

Original Article**Effects of Short-Term Exposure to Sublethal Concentrations of Silver Nanoparticles on Histopathology and Electron Microscope Ultrastructure of Zebrafish (*Danio Rerio*) Gills***Borhan Mansouri*¹, Seyed Ali Johari²**Received: 24.4.2015**Accepted: 10.06.2015***ABSTRACT**

Background: The increasing use of nanomaterials and nanoproducts has increased the possibility of contamination of the environment, which may have adverse effects on different organisms. The aim of this study was to evaluate the effects of silver nanoparticles on histopathology and gill ultrastructure of zebrafish (*Danio rerio*) under laboratory conditions.

Methods: Zebrafish were exposed to four concentrations of silver nanoparticles (0.0015, 0.00375, 0.0075, and 0.015 mg/l) for a period of 4 days. Gill ultrastructure and histopathological changes were studied using scanning electron microscope and haematoxylin - eosin staining.

Results: Exposure to silver nanoparticles significantly ($P < 0.001$) increased the diameter of gill filaments and secondary lamellae, while silver nanoparticles significantly reduced the length of the secondary gills in zebrafish. Moreover, other changes such as vacuolization, dilated and clubbed tips, hyperplasia, edema, fusion, swelling of mucocytes, hypertrophy, and necrosis were observed. The effects of silver nanoparticles in zebrafish gills were dose dependent.

Conclusion: Based on the adverse effects of AgNPs on zebrafish gills, silver nanoparticle solutions can be hazardous pollutants for the environment.

Keywords: Gills, Metal Nanoparticles, Silver, Water Pollution, Zebrafish.

IJT 2016; 15-20**INTRODUCTION**

Silver nanoparticles (AgNPs) are one of the most increasingly used nanoparticles in consumer applications including bacteriocides, sensors, inks, catalysts [1], nanocomposite films, and ultrafiltration membranes [2]. Approximately 500 tons of nanosilver is produced every year [3]. Moreover, this nanoparticle can be released into the environment from processes such as washing textiles or cosmetics, leaching from consumer products, and industrial wastes [4], and finally disposal to aquatic ecosystems. AgNPs have adverse impact on zebrafish and cause oxidative stress, cellular apoptosis, and chromosomal aberrations [5]. AgNPs have toxic effects during early life stages and adulthood of Japanese medaka [6]. However, there is still not enough information about the toxic effects of AgNPs, and comparing findings are difficult due to the variety of different AgNPs and stabilizing

surfaces utilized in various studies. Thus, it is of great importance to fully investigate the potential risks of environmental pollution by nanosilver.

The gills are multifunctional organs and are responsible for gas exchange, water and ion regulation, hydromineral and acid-base balance [7]. Moreover, fish gills are the first organs to encounter pollutants and are considered the main targets for waterborne nanoparticles uptake [8]. Thus, gills are useful bioindicators of the health of fish and assessors of chemical contaminants potential. To determine the severity of these effects, special techniques are required. Histopathology, as one of the most useful techniques in aquatic toxicology, can provide useful information for identifying target tissues and the effects of exposure of aquatic organisms to toxins present in water [9]. Histological changes can be used as indicators to identify effects of various chemical contaminations on

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organisms such as fish, and are reliable tools in controlled experiments and field studies [10]. Various histopathological alterations have been reported in the gills of many kinds of fish including: hyperplasia, aneurisms, edema, epithelial lifting and fusion of the lamellae during acute toxicity, and changes in the proportions of epithelial cells and mucocytes in chronic exposure [8,11].

Numerous studies have evaluated susceptibility of aquatic organisms such as fish to toxic effects of exposure to nanoparticle [2, 11-13]. However, only a few reports have documented histopathological effects and gill ultrastructural changes in fish exposed to nanoparticles such as TiO₂ NP, CuNPs, AgNPs, and ZnNPs [6,11,14,15].

Therefore, the aim of this study was to investigate the effects of silver nanoparticles on gill histology and ultrastructure in zebrafish (*Danio rerio*) under laboratory conditions.

MATERIAL AND METHODS

Zebrafish (*Danio rerio*) with a size range of 2–4 cm in length and weighing 2 - 3 g were procured from local a farm in 2015 and were acclimatized under laboratory conditions for 10 days prior to the experiment in Fisheries Department of Kurdistan University, Iran. The experimental period was four days and for preserving the level of AgNPs in all aquariums, water was changed every 48 h. Fish were not fed 24 h before or during the experiment.

Colloidal AgNPs was purchased from Nano Nasb Pars Co. (Tehran, Iran). The complete characterization of these particles was previously reported by Johari et al. [12]. Based on LC₅₀ (0.015 mg/L) results [13], four concentrations of AgNPs (0.0015, 0.00375, 0.0075, and 0.015 mg/L) were chosen. The fish were divided into 5 groups of 7 each; the first group served as the control and the others as the experimental ones. Then, fish were exposed to different concentrations of AgNPs in aquaria containing 20 L of water. The aquariums were inspected after 24, 48, 72 and 96 h for dead fish.

The right and left gills in each fish were selected for ultrastructural and routine histological studies, respectively. The left gills from each fish was surgically removed and immediately fixed in 10% buffered formalin solution for histopathological examinations.

Histological examinations were performed as described by Bernet et al. [16] with some minor modifications. After fixation, the samples were dehydrated, inserted in paraffin wax and sliced at 4-6 µm using a microtome and then stained with haematoxylin-eosin and examined microscopically. The diameter and length of secondary gill lamellae as well as diameter of gill filaments were measured using the Axio Vision (Release 4.8.2).

For ultrastructural studies, the right gill arches were cleaned several times by cool phosphate buffer and were fixed overnight in 2.5% glutaraldehyde, and post-fixed in 1% osmium tetroxide at 4 °C for 1 h. Thereafter, samples were washed by deionised water. Post-osmium fixed gills were subjected to serial dehydration in 80 and 90% ethanol (three times in each concentration for five minutes) and 100% methanol (two times in 100% concentration for five minutes). Finally, the samples were washed two times in acetone for five minute and were air dried overnight [15]. Processed gill tissues were examined using scanning electron microscope (MIRA FEG-SEM, Scanning Electron Microscopy, TESCAN Company).

The statistical package SPSS, version 16, (Chicago, IL, USA) were used for data analysis. To compare the diameters and lengths of secondary lamellae and diameter of gill filaments following exposure to different concentrations of AgNPs, one-way analysis of variance (ANOVA) was used. Data were log transformed to obtain normal distributions that satisfied the homogeneity of variance required by ANOVA. Values are given in means ± standard deviation (SD). Ethical considerations and animal rights in this paper were considered and the study was approved by Ethics Committee of the university (MUK.REC.1393.98).

RESULTS

No mortality was observed during the experimental period in control and treatment groups. The changes in the size of the zebrafish gill filaments and secondary lamellae are presented in Table 1. As can be seen, exposure to highest tested concentration of AgNPs (0.015 mg/L) significantly increased the diameter of gill filaments and secondary lamellae ($P < 0.001$). In

contrast, AgNPs significantly reduced the length of secondary lamella in zebrafish ($P < 0.01$). These findings indicated that the extent of damage to zebrafish gills increased with higher concentration of silver nanoparticles.

The histopathological results of control and silver nanoparticles exposed fish gills are displayed in Figure 1. In control group, gill filaments and primary lamellae appeared normal and mucus free with well-defined secondary lamellae branching from them (Figure 1, A). Four days exposure to silver nanoparticles caused injuries that mainly included vacuolization, dilated and clubbed tips,

aneurism, hyperplasia, edema, fusion, swollen mucocytes, hypertrophy, and necrosis (Figure 1, B to E2). These damages to fish gills increased with increasing concentrations of AgNPs. The most severe injuries such as fusion and necrosis in zebrafish gills were observed in 0.0015 mg/L (Figure 1, E1 and E2). Ultrastructural results from scanning electron microscope (SEM) studies in control and treatment groups are displayed in Figure 2. At 0.0015 mg/L AgNPs, cell proliferation resulted in lengthening of the filament epithelium. The thickening of secondary lamellae appeared more irregular than the control group (Figure 2, a).

Table 1. Mean (\pm SD) of the secondary lamellae length and diameter and mean of the gill filaments diameter (μ m) following 4 days exposure of zebrafish to different concentrations of AgNPs.

AgNPs concentration	Diameter of gill filaments	Diameter of secondary lamellae	Length of secondary lamellae
Control group	16.13 \pm 1.16 ^a	6.31 \pm 1.38 ^a	89.33 \pm 7.13 ^a
0.0015 mg/L	19.35 \pm 3.86 ^a	8.51 \pm 1.61 ^a	80.51 \pm 25.34 ^a
0.00375 mg/L	17.62 \pm 1.64 ^a	9.59 \pm 1.63 ^a	73.04 \pm 23.57 ^a
0.0075 mg/L	22.83 \pm 8.15 ^a	10.15 \pm 3.97 ^a	63.50 \pm 10.34 ^a
0.015mg/L	27.93 \pm 5.78 ^b	10.97 \pm 2.87 ^b	54.74 \pm 18.51 ^b
* <i>p</i> value	0.001	0.001	0.01

* *P* value for one-way ANOVA

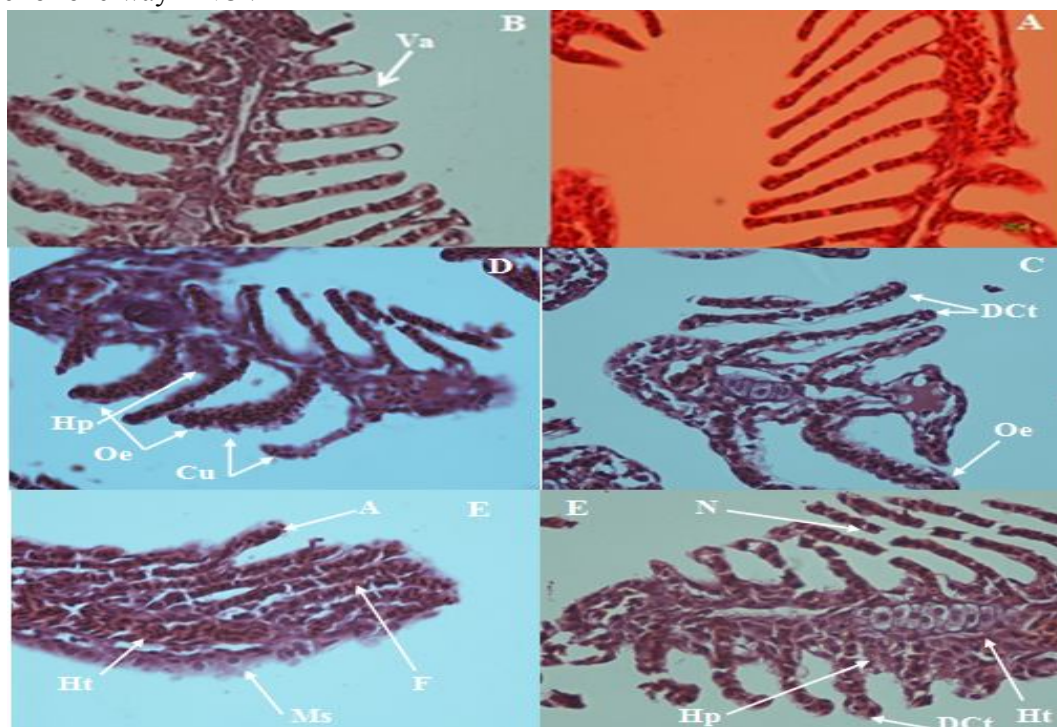


Figure1. Gill morphology in zebrafish exposed to silver nanoparticles for 4 days. The panels include (A) control group, (B) 0.0015 mg/L, (C) 0.00375 mg/L, (D) 0.0075 mg/L, and (E) 0.015 mg/L. The gills of control fish indicated normal histology, whilst all treatment groups showed injuries that included (B) vacuoles (Va); (C) dilated and clubbed tips (DCt) and hyperplasia (Hp); (D) oedema (Oe), hyperplasia (Hp), and curling lamellae or curvature (Cu); (E) fusion (F), increase of mucous secretion (Ms), aneurism (A), hyperplasia (Hp), hypertrophy (Ht), and necrosis (N).

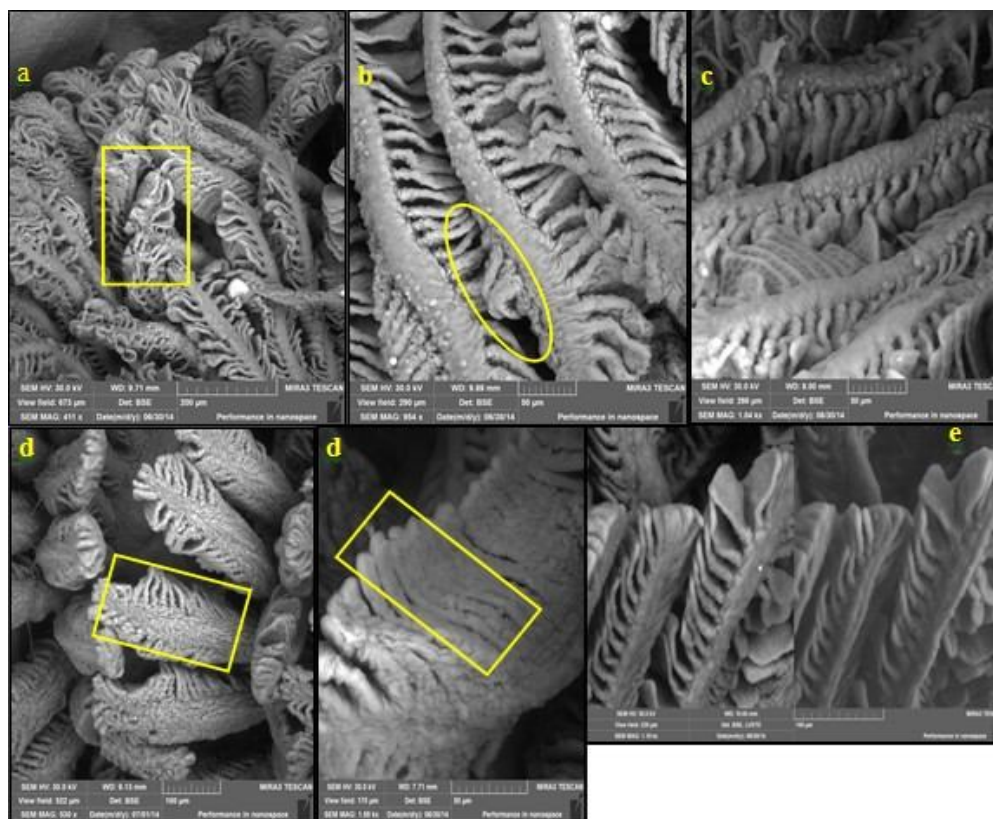


Figure 2. Scanning electron micrographs of zebrafish gills after 4 days exposure to different concentrations of silver nanoparticles (**a** 0.0015 mg/L, **b** 0.00375 mg/L, **c** 0.0075 mg/L, **d** 0.015 mg/L, and **e** control group).

DISCUSSION

Fish gill is the primary organs to encounter chemical pollutants in the aquatic environments; moreover, this organ is an important site for the entry of exogenous toxicants that provoke lesions and gill injury [17]. The toxicant levels in gills reflect the pollution levels in the waters. Fish gills are also the main site for gas exchange and previous studies have shown them to be sensitive to manufactured nanoparticles. Al-Bairuty et al. [14] demonstrated that exposure to copper nanoparticles resulted in edema, lamellar fusion, clubbed tips, and hyperplasia, aneurisms, and necrosis in the secondary lamellae of the gills filaments of rainbow trout. Govindasamy and Rahuman observed similar findings with AgNPs [18]. In other study, exposure to copper nanoparticle suspension caused damage to gill lamellae characterized by proliferation of epithelial cells as well as edema of primary and secondary gill filaments of zebrafish [19]. Similarly, we observed several histological changes, such as mucus secretion, hyperplasia, edema, fusion, hypertrophy, and necrosis in the

gills' primary and secondary lamellae of AgNPs-exposed fish (Figure 1). Hyperplasia and hypertrophy of lamellae might be a necessary response of gill tissue to counteract the effects of AgNPs.

Mucus secretion is the first mechanism of defense against the effects of metal injury in the gill tissue and underlying epithelium [20]. As in our study (Figure 1, E1), in other similar studies, exposure to silver nanoparticles [6], titanium dioxide nanoparticles [21] and single-walled carbon nanotubes [22] increased mucus secretion from the gills. However, in our study it seems that mucus secretion was not adequate to defend the gill tissue from the histological changes arising from AgNPs exposure. Increased secretion of gill mucus increases oxygen blood-to-water diffusion path [23] and elevates critical oxygen tension. Silver exposure may lead to hyperplasia and hypertrophy of gill epithelial cells. Finally, silver nanoparticles will precipitate in gill branches.

SEM is an important technique for assessing the effects of environmental stressors

on gill structures, and has ability to show damage of surface ultrastructure of the branchial epithelium that cannot be revealed by light or transmission electron microscope [24]. In our study, alterations observed on the surface of gills of zebrafish using SEM were in agreement with previous results [25, 26]. Several authors have reported histopathological anomalies in the gills of other species exposed to nanoparticles. Exposure to TiO₂NPs resulted in edema and thickening of gill lamellae as well as gill filaments of rainbow trout and carp [21, 27]. Moreover, Griffitt et al. [19] reported filament and lamellar fusion in the gills of zebrafish exposed to copper NP suspensions; and observed edema of primary and secondary gill filaments of zebrafish. In another study, gill filaments were threefold thicker than controls in CuNPs exposed fish, but were not significantly altered by TiO₂NPs or AgNPs [28]. In our study, exposure to AgNPs caused damage to gill lamellae as well as gill filaments of zebrafish. Moreover, in this study similar to another one [29], filament cell proliferation displayed heightening of the filament epithelium. Effects of AgNPs in zebrafish gills were dose dependent, with significantly greater damage observed at higher AgNPs concentrations (Table 1).

Exposure to AgNPs resulted in thickening, edema, and fusion in the gill lamellae and filaments of zebrafish that impaired the structure and function of gills with increasing concentrations of nanoparticles (Figure 2d).

CONCLUSION

Short-term exposure to sublethal concentrations of silver nanoparticles (AgNPs) adversely affects histology and ultrastructure of gills of zebrafish (*D. rerio*). Adverse effects of AgNPs on zebrafish gills were dose dependent and greater damage was observed at higher concentrations of AgNPs. Exposure to AgNPs increased the diameter of gill filaments and secondary lamellae, in contrast, the length of the secondary gills were reduced in zebrafish. Moreover, several additional adverse effects such as vacuolization, dilatation and clubbing of the tips, hyperplasia, edema, fusion, swelling of mucocytes, hypertrophy, and necrosis were observed. Based on the adverse effects of AgNPs on zebrafish gills in this study, silver

nanoparticle solutions can be hazardous pollutants for the environment.

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