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Research Article

Histological study and evaluation of Hsp70 gene expression in gill and liver tissues of goldfish (*Carassius auratus*) exposed to Zinc oxide nanoparticles

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Abstract

In parallel with the increased use of nano-sized metallic materials, the possibility of their release into the environment has increased in recent decades. However, there is not sufficient information on the toxic potential of these materials on different aspects of the life history of aquatic organisms. In this study, we investigated the impact of zinc oxide nanoparticles (ZnO-NPs) on goldfish by studying histopathological alteration and Hsp70 gene expression in gill and liver tissues. The results showed that after exposure to different concentrations of ZnO-NPs, severe lesions were observed in the gill and liver tissues of goldfish. Expression of Hsp70 significantly increased in comparison with the control group and this increase was observed especially in the initial sampling times. Also expression pattern of Hsp70 is relatively similar in gill and liver tissues of goldfish. Based on the results of the present study, ZnO-NPs, as widely used nanoparticles can endanger the health of goldfish and further studies are needed to evaluate its potential toxic effects.

Keywords: ZnO-NPs, Gene expression, Histopathology, Toxicity, Goldfish

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Introduction

The unique characteristics of nano-sized materials, such as large surface area, a greater number of active sites, ultrahigh reactivity, and quantum effects, allow them to produce many specific effects different from their bulky states (Huang *et al*., 2003; Macwan *et al*., 2011). Due to these characteristics, these materials have been exploited in many fields such as aerospace engineering, nanoelectronics, environmental remediation, medical healthcare and consumer products (Singh *et al*., 2009). Because of the widespread application of nontechnology, its global market was US $10.5 billion in 2006 and is predicted to increase significantly to $3 trillion by 2016 (Farré *et al*., 2009).

In parallel with the increased application of nanoparticles (NPs), the likelihood that they will be released into the environment, is high. Although few studies have been conducted on real concentration of engineering NPs in the environment (air, soils, or water), the existence of some of these NPs in the environment has been confirmed in some studies (Praetorius, *et al*., 2012; Chowdhury *et al*., 2013; Gottschalk *et al*., 2013). However, there is now a wider debate about the environmental fate of nano-materials and their toxicity to humans and impact on the environment. Because the aquatic environment is considered as a sink for most environmental contaminants, it is particularly at risk of exposure to engineering NPs (Mills and Chichester 2005). Many studies have been conducted to investigate the effects of NPs on aquatic organisms. In a study conducted by Yeo and Kang (2008), it was found that the hatching rate of zebrafish (*Danio rerio*) decreased in the silver NPs exposed groups (10 and 20 ppt). Also in another study, it was seen that after 13 weeks of TiO_2 NPs exposure, the number of zebrafish eggs fell by 29.5% (Wang *et al*., 2011). After exposure to sub-lethal concentrations of ZnO NPs, expression of genes involved in cytoskeletal transport, cellular respiration, and reproduction including multicystatin, ferritin, and C1q, respectively was suppressed in *Daphnia magna* (Poynton *et al*. 2010). Multi-walled carbon nanotubes (MWCNTs) cause damage to the reproduction potential in zebrafish (Cheng *et al*., 2011). 100 µg/L Cu NPs causes gill injury in adult zebrafish (Griffitt *et al*., 2007).

Due to the properties of zinc oxide NPs (ZnO-NPs), they have been widely applied in optoelectronics, cosmetics, catalysts, ceramics, and pigments, etc. (Poynton *et al*., 2010). In different studies the adverse effects of ZnO-NPs have been proved (Zhu *et al*., 2009; Poynton *et al*., 2010). ZnO-NPs are easily bio-accumulated by aquatic organisms; therefore they can exert their toxic effects in these organisms.

According to the position of gills in fish, this tissue is the first inner organ to react to environmental factors (Katuli *et al*., 2014). Also, the liver is an important organ that contains detoxification enzyme systems (Liska, 1998) and in a different study, lesions

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of these organs were considered as appropriate indicators of environmental contamination (Olojo *et al*., 2005; Mishra and Mohanty 2008; Vinodhini and Narayanan 2008; Katuli *et al*., 2014).

Different environmental stress such as alterations in environmental salinity, temperature and exposure to pollutants can alter heat shock protein expression 70 (Hsp70), so Hsp70 considered as a general and reliable stress response (Planelló *et al*., 2008; Miandare *et al*., 2016). Induction of Hsp70 by heavy metals, 4-Nonylphenol (4-NP) and pesticide in different tissues of fish has been found (Boone and Vijayan, 2002; Eder *et al*., 2004; Palermo *et al*., 2012; Rajeshkumar *et al*., 2013).

Goldfish (*C. auratus*) is a species that is preferred as an experimental model organism for screening environmental pollution and following toxicity (McCarty *et al*., 1978; Abarghoei *et al*., 2016, Golshan et al., 2020). This study aimed to describe the effects of ZnO NPs on histological and transcriptional alterations of Hsp70 gene in gills and liver tissues of the goldfish in relation to a different concentration and exposure time.

Materials and methods

Zn-NPs powder was purchased from Nonaka Company, Iran. Based on the manufacturer's information, the average size of Zn NPs was 70 nm with a purity of ~ 99% purity. To prepare the stock solution, Zn-NPs dry powder was dispersed in Milli-Q water and sonicated for 6 h (50 W, 40 KHz, Bandeline, Germany). Different concentrations of Zn NPs were obtained by dilution of stock solution in Milli-Q water and then, these suspensions were sonicated again for 30 min. To determine particle size distribution and zeta potential of Zn NPs in suspension, dynamic light scattering (DLS; zetasizer, Stabisizer, Particle Matrix, Germany) was used. The particle size and shape in the exposure suspension were also confirmed by transmission electron microscopy (TEM) (Hitachi, Japan) In the present study, for determination of dissolution, 5 ml of suspension was added to Amicon Ultra Centrifugal Filters (Millipore, USA), then centrifuged for 30 min at 8000 rpm at room temperature (24 °C). After centrifugation, the supernatant was collected and analyzed using the Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Elan 6000 DRC, Perkin Elmer, USA).

Goldfish (*C. auratus*), weighing from 5±3 g, were obtained from the Fish Breeding and Rearing Center (Guilan Province, Iran) in summer 2015. The fish samples were then transported to ornamental fish culture center in Gorgan (Golestan Province, Iran), for acclimatization under laboratory conditions (T~ 23°C; pH~ 7.5; DO~ 7.89 mg O2 L^{-1}) and fed with commercial food of ornamental fish (~5% of their body weight daily) for two weeks before the experiments. There were no significant differences between tanks for water quality.

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In total, 240 specimens of fish were randomly distributed into 12 tanks of 300 L (20 fish per tank), and exposed during 14 days to 5, 20 and 50 mg/L ZnO NPs. These concentrations were selected according to the study of Hao *et al*. (2013), who used the same concentrations and reported these concentration had no acute toxicity. Three replicate tanks were allotted to each treatment. During the exposure time, the water system was continuously inspected for temperature, dissolved oxygen, pH and conductivity. Two-thirds of the total water was renewed every 24 h by adding ZnO NPs stock solution to minimize the metal loss after feeding and thus reduce contamination of tanks by food remains. In each sampling time, five fish per treatment were rapidly anesthetized with clove powder (300 mg/L) and tissue samples were collected and used for histology experiment and gene expression (RNA extraction). For histological method, tissues were located in buffered formalin and for gene-expression analysis, gill and liver tissue samples were immediately deep-frozen in liquid nitrogen and stored in a -80°C freezer and were kept frozen until analysis. All tests were carried out under a protocol approved by the Ethics Committee of the Faculty of Sciences of Tehran University (357; 8 November 2000).

The protocol for histological analysis has been reported previously (Katuli *et al*., 2014). Briefly, the second right gill arch of goldfish was sampled and fixed in 10% buffered formalin solution. After deparaffinization, sections were stained with haematoxylin and eosin (H&E staining). The images were captured with Nikon EC 600 Eclipse microscope.

RNA extraction

RNA extraction steps were done using the acid guanidinium thiocyanate-phenolchloroform method modified according to Awad *et al*. (2011). Total RNA isolation was done based on BIOZOL Reagent protocol (Bioflux-Bioer, China). Quantity of extracted total RNA was evaluated by spectrophotometer at 260/280nm and RNA integrity was determined by 1 % agarose gel electrophoresis. DNA of samples was removed by treatment with DNase I (Fermentas, Lithuania). Afterwards, RNA from three samples of each replicate were pooled (Roy and Bhattacharya, 2006). Synthesis of cDNA was carried out according to the Fermentas protocol. Briefly, 1µg RNA was denatured and annealed at 70°C for 5 min with 0.2 µL of oligo (dT) 20. After chilling on ice, 4 µL 5X reaction buffer, 1 µL Ribo Lock Ribonuclease inhibitor (20 U/µL) and 2µL 10 Mm dNTP were mixed and incubated at 37°C for 5 min. This mixture was added to 1µL M-MuLV RT (200U/µL) in final volume of 20 µL, incubated at 37°C for 10 min, and then at 42°C for 60 min, finally at 70°C to stop the reaction (Fermentas, Lithuania).

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Primers for Hsp70 and reference (RPL6) in goldfish were designed by using Primer-Blast (Table 1). The expression of housekeeping gene β-actin, as a reference gene, was used for normalization of mRNA levels of the genes. The synthesized cDNA from samples with reference and target genes primers designed for qPCR were reproduced using standard PCR procedures. cDNA products were electrophoresed on a 1.5% agarose gel.

Real-time Quantitative PCR (RT-qPCR) was performed by applying SYBR Green I technology on iQ5 System (BioRad, USA). 1x SYBR Green PCR Master Mix (SYBR biopars, GUASNR, Iran), 300 nm of each specific forward and reverse primers, 10 ng of cDNA template, and nuclease free water to a final volume of 20 µL were used for each reaction. PCR reaction mixtures were exposed to the following thermal profile: 94°C during 5 min, 40 cycles at 10 s at 94 °C followed by 10 s at 56°C and 10s at 72°C and each reaction was amplified in triplicate. To validate the real-time PCR primers, a specific primer pair was run in duplicate, along a temperature gradient (55-65ºC) in the same plate. A melting curve analysis was performed after every amplification program for specificity of target and the absence of primer dimers, and a no template control (NTC) was included with each assay to verify that PCR master mixes were free of contamination. Standard curves were constructed with dilution series of pooled cDNA (including seven dilutions from 1/10 to 1/1000). The PCR efficiency was calculated using the following equation: E%=(101/Slope-1)*100 (Radonic *et al*., 2004; Kolangi Miandare *et al*., 2017). The fold change in expression of of relative mRNA of Hsp70 was presented using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The obtained data were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad, USA).

Table 1: qPCR primer used in this study.

Primer name	Seq	Size (bp)	Reference
Hsp70 Forward	GAAGGTCCTGCACTGCACT	180	
Hsp70 Reverse	CTTGGTGCACAAGTTCTGGT		Rytkonen *et al*., 2012
β-Actin Forward	ACAGAGCTGATGGGATACCAG	210	
β-Actin Reverse	CCTGTGATGGCTTGTCCTTT		Rytkonen *et al*., 2012

Statistical analysis

The data regarding the effect of ZnO NPs on levels of mRNA gene expression of Hsp70 were analyzed using $2^{-\Delta\Delta ct}$ in Excel software version 2013 (Pfaffl *et al*., 2002). All data are presented as means with standard deviation (SD) and in all cases *p*-values<0.05 were considered significant.

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Results

The results of Transmission Electron Microscope (TEM) image showed that the diameter of ZnO-NPs ranged between 70 and 90 nm (Fig.1 A) and with regard to results from dynamic light scattering (DLS), average diameter of NPs was 90 nm (Fig.1 B). Also the results of this study showed that ζ-potential was -15.13±2.3 mV, as well as dissolution value in suspension of ZnO-NPs was 0.7% and this indicated appropriate stability of NPs in suspension.

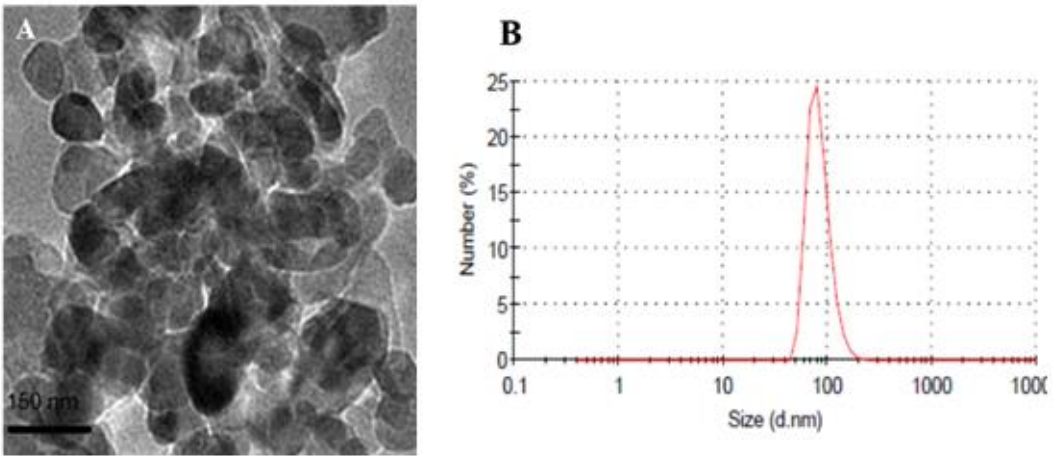


Figure 1: The dynamic light scattering (TEM) image of diameter of ZnO NPs (A) and the results of transmission electron microscopy (DLS) (B).

The histological examination indicated a low to moderate incidence of damage on tissues of goldfish after the exposure to different concentrations of ZnO-NPs for 14 days. Histopathological changes recorded in gill and liver tissues are summarized in Tables 2 and 3 and shown in Fig.s 2 and 3. The control group did not display histological changes in both tissues at sampling time. In the ZnO-NPs - exposed fish, the destruction of the secondary lamella, necrosis, adhesion of secondary lamellae, shortening of secondary lamellae, club shaped lamellae and hyperplasia were observed (Table 2 and Fig. 2 A-D). Histopathological changes were also noticed in the liver tissue of ZnO-NPs-exposed fish. Microscopic analyses revealed that the most noticeable changes include, cell enlargement, degeneration in liver, necrosis, obstruction of blood flow and intercellular edema in exposed fish (Table 3 and Fig. 3 A-D). Sinusoid Dilution and nuclear vacuolization are visible in exposed fish Fig. 3 E and G). Exposure to ZnO NPs destroyed tissue structures in a time and dose-dependent manner so that observed histological alterations were more evident in fish exposed to 20 and 50 mg/L ZnO NPs for 7 and 14 days.

As shown in Fig. 2 and 3, ZnO-NPs affected the Hsp70 in gill and liver of

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goldfish. In gills, highest gene expression was shown 1 and 3 days after exposure to 50 mg/L of ZnO NPs but there wasn't significant difference between the control and 5 mg/L of ZnO NPs on days 1 and 3 of sampling (Fig. 4). Highest expression of gene in the liver was observed on dys 1 and 3 after exposure to ZnO NPs. Gene expression pattern on day 7 of sampling was irregular. Lowest expression of Hsp70 was observed on day 14 of sampling and there was no comparable difference between the control and exposed groups (Fig. 5). Gene expression at different sampling times in the gills has more similarities.

Table 2: Summarized gill lesions of goldfish exposed to ZnO NPs.

Exposure time (day)	Concentration (mg/L)	Lesions						
		Shortening of secondary lamellae	Destruction of the secondary lamella	Dilation of capillaries	Adhesion of secondary lamellae	Bulge epithelium of secondary lamellae	Necrosis	Hyperplasia
0	0	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	20	-	-	-	-	-	-	-
	50	-	-	-	-	-	-	-
1	0	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	20	+	-	+	-	-	+	-
	50	++	-	+	+	-	+	-
3	0	-	-	-	-	-	-	-
	5	-	+	+	-	+	-	+
	20	-	+	++	+	+	-	+
	50	+	-	-	+	-	+	-
7	0	-	-	-	-	-	-	-
	5	++	+	++	+	++	++	+
	20	++	++	++	+++	+	++	++
	50	+++	++	++	++	+	+	++
14	0	-	-	-	-	-	-	-
	5	++	+++	++	++	++	+++	++
	20	+++	++	++	++	++	+++	++
	50	++	+++	++	++	++	+++	+++

Score value: None (−), mild (+), moderate (++), severe (+++).

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Table 3: Summarized liver lesions of goldfish exposed to ZnO NPs.

Exposure time (day)	Concentration (mg/L)	Lesions								
		Degeneration	Obstruction of blood flow	Intercellular edema	Cell enlargement	Lateral nucleus	Necrosis	Sinusoid Dilution	Nuclear vacuolization	Proliferation of oval cells
0	0	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-
	20	-	-	-	-	-	-	-	-	-
	50	-	-	-	-	-	-	-	-	-
1	0	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-
	20	-	-	-	-	-	-	-	-	-
	50	-	-	-	-	-	-	-	-	-
3	0	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-
	20	+	-	-	+	-	+	-	-	-
	50	+	-	-	+	-	+	-	-	-
7	0	-	-	-	-	-	-	-	-	-
	5	+	-	-	-	+	+	-	-	-
	20	+	+	-	++	++	+	+	-	++
	50	+	+	+	++	++	+	+	+	++
14	0	-	-	-	-	-	-	-	-	-
	5	++	+	++	++	++	+	+	++	++
	20	+	+	+	++	++	++	++	+	++
	50	++	++	+	++	++	+	++	++	++

Score value: None (−), mild (+), moderate (++) and severe (+++).

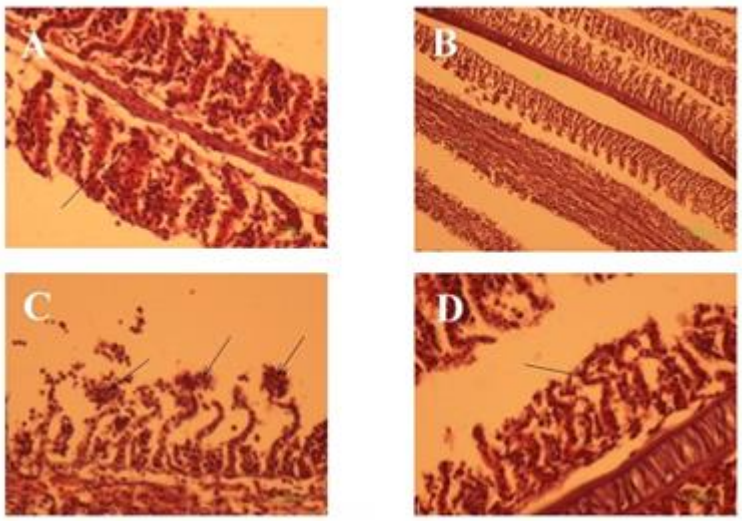


Figure 2: Representative images of histopathological changes in gill tissue of goldfish exposed to ZnO-NPs. (A) Destruction of the secondary lamella, epithelial hyperplasia (arrow), (B) regular shaped secondary gill lamellae in control fish, (C) necrosis and filament epithelium proliferation (arrows), (D) fusion of the secondary lamellae (arrow) (H&E staining).

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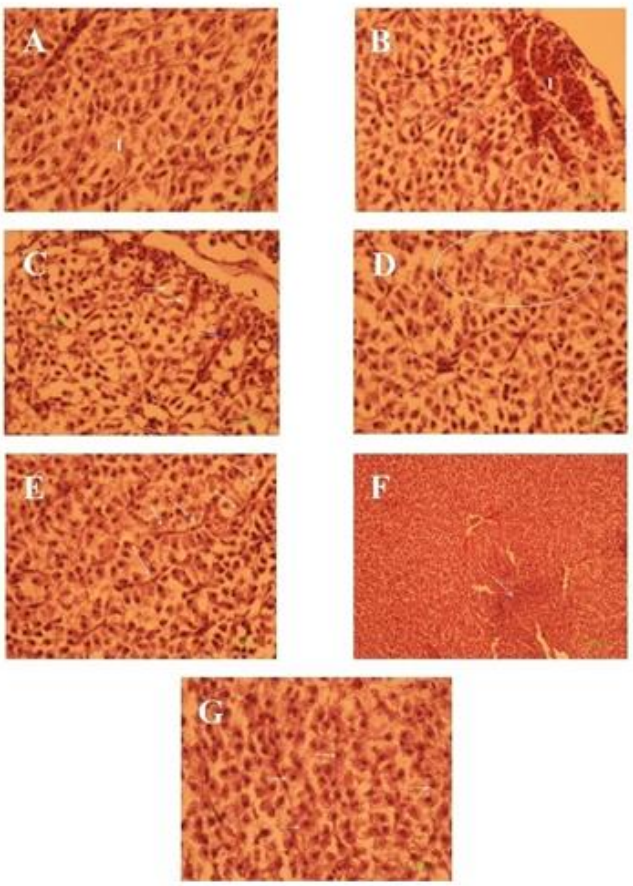


Figure 3: Histopathological changes and microscopic analyses of liver tissue of ZnO-NPs -exposed goldfish. (A-D) cell enlargement, oedema, degeneration and necrosis in the liver tissue (arrows), (E-F) Obstruction of blood flow and intercellular edema in exposed fish to ZnO-NPs (arrows), (G) vacuolization in hepatocytes along with lateral nuclei (arrows) (H&E staining).

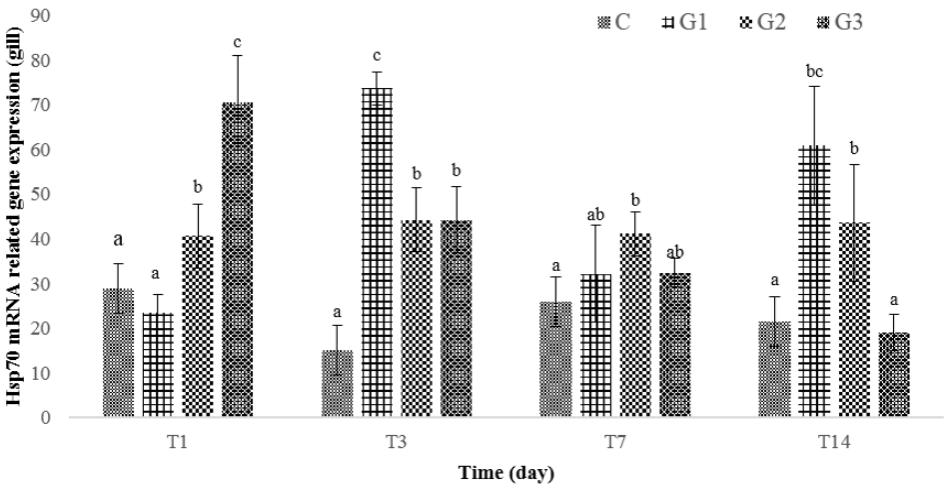


Figure 4: Expression pattern of Hsp70 gene in gill of goldfish. C, Control; G1, Gill tissue at treatment 5 mg/L ZnO-NPs; G2, Gill tissue at treatment 20 mg/L ZnO-NPs, and G3 Gill tissue at treatment 50 mg/L ZnO-NPs.

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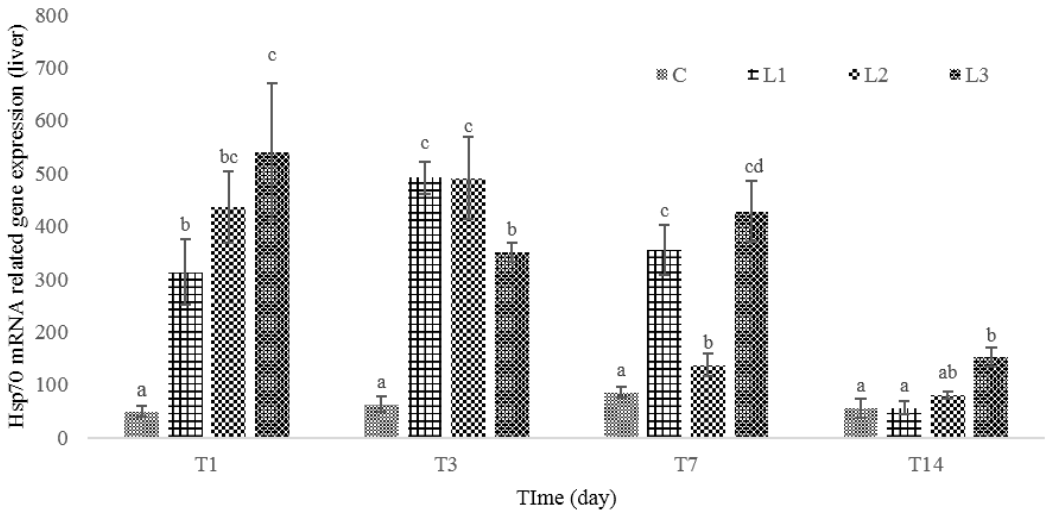


Figure 5: Expression pattern of Hsp70 gene in liver of goldfish. C, Control; L1, Liver tissue at treatment 5 mg/L ZnO-NPs; L2, Liver tissue at treatment 20 mg/L ZnO-NPs, and L3, Liver tissue at treatment 50 mg/L ZnO-NPs.

Discussion

In this study adult goldfish were exposed to sub-lethal concentrations of ZnO-NPs for up to 14 days. The organisms were examined for histological alterations and Hsp70 gene expression profiles in liver and gill tissues. Although a high concentration of ZnO-NPs was used in the present study, no mortality was observed during the experiment. A number of other studies confirmed ZnO-NPs was not very toxic for the survival of different fish species (Bai *et al*., 2010, Johnston *et al*. 2010, Wong *et al*.,2010), and due to the low dissolution value of ZnO-NPs (0.7%), it can be said that zinc ion contributes less to the toxic effects of ZnO-NPs.

Fish gills are generally considered a good indicator of water quality for studies of environmental impact (Cengiz and Ünlü, 2003). Several studies have demonstrated that exposure to NPs resulted in lifting up of epithelium, hyperplasia of the gill filaments epithelium and intraepithelial oedema of gill lamellae of common carp (Linhua *et al*., 2009), zebrafish (*Danio rerio*) (Griffitt, *et al*., 2007; Chen *et al*., 2016), and rainbow trout (*O.mykiss*) (Federici *et al*., 2007).

In the study conducted by Subashkumar and Selvanayagam (2014) the sub-lethal concentrations of ZnO-NPs in common carp, (C*yprinus carpio*) were evaluated. They reported epithelial lifting, desquamation and necrosis, swelling and hyperplasia in gills after 21 days of exposure. Squamous epithelium in pavement cells, chloride cells, and mucous cells, capillary vessels in epithelium and desquamated structure of gill filament were observed in medaka (*Oryzias latipes*) exposed to iron NPs. Our

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results of gill histopathological alteration were consistent with those reports. With respect to these cases, the current results indicate that after 14 days exposure of goldfish to ZnO-NPs, some histopathological lesions in gills were visible. Major alteration in this study includes shortening of secondary lamellae, adhesion of secondary lamellae, necrosis and hyperplasia. These changes can decrease the contact surface between the gills and ZnO-NPs and therefore increase time effect of ZnO-NPs (Cengiz and Ünlü 2003; Katuli *et al*., 2014; Safari *et al*., 2016). In the present study, in addition to the gill tissue, structural changes in liver tissue of goldfish were also investigated. The liver of fish is the main organ of active metabolism and detoxification and is extremely sensitive to pollutants (Choi *et al*., 2010) and different studies used liver as a biomarker that indicate the impact of exposure to environmental stressor. Damage of liver in fish as a consequence of exposure to nanoparticles is reported in previous studies (Federici, *et al*., 2007; Smith *et al*., 2007; Linhua *et al*., 2009). However, it is well known that organ pathology is not necessarily associated with direct ZnO-NPs accumulation in the gill and liver tissue of goldfish, because of secondary oxidative stress and hypoxia during the metal exposure. Because of their external position and large surface of gills, it is likely that the effects of ZnO-NPs on the gill tissue are more than on the liver. In this study accumulation of ZnO-NPs in tissues was not measured, but according to previous findings from related studies, tissue specific accumulation trends of ZnO-NPs (Al-Bairuty *et al*., 2013; Hao *et al*., 2013) could be another reason for more abnormalities in gills of goldfish. However, there are not enough studies on the specific accumulation of ZnO-NPs in tissues of aquatic organisms and their specificity needs to be better understood.

Exposure of goldfish to ZnO-NPs was accompanied by the induction of Hsp70 in gills and liver tissue. These stress proteins are commonly used as a general biomarker to indicate environmental stressors due to chemicals or heat (Vijayan *et al*., 1998; Hansen *et al*., 2007; Farmen *et al*. 2012). A number of studies have described heat shock protein induction by metallic NPs (Ahamed *et al*. 2010; Hu *et al*. 2011). However, none of them have ever dealt with ZnO-NPs- induced Hsp70 induction. In the present study a rapid increase in mRNA levels in the first sampling times (1 and 3 days) was observed. The expression pattern is relatively similar to that in the gills and liver of goldfish.

The results of Hsp70 expression are similar to that of data reported by Williams *et al*. (1996) and Hansen *et al*. (2007). They found Hsp70 up-regulation in fish gills following various heavy metal (such as Cd, Cu, Pb, Zn) exposure. In another study, Ryan and Hightower (1994) reported that expression of Hsp70 in the gills of

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winter flounder (*Pseudopleuronectes americanus*) were evaluated after exposure to Cd and Cu. In contrast to this study some other studies reported that induction patterns of heat shock proteins in fishes are tissue-specific (Dietz and Somero, 1993; Yoo and Janz, 2003).

Based upon these results, induction of the Hsp70 mRNA levels in the gill and liver is abrupt, indicating a general stress response induced from exposure to ZnO-NPs, but not pro-longed, showing an initial increase during the first couple of days and then a decrease back to the basal level expression with longer exposure times (7 and 14 days). In previous studies it was found that nanoparticles can enter cells and cause increased genotoxicity independent of oxidative stress (Kiang and Tsokos, 1998; Wong *et al*., 2010; Yang *et al.*, 2011). It seems that, due to the increase of reactive oxygen species (ROS) in exposed fish, an increase of Hsp70 gene expression can prevent the inappropriate increase in the levels of antioxidant enzymes and their activities, followed by the increase in cellular defense (Currie and Tanguay, 1991; Yuan *et al*., 2016). This gene can prevent apoptosis that results from oxidative stress, by interfering with the apoptotic-signaling pathway (Garrido *et al*., 2001). The results of the present study are consistent with those of Wong *et al*. (2010), who found Hsp70 up-regulated in relation with antioxidant enzyme in medaka (*Oryzias melastigma*) exposed to ZnO-NPs.

Due to lesions in the gills and liver in treated goldfish, cellular stress and potential damage was expected. ZnO-NPs exposure was known to induce oxidative stress and enhanced activities of SOD and CAT (Xiong *et al*., 2013; Trevisan *et al*., 2014). In the current study increasing expression of Hsp70 after exposure to ZnO-NPs can be related to oxidative damage that probably occurs through ZnO-NPs exposure. In contrast, a decrease in Hsp70 gene expression was reported previously by Chae *et al*. (2009). They reported that after 2 days of exposure to Ag NPs, expression level of Hsp70 decreased in Medaka. In this study expression of Hsp70 in gills and liver decreased within 7 and 14 days after exposure (especially in liver). Previous studies suggested that there is a relation between tissue lesions and Hsp70 expression in the same tissue (Schett, *et al*., 1999; Sreedhar and Csermely, 2004) and this could be because of the decrease in the expression of Hsp70 on days 7 and 14.

In conclusion, this study has demonstrated from the viewpoint of pathology that the target organs for ZnO-NPs are similar to those for known nanoparticles. It has also demonstrated that the types of pathologies caused by ZnO-NPs are similar to those known for nanoparticles. There are also some organ-specific material-type effects with ZnO-NPs causing more injury in the gill and liver tissues. The data also indicate that histological analysis is a highly sensitive endpoint for evaluating

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ZnO-NPs toxicity in goldfish. Furthermore, expression patterns of Hsp70 are relatively similar in both the gills and liver, so that after initial sampling times, gene expression was significantly increased in treated fish but did not continue until final sampling times. The results of the present work clearly demonstrated that these tissues and gene expression levels respond to the presence of ZnO-NPs. Further studies are underway to elucidate the mechanism of ZnO-NPs accumulation and determine the involvement of other parameters including free radicals and ROS in ZnO-NPs toxicity.

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