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Influence of Copper Oxide Nanoparticle on Hematology and Plasma Biochemistry of Caspian Trout (*Salmo trutta caspius*), Following Acute and Chronic Exposure

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ABSTRACT: The Caspian trout is an endangered and quite vulnerable fish, considered for a natural protection program in the southern area of the Caspian Sea. Copper oxide nanoparticles (CuO-NPs) are toxic substances, which induce oxidative stress, not to mention other pathophysiological states. The toxicity of nanoparticles on fish needs more characterization for short- and long-term effects. Thus, the present paper examines the acute and chronic effects of CuO-NPs on hematology and plasma biochemistry of juvenile Caspian trout. After determining the lethal concentrations (LC50), juvenile Caspian trout is exposed to 0.1 $LC50_{96}$ CuO-NPs for 28 days in three replicates. The blood samples are then collected from fish after 24, 48, 72, and 96 hours as well as 1, 2, 3, and 4 weeks of exposure to the CuO-NPsto deal with short- and long-term effects, respectively. Analysis of these samples shows that some hematological factors like hemoglobin (Hb), red blood cells (RBC), and hematocrit (Hct) are significantly increased after acute exposure, compared to the control group ($p<0.05$). The number of white blood cells (WBC), neutrophilis, and monocytes are also increased after acute and chronic exposure with significant differences ($p< 0.05$). Furthermore, the levels of lactate dehydrogenase after acute and alkaline phosphatase along with aspartate aminotransferase after acute and chronic exposure are significantly increased ($p<0.05$). Thus, results indicate that the presence of even a tiny amount of CuO-NPs can affect most haematological and metabolic enzymes of the Caspian trout in the short and long-term exposure. It is therefore essential to prevent these nanomaterials from entering the aquatic environment.

Keywords: Copper oxide nanoparticle, *Salmo trutta caspius*, Aquatic Nanotoxicology, Lethal concentration

INTRODUCTION

The characteristics of engineered nanoparticles, which make them suitable in an extensive variety of industrial uses, have led to considerable concern about their possible effects on human health and the environment (Scown et al., 2010). Copper is an important and vital element for the health of all living organisms as it is involved in some basic biological processes (Isani et al., 2013). Copper oxide nanoparticles (CuO-NPs) have been utilized in various industries like batteries, solar energy conversion, gas sensors, field emission emitters, high temperature superconductors, and catalysis (Dar et al., 2008). There are many reports about the contamination of

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aquatic ecosystems by nanoparticles (Al-Bairuty et al., 2013). In aquatic organisms, NPs can enter organisms from different paths such as direct transmission across the gills and other external epithelial surfaces (Isani et al., 2013). Denominating biomarkers of these biological reactions can be used to recognize the health status of organisms and to acquire the earliest signal of environmental contamination (Binelli et al., 2009). Haematological studies provide an indicator of physiological changes in fish (Suvetha et al., 2010), and the fish blood acts as an effective tool to find the variations in the examined organism (Adhikari et al., 2004). The most common hematological variables, measured during stress, include red and white blood cells count, hematocrit value, hemoglobin content, and red blood cells indices (Ololade & Oginni, 2010). An evaluation of biochemical factors could assist recognizing organisms' common health condition. It may also serve as a primary alarm indicator of stress in animals (Dube et al., 2014).

One of the nine subspecies of *Salmo trutta* (known as the brown trout) is the Caspian trout, aka *Salmo trutta caspius* (Quillet et al., 1992). Caspian trout are considered critically endangered (CR) or severely endangered species, according to the international union for conservation of nature (IUCN) criteria (Vera et al., 2011). In Iran, during the past two decades, the populations of Caspian trout have reduced severely due to habitat and environmental pollution, overfishing, and decline of spawning areas. Meanwhile, sufficient research attention has not been paid to toxicological dangers, faced by this species (Barannik et al., 2004; Niksirat and Abdoli, 2009; Adel et al., 2017). Some studies have been conducted on the ecotoxicity of CuO-NPs on biochemical and hematological indices of fish, e.g. Khabbazi et al. (2015) studied the effect of CuO nanoparticles for 96h on some hematological indices of rainbow trout *Oncorhynchus mykiss*. Also, effects of sub-lethal concentrations of CuO nanoparticles on blood parameters in *Rutilus rutilus* was studied by Jahanbakhshi et al. (2015) for a period of seven days. Another study investigated the toxic effects of copper sulfate and copper nanoparticles for a period of 14 days on minerals, enzymes, thyroid hormones, and protein fractions of plasma and histopathology in *Cyprinus carpio* (Hoseini et al., 2016). However, data on the chronic impacts of copper oxide nanoparticles on biochemical and hematological factors of fish is limited (Amr et al., 2015). Additional studies are needed to deal with long term and chronic effects of environmentally-relevant amounts of engineered nanoparticles on fish health (Perera and Pathiratne, 2012).

Therefore, the objectives of this study is to evaluate both acute and chronic impacts of CuO-NPs on biochemical and blood parameters of *Salmo trutta caspius*, and determine whether these indicators can be assessed at short or long-term exposure. Such information could be valuable in environmental protection and aquatic toxicology management.

MATERIALS AND METHODS

Juvenile Caspian trout with an average weight of 25±5 g and the average length of 20 ± 4 cm were obtained from the Breeding Center of Salmonids (BCBCS) in Kelardasht, Mazandaran, Iran. The fish were allowed to acclimatize for 10 days in 1000 L tank prior to the experiments. They were fed with commercial trout pellets, used every day at a rate of 5% body weight, and their water got changed daily (Imani et al., 2015). During the period of acclimatization and experiment, the fish were maintained in 12 h light/dark cycle of photoperiod, with the temperature of the test water kept at approximately 14± 1°C. The pH was 7.5 ± 0.2; the dissolved oxygen, 8 mg/l; and water hardness or concentration of CaCO3, 230 mg/l (Shirdel and Kalbassi, 2016).

CuO-NPs were prepared from the Iranian Nanomaterials Pioneers Company with 99%

purity. The properties of the nanoparticles were studied by SEM analysis (Company: Tescan, model: MIRA3) in Razi Metallogy Research Center and the density and crystal structure of the purchased NPs got determined by analyzing X-ray (Company: Philips, model: Xpert MPD) in the X-ray laboratory of Tarbiat Modarres University. A stock suspension of 400 mg/L of CuO–NPs was prepared by dispersing 40 mg of the powder in 100 mL of distilled water, followed by 15 min of sonication, by means of ultrasound device (QSonica, model: S3000) at room temperature (Johari et al., 2014).

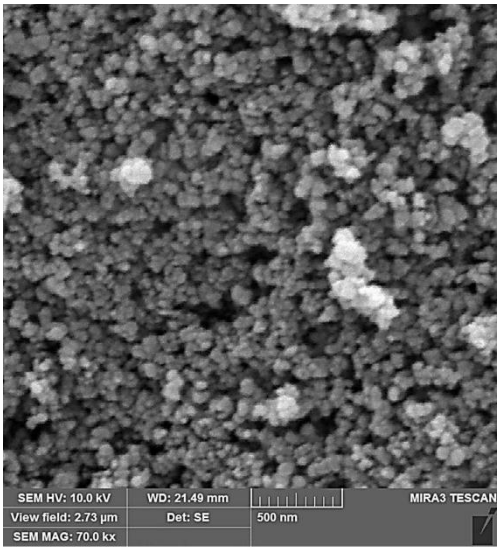
The lethal concentration of CuO-NPs on juvenile Caspian trout was determined according to the method, explained in the Organization of Economic Cooperation and Development guideline (OECD guideline No. 203, 1992). The experiment was planned and performed in 50-liter tanks, containing 20 liters of aerated water. The feeding stopped 24 hour before the start of the experiment and a preliminary experiment was performed in order to determine the lethal concentration of CuO-NPs. In the preliminary experiment, the fish were exposed to 0, 10, 25, 50, 100, 150, 200, 300, and 500 mg/l CuO-NPs for 96 hours. The study was consisted of six treatment tanks, one control group and five experimental ones, with each treatment replicated three times. Thus, a total of 18 tanks were used and the number of dead fish was recorded every 24 hours.

For acute (96 hours) and chronic (28 days) experiments, after the adaptation period, a total of 90 juvenile Caspian trout were allotted to one treatment group, containing 28.647 mg/l (10% of the LC_{50}) CuO-NPs, as well as one control group (without CuO-NPs) with three replicates. After 24, 48, 72, and 96 hours, on one hand, and 7, 14, 21, and 28 days, on the other, three fish were randomly sampled from each of the six tanks. They got anesthetized with powdered clove and heparinized insulin syringe was used to sample blood from the tail vein. Blood samples were transferred to 0.5-mL micro-tubes, containing heparin solution. To analyze the enzymes, part of the blood samples got centrifuged for 10 min at 4500 g (Centurion Scientific, U.K.). After separating plasma with pipette, it was stored at $-70^{\circ}C$ until analysis for preservation (Affonso et al., 2002).

The number of RBC and WBC was manually counted via haemocytometer method. Hemoglobin level was examined by the cyanmethemoglobin method spectrophotometrically at 540 nm (Blaxhall et al., 1973) and hematocrit (Hct) was determined via microcentrifuge method, utilizing standard and small heparinized hematocrit capillary tubes at 7000g for 10 minutes after preparing thin blood smears slides. These were stained with Wrighte Giemsa. The percentages of leukocyte types were calculated (Blaxhall et al., 1973). The hematological indices of mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) by means of the total RBC count, Hct, and Hb concentration were calculated (Lee et al., 1998).

Biochemical auto analyzer instrument (Eurolyser, Belgium) and commercial kits of Parsazmoon (Tehran, Iran) were employed to estimate the levels of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) enzymes (Shahsavani et al., 2010).

The results were reported as means ± SD. Analysis of variance (one-way ANOVA) technique with Tukey's test multiple comparisons was used to determine whether there was any significant difference between the measured values or not. Differences were considered statically significant at $p < 0.05$. To determine the median lethal concentration of CuO- NPs, SPSS software program (IBM SPSS Statistic 20) was used through probit analysis with a confidence level of 95%.



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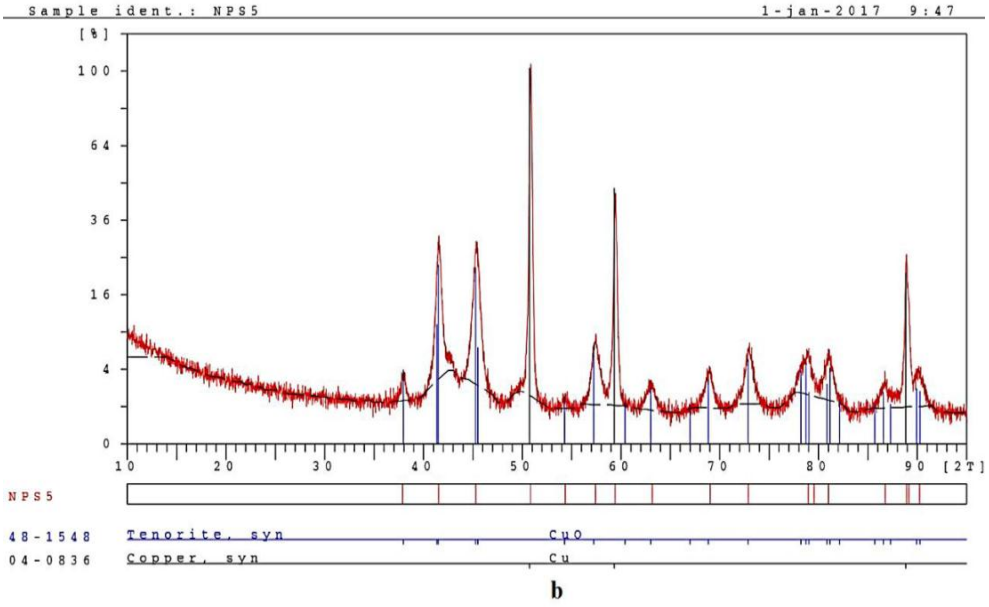

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Fig. 1. Characterization of CuO nanoparticles: a: SEM image of CuO –NPs, b: X-ray image of CuO –NPs

Table 1. Properties of CuO-NPs, used in this study

True density	Bulk density	Morphology	(SSA)	(APS)	color	Purity
6.4 g/m^3	0.79 g/cm^3	Towards spherical	20 m^2/g	40 Nm	black	99.9%

RESULTS AND DISCUSSION

According to SEM (Fig. 1a) and XRD (Fig. 1b) analyses, characteristics of copper oxide NPs were as follows: purity was 99.9%; specific surface area, 20 m^2/g; and bulk density, 0.79 g/cm^3. Table 1 shows other properties of CuO-NPs.

Lethal concentration (LC50) was based on fifty percent mortality of the experimental animals in a fixed time (96

hours). It was utilized to recognize the relation between a specific effect of a chemical material and the dose at which it took place; Therefore, it has ecological and biological significance (Kumar et al., 2018). Table 2 summarizes the obtained result for LC_{50}-$_{96h}$ of the CuO-NPs for the Caspian trout. No mortality was observed in the control tanks and the CuO-NPs concentrations up to 150 mg/l. The 96-hour LC50 of CuO NPs was 286.47 mg/l. Zhao et al. (2011) studied copper oxide NPs and bulk particles (CuO BPs) toxicity (at the concentrations of 10, 50, 100, 200, 300, 500, and 1000 mg/l), indicating that the mortality rates at all exposure concentrations were below 30%, which suggested that CuO nanoparticles up to 1000 mg/l had no obvious acute toxicity to carp. Jevgenij et al. (2013) investigated acute toxicity (LC_{50} values) of 31 different nanoparticles to zebrafish (*Danio rerio*). They reported that the 96-hour median lethal concentration of CuO-NPs was 400 mg/l. Furthermore, a study on the acute toxicity of CuO-NPs on rainbow trout (*Oncorhynchus mykiss*) at the concentrations of 1, 5, 20, and 100 mg/l showed no mortality (Khabbazi et al. 2015). These data showed that copper oxide nanoparticles had low acute toxicity for fish. However, metal ions, dissolved in CuO nanoparticles, could be taken up and accumulated by the fish, resulting in chronic and sub-chronic toxic effects (Zao et al., 2011; Studer et al., 2010).

In the present study, after 96 hours of exposure to CuO-NPs, the number of RBC, Hb, and Hct% showed a significant increase ($p < 0.05$), yet indices like MCH, MCHC, and MCV did not show any significant difference, compared to the control group (Table 3). Increasing RBC, Hb, and Hct after short-term exposure to copper has been demonstrated in other studies. For example, Serezli et al. (2011) found a significant rise ($p < 0.05$) in the levels of erythrocytes, hemoglobin, and hemoglobin amounts of the peripheral blood of Coruh trout *(Salmo coruhensis*) when exposed to 10 mg/l of Cu after 48 hours (Serezli et al., 2011). Furthermore, in *Oreochromis mossambicus* after 24 hours of exposure to 100 and 200 mg/l of Cu, a significant increase ($p < 0.05$) was observed in hematocrit and hemoglobin concentration (Cyriac et al., 1989). Also, in common-carp *Cyprinus carpio* and *Prochilodus lineatus*, exposure to copper induced blood alterations, characterized by a significant rise in the RBC count and hemoglobin levels ($p < 0.05$) (Witeska, 2005; Caravalho & Fernandes, 2006). Higher RBCs' count may be due to an increase in the blood cell reserve, combined with cell shrinkage due to NP-induced osmotic alteration of blood (Faeiz et al., 2015).

Table 2. Acute toxicity of the Caspian trout (*Salmo trutta caspius*), exposed to different concentrations of CuO-NPs for 96 h

Concentration CuO-NPs mg/l	No. of animals exposed	Mortality	LC_{50} (mg/l)
0	10	0	
10	10	0	
25	10	0	
50	10	0	
100	10	0	
150	10	0	
200	10	3	
300	10	5	(mg/l) 286.47
400	10	7	
500	10	10	

Table 3. Hematological parameters of the Caspian trout, exposed to CuO-NPs during acute period. Each value is a means ± standard error. Different letters show statistically significant differences (p <0.05)

Hematological Parameter	control	24h	48h	72h	96h
RBC (10^6 mm^3)	1.16±0^a	1.17±0.02^a	1.28±0.02^b	1.38±0.03^c	1.38±0.05^c
WBC (10^3 mm^3)	5±0.001^a	5.03±0.054^a	6.03±0.28^b	6.03±0.18^b	5.91±0.09^b
MCV (fl)	315.75±0.46^a	316±4.09^a	316.66±1.36^a	317±0.89^a	316.66±2.25^a
MCH (pg)	67.42±0.27^a	67.66±0.51^a	68.33±1.36^a	68.66±1.03^a	68.33±1.36^a
MCHC (g/dl)	21.37±0.44^a	21.33±0.51^a	21.66±0.51^a	21.66±0.51^a	21.33±0.51^a
HB (g/dl)	6.5±0.31^a	6.7±0.35^a	8.3±0.13^b	9.76±0.6^c	9.6±0.47^c
HCT (%)	33.32±1.05^a	33.66±0.51^a	41.66±0.51^b	43±0.89^b	40.83±1.02^b
Neutrophil (%)	18.98±0.31^a	19.66±0.51^a	23±0.89^b	25±0.89^b	23.66±1.36^b
Lymphocyte (%)	79.62±0.44^a	80±0.89^a	80.66±2.06^a	80.66±1.36^a	80.33±1.36^a
Monocyte (%)	2.8±0.62^a	3±0.44^a	3.33±0.51^{ab}	4.3±0.51^b	4.16±0.25^b

Table 4. Hematological parameters of Caspian trout, exposed to CuO-NPs during chronic period. Each value is a means ± standard error. Different letters show statistically significant differences (p <0.05)

Hematological Parameter	control	W1	W2	W3	W4
RBC (10^6 mm^3)	0.95±0^a	0.95±0.01^a	0.95±0.008^a	0.94±0.01^a	0.92±0.02^a
WBC (10^3 mm^3)	5.07±0.001^a	5.1±0.09^a	5.83±0.36^{ab}	6.33±0.54^b	6.36±0.33^b
MCV (fl)	312.75±0.49^a	312±1.78^a	310.83±1.12^a	310±1.18^a	309.83±1.57^a
MCH (pg)	67.67±0.49^a	67.33±0.51^a	67±0^a	66.66±1.03^a	68.66±0.51^a
MCHC (g/dl)	25.67±0.49^a	21.66±0.51^b	21±0^{bc}	20.66±0.51^{bc}	20.33±0.51^c
HB (g/dl)	6.72±0.08^a	6.83±0.13^a	6.83±0.26^a	6.1±0.38^a	5.76±0.74^a
HCT (%)	31.37±1.15^a	31±0.89^a	30.16±0.93^{ab}	27.83±1.69^{ab}	26.66±1.86^b
Neutrophil (%)	21.67±0.49^a	23±1.54^a	28±0.89^b	30±2.36^{bc}	32.33±0.51^c
Lymphocyte (%)	71.37±0.44^a	71.33±1.36^a	73.66±1.36^a	75±1.54^a	75.66±1.36^a
Monocyte (%)	3.18±0.22^a	3.33±0.51^a	4±0.89^a	4.66±0.51^{ab}	5.66±0.26^b

In the current study, after 28 days of exposure to CuO-NPs, Hct% was significantly decreased ($p < 0.05$), in comparison to the control group (Table 4). Some studies reported significant reduction in hematocrit indices after chronic exposure to copper oxide NPs. Amr et al. (2015) studied the effects of 1/10 and 1/20 $LC_{50\text{-}96\ h}$ of nano and bulk CuO on *Oreochromis niloticus* (Nile tilapia) for 30 days and found out a decrease in Hct%, Hb, and RBC amounts. In addition, Dhanapakiam and Ramasamy (2001) reported a significant decrease in Hct, Hb, and RBCs content in the *Cyprinus carpio* (common carp) after 30 days of exposure to Cu. The continuous exposure to copper caused a reduction in Hct% and Hb content through accelerating the disintegration of RBC membranes and damaging the hemopoietic processes (Amr et al., 2015).

Statistical analysis indicated that white blood cells, neutrophils, and monocytes were significantly increased ($p < 0.05$) after acute and chronic exposure to CuO-NPs (Table 3 and 4). These results were in agreement with Jahanbakhshi et al. (2015) who reported a significant rise in WBC, neutrophils, monocytes, and lymphocytes of *Rutilus rutilus*, following exposure to CuO-NPs after 7 days ($p < 0.05$). According to Ramyla et al. (2008) increase in WBC numbers might occur to overcome stressful states. Alternations in the WBC numbers can be used as a sensitive indicator of stress in fish (Barton et al., 1991). Since monocytes, neutrophils, and lymphocytes as phagocytes, are involved in the immune response, their increase is suggestive of the immune system reaction to nanoparticles as a foreign factor (Al-Bairuty et al., 2013).

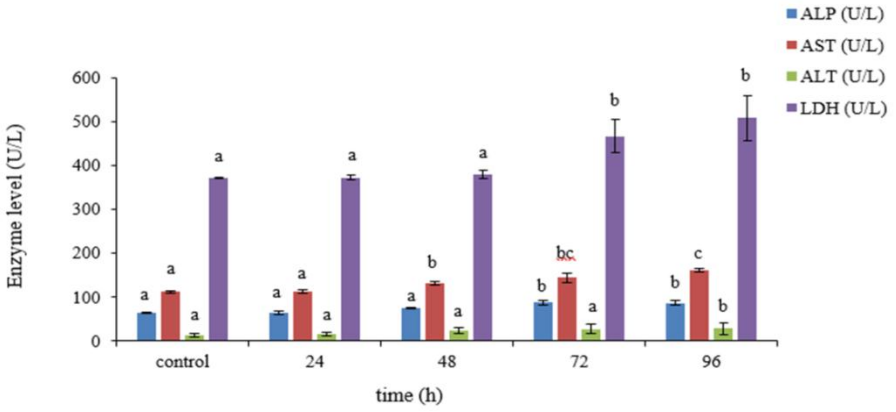

Fig. 2. Relation of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) to time during acute exposure to CuO-NPs. Different letters in different columns indicate a significant difference (p <0.05). U/L: Unit/ Liter

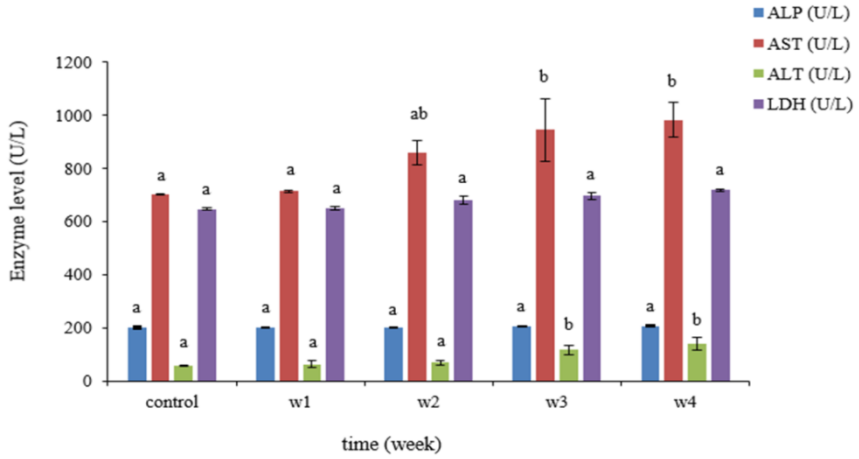

Fig. 3. Relation of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) to time during chronic exposure to CuO-NPs. Different letters in different columns indicate a significant difference (p <0.05). U/L: Unit/ Liter

Serum enzymes and biochemical indices like AST, ALT, LDH, and ALP can be utilized as sensitive and suitable biomarkers in aquatic ecotoxicology, as they provide a primary alarm for potentially dangerous variations in polluting aquatic organisms (Nel et al., 2009). The present study showed a significant increase in the levels of ALP, AST, and LDH serum enzymes after 96 hours (Fig. 2). Also, the activity of ALT and AST after 28 days of exposure to 10% $LC_{50\text{-}96\ h}$ of CuO-NPs was significantly elevated ($p < 0.05$) (Fig. 3). Results from this study were quite similar to those of Amr et al. (2015), who studied the effects of 5% and 10% $LC_{50\text{-}96\ h}$ of CuO-NPs on Nile Tilapia (*O. niloticus*) for 30 days, showing a significant rise in AST, ALT, and ALP levels. Furthermore, Abdel-Khalek et al. (2015) showed that ALP, AST and ALT concentrations in *Oreochromis niloticus* increased after 96 hours of exposure to CuO-NPs. In another study, three species of fish, namely *Oreochromis niloticus*, *Tilapia zillii*, and *Clarias gariepinus* were exposed to copper metal for 30 days and showed a significant increase in the levels of ALP, AST, and ALT enzymes ($p < 0.05$) (Zaghloul et al., 2006). The differences in enzyme responses may be a result of differences in mode of toxin action (Tencalla et al., 1994). Variations in the levels of ALP could be a result of functional and physiological changes in metal-exposed

fish (Jiraungkoorskul et al., 2003). Non-functional serum enzymes such as ALT and AST, localized within the cells of numerous organs, included the liver. They act as a significant indicator for evaluation of kidney and liver status and tissue injury or organ dysfunction (Louei Monfared et al., 2013).

Khosravi-Katuli et al. (2018) reported that LDH levels, following exposure to ZnO NPs in Caspian Roach (*Rutilus rutilus caspius*), was elevated. They also showed LDH concentration was higher for acute exposure (96 hours) than the sub-acute one (28 days) (Khosravi-Katuli et al., 2018). LDH is the final enzyme in the glycolysis passageway in vertebrates and one of the enzymes, used in injury discovery of contaminants in tissues such as the liver, muscle, and gills of the fish (Neff, 1985; Heath, 1995). Damage to the cell membrane or cell necrosis leads to the release of such enzymes, consequently increasing the serum levels (Costillas and Smith, 1977).

CONCLUSION

Results from this study indicated that CuO nanoparticles could significantly change most of the studied haematological factors and plasma enzymes levels of the Caspian trout after acute and chronic exposure. Moreover, chronic exposure had not much effect on the studied haematological and biochemical parameters than short-term exposure to copper oxide nanoparticle in the Caspian trout. The CuO-NPs toxicity may also vary significantly among fish species due to other factors, such as fish size, exposure dose and time, species unique mechanisms for metabolism of copper ion, individuals' physiological conditions, and water physicochemical parameters. Therefore, further researches on other species are needed to compare the physiological and biochemical effects of CuO-NPs in the short and long-term exposure and to indicate the exact toxicity mechanisms. These responses can be used to evaluate the health status of aquatic organisms.

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