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Effects of cobalt oxide nanoparticles and cobalt ions on gill histopathology of zebrafish (*Danio rerio*)

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Abstract. The purpose of this study was to determine acute toxicity of cobalt ions (as $CoCl_2.6H_2O$) and cobalt oxide nanoparticles (as Co_2O_3 NPs) as well as their effects on histopathology of gills of zebrafish (*Danio rerio*) with average weight of 2–3 g. The results of this study showed that the 96-houre LC_{50} values for both $CoCl_2.6H_2O$ and Co_2O_3 NPs were > 100 mg L^{-1}. On the other hand, the results of histopathological studies indicated that exposure of zebrafish to cobalt nanoparticles and cobalt ions caused several gill injuries such as aneurism, dilated and clubbed tips, hyperplasia, oedema, curvature, fusion of lamellae, increase of mucous secretion, hypertrophy, and necrosis. In conclusion, the results of this study showed that the zebrafish is sensitive to both cobalt oxide nanoparticles and cobalt (II) chloride hexahydrate.
Key Words: cobalt, toxicity, zebrafish, nanotoxicology, nanoparticle, ion.

Introduction. Since most of the metals have potentially toxic effects on organisms, contamination of aquatic ecosystems by metals has become a serious problem worldwide (Yilmaz 2009). Metals such as cobalt may present environmental risk when occurring at raised levels (Mansouri et al 2011, 2012), although cobalt is of relatively low abundance in the earth's crust and in natural waters. The concentration of cobalt in organs of fish is low and accumulation of this metal in fishes was not detected in places where levels of cobalt in water were near to the background values (Güner 2010). Results of acute toxicity tests on marine fish have shown that cobalt has a low toxicity, with 96-h LC_{50} ranging from 52.5 to more than 1000 mg L^{-1} (WHO 2006). The several studies reported the 96-h LC_{50} values of $CoCl_2$ on *Pimephales promelas*, and *Carassius auratus*, rainbow trout (*Oncorhynchus mykiss*) were 21.8 mg L^{-1} by Ewell et al (1986), and 333 mg L^{-1} by Das & Kaviraj (1994), and 1.4 mg L^{-1} by Marr et al (1998) respectively. On the other hand, metal oxides nanoparticles (NPs) such as cobalt oxide, play an important role as antimicrobial agents and can be used because of its effectiveness on resistant strains of microbial pathogens, less toxicity and heat resistance (Karvani & Chehrazi 2011). Moreover, cobalt oxide NPs are used in pigments, catalysis, sensors, electrochemistry, magnetism, energy storage, etc (Liu et al 2005). This nanomaterial may release to aquatic ecosystems and cause toxic effect on organs of aquatic organisms such as fish. However, there is still not enough information existing to conclude what the toxic effects of cobalt oxide nanoparticles are on aquatic biota. Therefore, it is of great importance to know the potential risks of environmental pollution by cobalt oxide NPs.

Histological lesions can be used as indicators to identify effects of various chemical contaminations on organisms such as fish, and reliable tool in controlled experiments and field studies (Lee et al 2012). Histopathology can show various injuries such as epithelial lifting and fusion of the lamellae, hypertrophy, hyperplasia, edema, and necrosis in fish

tissues following exposure to pollutants (Karvani & Chehrazi 2011; Johari et al 2013). Numerous researches have assessed susceptibility of aquatic organisms such as fish to the toxic effects of NPs (Lee et al 2012; Johari et al 2013, 2014a; Al-bairuty 2013). In this regards, Lee et al (2012) evaluated toxicity of citrate-capped silver NPs in common carp (*Cyprinus carpio*), Johari et al (2013, 2014a, b) reported toxicity comparison of colloidal silver NPs on rainbow trout and zebrafish (*Danio rerio*), and Al-bairuty (2013) assessed histopathological effects of metal and metallic NPs on the body systems of rainbow trout. Although toxicity of cobalt oxide NPs on histopathology of fish gills has been largely ignored, some reports have documented acute toxicity effects of cobalt ions (as $CoCl_2$) on fish (Al-bairuty 2013; Lee et al 2014; Wu et al 2010). Therefore, the aim of this study was to investigate the toxicity effects of cobalt oxide NPs and cobalt ions on gill histopathology of zebrafish under controlled laboratory condition.

Material and Method. In the present study, the cobalt (III) oxide (Co_2O_3) nanoparticle was purchased from Nanosany Co. (Mashhad, Iran). According to the information provided by the manufacturer, this black powder was 99.7% pure nearly spherical cobalt oxide NPs with an average size of 50 nm (Figure 1). The other characteristics of these NPs were 75.8 $m^2 g^{-1}$ for specific surface area (SSA), 0.21 for loss of weight in drying, 0.38 loss of weight on ignition, 5.3-6.5 for pH, and 0.732 g cm^{-3} for bulk density. To provide 1000 mg L^{-1} stock suspension of cobalt oxide NPs, one gram NP was added to 1000 mL distilled water firstly, then to achieve uniform NP dispersion, this mixture was sonicated in a bath type sonicator (Elmasonic P) for three times (30 minutes each). Also stock solution (1000 mg L^{-1}) of cobalt ions was prepared by dissolving analytical-grade $CoCl_2.6H_2O$ in distilled water.

From October to December of 2014, zebrafish with a mean total length of 3±0.5 cm and mean weight of 0.5±0.04 g were procured from a local aquarium shop and were acclimatized to laboratory conditions (Ecotoxicology labratory, Health Department) for 10 days prior to beginning of the experiments. All the acute toxicity tests were conducted according to OECD guideline for testing of chemicals No. 203 (OECD 1992). Preliminary acute tests were conducted using several concentrations (up to 100 mg L^{-1}) of cobalt ions and cobalt oxide NPs to estimate the minimum lethal and maximum nonlethal concentrations. Accordingly, fish were divided into 5 groups of 7 each; the first group (0 mg L^{-1}) served as the control group and the others (10, 40, 70, and 100 mg L^{-1}) as the experimental ones. These concentrations of cobalt ions and cobalt NPs were selected based on LC50 in this study that was > 100 mg L-1. Each exposure was conducted in aquariums with 20 L dechlorinated and aerated tap water in triplicates. Fish were not fed 24 h before or during the exposure experiments. The aquariums were inspected after 24, 48, 72 and 96 h for finding any dead fish.

To assess histopathological effects of tested chemicals on fish gills, acute toxicity tests were prolonged to eight days. To preserving the constant levels of cobalt ions and NPs in the aquariums in this stage, exposure water were changed every other day. At the end of eighth day of exposure of fish to chemicals, the gills from each fish were surgically removed and immediately fixed in 10% buffered formalin for further histopathological examinations. Tissue preparations were performed as described in Bernet et al (1999) and prepared slides were photographed by a light microscope equipped with a digital camera (Nikon Eclipse E200). During the experiments, dissolved oxygen (mg L^{-1}), temperature (°C), and pH were recorded individually in each test aquarium (Table 1). Total hardness, nitrite, and chloride (mg L^{-1}) were determined before starting the experiments by standard methods (APHA 1989).

Table 1

Physico-chemical properties of the aerated tap water used for toxicity tests

Parameter	*Measured value*
Total hardness (as $CaCo_3$, mg L^{-1})	220.9±18
pH	8.4±0.1
Temperature (°C)	26±0.2
Dissolved oxygen (mg L^{-1})	7.1±0.2
Nitrate (mg L^{-1})	0.60±0.41

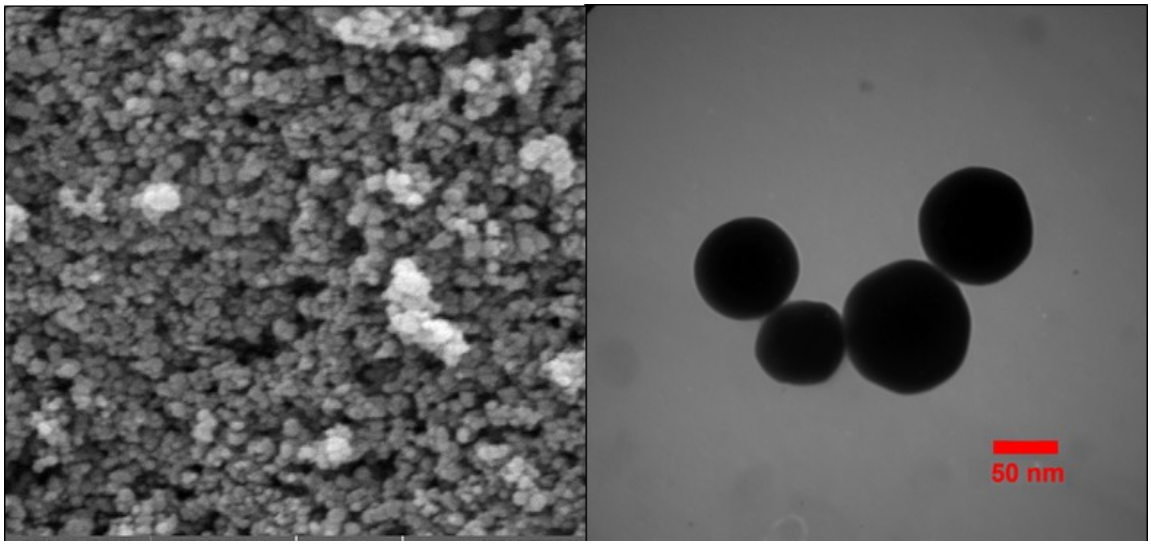

Figure 1. SEM (left) and TEM (right) images of tested cobalt oxide nanoparticles.

Results and Discussion. The results of this study showed that the LC_{50} for cobalt ions and cobalt oxide NPs for zebrafish in 96-hours was > 100 mg L^{-1} (LC_{50} for cobalt ions and cobalt oxide NPs were 300 mg L^{-1} respectively). Moreover, results of this study indicated no mortality during the experimental period in controls and treatments. According to aquatic hazard classification of USEPA, if the LC_{50} of a chemical be higher than 100 mg L^{-1}, it don't have acute toxicity but may cause long lasting harmful effects to aquatic life (EPA 2004). It seems that lake of acute toxicity of cobalt oxide NPs in this study is related to its rapid sedimentation. The cobalt NPs and cobalt ions in solution were approximately deposited after 4 hours and were visible to the naked eye. In this condition, an orange layer of cobalt ions and or a black layer of cobalt NPs was found as a consequence of aggregation and sedimentation of particles at the bottom of the aquariums. Finally, according to the results of acute toxicity tests in this study, we found that Co NPs and Co-ions is low toxic to the zebrafish. Pourkhabbaz et al (2011) reported a 96-h LC_{50} of cobalt (as $CoCl_2$) as 204.8 mg L^{-1} for *Capoeta fusca*. Moreover in another study, Dave & Xiu (1991) determined the toxicity of cobalt in embryos and larvae of the zebrafish under standardized conditions, and reported a nominal "no effect" concentration for effect on hatching time was 3840 mg L^{-1}.

Several studies have been shown the harmful effects of NPs with endpoints such as genotoxic, cytotoxicity, membrane damage, inflammatory response, oxidative stress, apoptosis, etc. on various aquatic organisms (Zhu et al 2009; Kalbassi et al 2011; Asghari et al 2012; Salari Joo et al 2012, 2013; Johari 2014; Tavana et al 2014). In this regard, fish gills are good target organs to show fish health and assessment of chemical contaminants potential in aquatic ecosystems. To determine the severity of these effects, special techniques are needed and histopathology of fish tissues is one of the useful techniques in aquatic toxicology which can provide useful information for identifying toxicity of metals and NPs. The histopathological changes in zebrafish gills after exposure to cobalt oxide NPs and cobalt ions are displayed in Figures 2 and 3. As can be seen, in the control groups, gill filaments and primary lamellae showed only some small histopathological alterations (Figures 2 and 3, E). But eight days exposure to cobalt oxide NPs and cobalt ions caused injuries that were mostly include aneurism, dilated and clubbed tips, hyperplasia, oedema, curvature, fusion of lamellae, disruption of epithelial cells, increase of mucous secretion, hypertrophy, and proliferation in the erythrocytes of cartilaginous core, and also necrosis (Figure 2, B to E2). These kinds of histopathological alterations show that the gills are affected by both cobalt oxide NPs and cobalt ions. Winkaler et al (2001) reported the histopathological changes due to fish response to the toxic agents present in the water and in the sediment. Moreover, Subashkumar & Selvanayagam (2014) reported increase of some histopathological changes including hyperplasia, epithelial lifting, and fusion of lamellae in the fish gill following exposure to toxins, resulting in breathing disorder and fish death.

In this study we observed fusion of lamellae and formation of aneurism in zebrafish gills exposed to cobalt oxide NPs and cobalt ions. The aneurism is blood-filled and swelling blood vessel and this condition in the gill tissue may lead to disturbances in blood flow in the gills, risk of rupture and bleeding or death (Flores-Lopes & Thomaz 2011; Rajkumar et al 2015). Rajkumar et al (2015) reported similar results of aneurism lesions in the gills of fish following exposure to silver NPs. Al-bairuty (2013) indicated that exposure to copper NPs resulted in some increases in the incidence of edema in the secondary lamellae, lamellar fusion, clubbed tips, and hyperplasia, aneurisms, and necrosis in the secondary lamellae of the gills filaments of rainbow trout. The results of present study showed that the severity of damage to the fish gills increased with increasing concentration of cobalt oxide NPs and cobalt ions, but severity from the cobalt ions was higher than cobalt oxide NPs. The highest injuries such as necrosis in zebrafish gills were observed in 100 mg L^{-1} in both of tested chemicals (Figures 2, and 3, D).

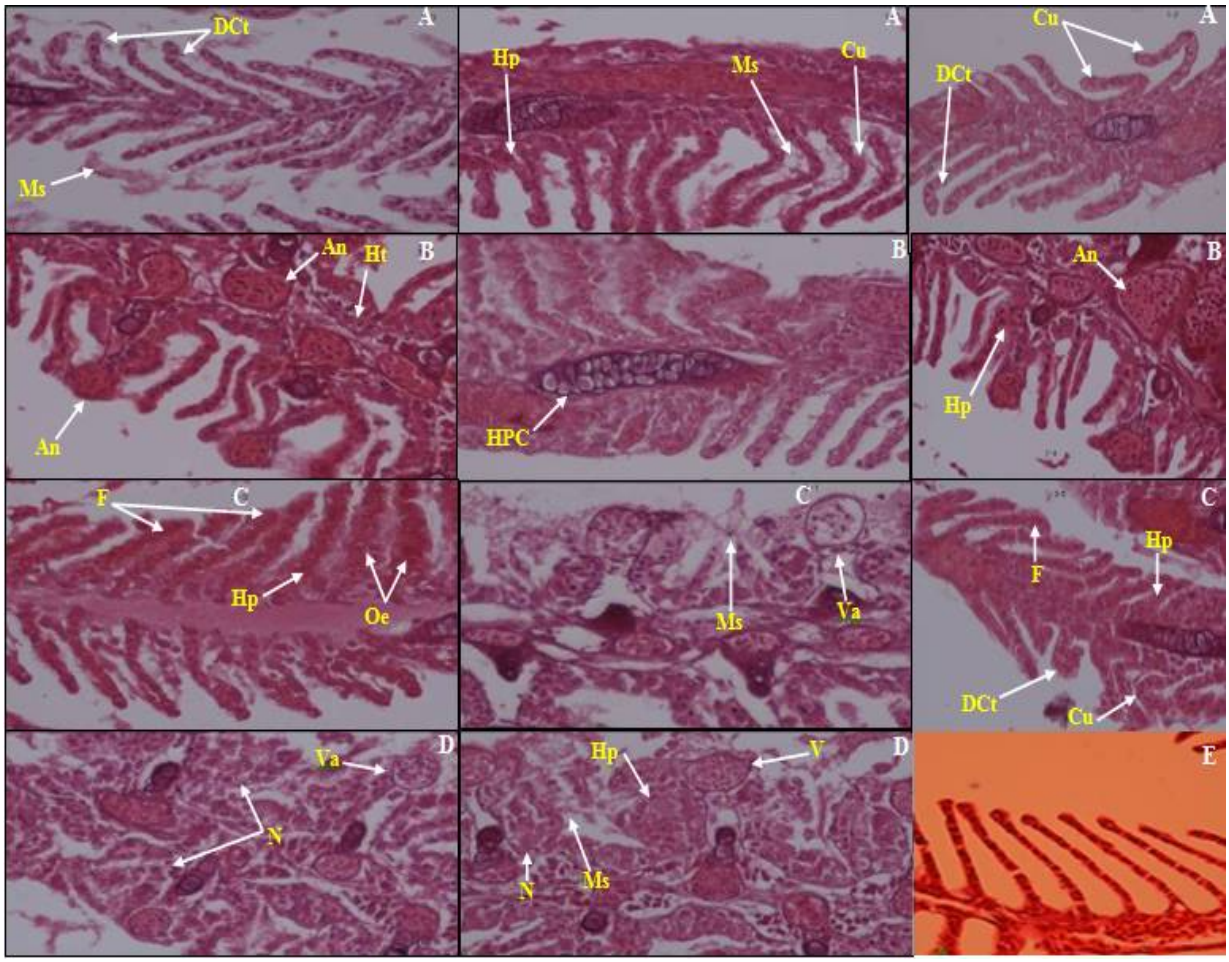

Figure 2. Gill morphology in zebrafish exposed to cobalt NPs for eight days (x40). The panels include A: 10 mg L^{-1}, B: 40 mg L^{-1}, C: 70 mg L^{-1}, D: 100 mg L^{-1}, and E: control group. The gills of control fish indicated normal histology, whilst all treatments showed injuries that include vacuoles (Va), aneurism (An), dilated and clubbed tips (DCt), hyperplasia (Hp), oedema (Oe), curvature (Cu), fusion of lamellae (F), increase of mucous secretion (Ms), hypertrophy (Ht), hypertrophy and proliferation in the erythrocytes of cartilaginous core (HPC), necrosis (N).

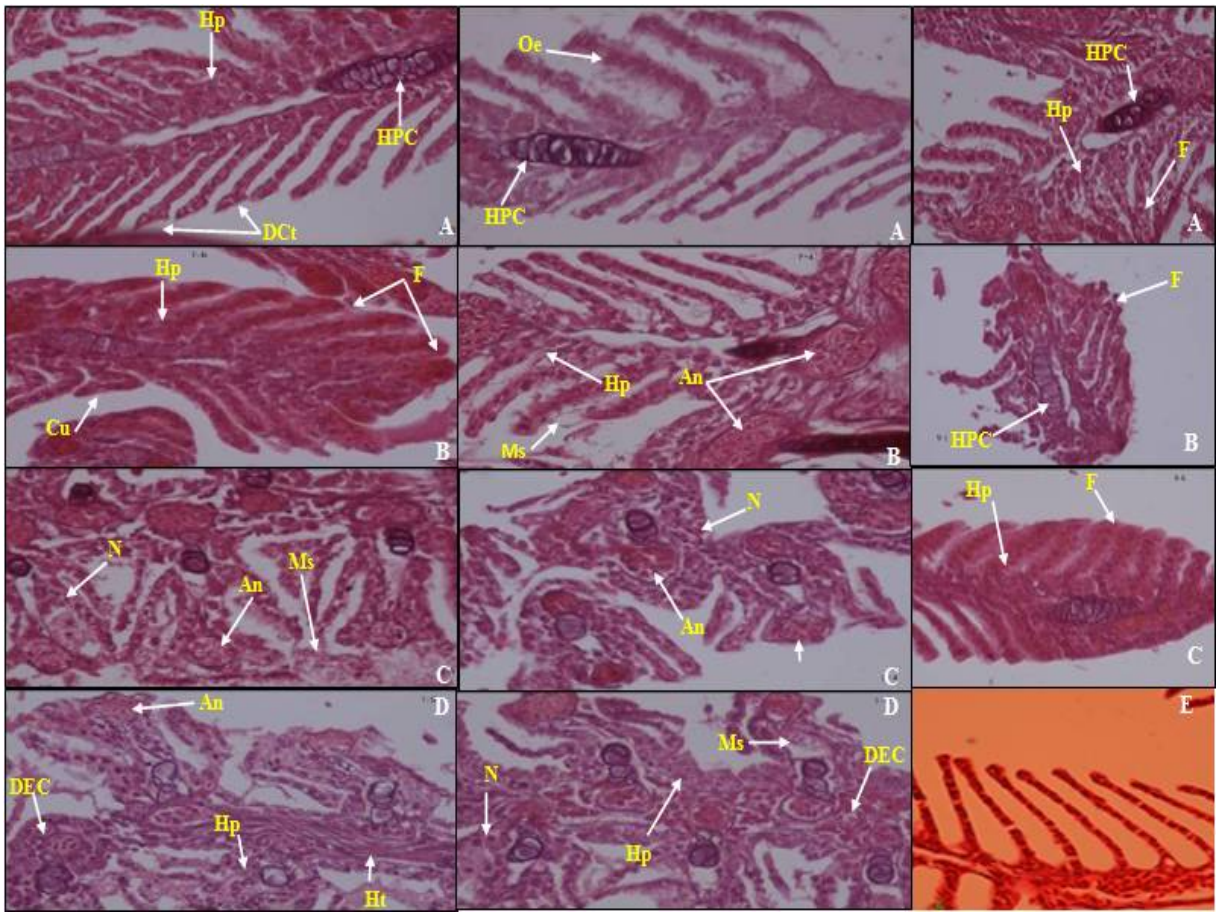

Figure 3. Gill morphology in zebrafish exposed to cobalt ions for eight days (x40). The panels include A: 10 mg L^{-1}, B: 40 mg L^{-1}, C: 70 mg L^{-1}, D: 100 mg L^{-1}, and E: control group. The gills of control fish indicated normal histology, whilst all treatments showed injuries that include vacuoles (Va), aneurism (An), dilated and clubbed tips (DCt), hyperplasia (Hp), oedema (Oe), curvature (Cu), fusion of lamellae (F), disruption of epithelial cells (DEC), increase of mucous secretion (Ms), Hypertrophy (Ht), hypertrophy and proliferation in the erythrocytes of cartilaginous core (HPC), necrosis (N).

Conclusions. According to the lethality results of this study, LC_{50} values were higher than 100 mg L^{-1}, threfore cobalt oxide NPs and cobalt ions didn't have acute toxicity effect on zebrafish. In contrast, some histopathological lesions in gill of zebrafish such as aneurism, hyperplasia, oedema, curvature, increase of mucous secretion, fusion, and necrosis were observed for both of tested chemicals during eight days of exposure. Moreover, effects of these materials in zebrafish gills were dose dependent.

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