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Irreversible Nanotoxicity of Silicon Dioxide Nanoparticles in the Freshwater Fish, *Oreochromis mossambicus* (Peters 1852)

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Abstract

Silicon dioxide nanoparticles (SiO_2-NPs), is widely used in the fields of medicine, engineering and industries. In the present study, sublethal concentration (12 $mg.L^{-1}$) of SiO_2-NPs was exposed to the freshwater fish, *Oreochromis mossambicus* (Peters 1852) for short-term (24, 72 and 96 h) and long-term (15, 30 and 60 days) durations, maintaining a control group. Gill tissues showed significant ($P < 0.05$) reduction in the activities of antioxidant enzymes, superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase with a concomitant increase in the production of hydrogen peroxide and lipid peroxidation. Liver tissue also showed induction of oxidative stress which was evident by the significant decrease in the activities of antioxidant enzymes and generation of hydrogen peroxide and lipid peroxidation. In brain tissue, the alteration of antioxidant defence system was more prominent only after 96 h. The activities of tissue marker enzymes were also decreased in gill, liver and brain tissues indicating tissue-specific nanotoxicity. The reversible of treatment for 60 days showed no withdrawal effect of nanoparticles in all tissues. The present results conclude that exposure to silicon dioxide nanoparticles induced oxidative stress and the effects are found irreversible in the gill, liver and brain tissues of the fish, *O. mossambicus*.

Keywords: silicon dioxide nanoparticles, oxidative stress, gill, liver, brain, *Oreochromis mossambicus*

Introduction

Nanotechnology is the rapidly developing multidisciplinary science that includes the fields of physics, chemistry, biology and engineering, which involves the production and release of several nano-sized particles into the environment. Some nanoparticles are known to exist naturally and are therefore, in direct and continuous contact with the living systems.

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While several nanoparticles are synthesised artificially and the increase in the rate of synthesis and the intentional or unintentional release of nanoparticles into the ecosystem could adversely affect living organisms including bacteria, algae, insects, birds and mammals. The properties, behaviour and environmental fate of engineered nanoparticles are different, which lead to an unforeseen impact on environment and living system (Oberdörster et al. 2005). Engineered nanoparticles include nanotubes, nanospheres, nanowires, quantum dots and are characterized for its novel physical, optical, thermal and biological properties. Among the creation of manipulated nanoparticles, silicon dioxide nanoparticles (SiO_2-NPs; nano silica) are widely used in engineering, industries and biomedicine, particularly as tools for targeted drug and gene delivery. However, the toxicity of silica nanoparticles may be due to altered physicochemical properties such as size, cell type, dose and even specific coatings, ligand incorporation, or surface modifications, which lead to health hazards in non-target organisms (Nowack and Bucheli 2007).

Silicon dioxide nanoparticles are the intentionally produced nanoparticles having applications in the biomedical fields. It is widely used for *in vitro* and *in vivo* drug and gene delivery, siRNA delivery, biosensing, sunscreen lotions, food, nanomedicine, cancer therapy and chemical industries (Slowing et al. 2007; Li et al. 2012).The main route of exposure is from different sources like manufacturing laboratories, sewage treatment plants, landfills and runoff water. Owing to the small size, silica nanoparticles have possibilities of internalisation through different penetration routes and it has been reported that nanosized particles are more toxic than the bulked size (Kim et al. 2014). Silica nanoparticles are highly stable and persistent, where the particle size influences the toxicity, tissue distribution, metabolism and excretion. One of the studies has observed that submicron-sized silica particles (100 or 200 nm diameter) significantly increased the incidence and severity of liver inflammation, whereas the effects of nano-sized particles (50 nm diameter) were non-significant (Cho et al. 2009). On the contrary, silica nanoparticles of 7–14 nm size has been shown to cause potentially harmful effects on fish hepatocytes by the generation of reactive oxygen species (Vidya and Chitra 2015).

Therefore, the toxicity and environmental persistence of nanoparticles in living organisms depend on various factors such as stability, bio-persistence and bioaccumulation properties (Reeves et al. 2010; Zhu et al. 2010; Dobias and Latmani 2013).Once released into the environment, the particles will interact with the surroundings such as air, water and land. It has been earlier reported that silicon dioxide nanoparticles also cross the blood-brain barrier in mice (Kim et al. 2006). *In vitro* studies, using human cell line models also reported that SiO_2-NPs induced mitochondrial dysfunction, oxidative stress and apoptosis (Wang et al. 2009). In endothelial EAHY926 cell line, SiO_2 exposure resulted in decreased cell viability and cytotoxicity (Napierska et al. 2009). In the rat, intranasal/administration of silicon dioxide nanoparticles resulted in hepatotoxicity characterized by depletion of antioxidant system and loss of normal liver architecture (Parveen et al. 2012). Similarly, one of the nanomaterials fullerene C_{60} has been shown to induce oxidative stress in hepatocytes of the freshwater fish, *Pseudetroplus maculatus* (Bloch, 1795) (Sumi and Chitra 2017a).

Green synthesis of silver nanoparticles from *Piper nigrum* at sublethal concentrations has led to the accumulation of silver and induced oxidative stress and histopathological alterations in gill, liver and kidney of the fish, *Labeo rohita* (Hamilton 1822) (Shobana et al. 2018). Aquatic organisms, especially fishes, are highly susceptible to the uptake of nanoparticles from the exposed aquatic environment. However, the cells of fish are highly equipped with antioxidant defence system to eradicate the toxicant-induced oxidative stress. The antioxidant defence system consists of both enzymatic and non-enzymatic defensive mechanisms. The non-enzymatic antioxidant system for example consists of vitamin C, vitamin E, carotenoids, amino acids and the enzymatic antioxidant system includes superoxide dismutase, catalase, glutathione reductase and peroxidase and others (Martinez-Alvarez et al. 2005). Impairment of antioxidant system leads to the generation of reactive oxygen species and oxidative stress, which are responsible for the peroxidation of membrane lipids and thereby causing tissue damages (Fridovich 1978).

The present study was carried out to investigate the toxicity of silicon dioxide nanoparticles at sublethal concentration on the antioxidant system in the freshwater fish, *Oreochromis mossambicus* (Peters 1852). The study critically evaluated the toxic effects of SiO_2 nanoparticles in different vital tissues such as gill, liver and brain. In addition, the reversal of treatment was also performed in order to determine if the nanotoxicity is withdrawn after a specific time interval. The results obtained from the fish model can be considered as an indirect endpoint to extrapolate the impact of such nanoparticles on higher organisms, including human as fish occupy prime role in the food chain of humans.

Materials and Methods

Test animal

Oreochromis mossambicus weighing 6 ± 1.5 g and length 6.5 ± 1 cm were collected from the local fish farm, Safa Aquarium, Kozhikode, Kerala (11º22'N, 75º85'E). Fishes were acclimatised in glass tanks of 40 L capacity, supplied with good aeration, light system (12h:12h; light:dark) and dechlorinated water prior to the experiment for 2 weeks. The physico-chemical features of the tap water such as temperature (28 ± 2 °C), oxygen saturation (70 and 100 %), pH (6.5 to 7.5) was maintained throughout the experiment in both control and treated groups according to APHA guidelines (1998).

Test chemical and experiment design

SiO_2-NPs (Cat. No: 1940323) was purchased from SISCO Research Laboratory (SRL), India. The pre-characterised SiO_2 nanoparticles of 1 nm size are used for toxicity testing (Vidya and Chitra 2017). The nanodispersions were prepared just before exposure by ultra-sonication at 100 kHz for 10 min using double distilled water and maintained as stock. Median lethal concentration (LC_{50}-96 h) of SiO_2-NPs determined by probit analysis was 120 $mg.L^{-1}$ (Vidya and Chitra 2017).

Sublethal concentration, i.e., $1/10^{th}$ of LC_{50} (12 $mg.L^{-1}$) was selected in the present study for the toxicity evaluation. The concentration was exposed for short-term (24, 72 and 96 h) and for long-term (15, 30 and 60 days) durations, maintaining a control group. For testing treatment withdrawal, after exposure to nanoparticles for 60 days, the group was maintained without toxicant for another 60 days so as to evaluate the reversal of nanotoxicity.

Preparation of test samples

Health conditions of fish were continuously monitored throughout the study in all experimental groups. In each group, 10 fish were maintained along with a control group. At the end of every treatment period, fish were caught from glass tanks using small dip nets with least disturbances in order to avoid stress. Fishes were killed by decapitation and the tissues such as gill, liver and brain were dissected out to cold normal saline and cleaned. The weights of fish along with tissue weights were recorded and 1 % tissue homogenates were prepared in ice cold saline using a motor driven tissue homogeniser. The homogenates were centrifuged at 800 *g* for 15 min at 4 °C to obtain supernatants, which were then used for various biochemical analyses.

Biochemical analysis

Total protein was estimated by the method of Lowry et al. (1951). Activities of antioxidant enzymes as superoxide dismutase -EC 1.15.1.1 (Marklund and Marklund 1974), catalase - EC. 1.11.1.6 (Claiborne 1985), glutathione reductase - EC. 1.6.4.2 (Carlberg and Mannervik 1985), glutathione peroxidase - EC.1.11.1.9 (Mohandas et al. 1984), levels of hydrogen peroxide generation (Pick and Keisari 1981) and lipid peroxidation (Ohkawa et al. 1979) were determined in gill, liver and brain tissues. The activities of alkaline phosphatase -EC.3.1.3.1 (Bessey et al. 1946) and acetylcholinesterase - EC.3.1.1.7 (Ellman et al. 1961) was also evaluated in respective tissues as markers.

Statistical analyses

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test using statistical package SPSS 17.0. Differences were considered to be significant at $P < 0.05$ against the control group. Data represented as mean ± SD for 10 animals per group and all biochemical estimations were carried out in duplicate to reduce statistical error.

Results

Body weight and organ weights

Exposure to silicon dioxide nanoparticles showed significant ($P < 0.05$) reduction in the body weights of the animal only after 30 and 60 days when compared to the control group (Table 1). The reversal of treatment for 60 days showed a slight increase in the weight of the fish (Table 1).

The weight of gill showed significant ($P < 0.05$) decrease in 30 and 60 days of SiO_2-NPs exposure, whereas no changes were observed after short-term exposure (Table 1). Hepatosomatic index of silicon dioxide-treated fish showed a significant ($P < 0.05$) time-dependent decrease from 96 h of exposure onwards (Table 1). The weight of the brain tissue decreased significantly ($P < 0.05$) only after long-term exposure of nanoparticles and no significant changes were observed in the short-term exposure groups (Table 1). The treatment withdrawal showed a slight increase in the weights of all tissues, but the changes were not significant when compared to the corresponding control group (Table 1).

Table 1. Effect of silicon dioxide nanoparticles (SiO_2-NPs) on the body weight and tissue weights in the fish, *Oreochromis mossambicus* (Mean ± SD; n = 10/group; *$P < 0.05$).

Parameters	SiO_2-NPs (12 mg.L^{-1})							Treatment withdrawal (60 days)
	Short-term exposure				Long-term exposure			
	Control	24 h	72 h	96 h	15 days	30 days	60 days	
Body weight (g)	6.71 ± 0.09	6.68 ± 0.07	6.63 ± 0.08	6.64 ± 0.06	6.68 ± 0.04	6.59 ± 0.04*	6.52 ± 0.05*	6.81 ± 0.04
Gill weight (mg)	143 ± 11.9	142 ± 7.71	141 ± 4.89	140 ± 4.86	138 ± 6.90	135 ± 5.93*	132 ± 4.06*	151 ± 4.32
Hepatosomatic index (%)	1.31 ± 0.23	1.30 ± 0.13	1.28 ± 0.14	1.24 ± 0.22*	1.21 ± 0.20*	1.20 ± 0.23*	1.16 ± 0.14*	1.37 ± 0.11
Brain weight (mg)	16.5 ± 1.81	16.5 ± 1.38	16.4 ± 0.62	16.4 ± 0.88	16.1 ± 0.45*	15.9 ± 1.21*	15.6 ± 0.76*	17.2 ± 1.12

Antioxidant status in gill

Fish exposed to SiO_2-NPs showed a significant ($P < 0.05$) decrease in the activities of antioxidant enzymes as superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase in the gill tissue in both short- and long-term exposure groups in time-dependent manner when compared to the control group (Fig. 1A–D). The levels of hydrogen peroxide and lipid peroxidation showed a significant increase after short-term and long-term exposure in a time-dependent manner when compared to the control group (Fig. 1E and F). Treatment withdrawal also showed decrease in the activities of antioxidant enzymes and increase in the levels of hydrogen peroxide generation and lipid peroxidation in gill tissue (Fig. 1A–F).

Antioxidant status in liver

During the short-term exposure of nanoparticles, liver tissue showed a remarkable increase in the activities of superoxide dismutase and catalase after 24 h, however, at the end of 72 h onwards it showed a significant ($P < 0.05$) decrease in duration-dependent manner till the end of 60 days of treatment (Fig. 2A and B). The activities of glutathione reductase and glutathione peroxidase showed significant decrease in all treatment durations when compared to the corresponding control group (Fig. 2C and D).

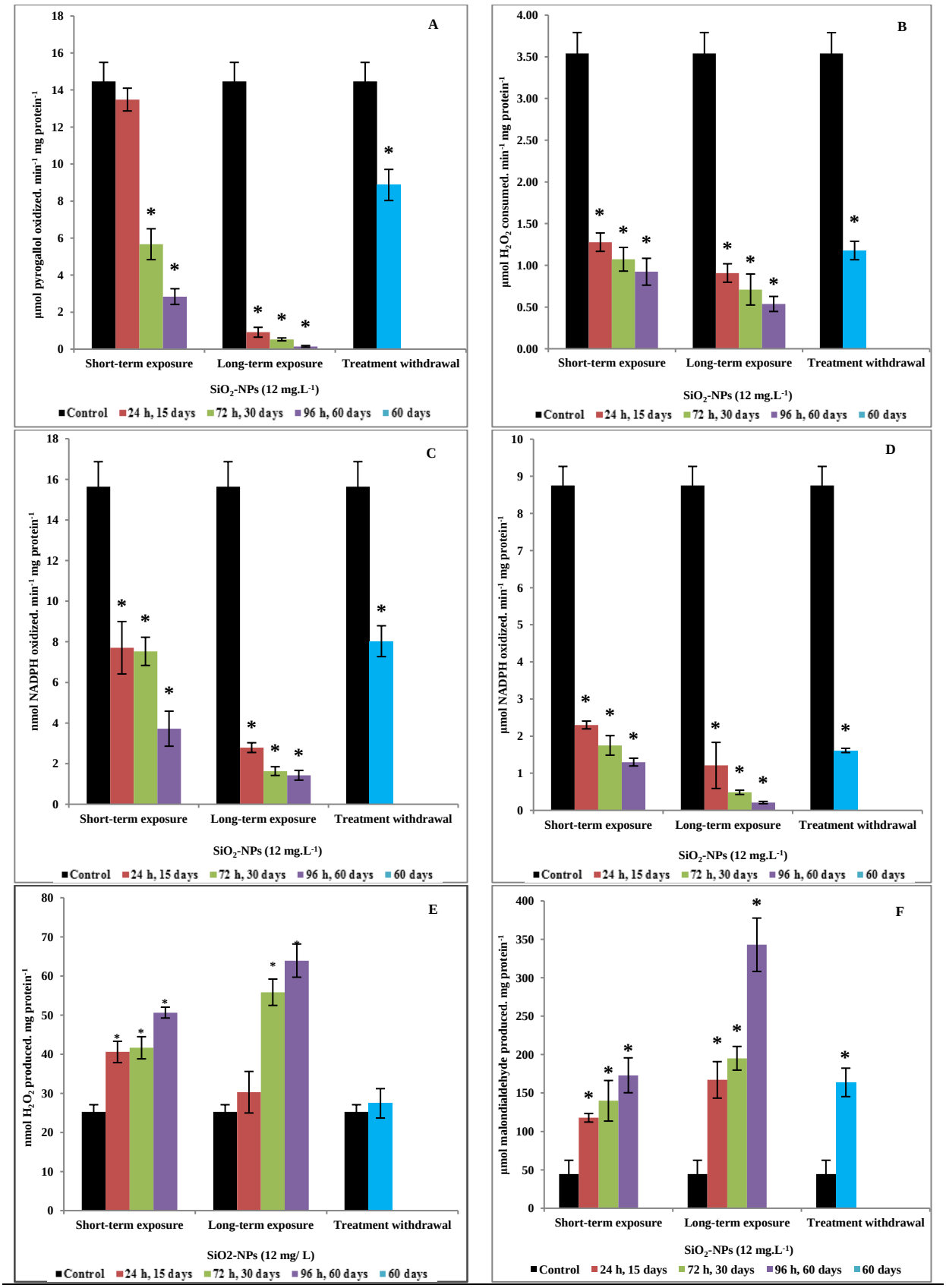

Fig. 1. Effect of silicon dioxide nanoparticles on the activities of A) superoxide dismutase; B) catalase; C) glutathione reductase; D) glutathione peroxidase; E) levels of hydrogen peroxide generation; F) lipid peroxidation in the gill tissue of *Oreochromis mossambicus.* * denotes significant difference at $P < 0.05$ against the control group.

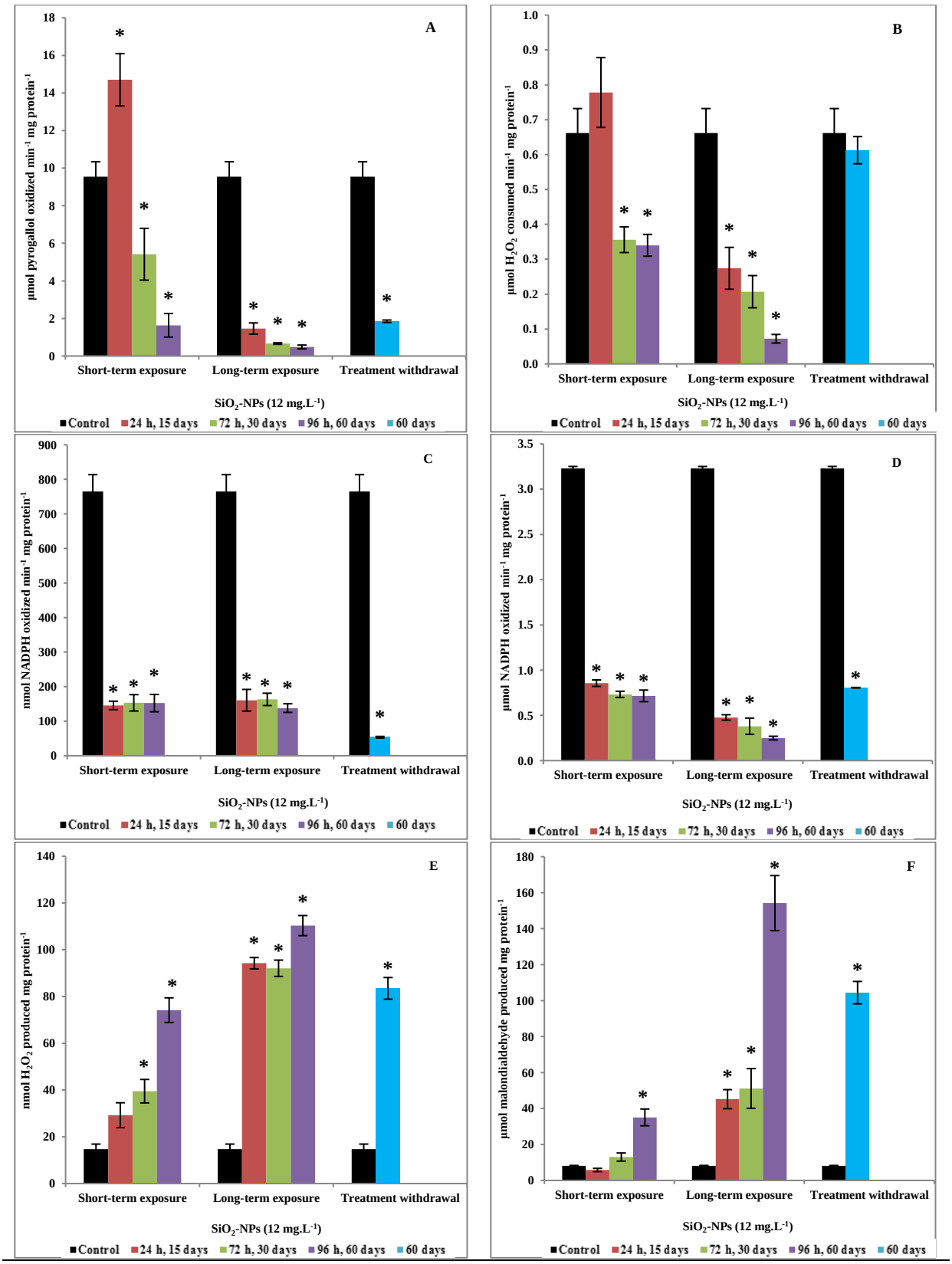

Fig. 2. Effect of silicon dioxide nanoparticles on the activities of A) superoxide dismutase; B) catalase; C) glutathione reductase; D) glutathione peroxidase; E) levels of hydrogen peroxide generation; F) lipid peroxidation in the liver tissue of *Oreochromis mossambicus.* * denotes significant difference at $P < 0.05$ against the control group.

The levels of generation of hydrogen peroxide increased significantly ($P < 0.05$) in all treatment durations, except 24 h whereas the level of lipid peroxidation showed significant increase after 96 h in time-dependent manner (Fig. 2E and F). The treatment withdrawal also showed similar changes in the antioxidant defence system of hepatocytes (Fig. 2A–F).

Antioxidant status in brain

Brain tissue showed significant ($P < 0.05$) decrease in the activities of superoxide dismutase, glutathione reductase and glutathione peroxidase after 96 h in short-term exposure group and in all durations in long-term exposure groups (Fig. 3A, C and D). However, the activity of catalase showed no significant changes in short-term groups, while a time-dependent significant ($P < 0.05$) increase was observed in all long-term exposure groups (Fig. 3B). The level of hydrogen peroxide increased significantly ($P < 0.05$) in 96 h of short-term exposure and a time-dependent increase in all durations was observed in long-term exposure groups (Fig. 3E). However, the induction of lipid peroxidation showed significant increase only after 30 and 60 days of the long-term exposure (Fig. 3F). Withdrawal of silicon dioxide nanoparticles for 60 days showed similar effects as in the case of treatment groups (Fig. 3A–F).

Activities of tissue-specific marker enzymes

The activity of alkaline phosphatase in gill showed significant decrease only after 96 h of SiO_2-NPs exposure whereas no significant changes were observed in the short-term exposure groups in hepatocytes of fish (Fig. 4A and B). Long-term exposure of nanoparticles significantly ($P < 0.05$) decreased the activity of alkaline phosphatase in both gill and liver tissues when compared to the corresponding group of control tissues (Fig. 4A and B). Similarly, the activity of acetylcholinesterase enzyme in brain tissue showed no changes after short-term exposure, while a drastic significant ($P < 0.05$) decrease was observed in long-term exposure (Fig. 4C). No reversal effects on the activities of alkaline phosphatase and acetylcholinesterase was observed when the treatment was withdrawn for 60 days (Fig. 4A–C).

Discussion

There is an increasing concern among the ecotoxicologists regarding the entry of engineered nanoparticles into the aquatic environment. Nanoparticles readily conjugate with biological molecules in the aquatic ecosystem and become water soluble, finally could cause adverse effects on aquatic organisms mainly through oxidative stress-mediated nanotoxicity. The present study evaluates the sublethal effects of silicon dioxide nanoparticles in gill, liver and brain tissues exposed for short-term and long-term durations in the fish, *O. mossambicus*. The health status of the fish was monitored by assessing the parameters like changes in the body weight, tissue weights along with an alteration in antioxidant defence system in the selected tissues. The response of each tissue to the exposed nanoparticles was different, which dependents on the mode of entry, target specificity, detoxification mechanisms of tissues and others.

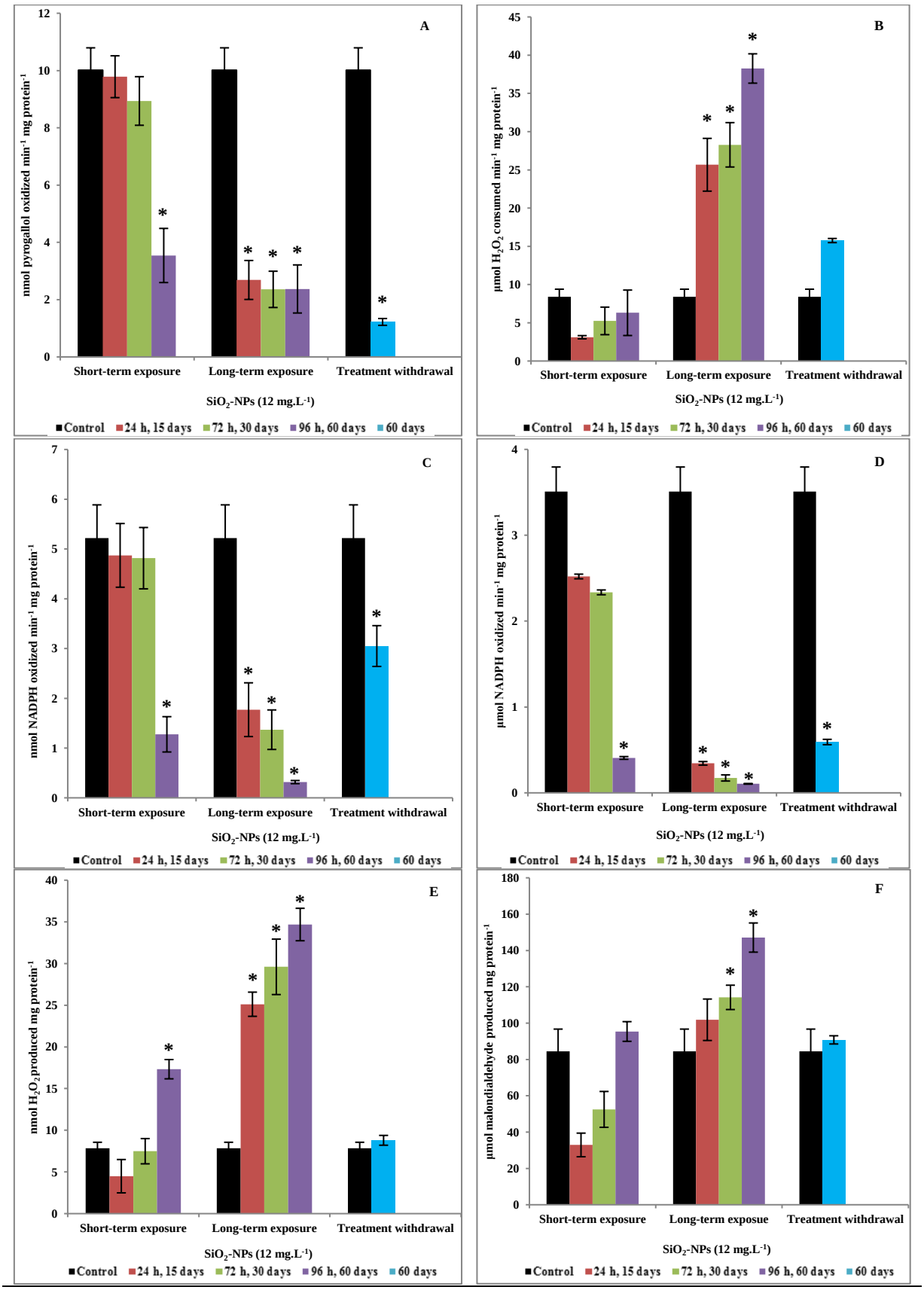

Fig. 3. Effect of silicon dioxide nanoparticles on the activities of A) superoxide dismutase; B) catalase; C) glutathione reductase; D) glutathione peroxidase; E) levels of hydrogen peroxide generation; F) lipid peroxidation in the brain tissue of *Oreochromis mossambicus*. *denotes significant difference at $P < 0.05$ against the control group.

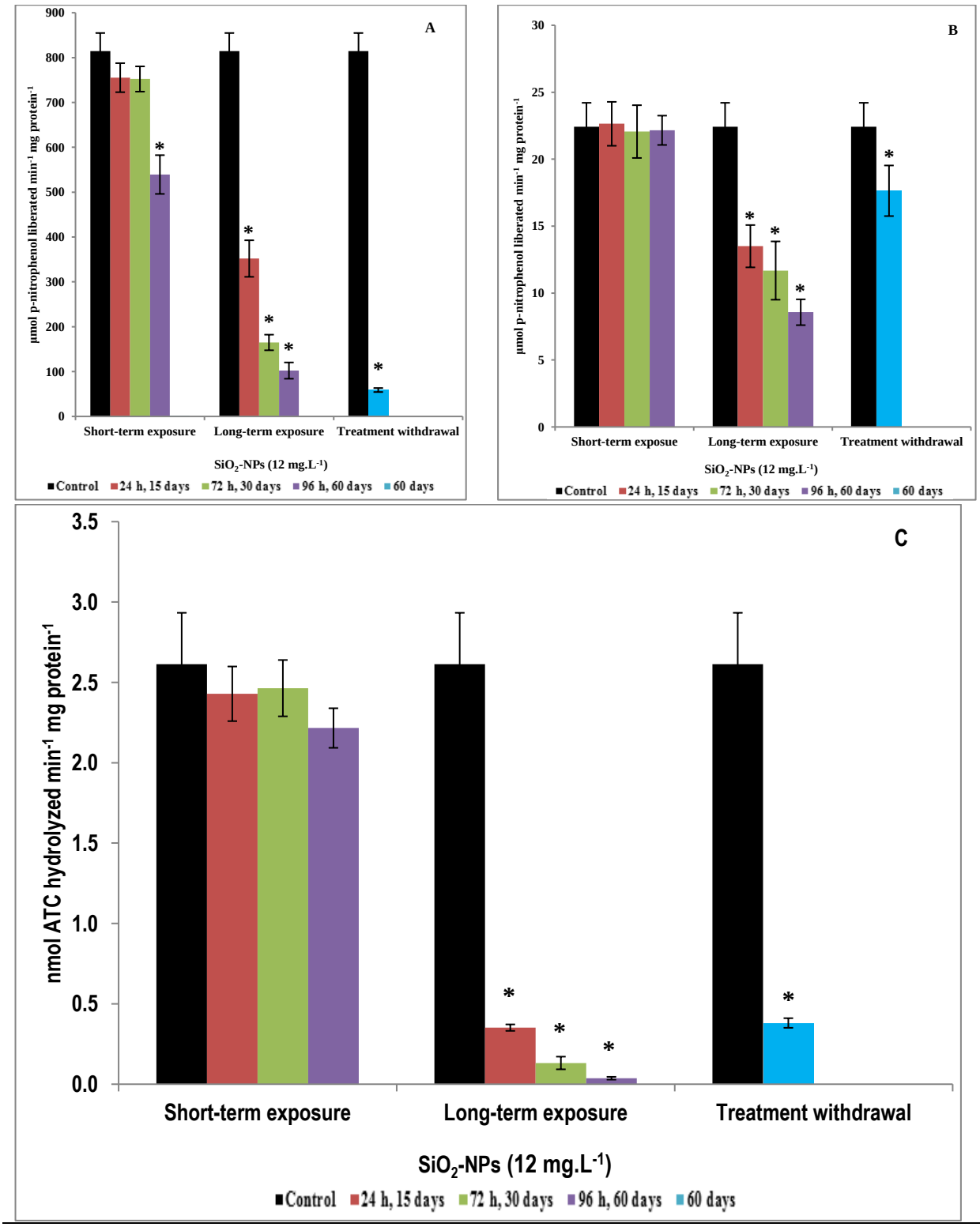

Fig. 4. Effect of silicon dioxide nanoparticles on the activities of A) alkaline phosphatase in gill tissue; B) alkaline phosphatase in liver tissue; C) acetylcholinesterase in brain tissue of the fish, *Oreochromis mossambicus.* * denotes significant difference at $P < 0.05$ against the control group.

Sublethal exposure of silicon dioxide nanoparticles showed a reduction in the weight of the fish after 30 and 60 days of treatment. The reduction in weight could be due to treatment-related anorexia or stress-induced reduction and seems to be coincident with the decrease in the weights of tissues such as gill, brain and hepatosomatic index.

One of the major toxicological mechanisms of nanoparticles includes the generation of reactive oxygen species (ROS) and induction of oxidative stress (Ahamed et al. 2010). During aerobic respiration and energy metabolism, small amounts of free radicals such as superoxide anion (O_2^-), hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2) are formed inside the cell and subcellular compartments. The free radicals together with unstable intermediates in the peroxidation of lipids are referred to as reactive oxygen species (ROS) (Sikka 2001).

Nano-sized particles have easy access to the flowing water which easily enters into the gill surface layers, penetrate into the secondary lamellae and thus fish gills are sensitive to nanoparticles (Federici et al. 2007; Sumi and Chitra 2016). The gill tissue consists of efficient radical scavenging system such as antioxidant enzymes that effectively removes the ROS. Sublethal exposure of silicon dioxide nanoparticles decreased the activities of antioxidant enzymes like superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase in the gill tissue of both short-term and long-term exposure groups. Fish under toxicant stress are susceptible to the effects of reactive oxygen species (Lushchak 2011). In the present study, the rate of ROS generation exceeds the antioxidant defence system which is evident by the decrease in the activities of antioxidant enzymes. Superoxide dismutase (SOD) is an enzyme that catalyses the dismutation of superoxide to hydrogen peroxide (H_2O_2) and oxygen (O_2). The conversion of H_2O_2 to $2H_2O$ occurs by the enzymes glutathione reductase and peroxidase whereas catalase converts H_2O_2 to O_2 and H_2O (Sikka 2001). The deleterious effects of ROS generation include oxidation of proteins, DNA damage, peroxidation of unsaturated lipids in cell membranes (Ferreira et al. 2005). Silica nanoparticles exposure increased the levels of hydrogen peroxide and lipid peroxidation in both short-term and long-term groups. Thus the imbalance between radical-generating and radical-scavenging systems resulted in a condition called oxidative stress (Sikka 2001).The present study showed good similarity with fullerene-induced lipid peroxidation in the gill of the cichlid fish, *Etroplus maculatus* (Bloch 1795) (Sumi and Chitra 2016). Alteration in gill antioxidant system also indicates impaired osmoregulation, ionic balance, circulatory and respiratory systems (Evans 1987; Vidya et al. 2016).

The liver is the prime organ for detoxification and also the major target site for toxicants. Major accumulation and metabolism of toxicants mainly take place in the liver. Exposure to toxicants can, therefore, promote the formation of ROS and oxidative stress in hepatocytes. Short-term exposure of silicon dioxide nanoparticles showed an increase in the activities of superoxide dismutase and catalase after 24 h. The results suggest that liver tissue attempted to convert the formation of free radicals, however, after 72 h onwards it showed a significant decrease in the activities of SOD and catalase, which reflects the failure of antioxidant enzymes in the suppression of free radicals formed. The activities of glutathione reductase and glutathione peroxidase showed a significant decrease in all treatment durations with concomitant increase in the levels of generation of hydrogen peroxide and lipid peroxidation. The present results showed an agreement with the observations that have found the induction of lipid peroxidation after 7 days of exposure to silicon dioxide nanoparticles in zebra fish (Ramesh et al. 2013).Similarly hepatotoxicity has been documented in the fish, *P. maculatus* after acute exposure to fullerene C_{60} nanoparticles by the alteration of the antioxidant defence system and induction of lipid peroxidation (Sumi and Chitra 2017a).

The brain tissue is responsible for receiving and interpreting signals from the peripheral nervous system and also sends a signal to other parts of the body. Brain is another important target organ for the nanoparticles as it has been reported to cross the blood-brain barrier and get deposited (Kim et al. 2006). The present study showed a significant decrease in the activities of superoxide dismutase, glutathione reductase and glutathione peroxidase after 96 h in short-term exposure group and in all durations in long-term exposure groups. However, the activity of catalase showed no significant changes in short-term groups, while a time-dependent significant increase was observed in all long-term exposure groups. In general, blood-brain barrier has been shown to selectively prevent the entry of toxicants into the brain, as it is the centre of coordination of all physiological activities. It is well known that SiO_2-NPs can cross biological membranes and are widely used for drug and gene-targeted therapy (Slowing et al. 2007). The present results suggest the imbalance of antioxidant defence system as a result of exposure to silicon dioxide nanoparticles. The level of hydrogen peroxide increased after 96 h of short-term silica nanoparticles exposure whereas a time-dependent increase was observed in all durations of long-term exposure. However, the induction of lipid peroxidation was increased only after 30 and 60 days of the long-term exposure. In short-term of nanoparticles exposure, the brain tissue facilitated to withstand the oxidative damage and the failure of defensive mechanism could have resulted in the oxidative degeneration of polyunsaturated fatty acids at the end of the exposure period. It was further proved by the imbalance of pro-oxidant and antioxidant defence system in brain tissue after chronic exposure.

Some tissue marker enzymes as the activity of alkaline phosphatase in gill and liver and acetylcholinesterase activity in brain tissue were also evaluated to assess the tissue-specific effects of nanoparticles. It was observed that treatment with SiO_2-NPs decreased the activity of alkaline phosphatase from 96 h onwards in a time-dependent manner. This could be due to the decreased state of inter and intracellular membrane transport across the gill and liver tissues (Leohner et al. 2001). The activity of acetylcholinesterase enzyme in brain tissue showed no changes after short-term exposure, while a drastic decrease in the activity of the enzyme was observed in long-term exposure. The results indicate that prolonged exposure of nanoparticles caused failure of neurotransmission due to the high accumulation of acetylcholine in neurojunction and proved silicon dioxide as neurotoxic in fish, *O. mossambicus*. The findings are in agreement with the observations of fullerene nanoparticles exposure in the brain of the fish, *P. maculatus* (Sumi and Chitra 2017b).

Reversal of SiO_2 exposure was also performed by treatment withdrawal after 60 days of exposure where fish was maintained in normal water for another 60 days. The activities of antioxidant enzymes, levels of hydrogen peroxide generation and lipid peroxidation and the activities of tissue marker enzymes did not reverse to normal thereby proving the nanotoxicity of silicon dioxide is irreversible. This could be due to the stable and persistent nature of nanoparticles or due to the failure of elimination from the body as the detoxification mechanism is irreversibly damaged and sometimes the withdrawal period of 60 days may not be adequate to reverse the toxic effects.

Conclusion

The sublethal concentration of silicon dioxide nanoparticles had a profound effect on the antioxidant defence system of fish, which proved as effective potential biomarkers to assess the nanotoxicity of engineered nanoparticles in fish. The present study also reveals that nanoparticles-induced oxidative stress in gill, liver and brain tissues remained irreversible. From this study, all tissues were equally targeted by the nanoparticles; however, more profound effect was evident in gill tissue followed by liver and brain. Thus the need for limited use and safe disposal of nano-sized particles warrant for the safety of aquatic organisms and of course to humans.

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