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Acute toxicity and effects of titanium dioxide nanoparticles (TiO_2 NPs) on some metabolic enzymes and hematological indices of the endangered Caspian trout juveniles (*Salmo trutta caspius* Kessler, 1877)

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Abstract

Titanium dioxide nanoparticles (TiO_2 NPs) have been incorporated into a large range of materials for different usages and they are very likely to come in wastewater and sewage, finally reaching the aquatic ecosystems. Therefore, valuating the impact of TiO_2 NPs on aquatic environment is a major concern. The aim of this work was to study the effects of TiO_2 NPs on metabolic enzymes activity and haematological indices of the Caspian trout juveniles. After determining 96h-LC_{50}, juveniles have been exposed to 0.1 $LC_{50\text{-}96h}$ TiO_2 NPs in three replicates for 28 days. The blood samples were collected from fish after acute (24, 48, 72, 96 hours) and sub chronic (7, 14, 21 and 28 days) exposure to the TiO_2 NPs. The analysis showed that the red blood cells count (RBC), haemoglobin (Hb), haematocrit (Hct), white blood cells count (WBC) and lymphocytes have been increased after acute and sub chronic exposure to TiO_2 NPs. Levels of neutrophils and monocytes were increased mostly in acute treatments. Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) showed no significant differences. According to analysis of metabolic enzymes activities, levels of Alkaline Phosphatase (ALP) and Aspartate Amino Transferase (AST) after acute and sub chronic exposure as compared to control group were increased/decreased, respectively. Alanine Aminotransferase (ALT) levels showed significant decrease (p<0.05) after 28 days. Lactate Dehydrogenase (LDH) enzyme level increased mostly after acute exposure. The obtained results indicated that the presence of very low amount of TiO_2 NPs could affect most haematological and metabolic enzymes of Caspian trout juvenile.

Keywords: Metabolic enzyme, Haematological indices, Titanium dioxide nanoparticles, Caspian trout

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Introduction

Nanotechnology is the ability of producing materials, devices and new systems production at the molecular and nuclear level (1 to 100 nm) which takes advantage of the properties of that scale (Amelia *et al.*, 2012; Yan *et al.*, 2012). Aquatic nano toxicology is facing the challenge of evaluating the impact of nanoparticles on diverse biological endpoints (Purushothaman *et al.*, 2014). The nanoparticles have widespread usages in many industries especially in paints, materials and cosmetics, which make them possible for them to be released in environment. Therefore, it is necessary to determine their potential risk for living organisms in terrestrial and aquatic environments (Nicolas *et al.*, 2015).

Metal oxide nanoparticles are widely used in many fields of science like food, materials, chemicals and biological sciences (Aitken *et al.*, 2006). Titanium dioxide nanoparticles (TiO2-NPs), as one of the metal oxide nanoparticles, have been widely used in recent years. It has many applications in paint and coating textile industry. This nanoparticle has many usages due to the extraordinary and unique properties like photocatalytic properties. The extensive use of TiO_2 NPs can lead to release the significant amounts of these substances with potentially dangerous consequences to the environment (Hall *et al.*, 2009) which raise the concern of the risk to both ecosystems and humans (Ali, 2015). It is reported that TiO_2 NPs cause toxic effects, including inflammation and cell toxicity in mammalian, plants and microorganisms (Warheit *et al.*, 2007; Wang *et al.*, 2007, 2009).

Salmonids are the most valuable aquatic species, which are distributed widely in the most parts of the world. *Salmo trutta caspius*, is an anadromous fish that is living in the western and southern parts of the Caspian Sea. It is considered as critically endangered species according to the IUCN criteria (Coad, 2000). Most of the rivers of the Caspian Sea have lost their ecological values because of losing their sand and gravel, making dams and barriers development in the path of migrating fish, and overfishing, especially the emission of pollutants and toxins, as a result, these fishes are endangered (Coad, 2000). Therefore, studying the effects of pollutants to protect this species is necessary.

Many reports have been published on the ecotoxicity effects of nanoparticles on fish (Boyle *et al*., 2013; Abdel-Khalek *et al*., 2015; Louei Monfared *et al*., 2015), but findings about the consequences of TiO_2 NPs on blood indices of salmonid fishes are scare (Federici *et al*., 2007) and as far as it is known no investigation has been conducted on the effects of TiO_2 NPs on haematological indices and metabolic enzymes of Caspian trout.

To determine the health of an organism, blood is utilized as a useful indicator, and the haematological parameters are very important in diagnosis of functional status of animals, which are exposed to the toxic materials (Joshi *et al*., 2002).

Nanoparticles are very reactive and they have the ability to pass through

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cell membranes of organisms. Furthermore, their interactions with biological systems are relatively unknown (Khabbazi *et al.,* 2014). Therefore, the aim of this study was to assess the TiO_2 NPs acute and sub-acute effects on metabolic enzymes activities and haematological parameters in *S. trutta caspius*. The results of the present study can be useful in aquatic toxicity management and environmental health.

Materials and methods

Test organisms

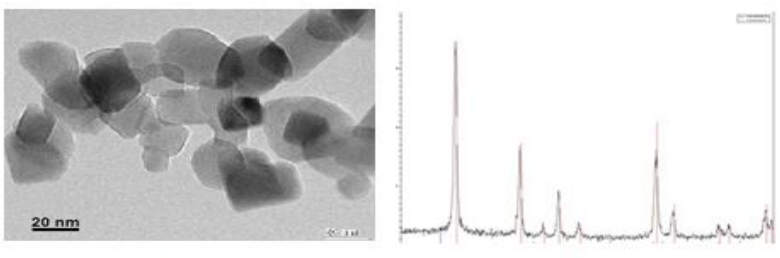
Juveniles of Caspian trout *(Salmo trutta caspius)* with average length of 20 ± 4 cm and average weight of 25±5 g were obtained from Breeding Centre of Salmonids (BCBCS) in Kelardasht, Mazandaran, Iran. Prior to the experiments, the fish were adapted to the laboratory conditions in 1000 L fiberglass tanks prior to the experiments for 10 days. Fish were fed daily with a commercial trout pellets and the total volume of water was renewed every 24 h. During the period of adaptation and experiment, fish were maintained in 12 h light/dark cycle of photoperiod with average temperature of (14 ± 1 °C) and pH (7.5±0.2). The water hardness or the concentration of CaCO3 (230 mg L^{-1}) and dissolved oxygen (8 mg L^{-1}) were controlled every day.

TiO_2 –NP characterization

TiO_2 NPs with purity of 99% were purchased from Iranian Nanomaterials Pioneers Company. TiO_2-NPs were mixed with certain amount of distilled water to obtain a uniform suspension, then sonicated for 15 minutes via ultrasound device (Company: QSonica, model: S3000) at room temperature. The morphology of the nanoparticles was studied by analysis of SEM (Company: Tescan, model: MIRA3) at Razi Metallogy Research Center (Fig. 1a). The density and crystalline structure of the purchased nanoparticles were studied by analysis of X-ray (Company: Philips, model: Xpert MPD) in the X-ray laboratory of Tarbiat Modarres University (Fig. 1b). The properties of the utilized TiO_2 NPs are presented in Table 1.

Table1: Properties of used TiO_2 - NPs.

Bulk density	Specific surface area (SSA)	Color	Purity
0.64 g cm^{-3}	10-45 m^2 g^{-1}	white	99%



a b

Figure 1: Characterization of TiO_2 nanoparticles: A: SEM image of TiO_2 –NPs, B: X-ray image of TiO_2 –NPs.

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Acute lethal assay

For determination of the acute toxicity in Caspian trout juveniles, the experiment was planned and executed in 50-liter tanks containing 20 litres of aerated water (Static method) according to the procedure, which was described in Organization of Economic Cooperation and Development guideline (OECD guideline No. 203, 1992). The feeding process was stopped 24 h prior to beginning of the experiment. An initial test was done to determine the lethal range of TiO_2-NP. To obtain the results of the preliminary test, the fish have been exposed to TiO_2 NPs at concentrations of 50, 100, 125, 150, 175, 200, 225, 250, 275 and 300 mg L^{-1} of TiO_2 NPs for 96 hours. In this study, 18 tanks were prepared, which were consisted of three tanks as control group and fifteen ones as treatment groups with different concentrations of TiO_2-NPs (five groups in three replicates of each group). The number of fish in each tank was 10 (total number=180). Every 24 h the dead fish collected and recorded and the probit analysis has been used to determine the median lethal concentration of TiO_2-NPs for Caspian trout (Finney, 1978).

Experimental exposure

After acclimatization, juveniles of Caspian trout (n=90) were transferred to six tanks. The tanks containing 16.054 mg L^{-1} (10% of the $LC_{50\text{-}96h}$) of TiO_2 NPs and the control thanks with no TiO_2 nanoparticle (in three replicates). The fish has been exposed to TiO_2 - NPs for 28 days. Sampling was done at 24, 48, 72 and 96 hours, and 7, 14, 21 and 28 days after exposure to the nanoparticles.

Blood samples collection

At each sampling time, 3 fish were randomly sampled from the six tanks and anesthetized with powdered clove (150 mg L^{-1}) (Adel *et al*., 2015). Blood samples were taken from the tail vein by heparinized insulin syringe and they were transferred to micro-tubes 0.5 ml containing 0.01 ml heparin. Half of the blood samples for the enzymes analysis were centrifuged for 10 min at 4500 g (Centurion Scientific, UK).The plasma were separated, and stored at −70 °C until analysis (Adel *et al*., 2015; Abdel-Khalek *et al*., 2016).

Haematological indices

The total number of red and white blood cells was manually counted by haemocytometer. The haemoglobin (Hb) level of blood was performed by the cyamethaemoglobin method. Cyanomethemoglobin was measured spectrophotometrically at 540 nm wavelength (Abdel-Khalek *et al*., 2016). The haematocrit (Hct) was determined by micro centrifuge technique, using standards heparinized microhematocrit capillary tubes (75 mm at 7000 g for 10 min) (Adel *et al*., 2015). Thin blood smear slides were prepared and stained with Wright Giemsa for measuring differential leukocyte cells. To calculate the percentages of leukocyte types, at least 100 leucocytes were counted under a light microscope (Blaxhall and Daisley, 1973; Adel *et al*., 2015). The haematological indices of the mean cell

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haemoglobin concentration (MCHC), mean cell haemoglobin (MCH) and mean cell volume (MCV) were computed using the total RBC count, Hb concentration and Hct (Lee *et al.*, 1999).

Biochemical analysis

The activity levels of AST, ALT, LDH and ALP were estimated using commercial kits (Pars Azmoon, Tehran, Iran) and a biochemical auto analyser instrument (Eurolyser, Belgium) (Shahsavani *et al.*, 2010).

Statistical analysis

All of the data were reported as means±standard deviation (SD). A one-way analysis of variance (ANOVA) with Tukey's multiple comparisons was performed to see if there was any significant difference among the groups. It was found that a difference was statistically significant when $p<0.05$.

Results

Acute lethal assay

The result of 96 h median lethal concentration values for Caspian trout juveniles were calculated and summarized in Table 2. By increasing the concentration of TiO_2-NPs in the Caspian trout, the acute toxicity increased. No mortality has been observed in the control tanks. By using probit analysis, it was found that the mortality of the 50% of the fish was observed in 160.54 mg L^{-1}. Then, the fish were exposed to 0.1 $LC_{50\text{-}96h}$ (i.e. 16.054).

Table 2: Acute toxicity of Caspian trout juveniles (*Salmo trutta caspius*) exposed to different concentrations of TiO_2 NPs for 96 hours.

Concentration TiO_2 NPs (mg L^{-1})	No. of fish exposed	Mortality (%)	LC50 (mg L^{-1})
50	10	0	
100	10	10	
125	10	20	160.54 (mg L^{-1})
150	10	50	
175	10	60	
200	10	70	
225	10	80	
250	10	90	
275	10	90	
300	10	100	

TiO_2 - NPs acute effects on blood indices

In the present study, red blood cells, haemoglobin and haematocrit were significantly increased when compared to control group at all treatments except 48 h group (Fig. 2 A, B, C). The MCV, MCH and MCHC indices of the samples did not have significant differences with the control groups (Fig. 3 A, B, C). After 48 hours of exposure to TiO_2, the number of white blood cells significantly increased in comparison with control group (Fig. 4A). The amount of the neutrophil at all times except 96 h group have been showed significant increase compared to control group ($p<0.05$) (Fig. 4B). The amount of lymphocyte at 48h group showed significant increase compared to control group ($p<0.05$)

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(Fig. 4C). In the case of monocytes only at 24 and 48 h groups compared to the control group had a significant increase (p<0.05) (Fig. 4 D).

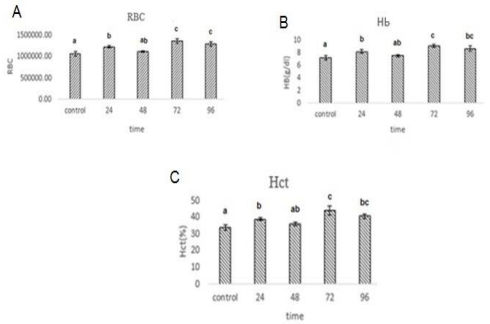


Figure 2: This figure indicates the relation of A: Red Blood Cell (RBC), B: Hemoglobin (Hb), C: Hematocrite (Hct) with time. Different letters in columns indicate a significant difference (p<0.05).

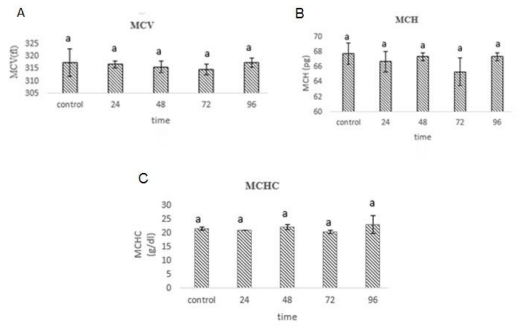


Figure 3: This figure indicates the relation of A: Mean cell volume (MCV), B: Mean cell haemoglobin (MCH), C: Mean cell haemoglobin concentration (MCHC) with time. Different letters in columns indicate a significant difference (p<0.05).

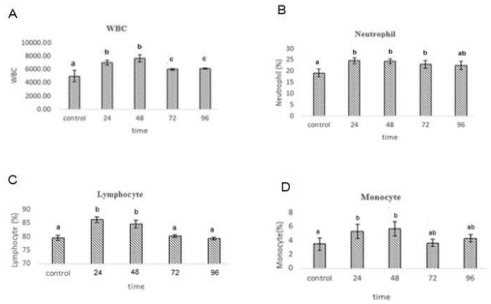


Figure 4: This figure indicates the relation of A: White Blood Cell (WBC), B: Neutrophils, C: Lymphocytes and D: Monocytes with time. Different letters in different columns indicate a significant difference (p<0.05).

TiO_2 NPs sub-chronic effects on blood indices

In the present study, the number of red blood cells, haemoglobin and haematocrit after 1 and 3 weeks of exposure were significantly increased in comparison with control group (Fig. 5 A, B, C). The MCV and MCH indices showed no significant differences. MCHC index only increased significantly compared to control group after 4weeks (Fig. 6 A, B, C). The number of white blood cells was significantly increased in comparison to the control group after 1 and 3 weeks of exposure (Fig. 7A). The amounts of neutrophil and monocyte did not show significant difference with the control group but after 4 weeks significant decrease has been observed in comparison to the first and third weeks of exposure (Fig. 7 B, D). Lymphocytes levels increased significantly compared to control at the end of 2, 3 and 4 weeks (Fig. 7C).

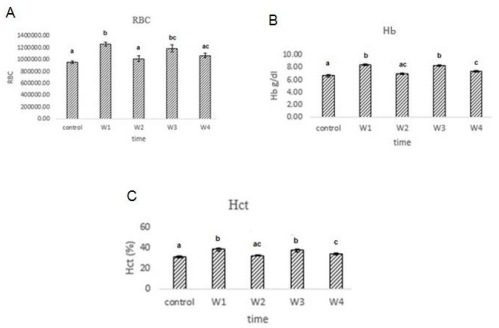


Figure 5: This Fig indicates the relation of A: Red Blood Cell (RBC), B: Haemoglobin (Hb), C: Haematocrit (Hct) with time. Different letters in different columns indicate a significant difference (p<0.05).

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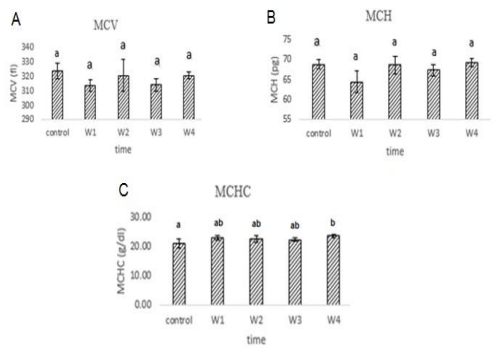


Figure 6: This Fig indicates the relation of A: Mean cell volume (MCV), B: Mean cell haemoglobin (MCH), C: Mean cell haemoglobin concentration (MCHC) with time. Different letters in different columns indicate a significant difference (p<0.05).

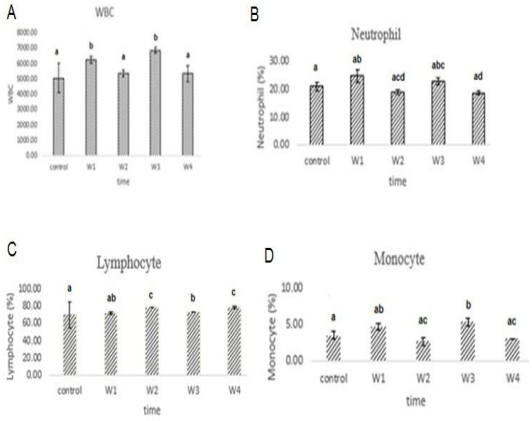


Figure 7: This Fig indicates the relation of A: White Blood Cell (WBC), B: Neutrophils, C: Lymphocytes and D: Monocytes with time. Different letters in columns indicate a significant difference (p<0.05).

TiO_2 NPs acute effects on blood enzymes

In the biochemical analysis of blood, ALP level was significantly increased compared to control group after 72 and 96 hours (Fig. 8A). AST amount in all of the acute treatments showed significant decrease compared to control (Fig. 8B). There were no significant differences in ALT amount (Fig. 8C). LDH showed significant increase in comparison with control enzyme after 72 and 96 hours of exposure (Fig. 8D).

TiO_2 NPs sub-chronic effects on blood enzymes

The levels of ALP were significantly increased in the biochemical analysis of blood, compared to control group after 1, 2 and 3 weeks of experiment (Fig. 9A). The amount of AST showed significant differences and were decreased compared to control group in all treatments except for week 1 treatment group (Fig. 9B). The ALT enzyme only showed significant decrease compared to control after 4 weeks (Fig. 9C). The LDH enzyme showed significant difference after 1 and 2 weeks of exposure in comparison to the control group. The highest level of LDH was observed after 1 week (Fig. 9D).

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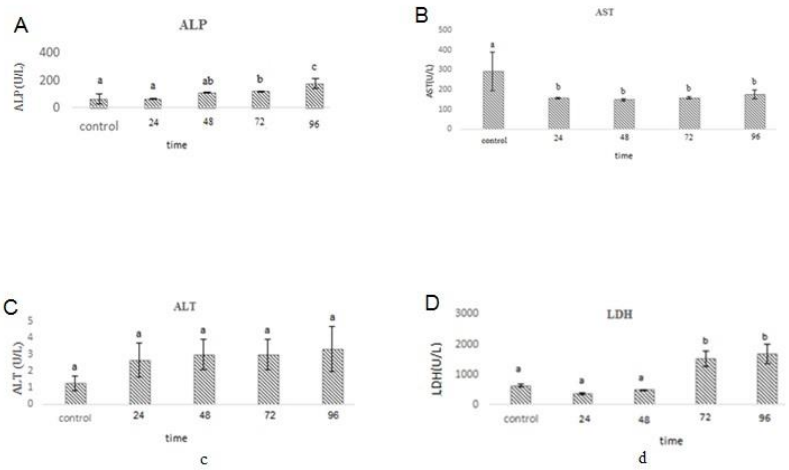


Figure 8: This Fig indicates the relation of A: Alkaline phosphatase (ALP), B: Aspartate aminotransferase (AST), C: Alanine aminotransferase (ALT) and d: Lactate dehydrogenase (LDH) with time. Different letters in columns indicate a significant difference (p<0.05).

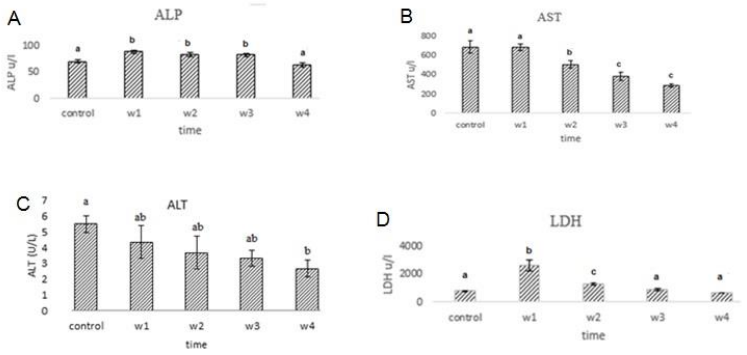


Figure 9: This Fig shows the relation of A: Alkaline phosphatase (ALP), B: Aspartate aminotransferase (AST), C: Alanine aminotransferase (ALT) and D: Lactate dehydrogenase (LDH) with time. Different letters in columns indicate a significant difference (p<0.05).

Discussion

According to the results of acute toxicity during the experimental period, 50 percent of the mortality ($LC_{50\text{-}96h}$) occurred at 160.54 mg L^{-1}. The obtained data of the acute toxicity of TiO_2 NPs on the tested fish species indicated low acute toxicity of TiO_2 NPs within the exposure period of studied: the 48 h LC_{50} for *Pimephales promelas* >500 mg L^{-1}, the 96 h LC_{50} for *Oncorhynchus mykiss* > 100 mg L^{-1} and no lethal effects on *Danio rerio* have been observed below 500 mg L^{-1} (Menard *et al.*, 2011). In another study, 14 days of exposure to 1 or 10 mg L^{-1} concentrations of TiO_2 NPs were not lethal to *Oreochromis niloticus* (Nile tilapia) (Perera and Pathiratne, 2012).

The haematological studies are valuable to assess to the environmental impacts of contaminants on fish (Stentiford *et al.*, 2003). In this study, acute exposure to TiO_2 NPs led to an

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increase in number of red blood cells, haematocrit and haemoglobin. The haematocrit and haemoglobin levels in the rainbow trout blood, which were exposed to 1 mg L^{-1} of TiO_2-NPs for 14 days, increased (Boyle *et al.*, 2013). In the other study, significant increases were found in erythrocytes count and haemoglobin levels of the peripheral blood of Nile tilapia, which were exposed to 1 and 10 mg L^{-1} of TiO_2 for 14 days (Perera and Pathiratne, 2012). The increased levels of haematocrit and haemoglobin in the fish blood, exposed to TiO_2-NPs, credited as a recompense for respiratory hypoxia related to the gill damages (Boyle *et al.*, 2013).

In the present study, sub-chronic (3 and 4 weeks) exposure to TiO_2 NPs did not show significant increase in RBC, haematocrit and haemoglobin levels. In addition, MCV, MCH and MCHC indices did not show significant differences at all treatments (except MCHC on 4^{th} week). In some studies, chronic and sub-chronic exposure to TiO_2 NPs did not show any significant changes in erythrocyte quantities of the blood (Federici *et al.*, 2007; Ramsden *et al.*, 2013; Ramsden *et al.*, 2009).

In this study, the WBC, neutrophils, lymphocytes and monocytes levels were significantly increased at acute TiO_2-NPs exposure. In sub-chronic treatments, neutrophil and monocyte levels did not show any significant change but white blood cells were increased significantly after 3 weeks and lymphocytes after 2, 3 and 4 weeks of exposure to NP. Some studies showed that the acute exposures of TiO_2-NPs to rainbow trout did not cause noticeable changes in the blood neutrophils count (Federici *et al.*, 2007; Ramsden *et al.*, 2009). However, more studies that are recent have described important effects of TiO_2 NPs on leucocytic factors of rainbow trout, zebra fish and fathead minnow after sub chronic exposure (Perera and Pathiratne, 2012). The increasing amount of leucocytes count in the peripheral blood of the Nile tilapia was related to the 14 days of exposure to 1 and 10 mg L^{-1} TiO_2 NPs (Perera and Pathiratne, 2012). The changes in leucocytes counts of the blood has been described in rainbow trout exposed to 1 mg L^{-1} TiO_2 NPs for 14 days (Boyle *et al.*, 2013) and subsequent exposure to 0.1 and 1 mg L^{-1} TiO_2 NPs for 14 days in zebra fish (Ramsden *et al*., 2013). Since lymphocytes, monocytes and neutrophils, as phagocytes, involved in the immune reactions, their high levels is indicative that the immune system responds to the nanoparticles as a foreign agent and the decrease in the number of WBC may be attributed to the unhealthy functioning blood-forming tissues (spleen and kidneys) or it can be a response to several infectious diseases (Al-Bairuty *et al.*, 2013).

The determination quantities of the fish blood biochemical parameters provide critical information for the diagnosis and management of contaminated and uncontaminated samples (Pincus, 1996). Analysis of data obtained from measurement of enzymes activities in the blood showed that, levels of ALP in most treatments and LDH (in 72, 96 h, W1 and W2)

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were increased significantly when compared to control group (p<0.05). The changes in the ALP activities could be resulted from functional and physiological changes in fish exposed to pollutants (Jiraungkoorskul *et al.*, 2003). Several studies have been reported increasing the levels of ALP after short or long terms exposure to nanoparticles. Abdel-Khalek *et al*., (2015) found that 96 hours exposure to CuO - NPs in *O. niloticus* significantly increase the levels of ALP enzyme when compared to the control groups. In addition, Louei Monfared *et al*. (2015) reported increasing of ALP activity in common carp exposed to Ag NPs after 8 weeks.

LDH is an enzyme found in almost all tissues, such as liver, kidney, heart, gill and erythrocytes. Measurement of LDH levels is currently used for tissue damage detection (Banaee *et al*., 2011). LDH activity may signal changes in carbohydrate and protein metabolism in fish under the stress of metals (Elia *et al*., 2017). The marked increase after treatment with TiO_2 NPs indicates that metal exposure induced a hypoxic condition, shifting from aerobic to anaerobic metabolism (Elia *et al*., 2017). Increasing the levels of LDH concentration has been showed after exposure to ZnO NPs and Ag NPs in common carp (Lee *et al*., 2014; Louei Monfared *et al*., 2015). In addition, exposure to Fe NPs significantly increased the levels of LDH activity in *O. niloticus* (Ates *et al*., 2016).

According to results, ALT levels show significant decrease after 4 weeks exposure to TiO_2 NPs and the levels of AST was significantly decreased in acute and sub-chronic (W_2, W_3 and W4) treatments (p<0.05). ALT and AST are non-functional enzymes of plasma, which are generally localized within the cells of numerous organs including liver. Furthermore, they are considered as significant indicators in evaluating kidney and liver status and tissue damage or organ dysfunction (Louei Monfared and Soltani, 2013). Therefore, reducing AST and ALT levels might be signs of liver cells insufficiency. Besides, ALT and AST enzymes play an important role in protein metabolism and catalyse the intermolecular transferal of the amino groups between amino acids and α-ketoacidosis. Decreasing the activity of AST show that glutamate and oxaloacetate are not appropriate for Krebs cycle via this regular transamination (Lee *et al*., 2006). Some studies reported effects of pollutant on decreasing ALT and AST enzymes activity in fishes (Özdemir *et al*., 2016).

According to Zhu *et al.* (2009), nanoparticle interaction with biological systems may lead to biochemical disorders or/and adaptive reactions. These reactions (biomarkers) can be used to evaluate the health condition of aquatic organisms (Wang *et al.*, 2009).

The results showed that the presence of low amount of TiO_2-NPs could affect the blood indices of the Caspian trout juvenile and subsequently, the processes of growth and fish physiology. It has been also showed that the most parameters of blood and metabolic enzymes have been affected even in the short term. The other

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observations in exposed fish were changes in the activity of the target enzymes in the fish blood parameters. The results showed that the blood parameters were very sensitive in monitoring the toxicity of TiO_2-NPs, especially at sub lethal concentrations and the changes in blood parameters was a useful tool to measure the early exposure to TiO_2-NPs. Thus, it can indicate the need for proper management of related organization to prevent the entering of the contaminants such as nanoparticles to aquatic environments. Further studies including the effects of TiO_2¬-NPs on histopathology, biochemical factors of different tissues and antioxidant gene expression in the Caspian trout need to be done to determine the effects of this nanoparticle.

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