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Histopathological studies and oxidative stress of synthesized silver nanoparticles in Mozambique tilapia (*Oreochromis mossambicus*)

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Abstract

To evaluate the potential environmental effects of engineered nano metals, it is important to determine the adverse effects of various nanomaterials on aquatic species. Adult tilapia (*Oreochromis mossambicus*) were maintained in 10 L glass aquaria, and exposed to a graded series of synthesized silver nanoparticles (Ag-NPs) at 25, 50 and 75 mg/L for eight days. The LC_{50} value was 12.6 mg/L. Reduced activities of antioxidant enzymes and the contents of antioxidants were lowered in the gills and liver of fishes treated with Ag-NPs, which resulted in heavy accumulation of free radicals. Histopathological results imply that the balance between the oxidative and antioxidant system in the fish was broken down during Ag-NPs exposure. The principal concern related with the release of nanomaterials and their smaller particle may change the materials transport and potential toxicity to aquatic organisms compared to larger particles.

Key words: silver nanoparticles; *Oreochromis mossambicus*; oxidative stress analysis; histopathological studies; toxic effect

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Introduction

Nanotechnology has been defined as using materials and structures with nanoscale dimensions, usually in the range of 1–100 nm (Masciangioli and Zhang, 2003). As interest in the potential benefits of nanomaterials have increased, the potential toxic effects resulting from use or unintentional release into the environment are concerned (Moore, 2006). Much of the toxicological research to date focus on atmospheric or inhalation exposures (Chen et al., 2006); however, use of nanomaterials are likely to result in releases into aquatic systems and may pose a risk to aquatic ecosystems (Moore, 2006).

Recently, significant concerns have been expressed about the potential risk of silver nanoparticles (Ag-NPs), due to the current and projected high exposure (Luoma, 2008) and their likely high hazard and toxicity in the environment (Klaine et al., 2008). Indeed, a number of ecotoxicology studies have been conducted to study the effect of Ag-NPs in algae bacteria, invertebrates, fish and humans in both *in vivo* and *in vitro* studies (Carlson et al., 2008; Gopinath et al., 2008).

Mechanisms of action including oxidative stress, binding to thiol groups on proteins, cell wall pitting, changes in membrane permeability and effects on the proton motive force and ATP generation (Choi and Hu, 2008; Lubick et al., 2008). A growing number of nanotechnology applications utilize metallic components, many of which can be toxic to aquatic organisms. The absence of a rapid and cost-effective approach for estimation of possible toxic effects of new chemicals has led to the unenforceable production and use of nano materials without sufficient information on their safety data. Histopathological studies have been conducted to help establish causal relationships between contaminant exposure and various biological responses. Johnson et al. (1993) and Schwaiger et al. (1996) have reported a rapid and sensitive tool to detect direct effects of chemical compounds within target organs of fish in laboratory experiments. The accumulation of metals in aquatic organisms has been linked to decrease in survival and reduction of reproductive ability of aquatic organisms (Pelgrom et al., 1995; Liao et al., 2003). Toxicity may vary with size, structure and composition of engineered nanoparticles (Griffitt et al., 2008; Klaine et al., 2008). Fish gills, food and skin are generally recognized as three possible routes for a substance to enter the fish. Owing to direct contact with ambient water, gills are proposed to be the first and most important targets of waterborne metals (Playle, 1998; Tao et al., 2000). Recent studies showed that both ionic Ag and particulate Ag influences toxicity in algae, *Chlamydomonas reinhardtii* (Navarro et al., 2008) and that both particulate and ionic Ag-NPs caused toxicity to zebrafish (*Danio rerio*) gills with the Ag-NPs causing greater toxicity (Griffitt et al., 2008). Many strategies have been developed for the preparation of Ag-NPs, including microemulsion techniques (Motte and Pileni, 1998), organic-water two phase synthesis (Korgel

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et al., 1998), and aqueous solution reduction (Rivas et al., 2001; Yonezawa et al., 2006).

In this study, we have assessed the toxicity of Ag-NPs in liver, skin and gill of *O. mossambicus* with the specific aim to study the role of oxidative damage and apoptosis. The analysis of histological changes markers of oxidative, skin, liver, tissues, and gill of Tilapia treated with Ag-NPs were studied. The extent to which chemical properties of nanomaterials deviate from that of larger particles is often inversely proportional to size (surface area) and is dependent on composition (surface energy). Here a one-step method for synthesizing monodisperse Ag-NPs with a controllable size is described.

1 Materials and methods

1.1 Synthesis of Ag-NPs

The 20 mL of 6.8 mol/L aqueous solution of tri-sodium citrate, containing increasing amounts of tannic acid (6.8–20.5 μmol/L), was heated to 60°C and added with vigorous stirring to 80 mL of 0.74 mmol/L $AgNO_3$ preheated to 60°C for the synthesis of Ag-NPs. The mixture was kept at 60°C for 3 min, or until it changed to yellow color. Then the mixture was boiled for 20 min, cooled down to room temperature and stored in a dark bottle at 4°C (Dadosh, 2009).

1.2 *Oreochromis mossambicus*

Adult Mozambique Tilapia (*O. mossambicus*) of both sexes were purchased from a local pet shop and acclimated in aerated recirculating tanks containing experimental media water. Tilapia were fed with a commercial fish food twice a day and kept at approximately 28°C with a 14 hr:10 hr light-dark cycle and held in 2 closed recirculating systems (200 L) for 15 days to acclimatise to laboratory conditions prior to experiments. In each closed recirculating systems, 50 fish were stocked. During the acclimation period and toxicity tests, about 20% of the water in each recirculating system was replaced daily and were fed 5% body weight twice a day with commercial trout pellets respectively. Fish measuring the average size about 5.0–6.5 cm in length and the average weight about 3.0–4.5 g. The water used to culture the fish was dechlorinated and continuously aerated tap water. After dissection of fish samples, parts of skin, liver, and gills were carefully removed and prepared for histological studies. The animals were handled under the guiding principles in the use of animals in toxicology (http://209.183.221.234/ai/air/air6.asp#).

1.3 Characterization of synthesized Ag-NPs

Ag-NPs were characterized for size and dispersity before exposure to tilapia fish (*O. mossambicus*) in the experimental media. This was accomplished using UV-vis spectroscopy and Transmission electron microscopy (TEM). UV-Vis absorption spectroscopy (1601 Schimadzu spectrophotometer, Canada) was used primarily to confirm the presence of an absorbance peak centered around 431 nm, consistent with that expected for the plasmon resonance of Ag-NPs in the 60–80 nm size range of TEM (model 1200EX, JEOL, Japan) measurements were operated at an accelerating voltage of 120 kV and later with an XDL 3000 powder.

1.4 Experimental design

Prior to toxicological tests, the fishes were determined to be free of external parasites (AFS-FHS, 2003). In the acute toxicity test, adult fish (8 per group) were maintained in 10 L glass aquaria, and exposed to a graded series of Ag-NPs at 25, 50 and 75 mg/L for eight days. Triplicates were performed for each concentration. A control was setup with deionized water. All fishes were fasted and the experimental media water was changed each day. Mortality was carefully recorded for calculating the LC_{50} values based on the Karber method (Yilmaz et al., 2004).

1.5 Histopathology studies

After dissecting the Ag-NPs media exposed fishes, small pieces of the liver, skin and gill tissues were fixed in neutral buffered formalin. The samples were dehydrated and embedded in paraffin. The liver, skin and gill tissues sections were taken (4 mm) and stained with hematoxylin and eosin. Portal inflammation, central necrosis and degeneration are histopathological signs are graded as zero: zero findings; 1: evidence of pathological signs; 2: mild pathological signs; 3: moderate pathological signs and 4: marked pathological signs (Park et al., 2005).

1.6 Biochemical tests

1.6.1 Oxidative stress parameters analysis

The Tilapia fish were exposed to 25, 50 and 75 mg/L of $AgNO_3$ and Ag-NPs separately for 8 days using a semi-static exposure test. The experiment was designed to allow for sub-lethal physiological effects over the exposure period. Four fish per treatment were randomly collected at day 1, 2, 4, 6 and 8, respectively for biochemical analysis. Gill, liver, and skin tissues were removed separately and immediately snap-frozen in liquid nitrogen and stored at –20°C until needed. The frozen tissues were rinsed in 9-fold chilled 100 mmol/L, pH 7.8 sodium phosphate buffer solution and homogenized by a hand-driven glass homogenizer. The homogenates were centrifuged at 10,000 r/min at 4°C for 20 min and the supernatant was stored in Eppendorf tubes at 4°C. The liver supernatant was diluted with 9-fold chilled sodium phosphate buffer solution to 1%. The prepared supernatants were analyzed for antioxidant enzymes, i.e., superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) activities to determine possible effects on oxidative stress and antioxidant defense, and lipid peroxidation (LPO) level were also measured for the content of malondialdehyde (MDA). All assays were preformed in triplicates. The SOD activity was estimated based on its ability to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated by xanthine/xanthine oxidase according to the modified method of Beauchamp and Fridovich (1971). One unit of SOD activity was defined as the quantity of SOD required to produce 50% inhibition of NBT reduction

under the experimental conditions and the specific enzyme activity was expressed as units per gram fresh weight of tissue per hour. The CAT activity was determined using the method of Beers and Sizer (1952) by measuring the initial rate of the decrease in absorbance at 240 nm as a consequence of H_2O_2 consumption over 1 min. Activity was expressed as a unit (one activity unit defined as absorbance at 240 nm changes 0.01 per min) per gram fresh weight of tissue. The POD activity was assayed using guaiacol as a hydrogen donor by measuring the change at 470 nm over 1 min as reported previously by Chance and Maehly (1955). Enzyme activity is defined as unit (one activity unit defined as absorbance at 470 nm changes 0.01 per min) per gram fresh weight of tissue. LPO was measured using the thiobarbituric acid (TBA) assay by the method of Buege and Aust (1978). The level of LPO was expressed as μmol MDA/g fresh tissue.

1.7 Tissue Ag content

Liver samples of approximately 300 mg were dried at 150°C and cooled. To each sample, 6 mL HNO_3 was added and samples were again heated at 150°C. Samples were re-weighed for measurement of dry weight and re-suspended in deionized distilled water to a mass of approximately 2.0 g. All the histopathological oxidative stress and biochemical parameters for the present study were maintained constant. Studies were made in triplicates for the concurrent results. A control was setup with deionized water.

1.8 Statistical analysis

Each treatment was replicated three times for statistical analysis. The results were expressed as mean ± standard deviation. The differences between the experimental and the control groups were tested for significance using one way analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$.

2 Results and discussion

2.1 Synthesis of Ag-NPs

A simple one-step synthesis method for the preparation of uniform and stable Ag-NPs was employed. The UV results of Ag-NPs showed a peak at 431 nm which is characteristic feature for the silver particles. The presence of increased amount of silver leads to tissue damage, cellular necrosis and release of intracellular components into the surroundings. Mostly the aggregates and clusters of spherical nanoparticles were seen in the Tilapia fish sections observed in TEM images (Fig. 1a, b). Indeed, the size distribution ranged from 60 to 80 nm (Fig. 1c).

2.2 Histopathological studies

The histopathology slides suggested the presence of the Ag NPs in the *O. mossambicus* tissues. Fish liver histopathology is an indicator of chemical toxicity and useful way to study the effects of exposure of aquatic animals to toxins present in the aquatic environment (Fernandes et al., 2008). In the liver tissues of exposed fish to Ag-NPs at 50

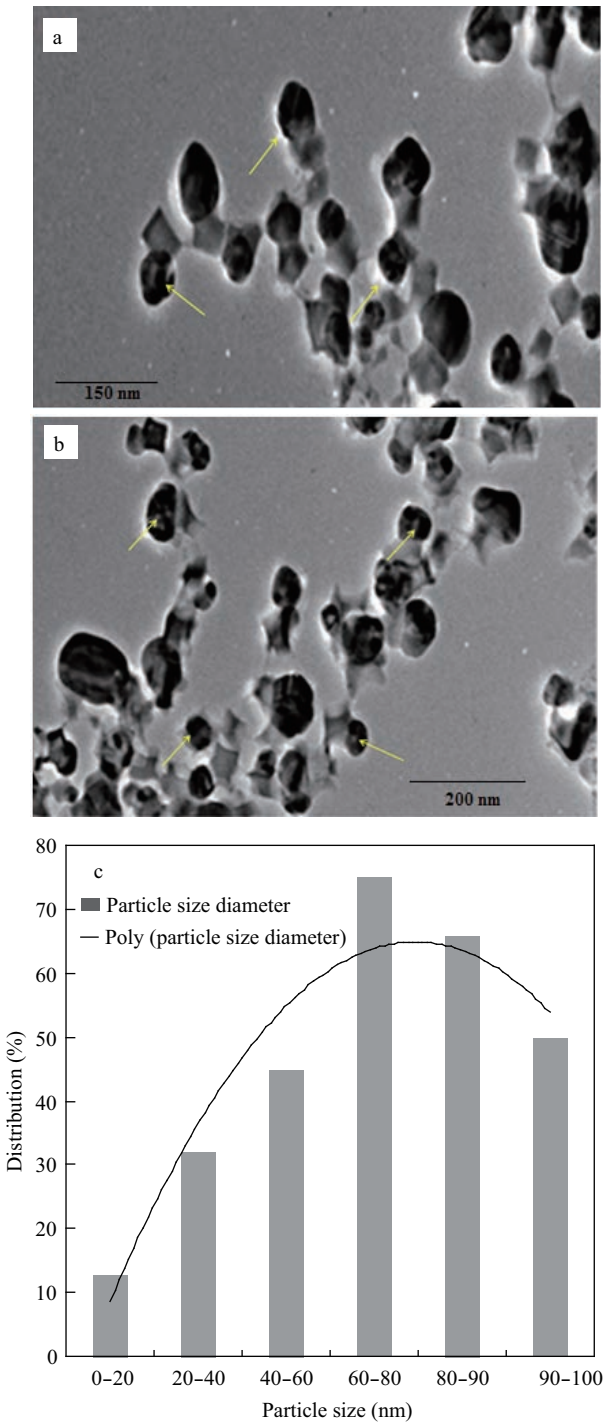

Fig. 1 Ag-NPs size distributions based on transmission electron microscopy (TEM). This figure illustrates the typical sizes of the as prepared Ag-NPs. (a) TEM illustrates uniform and stable Ag-NPs (150 nm scale); (b) comparison of the size distributions of particles (200 nm scale); (c) particle size distribution graph with polynomial trendline.

mg/L showed cloudy swelling of hepatocytes, congestion, vacuolar degeneration, karyolysis, karyohexis, dilation of sinusoids and nuclear hypertrophy (Fig. 2). Mild congestion of blood vessels were seen in the gill primary lamellae at 50 mg/L exposure level, showed a fusion of primary lamellae and marked hyperplasia of the branchial arch was evident at 50 mg/L concentration (Fig. 3). Most of the changes were observed in the skin due to direct contact with the Ag-NPs solution and reported the hyperplasia.

desquamation, and necrosis of epithelial, epithelial lifting, oedema, lamellar fusion, collapsed secondary lamellae and curling of secondary lamellae. No specific change could be observed in the skin and muscle damage at 50 mg/L exposure level.

However, at 25 mg/L muscle tissue exhibited dystrophic changes with marked thickening and separation of muscle bundles in addition to severe intramuscular oedema (Fig. 4). To test the concentration of dissolved Ag released from Ag-NPs and the toxic effects to fish was observed. The amount of Ag released into the test solutions from the Ag-NPs were tested in different concentrations in a way similar to those described for NPs. Since there were no observed differences in particle size and mortality in synthesized Ag-NPs. Gills are the primary sites of gas exchange, acid-base regulation and ion transfer (Randall, 1990). The gill epithelium consists mainly of three types of cells: pavement or respiratory cells, mucus cells, and chloride cells as pointed out by Laurent and Perry (1995).

2.3 Oxidative stress analysis (OSA)

The hazardous health effects of nanoparticles in Tilapia has been reported in toxicological studies. Reduced activities of antioxidant enzymes and lowered contents of antioxidants were also found in gills and liver of *O. mossambicus* treated with Ag NPs, which have resulted in the accumulation of heavy free metal radicals. The LC_{50} value was 12.6 mg/L (Fig. 5). Silver exists in a variety of forms, but ionic

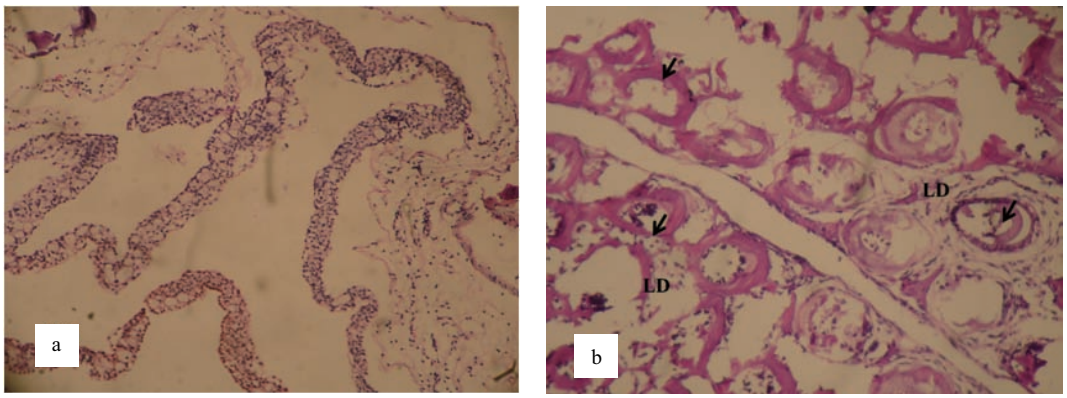

Fig. 2 Fish exposed Ag-NPs 50 mg/L for 4 days. (a) control; (b) degenerative changes in liver with congestion, vacuolar degeneration, karyolysis, karyohexis. LD: lamellar disorganization.

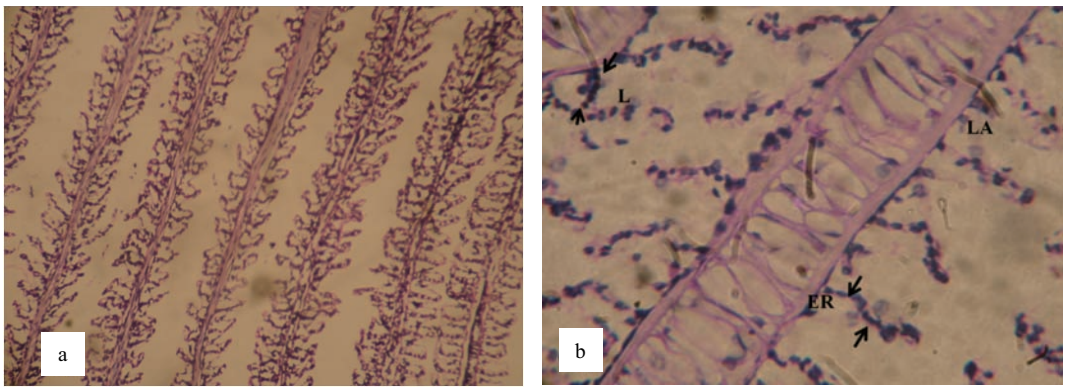

Fig. 3 Fish exposed Ag-NPs 50 mg/L for 4 days. (a) control; (b) degenerative changes in gill, such as intraepithelial edema in the secondary lamellae, thick coating of mucus covering the entire gill filaments and lamellae, erosion of secondary lamellae. LA: lamellar aneurysm; ER: epithelium rupture; L: lamellar disorganization.

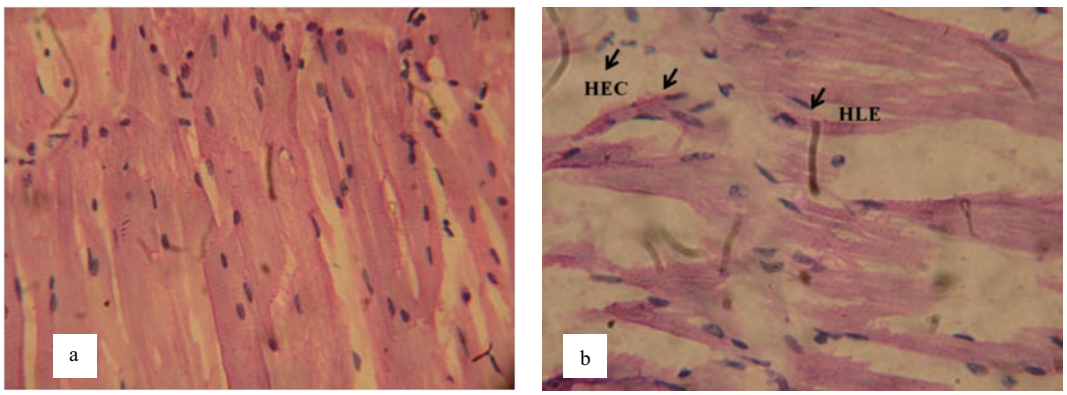

Fig. 4 Fish exposed to Ag-NPs 50 mg/L. (a) control; (b) degenerative changes in skin hyperplasia, desquamation, and necrosis of epithelial tissue. HEC: hyperplasia of epithelial cells; HLE: hypertrophy of the lamellar epithelium.

silver is particularly toxic (Lima et al., 1982; Nebeker et al., 1983; Morgan and Wood, 2004). The nanosilver had no impact on the basal metabolic rate, whereas exposure to 386 μg/L $AgNO_3$ resulted in a significant raise in basal metabolic rate and the critical oxygen tension increased approximately 50% after exposure to 300 μg/L nanosilver plus 31% and 48% by 39 and 386 μg/L $AgNO_3$, respectively against Eurasian perch, *Perca fluviatilis* (Bilberg et al., 2010). Griffitt et al. (2009) found that more silver was associated in the gills of zebrafish exposed to Ag-NPs compared with fish exposed to soluble $AgNO_3$. An enhanced toxicity of nanoparticulate silver, compared to silver ions (Ag^+), may result from their shape and/or size, the release of silver ions (silver ions are well known to be toxic to aquatic organisms) against rainbow trout, *Oncorhynchus mykiss* (Walker et al., 2008; Wood et al., 1996). There was no obvious damage to the gill filaments resulted due to exposures to silver particles, although some low level damage was noted in the gills of *O. mykiss* exposed to $AgNO_3$ (Scown et al., 2010).

In Japanese medaka (*Oryzias latipes*), the 96 hr LC_{50} has been demonstrated to be 34.6 μg/L for 50 nm uncoated silver particles (Chae et al., 2009). The commercial Ag-NPs suspension caused acute gill lamellae necrosis at high concentrations (100 μg/L), potentially giving rise to the substantial (73%) fish mortality at this concentration against juvenile Atlantic salmon, *Salmo salar* (Farmen et al., 2012). Embryos of *Pimephales promelas* were exposed to varying concentrations of either sonicated or stirred NP solutions for 96 hr and reported that the LC_{50} values for NanoAmor and Sigma Ag-NPs were 9.4 and 10.6 mg/L for stirred and 1.25 and 1.36 mg/L for sonicated NPs, respectively (Laban et al., 2010). Bilberg et al. (2012) have reported that the acute toxicity of nanosilver to zebrafish (*D. rerio*) was investigated in a 48h static renewal study and compared with the toxicity of $AgNO_3$ and the median lethal concentration (LC_{50}) values were 84 and 25 μg/L, respectively. The embryos of the zebra fish, *D. rerio* were exposed to varying concentrations of two Ag particle sizes, 12 and 21 nm, at time points 24 and 48-hr after fertilization and the 12 nm particles were found to be more bioactive

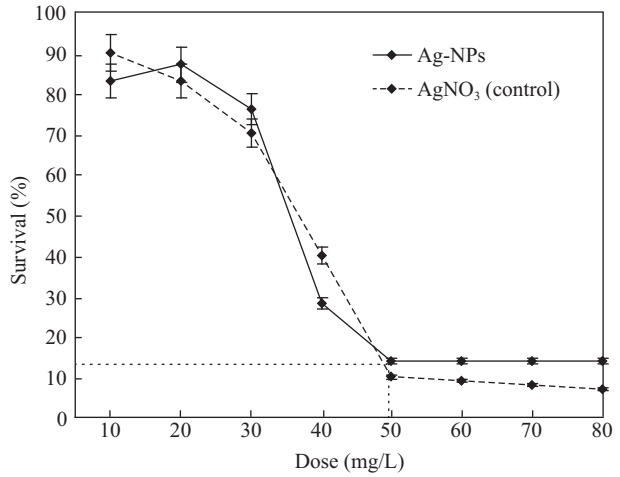

Fig. 5 Acute toxicity tests with the Ag-NPs and silver nitrate solution on adult Tilapia for eight days exposure. Drop lines indicate the LC_{50} concentrations for each material exposed.

with a lethal dose 50 (LD_{50}) concentration of 15.8 μg/mL compared to 50.1 μg/mL for 21 nm particles (Cowart et al., 2011). Kennedy et al. (2010) have reported that the lethal median concentrations (LC_{50}) were expressed as total silver, both *Daphnia magna* and *P. promelas* were significantly more sensitive to ionic Ag^+ as $AgNO_3$ (mean LC_{50} = 1.2 and 6.3 μg/L, respectively) relative to a wide range in LC_{50} values determined for the nanosilver suspensions (2–126 μg/L).

Recent studies demonstrate that Ag-NPs induce embryonic injuries and reduce survival of zebrafish (*Danio rerio*) (Asharani et al., 2008; Griffitt et al., 2008; Yeo and Kang, 2008). The 96-hr LC_{50} values for *Oryzias latipes* of 60 and 300 nm Ag NP suspensions were 28 and 67 μg Ag/L, respectively (Kim et al., 2011). Laban et al. (2010) reported the idea that silver ions dissociated from silver nitrate process a different toxicity than silver ions released from Ag-NPs. In the present study, it was not possible to fully differentiate between Ag-NPs and Ag ion modes of action with the biological response parameters included here, the observed differences in mortality could reflect a combined effect of an Ag-NPs specific reaction on gill osmoregulatory processes as well as an Ag ion effect, including release of Ag ions from Ag-NPs directly to the gill epithelia.

The OSA revealed that the release of enzymes and radicals are released from cellular organelles. Under normal conditions, free radical production and elimination are in a dynamic balance. Previous reports show that Ag-NPs can be toxic to fish. Earlier publication reported that 20–30 nm silver nanopowder induced a 48-hr LC_{50} of 7.07–7.20 g/mL in zebra fish (*Danio rerio*) depending on whether the exposure was to an adult or juvenile fish (Griffitt et al., 2008).

2.4 Superoxide dismutase levels (SOD) and catalase (CAT) and peroxidase (POD) analysis

SOD is the enzyme to deal with oxyradicals and responsible for catalyzing the dismutation of highly superoxide radical $O_2^{\bullet -}$ to O_2 and H_2O_2. In the present study, the SOD activities in the liver, gill and skin tissues of Tilapia exposed at different concentration with different exposure time (Fig. 6a). Exposure to 25 mg/L Ag-NPs, the SOD activities of different tissues were stimulated and showed a remarkable increase, which might be due to the synthesis of new enzymes or the enhancement of pre-existing enzyme levels under lower concentrations. These defense systems included the antioxidant enzymes (SOD and CAT), numerous low-molecular-weight and non enzymatic antioxidants (GSH) (Zhang et al., 2004, Van der Oost et al., 2003). The SOD-CAT system provides the first defense against oxygen toxicity. Superoxide dismutase catalyzes the transformation of O_2 and H_2O_2 (Kappus, 1985), and CAT contributes to convert H_2O_2 to water and oxygen (Stanic et al., 2006).

CAT and POD are the key enzymes in antioxidant defense systems to convert the resulting free radicals H_2O_2 to water and oxygen. In the present study, CAT and POD activities in different tissues of Tilapia fish at different

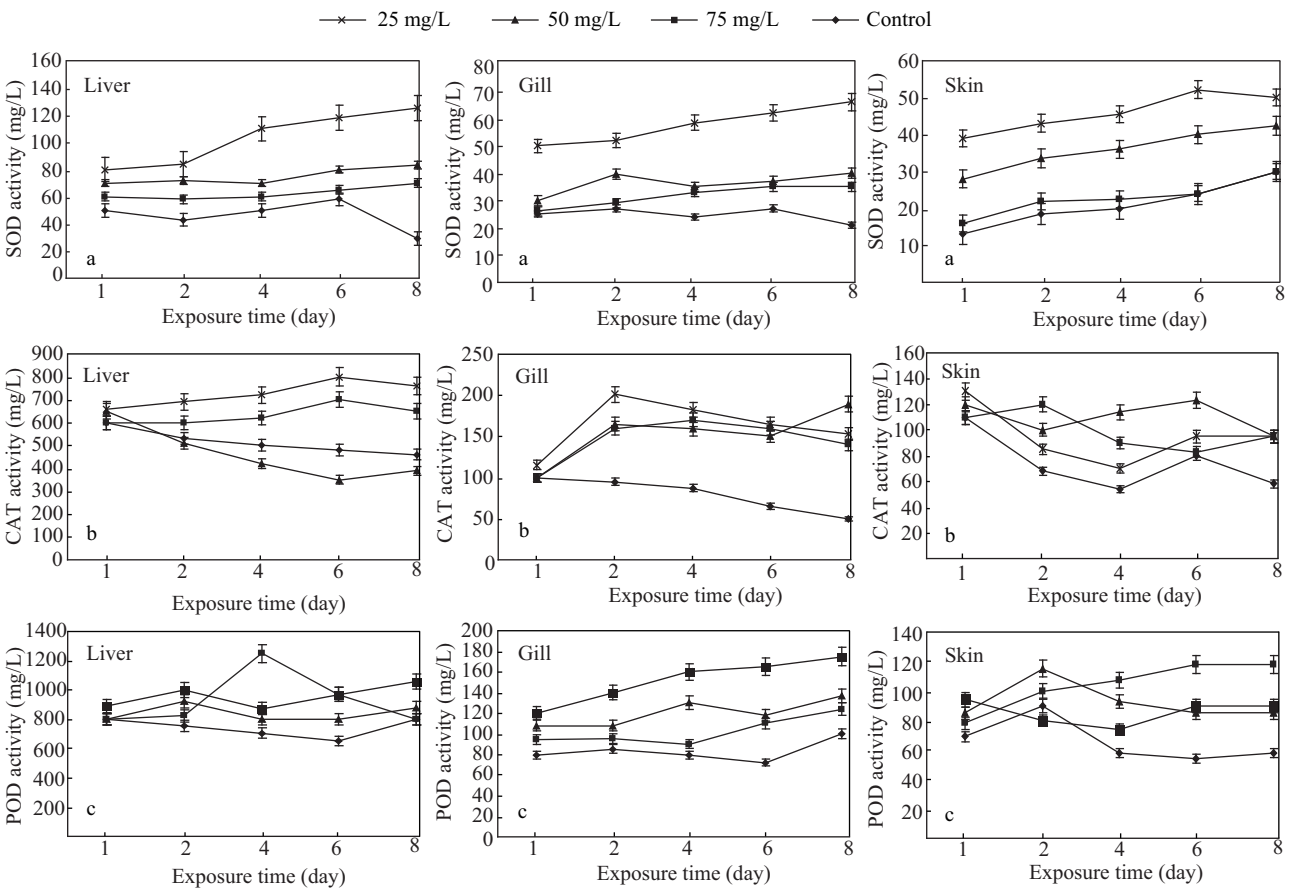

Fig. 6 SOD (a), CAT (b), and POD (b) activity in liver, gill and skin tissues of Tilapia after exposed at different concentrations and time to synthesized Ag-NPs.

Ag-NPs concentrations and an exposure at different time, respectively, as shown in Fig. 6b, c. The CAT activity of different tissues showed a slight decrease up to day 2 and then a remarkable elevation was observed at 50 mg/L. Results indicated that under the stress the CAT activity were inhibited, and the ROS scavenging was weakened and accumulated gradually in the major tissues of Tilapia. In addition, the CAT and POD activities in the liver were 2–3 folds and 5–10 folds of NPs accumulation in the gill and the skin at the same concentrations of experimental media exposure, respectively. Although Kashiwada (2006) reported the uptake of NPs using a fish model, have shown uptake of NPs in either unicellular organisms (bacteria) or invertebrate (*Daphnia*) species (Brayner et al., 2006; Zhu et al., 2006; Roberts et al., 2007).

2.5 Lipid peroxidation (LPO) detection by malondialdehyde (MDA) levels

Oxidative deterioration of cell membrane lipids and has been used extensively as a marker of oxidative stress in LPO detection method. In the present study, MDA contents in the liver, gill and skin tissues were not obviously different compared with the control after exposure of 25 and 75 mg/L Ag-NPs, however, the significant increase in MDA level was found after 8 days of exposure at 25 and 50 mg/L of Ag-NPs. It indicated that these tissues were undergoing oxidative stress, which was consistent with our results of higher concentration of Ag-NPs exhibiting more potent effects on disturbance to the antioxidant defense systems in Tilapia fish. In the present study, the content of MDA increased and the activities of antioxidant enzymes decreased in the liver of silver NPs-treated *O. mossambicus*. The results suggest that the balance between the oxidative and antioxidant system in the fish was broken during the exposure of Ag-NPs. Liver contains considerable amounts of polyunsaturated fatty acids, which are prone to damage by free radicals.

3 Conclusions

This study demonstrates that *O. mossambicus* adult fish can be used as a high-through put, highly efficient and cost-effective to investigate the toxicity of Ag-NPs. The LC_{50} data is useful and provides a good baseline for toxicity tests and molecular studies focused on the results from the phenotypic effects of *O. mossambicus* model. It can be concluded that analytical approaches to NPs in the aquatic environment are still in an initial phase of development. The formation of aggregates in water offers the opportunity for other organic materials, including toxicants, to become associated with the aggregates, which will change bioavailability of these materials and create additional toxicological concerns.

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