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Sublethal effects of silicon dioxide nanoparticles on the structure of gill, liver and brain tissues in the fish, *Oreochromis mossambicus* (Peters, 1852)

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Abstract
Nano-sized silica particles (SiO_2NPs) that are largely released into the aquatic environment are known to associate with naturally occurring sediments or colloids to find their way into cellular compartments, thereby causing harmful effects on aquatic organisms. Among several parameters used to assess the adverse toxic effects of nanoparticles on aquatic organisms, histology is the direct and sensitive tool to determine the tissue damage. In the present study SiO_2NPs at sublethal concentration i.e., 12 mg/ L were exposed to the freshwater fish, *Oreochromis mossambicus* for 96 h and 60 days. After the exposure period, histological alterations in the gill, liver and brain tissues were examined and compared with that of control tissues. Treatment withdrawal was also carried out for 60 days in order to determine if the exposed nanoparticles undergo recovery after certain period of time. Nanoparticles treatment showed gross damage in gill, liver and brain tissues and the extent of severity of tissue damages were time-dependent. Gill tissue showed pathological changes such as upliftment of gill epithelium along with mucous deposition, damage and vacuolization in gill arches, aneurysm, absence and curling of secondary lamella. Major hepatic lesions includes loss of normal tissue architecture, segmentation of hepatocytes, vacuolization, spindle shaped nucleus and necrosis. Brain tissue of the treated fish showed degeneration of nerve cells, formation of vacuolization, cerebral edema, necrosis of neurofibrillar region and lesion in choroid plexus. The morphological changes caused by nanoparticles remained unchanged after the treatment withdrawal period suggesting irreparable architectural damage. The study benefits to contribute for environmental screening and monitoring programs to restrict the production and use of nanoparticles that naturally cause environmental variations.

Keywords: SiO_2NPs, gill, liver, brain, histopathology, *Oreochromis mossambicus*, treatment withdrawal

1. Introduction
Silicon dioxide nanoparticles (SiO_2NPs) also known as silica nanoparticles or nanosilica possess wide range of biomedical applications based on its ability to functionalize with range of polymers and other molecules. According to the structure, SiO_2NPs are classified as P-type and S-type, where P-type particles have numerous nanopores and S-type particles have comparatively smaller surface area. SiO_2NPs have been widely used in enzyme immobilization, bioseparation, biosensors, immunoassays [1], DNA detection, separation and purification of drug and gene delivery [2-4] and cancer therapy [5]. The unique physico-chemical properties such as small size, large surface area, structural discrimination, active function, dose, treatment time and the cell type under study are some of the factors behind the toxicity of silicon dioxide nanoparticles [6]. Large surface area of nanoparticles signifies an increase in surface reactivity, which enables the nanoparticles to interact with cell biomolecules [7]. Besides, SiO_2NPs are also used in industrial purposes, dietary supplements, cosmetics, drugs and pharmacies; therefore, the risk of human exposure is high through food, medicine or personal care products. Thus the fate and effect of such ubiquitous nanoparticles exposed to the aquatic ecosystems is of great concern because the risks to aquatic organisms directly or indirectly affect the human health through the food chain.
Among the aquatic organisms, fish is considered as the good indicator of water quality as it is sensitive to the changes in water chemistry such as, pH, dissolved oxygen, salinity and temperature, which may be altered by several natural factors or pollutant exposure. Increase

in the use of nanoparticles pose risk to non-target organisms including fish. Several studies demonstrated toxicological responses of SiO_2NPs in various organisms and are reported as hepatotoxicant [8], neurotoxicant [9, 10], cytotoxicant [11], genotoxicant [12] and also altered oxygen consumption in freshwater fish, *Oreochromis mossambicus* [13]. Such alterations in the cellular, genetic and biochemical levels could possibly induce changes in the morphology and functional efficiency of vital organs. Therefore, to establish a casual relationship between the pollutants exposed and biological response, histopathological examination is considered as one of the sensitive and reliable biomarkers [14]. Over the past several decades, evaluation of histological changes in different tissues has been used as the semi-quantitative determination of tissue abnormalities in fish and also reflects the overall health status of entire population in the ecosystems.

In general, the mode of toxic response can be detected by concentration-response relationship. Exposure to sublethal concentration for long duration helps to predict the chronic toxic response of the pollutants whereas the short duration provides the immediate mode of toxic action. The present study determines both primary mode of toxic effect of nanoparticles after 96 h as well as compares the chronic toxicity after 60 days of toxicant exposure. SiO_2NPs has been earlier reported to induce histopathological alterations in rats when exposed for a period of 60 days [15]. In teleost fish, gill, liver and brain tissues are most frequently used in ecological, toxicological and pathological studies [16]. The present study also evaluates the histopathological alterations induced by SiO_2NPs in gill, liver and brain tissues of the freshwater fish, *Oreochromis mossambicus*. Recovery of functional and morphological impairment of tissues due to nanoparticles exposure was also carried out by the treatment withdrawal for a period of 60 days in order to prove the elimination of nanoparticles when the exposed fish is brought to the normal condition. Thus the study attempted to state the adverse toxic effects and its persistence in the gill, liver and brain tissues of the fish, *Oreochomis mossambicus* by using histology as biomarkers.

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). Prior to experiments, fish were acclimatized to the laboratory conditions providing good aeration, light and dechlorinated water (40 L capacity). The health status of the fish was observed frequently by maintaining the physico-chemical parameters of the tap water as per APHA guidelines [17]. Water temperature (28 ± 2 °C), oxygen saturation of water (70 and 100 %), pH (6.5 to 7.5) was maintained throughout the experiment in both control and treatment groups.

2.2 Test chemical and toxicity testing

SiO_2NPs (Cat. No: 1940323) were obtained from SISCO Research Laboratory (SRL), India. The pre-characterised SiO_2 nanoparticles of 1nm size were used for the present study [18]. The nanodispersions were prepared just before exposure by ultra-sonication at 100 kHz for 10 min using double distilled water and maintained as stock. One-tenth of median lethal concentration (LC_{50}-96 h) i.e., 12 mg/L was used as the test concentration and exposed to fish for 96 h (short-term) and 60 days (long-term) durations with respective control group and treatment reversal group for 60 days.

2.3 Histology of tissues

After the end of every exposure period, gill, liver and brain tissues were collected. Tissues 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 then transferred to molten paraffin wax for an hour 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 were observed under light microscope and were compared with that of control tissues. Photomicrographs were taken using Cannon shot camera fitted to the Carl Zeiss Axioscope 2 Plus Trinocular Research Microscope.

3. Results

3.1 Effects of SiO_2NPs on histology of gill tissue

Examination of gill histopathology reveals that exposure to SiO_2NPs for 96 h resulted in excess mucous deposition, gill epithelial upliftment, vacuolization in gill arches, aneurysm, absence and curling of secondary lamellae (Figure 1b). When the exposure period was increased to 60 days showed severe damages such as complete injury of gill filaments, mucous deposition, vacuolization, aneurysm, loss of secondary lamella and loss of chloride cells when compared to the control tissue (Figure 1a and 1c). Morphological alteration observed in gill tissue did not recover to normal when the treatment was withdrawn for 60 days (Figure 1d).

3.2 Effects of SiO_2NPs on histology of liver tissue

Control liver showed normal morphology and structure with centrally placed nucleus in the hepatocytes (Figure 2a). Liver tissue exposed to SiO_2NPs for short-term duration resulted in segmented hepatocytes and spindle shaped nucleus (Figure 2b). Exposure of SiO_2NPs for 60 days showed severe vacuolization, absence of nucleus and completely disorganized hepatocytes (Figure 2c). Reversal of the treatment for 60 days showed no changes in the deformities formed and the structural damage persist similar to the treatment groups (Figure 2d).

3.3 Effects of SiO_2NPs on histology of brain tissue

The brain tissue of short-term exposed fish resulted in the mild degeneration of nerve cells whereas long-term exposure to SiO_2NPs were observed with severe neurodegeneration, formation of vacuolization, cerebral edema, necrosis of neurofibrillar region and lesion in choroid plexus (Figures 3b and 3c). None of these lesions were observed in the brain of the control fish (Figure 3a). Treatment withdrawal group showed similar damages in brain tissue like that of treatment groups (Figure 3d).

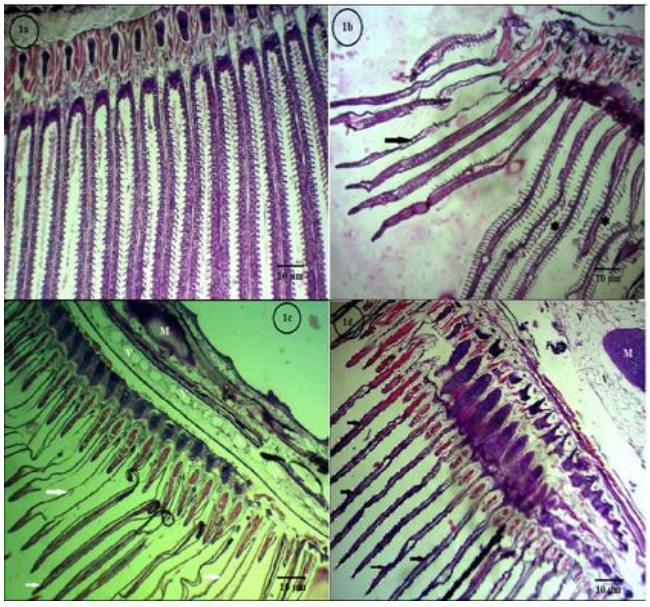

Fig 1: Histomorphology of gill tissue exposed to SiO_2NPs in *Oreochromis mossambicus*. 1a-Gill Control; 1b: SiO_2NPs at 12mg/L exposed for 96 h showing aneurysm (A), absence of secondary lamellae (→), curling of secondary lamellae (*); 1c: SiO_2NPs at 12mg/L exposed for 60 days showing mucous deposition (M), vacuolization (V), absence of secondary lamellae (→);1d: Treatment withdrawal showing mucous deposition (M), vacuolization (V), absence of secondary lamellae (→)

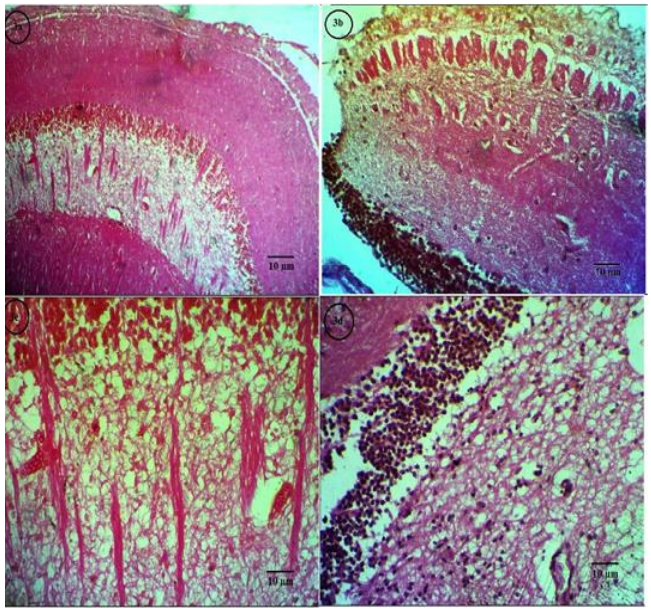

Fig 2: Histomorphology of brain tissue exposed to SiO_2NPs in *Oreochromis mossambicus*. 3a-Brain Control; 3b: SiO_2NPs at 12mg/L exposed for 96 h showing mild neurodegeneration; 3c: SiO_2NPs at 12mg/L exposed for 60 days showing severe neurodegeneration as lesion of choroid plexus; 3d: Treatment withdrawal showing vacuolization and severe neurodegeneration

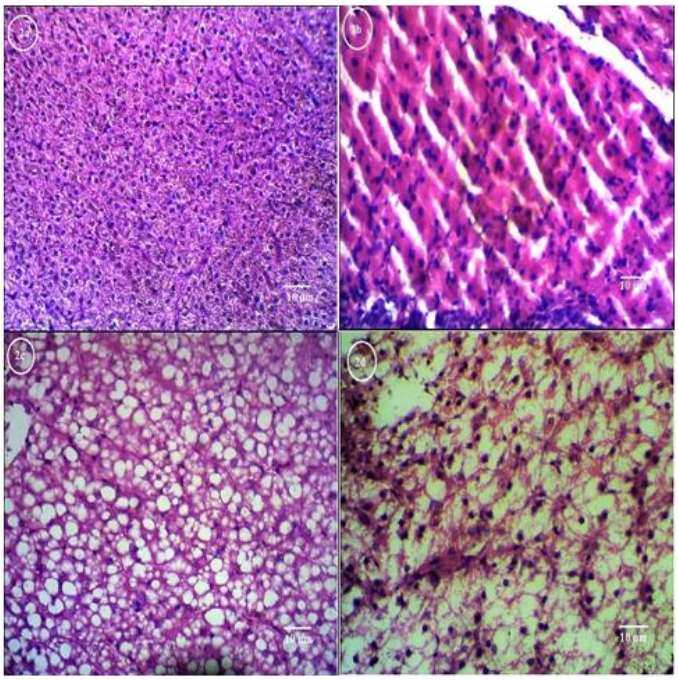

Fig 3: Histomorphology of liver tissue exposed to SiO_2NPs in *Oreochromis mossambicus*. 2a-Liver Control; 2b: SiO_2NPs at 12mg/L exposed for 96 h showing segmented hepatocytes and spindle nucleus; 2c: SiO_2NPs at 12mg/L exposed for 60 days showing vacuolization; 2d: Treatment withdrawal showing severe necrosis

4. Discussion

In toxicity studies, histopathology is the only reliable and unbiased tool that clearly discriminates between toxicant-induced lesions and normal variations in structure of tissues. Histological characteristic of specific organs is the easiest method to assess both short and long-term toxic effects of pollutants [19]. In the present study, the histopathological effects of SiO_2NPs was evaluated in the gill, liver and brain tissues of the fish, *Oreochromis mossambicus* for short-term (96 h) and long-term (60 days) duration. Nanoparticles toxicity was found to be irreversible when the fish was maintained without toxicant exposure after the treatment period for another 60 days.

In fish, gill is the primary target organ of many toxicants as the absorption of all contaminants occurs mainly through the gills that are continuously in contact with water [20]. The large surface area and presence of mucous cells are the salient features of gill, which largely help in avoiding toxicants. Gill secretes excess amount of mucous on contact with any foreign material thus mucous secretion is the first

level of defensive mechanism against the toxicant [21]. Gill epithelium is the major site for gaseous exchange, ionic regulation, nitrogenous waste excretion and acid-base balance in fishes [22, 23]. Hence they are supplied with blood directly from heart through ventral aorta, and the gill epithelium consisting of gill filaments and secondary lamella are active site of gas exchange between blood and water [24]. In the present study, gill exposed to SiO_2NPs for 96 h leads to excess deposition of mucous and upliftment of gill epithelium, which indicates that gill tissue tried to overcome the entry of toxicant by secreting mucous between the epithelial layers, and uplifting the epithelia. Severe damages including vacuolization in gill arches, aneurysm, absence and curling of secondary lamellae and loss of chloride cells was observed when the exposure period increased to 60 days. The results suggest that nanoparticles could adversely affect the gill tissue, which is involved to perform vital functions such as respiration, osmoregulation and excretion. Our previous study has reported that SiO_2NPs induced changes in the rate of oxygen consumption and impairment of respiratory and oxidative metabolism in *Oreochromis mossambicus* [13]. Likewise, consistent observations have been reported when *Pseudertroplus maculatus* was exposed to one of the nanoparticles, fullerene C_{60} at 0.1mg/L concentration for 96 h [25]. Treatment withdrawal for 60 days did not recover gill tissue from any morphological damage thereby suggests that nanoparticles caused permanent irreversible damage to gill tissue of the fish, *Oreochromis mossambicus.*

Liver is the major organ for metabolism, biochemical transformation of pollutants and detoxification, and hence histopathology of hepatocytes is used as reliable biomarkers in toxicity studies [26]. Fish liver is sensitive to the pollutant because of accumulation in liver at several orders of magnitude than in the environment which could in turn provoke severe histological lesions. SiO_2NPs exposure for 96 h showed histological alterations as segmented hepatocytes and spindle shaped nucleus. The severity of morphological tissue damage was increased when SiO_2NPs was exposed for 60 days with notable changes like severe vacuolization, absence of nucleus and completely disorganized hepatocytes. Vacuoles in the cytoplasm of the hepatocytes contain lipids and glycogen, which are associated to the normal metabolic function of the liver [27]. The vacuolization of hepatocytes, therefore, indicates an imbalance between the rate of synthesis of substances in the parenchymal cells and the rate of release into the systemic circulation [28]. Vacuolization is also considered as a signal of degenerative process that suggests metabolic errors possibly due to the exposure of pollutants [29]. Nuclear atrophy which is evident after SiO_2NPs exposure occurs as a result of hepatic vacuolization and this could be the cause of oxidative damage and induction of oxidative stress [30]. Similar observations has been reported when silver nanoparticles was exposed to rainbow trout, *Oncorhynchus mykiss* for 21 days [31]. Disorganization and degeneration or necrosis of hepatic parenchyma is the direct effect of toxicant exposure and are generally irreversible, and the persistence or progression may lead to a partial or total loss of organ function [32]. The results correspond to severe structural deformities as persistent necrosis in the hepatocytes of the fish when the treatment was withdrawn for 60 days.

The cerebrum of teleost consists of distinctive layer of neurons embedded on a neuropil that contained the processes of neuroglial and other neuronal cells [33]. In the present study, short-term exposure to SiO_2NPs showed mild degeneration of nerve cells whereas long-term exposure caused severe neurodegeneration, formation of vacuolization, cerebral edema, necrosis of neurofibrillar region and lesion in choroid plexus. Simple columnar epithelium of choroid plexus encloses neuronal cells which synthesize the components of cerebrospinal fluid that are secreted into the lumen of ventricles [34]. Nanoparticles-induced lesion of choroid plexus indicates pathological brain injury that more likely leads to sublethal neurological disturbance in the fish. The pathological lesions were found retained even after the treatment withdrawal thereby specifies neurotoxic effect of SiO_2NPs in the fish. Similar results have been reported in *Pseudetroplus maculatus* when exposed to sublethal concentration of chlordecone [35].

5. Conclusion

The alterations in the morphology of the gill, liver and brain tissues indicate direct effect of SiO_2NPs in the fish, *Oreochromis mossambicus*. Nanopaticles exert permanent pathological damages as evident by persistence of lesions after the treatment withdrawal. Therefore, more attention should be paid to reduce the excessive production and release of nanoparticles into the aquatic environment or else may seriously affect the fitness and survival of the fish population.

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