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Aluminium oxide nanoparticles induced irrevocable damages in gill, liver and brain tissues of the freshwater Fish, *Oreochromis mossambicus* (Peters, 1852)

PV Vidya, KC Chitra*
Endocrinology and Toxicology Laboratory, Department of Zoology, University of Calicut, Malappuram District, Kerala, India

Abstract
Aluminium oxide nanoparticles (Al_2O_3NPs) or nano-sized alumina is the engineered nanoparticles having wide range of application in engineering, science and technology. Histopathological alterations induced by Al_2O_3NPs were studied in the gill, liver and brain tissues of the freshwater fish, *Oreochromis mossambicus.* Al_2O_3NPs at 4 mg/ L ($1/10^{th}$ of LC_{50}-96 h) was exposed for short-term (96 h) and long-term (60 days) durations maintaining the control group. Treatment withdrawal for 60 days was also conducted in toxicant-free water to assess if the toxicity of nanoparticles revokes to normal. Histopathological changes in gill after 96 h showed epithelial upliftment, hypertrophy and hyperplasia of gill arches, aneurysm, lamellar curling and absence of secondary lamella. The degree of damages was more severe after 60 days of nano-alumina exposure showing severe mucous deposition, vacuolization, hyperplasia, aneurysm, and absence of secondary lamellae. Morphology of liver tissue showed pathological changes as segmentation and degeneration of hepatic cytoplasm with spindle shaped nucleus after 96 h of exposure. When the treatment period is increased to 60 days showed vacuolization and severe necrosis. Exposure to nanoparticles for 96 h showed lesions in brain tissue as indicated by mild vacuolization in neural cells, however, after 60 days more degenerative changes as intracellular edema was observed. Duration of exposure have profound effect on structural damage of gill, liver and brain tissues. Treatment of nanoparticles when withdrawn for 60 days also showed similar pathological alterations as the treatment groups thereby indicating Al_2O_3NPs induced irrevocable damages in gill, liver and brain tissues of the freshwater fish, *Oreochromis mossambicus.* The present observations clearly demonstrated that nanoparticles exhibited significant and permanent morphological changes in vital tissues, which could be one of the reasons behind the decline of fish population in toxicant-exposed aquatic environment.

Keywords: Al_2O_3NPs, gill, liver, brain, histopathology, *Oreochromis mossambicus*

1. Introduction
Nanotechnology is the combination of different branches of science, engineering and technology which is concerned with the manipulation of matter on an atomic, molecular and supramolecular scale. In nanotechnology applications, metal oxide nanoparticles exhibit unique physical and chemical properties due to the potential technological applications in various fields. Nanoparticles are natural and unintentionally produced having wider distribution of sizes, while engineered nanoparticles are artificially designed and intentionally produced, with more narrow defined sizes. Due to industrialization, nanotechnology is now reaping the potential benefits by increasing the production of nanoparticles. Ecotoxicological studies have recently listed plasticizers and pharmaceuticals as chemical contaminants and recent review of literatures focused engineered nanoparticles as new concerns on the health and stability of aquatic ecosystems. Aluminium oxide nanoparticles (Al_2O_3NPs) is one of the metal oxide engineered nanoparticles widely used in many products as abrasive, coatings, absorbant, catalyst, sensor and fuel additive (Colvin, 2003) [1]. It has been expected that the dispersed nanoparticles could easily enter and accumulate in aquatic, terrestrial and atmospheric environment, where the fate and toxicity on different organisms remain scanty. Nanoparticles can also interact with small organisms like algae, fungi and bacteria that enter into the food chain to reach higher animals, including fish in the aquatic ecosystems. Fish are considered as the non-target organism of accidental exposure to nanoparticles causing adverse effects on the health and life of the exposed animal.

Fish population represents almost half of all vertebrate species and gained much attention as laboratory model in ecotoxicological studies. In the present study, *Oreochromis mossambicus* was chosen as the model for histological analyses of nanoparticles toxicity. *O. mossambicus* provide a number of exceptional advantages as it is commercially important fish having wide range of tolerance to temperature, and can be bred and maintained in large numbers easily in laboratory condition at low cost (Garcia-Ulloa *et al*., 2007) [2]. Besides, fish have been documented as bioindicators of water quality because of the differential sensitivity to aquatic contamination. The present study analyzed histopathological alterations after exposure to Al_2O_3NPs in tissues as gill, liver and brain, which are good biomarkers of aquatic pollution. Biomarkers are biological indicators showing the effects of pollutants at a specific period of time, which can be monitored using several parameters, including histological analyses (Bernet *et al*., 1999) [3].

Histology is the scientific study of fine details of biological samples such as cells and tissues under microscope.

Histopathological techniques are sensitive, reliable, cheap and simple tool widely used in the field of disease diagnosis and other pathologic conditions. Histopathological studies are critical and sensitive parameter in toxicological studies, also it gives the direct impact of toxins on cell and tissue morphology and architecture. Thus it is used as an important tool in the toxicity studies of environmental contaminants in aquatic ecosystems. Al_2O_3NPs has been known to induce neurodegeneration, neurotoxicity and cause blood brain barrier damage in microvascular endothelial cells of human brain (Chen *et al*., 2008) [4] as well as in rat brain (Alshatwi *et al*., 2013) [5]. Genotoxicity of Al_2O_3NPs has been documented by the induction of micronuclei in peripheral blood of rat model (Balasubramanyam *et al*., 2009) [6] and in CHO-K1 cell lines (Di Virgilio *et al*., 2010) [7]. Al_2O_3NPs induced cytotoxicity by the induction of oxidative stress has been observed by the decline in mitochondrial membrane potential in human mesenchymal stem cells (Li *et al*., 2009) [8]. Exposure to Al_2O_3 nanoparticles of 40 nm size has been shown to cause histological alterations in the hepatic tissues of freshwater fish *Oreochromis mossambicus* (Murali *et al*., 2017) [9]. Size, surface properties, hydrodynamic behaviour, association with large sediments, binding to lipophilic organic compounds, routes of uptake are some important properties of nanoparticles to enhance the toxicity (Moore, 2006) [10]. Aluminium oxide nanoparticles induced irreversible alterations in the antioxidant defense system of gill, liver and brain tissues of the freshwater fish, *Oreochromis mossambicus* has been reported previously from our laboratory (Vidya and Chitra, 2018) [11]. Therefore, the aim of the present investigation was to evaluate the nanotoxic effects of Al_2O_3NPs of 16.7 nm size in histopathology of gill, liver and brain tissues in the fish *Oreochromis mossambicus.* The persistence effects of Al_2O_3NPs in organ toxicity were further evaluated by withdrawing the treatment in toxicant-free environment for 60 days after the maximum treatment period of two months.

2. Materials and Methods

2.1 Maintenance of 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 for two weeks in the laboratory conditions prior to the experiment. Fish were maintained in glass tanks (40 L capacity) supplied with dechlorinated and well aerated water having equal proportion of light and dark maintained for 12h: 12 h. The physico-chemical features of the tap water such as water temperature (28 ± 2°C), oxygen saturation of water (70 and 100 %) and pH (6.5 to 7.5) were maintained throughout the experiment in both control and treatment groups according to the standard guidelines as prescribed by APHA [12].

2.2 Test chemical and toxicity testing

Al_2O_3NPs (Cat. No: 0140408) was obtained from SISCO Research Laboratory (SRL), India. The characterization data confirmed the purity of nanoparticles as well as its size as 16.7 nm (Vidya and Chitra 2017) [13]. The nanodispersion was prepared just before the treatment exposure by ultra-sonication at 100 kHz for 30 min using double distilled water and maintained as stock. The test concentration i.e., 4 mg/L ($1/10^{th}$ of LC_{50}) was selected in this study according to the median lethal concentration (LC_{50}-96 h, i.e., 40 mg/L) as reported previously (Vidya and Chitra 2017) [13]. Experiments were performed for short-term (96 h) and long-term (60 days) durations along with control group maintained without toxicants. Treatment reversal for 60 days was also performed by maintaining another group without toxicant exposure after the long-term nanoparticles exposure. In each treatment groups ten fish were maintained, and the health conditions were monitored during the entire period. At the end of every treatment period, fishes were captured using small dip nets with minimum disturbances in order to avoid stress and killed by decapitation to collect gill, liver and brain tissues.

2.3 Histology of tissues

Gill, liver and brain tissues collected after the treatment period were fixed in 10% buffered formalin for 24 h and dehydrated 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. Then the blocks were cut in a rotary microtome to prepare sections of thickness 4 to 6 microns. The sections were stained with haematoxylin and eosin and mounted in DPx. The structural alterations of gill, liver and brain tissues were observed under light microscope and compared with those of control tissues. Photomicrographs were taken using Canon shot camera fitted to the Carl Zeiss Axioscope 2 Plus Trinocular Research Microscope.

3. Results

3.1 Effects of Al_2O_3NPs on gill tissue

Control gill tissue showed normal architecture having gill epithelium, gill arches, primary and secondary lamellae (Figure 1a). Exposure to Al_2O_3NPs for 96 h resulted in epithelial upliftment, hypertrophy and hyperplasia of gill arches, aneurysm, lamellar curling and loss of secondary lamella (Figure 1b). Long-term exposure of nanoparticles for 60 days showed severe mucous deposition, vacuolization, hyperplasia, aneurysm, and absence of secondary lamellae (Figure 1c). The severity of morphological alterations was found time-dependent. Reversal of treatment for 60 days in toxicant-free medium showed similar lesions as that of treatment groups such as severe mucous deposition, vacuolization, hyperplasia of gill arches, aneurysm, blebbing of primary lamellae and absence of secondary lamellae (Figure 1d).

3.2 Effects of Al_2O_3NPs on liver tissue

The fish used as control showed normal pattern of hepatocytes having homogenous cytoplasm with spherical nucleus (Figure 2a). However, exposure to Al_2O_3NPs for 96 h showed pathological changes as segmentation and degeneration of hepatic cytoplasm with spindle shaped nucleus (Figure 2b). Severe vacuolization and necrosis were observed after 60 days of nanoparticles exposure (Figure 2c). Reversibility of lesions was not observed after the treatment withdrawal for 60 days (Figure 2d).

3.3 Effects of Al_2O_3NPs on brain tissue

Histology of control brain was found normal and the cerebral

hemispheres or cerebrum viewed under the microscope showed outer granular layer, middle basophilic layer and the ganglionic layer possessing neuronal cells (Figure 3a). Al_2O_3NPs exposure for 96 h showed pathological lesions as mild degenerative changes on all regions with mild vacuolization in neural cells (Figure 3b). When the treatment period was increased for 60 days showed severe degenerative changes along with intracellular edema (Figure 3c). Treatment of nanoparticles when withdrawn for 60 days also showed similar pathological alterations like that of treatment groups (Figure 3d).

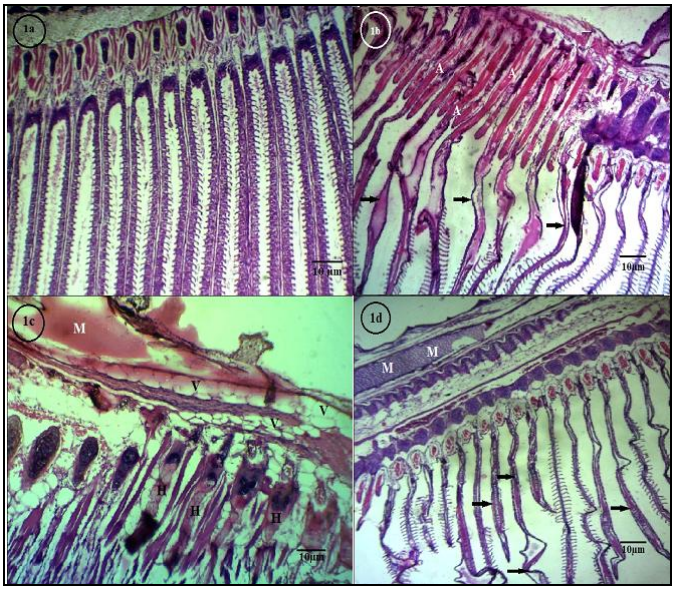

Fig 1: Histomorphology of gill tissue exposed to Al_2O_3NPs in *Oreochromis mossambicus*. 1a-Gill control; 1b: Al_2O_3NPs at 4mg/L exposed for 96 h showing aneurysm (A), absence of secondary lamellae (→); 1c: Al_2O_3NPs at 4mg/L exposed for 60 days showing mucous deposition (M), vacuolization (V), hyperplasia (H); 1d: Treatment withdrawal showing mucous deposition (M), absence of secondary lamellae (→)

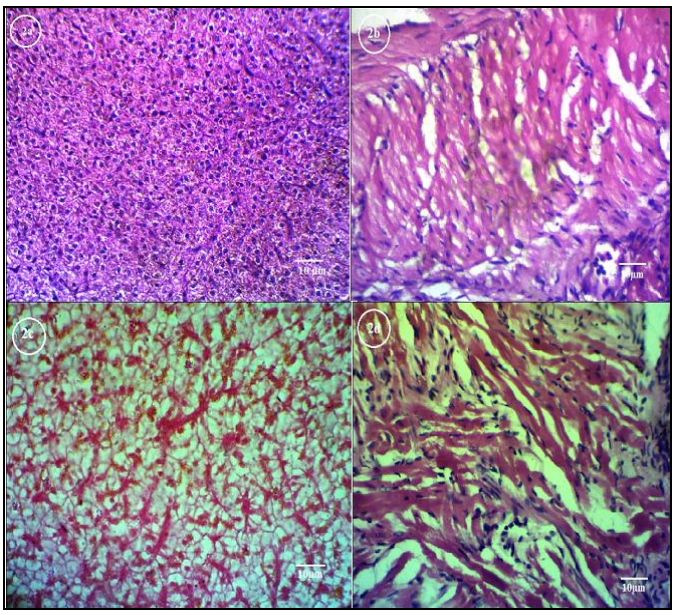

Fig 2: Histomorphology of liver tissue exposed to Al_2O_3NPs in *Oreochromis mossambicus*. 2a-Liver control; 2b: Al_2O_3NPs at 4mg/L exposed for 96 h showing segmented hepatocytes and spindle nucleus; 2c: Al_2O_3NPs at 4mg/L exposed for 60 days showing vacuolization and necrosis; 2d: Treatment withdrawal showing severe degenerated cytoplasm and spindle shaped nucleus.

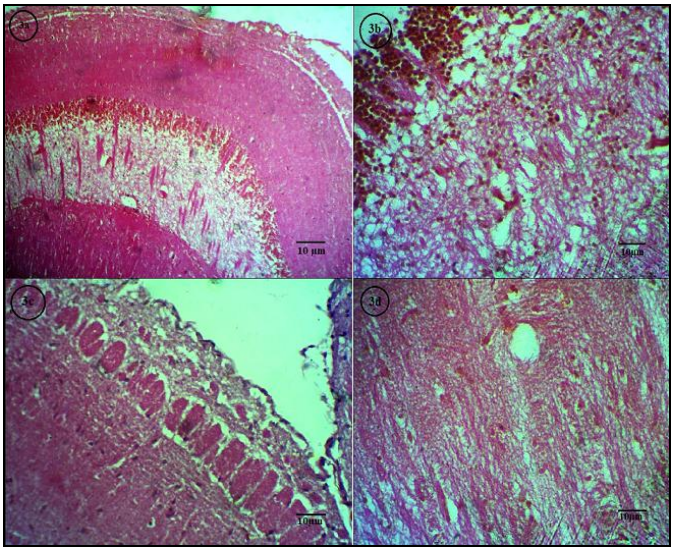

Fig 3: Histomorphology of brain tissue exposed to Al_2O_3NPs in *Oreochromis mossambicus*. 3a-Brain control; 3b: Al_2O_3NPs at 4mg/L exposed for 96 h showing mild neurodegeneration and vacuolization; 3c: Al_2O_3NPs at 4mg/L exposed for 60 days showing severe neurodegeneration; 3d: Treatment withdrawal showing vacuolization and severe neurodegeneration

4. Discussion

One of the global environmental issues of the recent years is aquatic pollution and its ecotoxicological impacts. There are several measures to detect the endpoints of pollutant toxicity, which also includes histology as biomarker. Histopathological changes in animal tissues are the most reliable and direct indicator of aquatic pollution and it is the easiest method for evaluating both short-term and long-term toxic effects of pollutants both in laboratory and in field conditions (Hinton, 1995) [14]. Morphological changes in tissues are considered as an early warning signs of disease in the organisms. In aquatic ecosystem, chronic exposure to contaminants at sublethal concentrations alters the structural architecture of tissues rather than causing mortality of the fish. Therefore, analysis of histological changes in different tissues of fish such as gill, liver, muscle, kidney and brain has been widely used for decades to assess aquatic toxicology and also to provide additional information to physico-chemical analyses (van Dyk *et al*., 2009) [15]. The primary objective of the present study was to determine the histological alterations induced by short-term and long-term exposure to Al_2O_3NPs in gill, liver and brain tissues of the fish *Oreochromis mossambicus* and in addition to examine if the structural changes induced by nanoparticles can be reversed on treatment withdrawal.

Fish gill respond to the toxicants primarily by secreting excess of mucous and this prevent the entry of pollutants into the body of animal by creating a physical barrier (Sheperd, 1982) [16]. Al_2O_3NPs exposure for 96 h caused lesions in gill morphology such as epithelial upliftment, hypertrophy and hyperplasia of gill arches, aneurysm, lamellar curling and loss

of secondary lamella. Lifting of the gill epithelium away from the basement membrane is the most common lesion noted as a result of toxicant exposure. This is the regular mechanism that occurs in gill tissues in order to reduce respiratory gas exchange by increasing diffusion and decreasing interlamellar distance. Mucous deposition, vacuolization, hyperplasia, aneurysm, and absence of secondary lamellae observed after 60 days of nanoparticles exposure is another commonly reported responses. The severity of morphological alterations is time-dependent and the reversal of treatment for 60 days in toxicant-free medium showed similar lesions as that of treatment groups. The persistence of nanoparticles induced toxicity was evident by the alterations in gill structure after treatment withdrawal. The same changes have also been reported in the gill of rainbow trout when chronically exposed to silver nanoparticles (Johari *et al*., 2015) [17], titanium dioxide nanoparticles (Federici *et al*., 2007) [18] and in zebrafish treated with copper nanoparticles (Griffitt *et al*., 2007) [19].

Detoxification process that occurs in liver plays a key role in biochemical transformations of pollutant. Due to the exposure and accumulation of toxicants either for short-term or long-term durations could cause histopathological changes in liver tissues. Since metabolism and excretion of toxicants occur in liver tissues, the nanoparticles exposure is expected to affect the hepatic architecture. There are some general pathological changes reported in fish liver that account for the toxicity and other functional impairment that includes vacuolization, swelling of hepatocytes, focal necrosis, disorganization of parenchyma, change in shape of nuclei (Hibya, 1982) [20]. In the present study, the liver of Al_2O_3NPs treated groups showed hepatic lesions after short-term and long-term exposures. Al_2O_3NPs treated for 96 h showed segmentation in hepatocytes and mild degeneration along with spindle shaped nucleus. Vacuolization is common in hepatocytes, which may contain lipids and glycogen that are important for normal metabolism (Camargo and Martinez, 2007) [21]. Increased vacuolization is also known as fatty change where the fatty vacuole formed in the cytoplasm drive the nucleus to the periphery of the cell as general response of hepatocytes towards toxicants (van Dyk *et al*., 2007) [22]. After 60 days of nanoparticles exposure, severe vacuolization and necrosis were observed and this could be due to the imbalance between rate of synthesis and rate of release of substances to the circulation. Reversibility of lesions was not observed after the treatment withdrawal for 60 days and this indicate irrevocable degeneration of hepatocytes which was evident by spindle nucleus and severe atrophy. Spindle shaped nuclei has been reported earlier in hepatocytes of Al_2O_3NPs treated fish, which could be due to the altered liver metabolism and increased biotransformation of toxicants in the liver (Braunbeck *et al*., 1990) [23]. Accumulation of Al_2O_3NPs in the hepatocytes along with histological anomalies like necrosis, vacuolization, aggregation of blood cells and melanomacrophages has been noted in *Oreochromis mossambicus* exposed to 120, 150 and 180 ppm of Al_2O_3NPs for 96 h (Murali *et al*., 2017) [9]. Nanoparticles induced hepatic lesions has been observed in the fish *Pseudetroplus maculatus* treated with fullerene C_{60} (Sumi and Chitra, 2017) [24].

In the present study, histological alterations observed in brain after 96 h of nanoparticles exposure includes mild degeneration in neural cells of cerebrum. In teleost fishes, the cerebrum is relatively large and contained distinct layer of neurons distributed throughout. After 60 days of Al_2O_3NPs treatment period showed severe degenerative changes and intracellular edema. This progressive toxicity clearly indicates that the nanoparticles can cross through blood brain barrier, which get accumulated in the tissues thereby causing damage to brain. The results coincides with induction of neurotoxicity and depletion of acetylcholinesterase enzyme observed after sublethal exposure of Al_2O_3NPs (Vidya and Chitra, 2018) [11]. Zinc oxide nanoparticles exposure for 96 h has been shown to cause neurotoxicity as evidenced by alteration in histopathology of brain tissue and impaired behavior in the fish, *Oreochromis mossambicus* (Suganthi *et al*., 2015) [25]. The persistent damage of brain tissue due to Al_2O_3NPs was marked by irrevocable morphological lesions after the treatment withdrawal for 60 days thereby proving the neurotoxic property of the nanoparticles.

5. Conclusions

In brief, the present study concludes that sublethal concentration of Al_2O_3NPs induced irreversible histopathological lesions in the gill, liver and brain of the fish *Oreochromis mossambicus*, which are time-dependent. The severity of morphological damage could cause several physiological and biochemical alterations thereby result in organ damage. The present results can contribute to environmental safety assessment in order to reduce the production, use and release of nanoparticles nearby the aquatic ecosystem so as to protect the fish population and also humans indirectly through food chain.

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