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# Combined effects of silver nanoparticles and mercury on gill histopathology of zebrafish (*Danio rerio*)

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## ARTICLE INFO

## ABSTRACT

**Objective:** To evaluate the combined effects of silver nanoparticles (Ag NPs) and Hg$^{2+}$ on the gill histopathology of zebrafish (*Danio rerio*) under the controlled conditions.
**Methods:** In this study, one non-lethal concentration of Ag NPs (0.1 mg/L), six concentrations of Hg$^{2+}$ (0.001, 0.005, 0.01, 0.05, 0.1 and 0.2 mg/L), and six mixture concentrations of Ag NPs and Hg$^{2+}$ (0.1 plus 0.001, 0.005, 0.01, 0.05, 0.1, and 0.2 mg/L) were used as the control group. After 4 days of exposure, samples were prepared for gill histology.
**Results:** The results showed that notable damages were observed in aneurism, such as vacuolation of secondary lamella, fusion, hypertrophy, mucus secretion and necrosis. Moreover, our findings indicated that the Hg$^{2+}$ and Ag NPs alone led to shorter secondary lamella length and smaller lamellae's diameter of gills compared to the mixture of Ag NPs and Hg$^{2+}$. However, the extent of damages in gill tissues after exposure to mixture of Ag NPs and Hg$^{2+}$ was lower than Hg$^{2+}$ ions and Ag NPs.
**Conclusions:** It appears that the presence of Ag NPs can potentially reduce the toxicity of Hg$^{2+}$ ions. However, to assess the toxicity mechanisms of nanoparticles in presence of pollutants, further studies should be encouraged.

## 1. Introduction

The global production of silver nanoparticles (Ag NPs) is estimated about 500 tons per year and among others, the use of Ag NPs is growing rapidly[1]. Ag NPs are used to control and eliminate various microorganisms. Applications of Ag NPs in bactericides, sensors, inks, catalysts, nanocomposite films and implanted ultrafiltration membranes are vast[2,3]. One consequence of this wide application is releasing Ag NPs into the environment through the processes such as washing textiles, leaching from consumer products and streaming from industrial waste[4]. Based on the current production rate, the concentration of Ag NPs in environment is modeled to be below 1 µg/mL[5]. The study conducted by Tiede *et al*. reported that the concentration of Ag NPs released to the aquatic environment was estimated at about 0.01 µg/L[6]. Thus, the potential effects of these nanomaterials on living organisms, especially those living organisms in water should be carefully studied.

Lee *et al*. studied the effects of Ag NPs on zebrafish embryos[7]. They found that by the chorionic channel, nanoparticles can penetrate into chorionic egg space and cause fish deformities. Increasing deformity, delayed egg hatching, edema, capillary blood flow and increasing mortality of zebrafish embryos as well as newly hatched larvae have been also reported[8]. However, the effects of nanoparticles in presence of other pollutants in environment have not been fully studied yet. Recent findings on hepatocytes have shown that nanoparticles of TiO$_2$ when accompanied with dichloro-diphenyl-tricgloroethane resulted in genetic toxicity, mainly due to increasing oxidative stress, oxidation compounds of DNA, DNA breaks and chromosomal damages[9].

Hg is an unnecessary element with high toxicity at low concentrations, so that the World Health Organization has stated a limit for daily intake of Hg in fish to $2.3 \times 10^{-4}$ mg/kg. For a person of 60 kg weight, the Joint Committee of the Food and Agriculture Organization and World Health Organization has advised the maximum weekly intake of 5 µg/kg Hg and 5.1 µg/kg (CH$_3$)$_2$Hg[10]. This metal has neurotoxic properties[11]. Studies link the reduction

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of $Hg^{2+}$ to Ag NPs in aqueous environments[12,13]. Sumesh *et al.* suggested that the water soluble Ag NPs supported on $Al_2O_3$ as an efficient system for removal of $Hg^{2+}$ from water[11]. The efficacy of Ag NPs on Hg in an aquatic environment has encouraged using its application in areas such as wastewater treatment. However, in spite of this promising efficacy, the effects of Ag NPs on toxicity of Hg in aquatic organisms are still unknown. Therefore, the purpose of this study was to address this issue by gill histopathology analysis of zebrafish under laboratory conditions.

## 2. Materials and methods

### 2.1. Material characteristics

This study was conducted using a colloidal Ag NPs (Nanocid®) which was commercially available in Iran. For more information about this product, readers are advised to refer to the study of Johari *et al.*[14]. A stock solution of Hg (1 000 mg/L) was prepared by dissolving $HgCl_2$ (Merck) in deionized water.

### 2.2. Experimental design

Zebrafish (*Danio rerio*) with a mean total length of (3.0 ± 0.4) cm and mean weight of (3.0 ± 0.4) g were obtained from a local aquaculture shop in Sanandaj City, west of Iran. Prior to beginning experiments, fish were acclimatized in 50 L tanks supplied with continuously aerated tap water [(22–27) ℃] under a 12 h light: 12 h dark photoperiod for 1 month. Fish were fed with commercially available fish food at a rate of 2% body weight per day. The characteristics of water used for the zebrafish were 7.5 ± 0.2 pH, (600 ± 10) μS/cm conductivity, 5° dGH hardness, (22.0 ± 3.0) ℃ temperature and (6.4 ± 0.6) mg/L dissolved oxygen content.

Fish were assigned randomly to one control group and 13 experimental groups as follows: one non-lethal concentration of Ag NPs (0.1 mg/L), six concentrations of $Hg^{2+}$ ions (0.001, 0.005, 0.01, 0.05, 0.1 and 0.2 mg/L) and six mixture concentrations of Ag NPs and $Hg^{2+}$ (0.1 plus 0.001, 0.005, 0.01, 0.05, 0.1 and 0.2 mg/L). There were 20 fish at each group (280 zebrafish in total). For each group, the exposure lasted 4 days for each aquarium (12 L of water). Half of the water in the tank was swapped with freshwater to keep concentration levels in daily basis. Moreover, during the exposure, aeration was supplied to the tanks to prevent the propensity of aggregation.

After 4 days of exposure, five fish were randomly sampled to assess the histopathological effects of tested chemicals. The gill of fish were carefully removed and fixed in Boiun's solution. Then, the tissues were dehydrated using a series of graded ethanol solutions. They were cleared in xylene and embedded in paraffin wax. Slices of 5 μm were separated from paraffin blocks by using a rotary microtome. These slices were then stained with haematoxylin-eosin and examined microscopically[15]. The primary lamella diameter and secondary lamellae length of gill tissues were measured using the Axio Vision Program (Release 4.8.2), Zeiss, Germany.

### 2.3. Data analysis

SPSS, version 16, (Chicago, IL, USA) was used for data analysis. To compare the primary lamella diameter and secondary lamellae length (μm) of zebrafish gill following exposure to different groups, One-way ANOVA was used. Data were log-transformed to obtain normal distributions and the homogeneity of variance required by ANOVA. Values were expressed as mean ± SD. Ethical considerations and animal rights were considered and the study was approved by Ethics Committee of the university (MUK.REC.1394.98).

## 3. Results

The histopathology alterations in gills exposed to experimental groups were presented in Figures 1, 2, and 3. While gills of fish from the control group showed only some minor histopathological damages and major injuries including aneurism, dilated and clubbed tips, hyperplasia, oedema, increasing mucus secretion, curvature, fusion of lamellae, lamellar synechiae, shortening epithelium and necrosis were observed in gills of fish from the other treatment groups. The extent of injuries in gills at various treatment groups was given in Table 1. The severity of damages, such as aneurism, hyperplasia, fusion of lamellae, necrosis in $Hg^{2+}$ group was higher than those at mixture of Ag NPs and $Hg^{2+}$ group (Table 1). Moreover, it appeared that exposure to $Hg^{2+}$ can significantly ($P < 0.05$) increase both the diameter of gill primary lamella and the length of secondary lamellae compared to exposing to the mixture of Ag NPs and $Hg^{2+}$ (Table 2). The primary lamella diameter and secondary lamellae length of the gill in cotrol group were (14 ± 2) μm and (89 ± 3) μm respectively, and the primary lamella diameter and secondary lamellae length of the gill in Ag NPs were (23 ± 1) μm and (45 ± 5) μm respectively.

**Table 1**

Histopathological effect of treatment groups on the gill of zebrafish with respect to the severity of injuries.

| Groups | Damages | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | An | DCt | Hp | Oe | Cu | F | LS | MS | N |
| Control | - | + | - | - | + | - | - | - | - |
| Ag NPs | ++ | + | + | + | + | + | + | + | + |
| $Hg^{2+}$ | +++ | + | +++ | + | ++ | +++ | ++ | ++ | +++ |
| Ag NPs and $Hg^{2+}$ mixture | ++ | + | ++ | + | + | + | + | + | ++ |

An: Aneurism; DCt: Dilated and clubbed tips; Hp: Hyperplasia; Oe: Oedema; Cu: Curvature; F: Fusion of lamellae; Ms: Increase of mucous secretion; N: Necrosis; -: None; +: Mild; ++: Moderate; +++: severe.

**Table 2**

The primary lamella diameter and secondary lamellae length of the gill treated with different concentrations of Ag NPs and $Hg^{2+}$ ions (μm).

| Groups | | Concentrations* | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| Primary lamellae diameter | $Hg^{2+}$ | 17 ± 3 | 23 ± 2 | 12 ± 3 | 31 ± 3 | 43 ± 5 | 36 ± 5 |
| | Ag NPs + $Hg^{2+}$ | 15 ± 1[a,b] | 18 ± 2[a,b,c] | 14 ± 3[b] | 26 ± 2[a,c] | 29 ± 3 [a,c] | 29 ± 4 [a,b,c] |
| | *P* value** | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| Secondary lamellae length | $Hg^{2+}$ | 60 ± 4 | 38 ± 7 | 45 ± 7 | 34 ± 5 | 43 ± 4 | 32 ± 2 |
| | Ag NPs + $Hg^{2+}$ | 66 ± 3[a,b,c] | 44 ± 2[a,c] | 50 ± 3[a,b,c] | 63 ± 6[a,b,c] | 59 ± 6[a,b,c] | 58 ± 4[a,b,c] |
| | *P* value | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |

All values were expressed as mean ± SD. *: Six concentrations of $Hg^{2+}$ ions (1: 0.001 mg/L; 2: 0.005 mg/L; 3: 0.01 mg/L; 4: 0.05 mg/L; 5: 0.1 mg/L; 6: 0.2 mg/L); six concentrations of Ag NPs and $Hg^{2+}$ mixture (1: 0.1 plus 0.001 mg/L; 2: 0.1 plus 0.005 mg/L,; 3: 0.1 plus 0.01 mg/L; 4: 0.1 plus 0.05 mg/L; 5: 0.1 plus 0.1 mg/L; 6: 0.1 plus 0.2 mg/L); **: *P* value for One-way ANOVA.
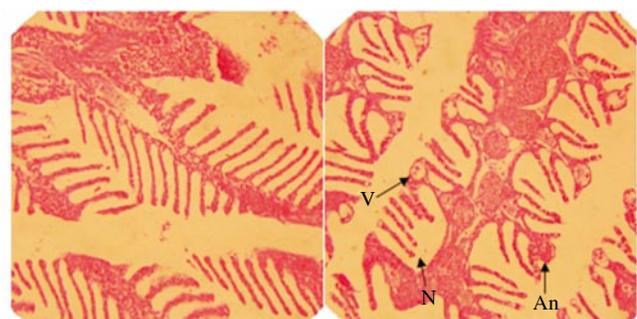


**Figure 1.** Gill morphology of the zebrafish in control group (left) and Ag NPs group (right).

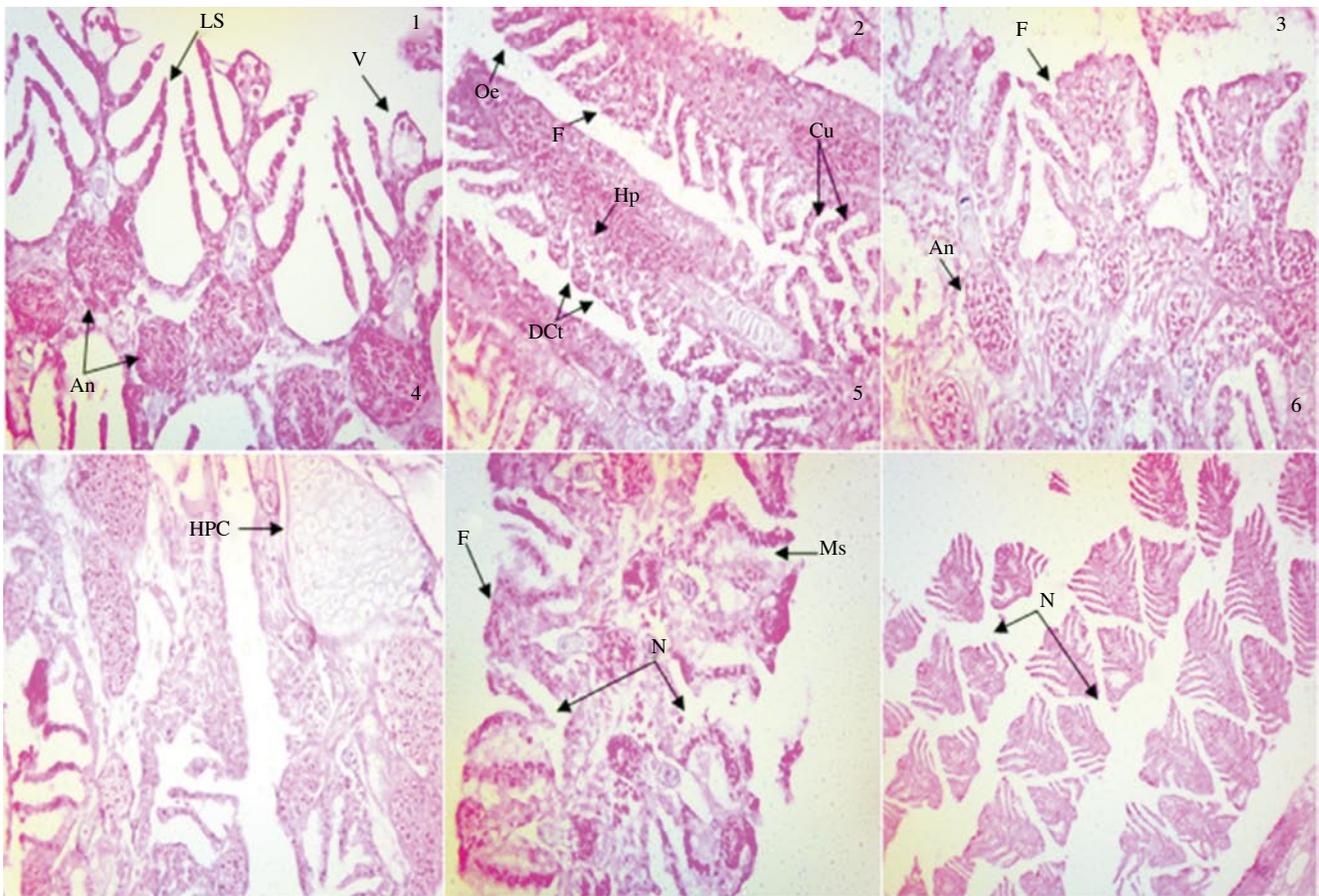An: Aneurism; V: Vacuoles; F: Fusion of lamellae; N: Necrosis.

**Figure 2.** Gill morphology in the zebrafish after exposure to $Hg^{2+}$ at different concentrations.
Gill showed the injuries at all treatment groups. An: Aneurism; DCt: Dilated and clubbed tips; Hp: Hyperplasia; Oe: Oedema; Ms: Mucus secretion; Cu: Curvature; F: Fusion of lamellae; LS: Lamellar synechiae; HPC: Hypertrophy and proliferation of erythrocytes of cartilaginous core; N: Necrosis.
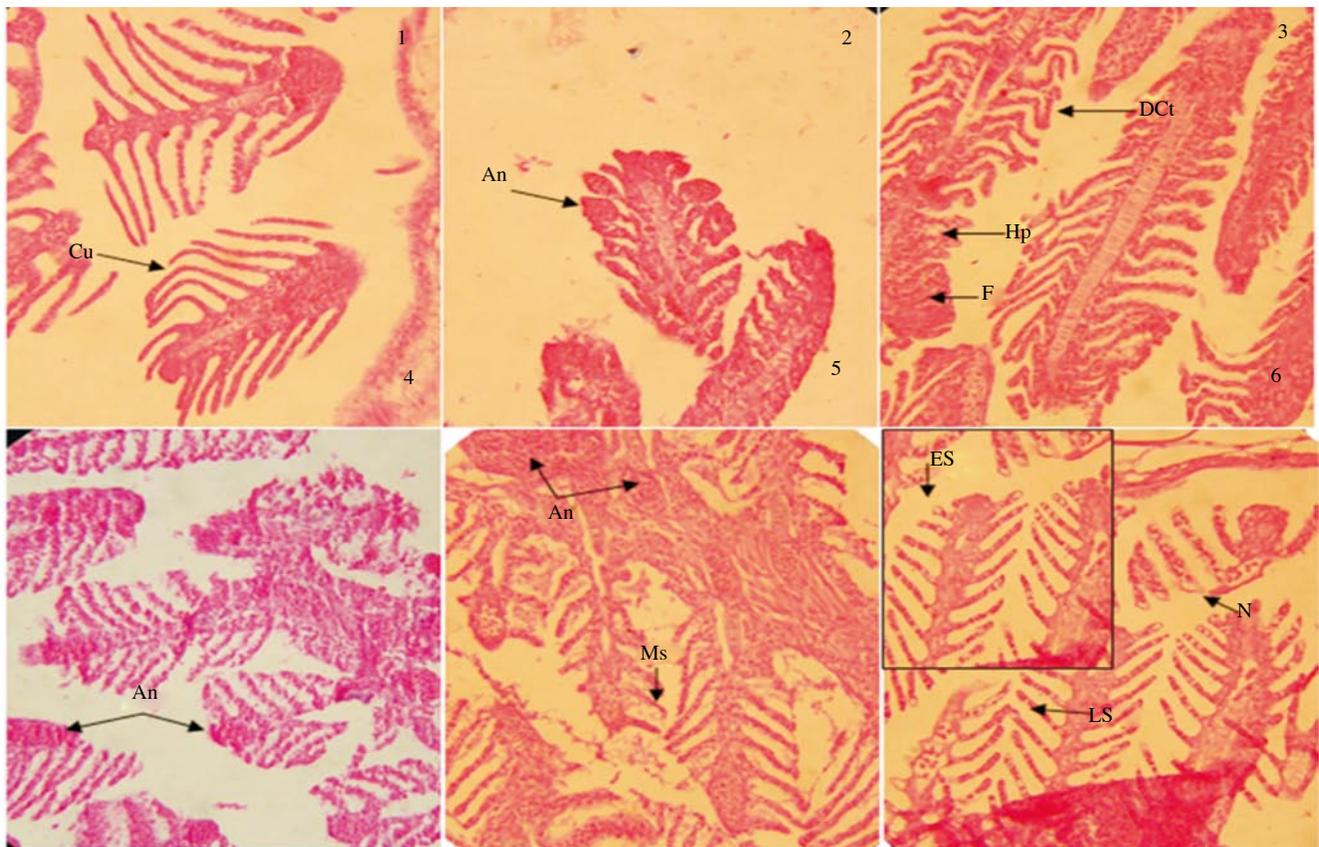


**Figure 3.** Gill morphology of the zebrafish at mixture of Ag NPs and $Hg^{2+}$ group at six concentration levels.
All treatments in gill organs showed injuries. An: Aneurism; DCt: Dilated and clubbed tips; Hp: Hyperplasia; Ms: Mucus secretion; Cu: Curvature; F: Fusion of lamellae; ES: Epithelium shortening; LS: Lamellar synechiae; N: necrosis.

## 4. Discussion

As a multifunctional organ, gill is an important organ for respiration, osmoregulation, acid-base balance and nitrogenous waste excretion. Any disorder in the gill's respiratory system can be thought of the first sign of stress exposing to pollutants[16]. Gill can be also used as an indicator of the aquatic environment quality making it appropriate for environmental toxicology studies. Environmental toxins can cause reversible and irreversible structural alterations in gills. Degeneration and necrosis are examples of irreversible changes whereas cellular hyperplasia is a reversible damage[17,18].

In this study, we used gills to assess the potential effects of chemical pollutants in an aquatic system. Histopathological analysis of gill showed significant damages in treated groups. We observed that the severity and extent of gill damage in a group treated simultaneously with Ag NPs and $Hg^{2+}$ was less critical than that observed in a group treated exclusively with $Hg^{2+}$. Most consequences in mixture-exposed group were aneurysm and hyperplasia whereas these were hyperplasia lesions, aneurysms, necrosis, and loss of lamellae in Ag NPs and $Hg^{2+}$ group. We believe histopathological changes such as aneurysm, fusion, hyperplasia, and mucus secretion that we observed were natural reaction of gill tissue in response to the presence of Ag NPs and $Hg^{2+}$ particles. Similar conclusions have been made by some researchers[19-21].

Curvature of the secondary gill lamellae is the first change resulted from environmental pollutants. Subsequent alterations include mucus secretion, inflammation and hyperplasia of gill tissue[22]. Expansion of mucus secretion, hyperplasia and adhesion of secondary lamellae are part of defense mechanism of gill against the absorption of nanoparticles and $Hg^{2+}$[23]. Mansouri and Johari also reported that Ag NPs can cause pathological changes such as excessive mucus secretion and hyperplasia in gill tissue of zebrafish[20]. In this study, sever alterations in zebrafish gills were an aneurysm at the head of the gill secondary lamellae. This could be due to the disturbance of blood flow in the blood channels and rupture of the pillar cells after exposure to Ag NPs and $HgCl_2$[24,25]. Rajkumar *et al.* reported similar findings on gill tissue after treating *Labeo rohita* fish with Ag NPs[21].

The mechanism of toxicity and behavior of nanoparticles in the presence of other particles or environmental pollutants are different. The results of this study showed that Ag NPs can partially reduce the toxicity of $Hg^{2+}$ on primary lamella diameter as well as secondary lamellae length of gill. In fact, nanoparticles may absorb other chemicals in a way to lessen environmental pollutants. For instance, it is known that Hg can bind to the surface of nanoparticles[11,13]. The toxicity of heavy metals in the presence of various nanoparticles such as C nanotubes, Ce and $TiO_2$ nanoparticles and $Al_2O_3$ nanoparticles is similar with the findings of some studies[26-28].

Kim *et al.* illustrated that the Ag NPs can reduce bioaccumulation of As and Cu and increase the acute toxicity and bioaccumulation of Cd in daphnia body[29]. Rosenfeldt *et al.* reported decreasing toxicity following joint use of $TiO_2$ nanoparticles and Cu in an aqueous environment on *Gammarus fossarum*[30]. Zou *et al.* found that mutual use of $TiO_2$ nanoparticles with Ag NPs reduces the environmental risk of Ag NPs[31]. In spite of these positive statements about reducing toxicity of heavy metals when combined with nanoparticles, there are different findings in some studies. Zhang *et al.* found the increasing bioaccumulation and toxicity of Cd in presence of $TiO_2$ nanoparticles in various tissues of common carp[32]. In another study, Shi *et al.* illustrated that $TiO_2$ nanoparticles can enhance synergism property of pesticide, dichloro-diphenyl-tricgloroethane on human hepatocyte cells[9]. In summary, the effects of nanoparticles on toxicity of other chemicals are still controversial. More extensive studies are recommended for deeper understanding of the mechanisms and effect of nanomaterial hazards when other nanoparticles and environmental pollutants are present.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgments

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