar structural degeneration of brain tissues has been observed when lethal concentration of organophosphate pesticide phorate was exposed to *Cyprinus carpio* for 4 days (Lakshmaiah, 2017). The neurodegenerative changes were found persistent as evident by the similar pathological lesions in the treatment withdrawal group (Fig. 3d) thereby designate permanent neurotoxic effect of the nanoparticles. Persistence of toxicity and damages in the brain tissue is also associated to the generation of reactive oxygen species and are the suspected reason behind neurotoxicity of nanoparticles (Farina *et al*., 2013; Vidya and Chitra, 2018). The increased severity of lesions is duration-dependent and the persistent toxic effects of nanoparticles are alarming as it caused permanent destruction to the vital tissues of the fish, *Oreochromis mossambicus*.

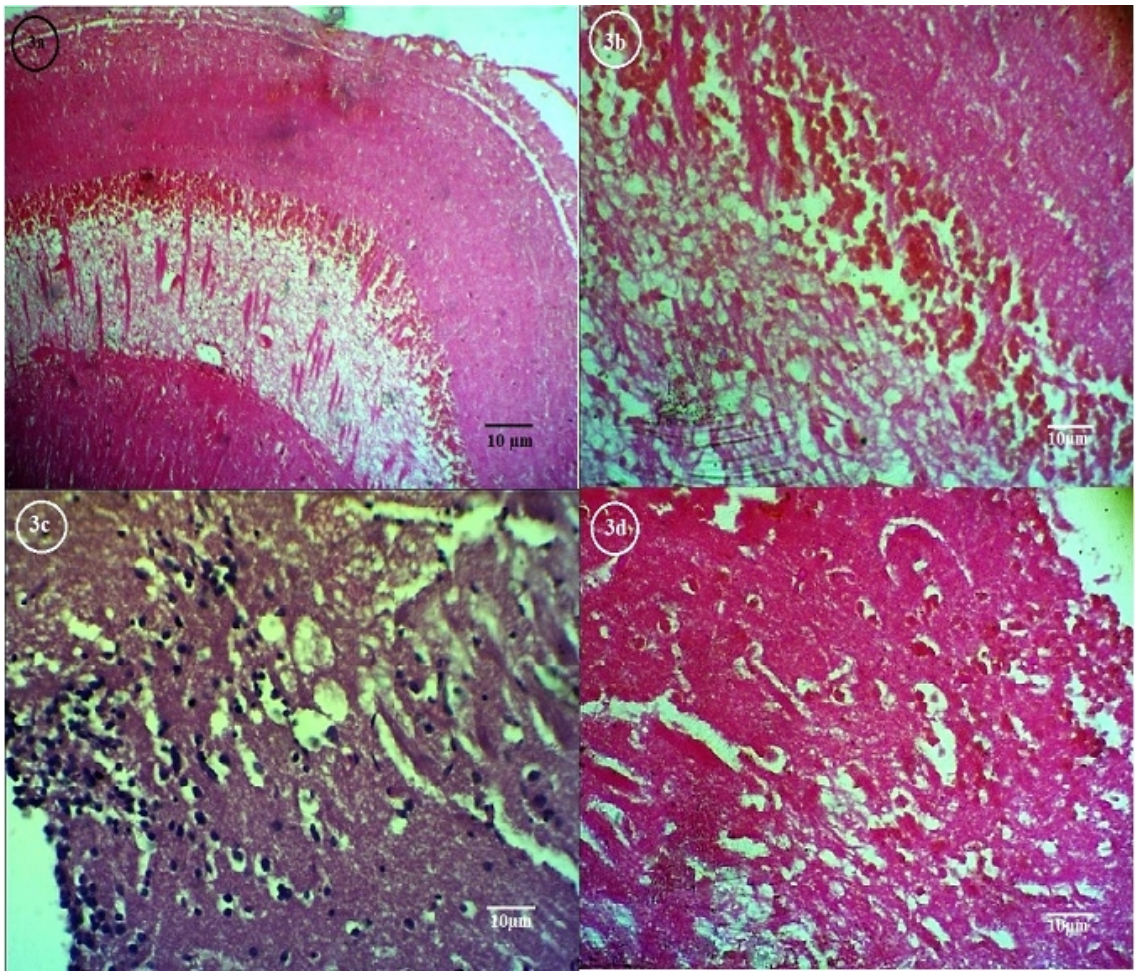

Fig. 3. Histomorphology of brain tissue exposed to Fe_3O_4NPs in *Oreochromis mossambicus*. 3a-Brain control; 3b: Fe_3O_4NPs at 15mg/L exposed for 96 h showing degeneration of neural cells and vacuole formation; 3c: Fe_3O_4NPs at 15mg/L exposed for 60 days showing vacuolization and intracellular edema; 3d: Treatment withdrawal showing severe neurodegeneration.

CONCLUSIONS

To brief, Fe_3O_4NPs at sublethal concentration caused pronounced tissue damage to the fish, which is irreversible. Thus proper measures should be taken to avoid the release of nanoparticles into the aquatic ecosystems, or else eventually leads to the change in physiological and behavioural modifications and finally results in the decline of fish population.

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