



# Fluctuation of biochemical, immunological, and antioxidant biomarkers in the blood of beluga (*Huso huso*) under effect of dietary ZnO and chitosan–ZnO NPs

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**Abstract** The objective of the present study was to investigate the effects of dietary supplementation with zinc oxide (ZnO) and chitosan–zinc nanoparticles (chitosan–ZnO NPs) on biochemical, immunological, and antioxidant biomarkers in blood of juvenile belugas (*Huso huso*). The beluga juveniles with initial weight of  $287 \pm 46$  g were fed with eight experimental diets containing  $0 \text{ g kg}^{-1}$  ZnO (the control diet); 10, 20, and  $40 \text{ mg kg}^{-1}$  ZnO; and 10, 20, and  $40 \text{ mg kg}^{-1}$  chitosan–ZnO NPs and  $36 \text{ mg kg}^{-1}$  chitosan. After 28 days of culture, the fish were fed with ZnO and chitosan–ZnO NP-supplemented diets showed a more significant increase in total antioxidant capacity (TAC), superoxide

dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione peroxidase (GPX), and glutathione *S*-transferase (GST) activity ( $p < 0.05$ ) compared to the control group. There were no significant differences ( $p > 0.05$ ) in malondialdehyde (MDA) and glucose level in all treatment groups. The results showed that with increasing levels of ZnO and chitosan–ZnO NPs, alternative complement activity (ACH50), and total immunoglobulin, total protein, albumin, and lysozyme had a significant increase in fish fed with ZnO and chitosan–ZnO NP-supplemented diets compared to the control group ( $p < 0.05$ ). ALP, ALT, and AST enzyme activities showed significant difference between control and treatment groups ( $p > 0.05$ ), while the levels of LDH enzyme activity, urea, and creatinine decreased by increasing both ZnO and chitosan–ZnO NP levels. These results demonstrated that dietary chitosan–ZnO NPs could improve the health status, immune function, and antioxidant capacity of the cultured beluga juvenile.

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**Keywords** *Huso huso* · Zinc oxide · Chitosan–zinc nanoparticles · Blood parameters

## Introduction

Fish like other animals require different micronutrients for survival, growth, and reproduction (Aliko et al. 2018). Minerals play an important role in fish living among the micronutrients which obtain their required minerals through diet or water (Faiz et al. 2015; Tawfik et al. 2017). Zinc as an essential element for fish

undertakes many important physiological actions in their life cycle that found in nature as various forms of elemental, inorganic, and organic (Huang et al. 2015; Abdel-Tawwab 2016; Pagano et al. 2017; Capillo et al. 2018).

The deficiency of this element can have dangerous and irreversible complications in metabolism, growth, and immunity (Faiz et al. 2015; Tawfik et al. 2017). The presence of zinc in most organs, tissues, and body fluids causes the membrane and cellular components to be stabilized (Fallah et al. 2018). Zinc has an effect on the activity of enzymes such as aldolase, peptidases, and phosphatases in the digestion process in the fish's gut (Hasnat et al. 2012). Constrain in growth and reducing feed intake is one of the proven complications of zinc deficiency in the diet (Feng et al. 2011). Over the past 50 years, zinc has been identified as one of the most influential factors in the immune system (Maares and Haase 2016).

One of the most important forms of zinc is zinc oxide which is in mineral form (Strnadová et al. 2011). In several studies of *Ctenopharyngodon idella* (Faiz et al. 2015), *Cyprinus carpio* (Chupani et al. 2017), *Oncorhynchus mykiss* (Connolly et al. 2016), and *Dicentrarchus labrax* (Fountoulaki et al. 2010), zinc oxide is used in fish diet and its positive role in the growth and immunity of aquatic animals has been proven. Although the zinc element is essential for natural metabolism, but excessive amounts of this element can also cause poisoning in human and animals, it is proven that high levels of zinc caused to produce cytopenia or decrease blood cells, reduce the copper element efficiency, and prevent the release of iron from storage sources and, as a result, disorder such as anemia, leukopenia, and neutropenia (Luo et al. 2011; Huang et al. 2015; Abdel-Tawwab 2016). On the other hand, researches have shown that the presence of phytate, calcium, and phosphorus in diets significantly affects the biological ability of fish to absorb and use zinc in the diet (Wang and Wang 2015; Taheri et al. 2017). Therefore, despite the high amounts of zinc supplementation in the diet, due to the reduced biological ability of fish to absorb zinc, this element is exited from the fish's access. Meanwhile, in recent years, the use of plant proteins such as soybean (containing phytate) has customized in the aquatic diet (Gatlin III et al. 2007). Due to the importance of adjusting the exact amount of zinc in the rations, the use of novel methods in the delivery of this element appears to be necessary.

Nowadays, for beneficial and effective use of nanoparticles in biological systems, various coatings such as albumin, dextran, polyethylene glycol, polyethylene oxide, and aspartic acid are produced on their surface (Fatahian et al. 2012; Raisi Dehkourdi et al. 2017). The presence of such coatings contributes to the stability of nanoparticles in biological solutions and blood circulation, tissue distribution, as well as the entry of these materials into cells and the reduction of their toxic effect (Raisi Dehkourdi et al. 2017).

Chitosan is one of the compounds that are extremely considered for the preparation of micro- and nanoparticles for the delivery of proteins, peptides, and genetic material (DNA and RNA), because of its cheapness (Wang et al. 2007a, 2007b). Chitosan is a cationic polysaccharide produced by N-deacetylation chitin from the unused parts of crabs and shrimp and has unique properties such as biocompatibility, biodegradability, low-immunogenicity, and non-toxicity (Kamil et al. 2002; Wang et al. 2007a, 2007b). On the other hand, chitosan has the ability to dissolve related to acidity (Romanazzi et al. 2009). The positive charge on the surface of chitosan tends to bind to the negative charge on the surface of cell membrane. The mucoadhesive properties of chitosan enhance absorption (or diffusion) of coated metal oxide nanoparticles such as zinc oxide by prolonging the gastric residence time (Alalawi et al. 2019). Chitosan nanoparticles were used to control delivery of vitamin C in rainbow trout (*Oncorhynchus mykiss*) (Alishahi et al. 2011), stable delivery of LHRH in common carp (*Cyprinus carpio*) (Rather et al. 2013), and control delivery of trypsin in the fish *Labeo rohita* (Kumari et al. 2013) in aquaculture.

Beluga (*Huso huso*) is the largest species of sturgeon inhabiting the Caspian Sea, the Black Sea, the Azov Sea, and the surrounding basins (Chebanov 1998). Beluga due to its rapid growth rate, fast adaptation to breeding conditions, high density in breeding conditions, habitability to commercial diet, and considerable resistance to low water quality is considered an appropriate option for breeding in fields (Doroshov 1985; Adel et al. 2016). In the production and cultivation of sturgeon, like other aquatic animals, food and nutrition are considered an effective and determining factor. The question to be raised at this stage is whether this or other amounts and type of zinc can have suitable biological and physiological changes in terms of blood parameters, immune, and antioxidant function in beluga as a unique and valuable species in aquaculture.

## Materials and methods

### Preparation of ZnO and chitosan–ZnO NP supplements

Reagent grade zinc oxide was obtained from Merck. Chitosan–zinc oxide nanoparticles supplement prepared according to Khajeh et al. (2014) method. For this aim, 10 g of ZnO was dissolved in 1000 ml solution of 1% glacial acetic acid to access Zn cations. Thereafter, 10 g chitosan (deacetylation degree 95.7%, MW  $1.6 \times 10^4$ ) was dissolved in this solution under ultra-sonic. Next, the solution pH was fixed to 10 with  $1 \text{ mol l}^{-1}$  NaOH. After 3 h heating in a  $60 \text{ }^\circ\text{C}$  water bath, the product nanoparticles were filtered and washed several times with distilled water and then dried in oven at  $50 \text{ }^\circ\text{C}$  for 3 h. The amount of Zn in the chitosan–ZnO NP supplement (final product) was  $0.1 \text{ g g}^{-1}$ . The scanning electron microscopy (SEM) image of chitosan–ZnO NP supplement is displayed in Fig. 1.

### Diet preparation

Commercially, the basal diet (Abzian<sup>TM</sup>, Iran) feed was supplemented with ZnO and chitosan–ZnO NPs at levels of 0 (control), 10, 20, and  $40 \text{ mg kg}^{-1}$  and chitosan at level  $36 \text{ mg kg}^{-1}$ . To preparation of experimental diets, a commercial pellet diet (containing 42% protein, 14% lipid, 10% ash, and  $21.71 \text{ MJ kg}^{-1}$  GE) was pestle, blend with the appropriate ZnO and chitosan–ZnO NP levels as well as 3% of dietary gelatin

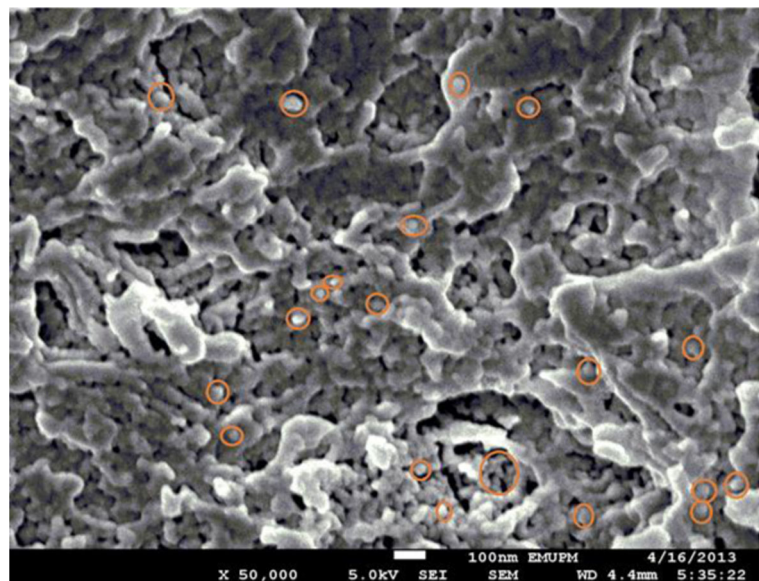
(for reduce the dissolving rate of pellets in water), and remade again the pellets. The control group diet contained only dietary gelatin. The food plates were dried on nylon screens at  $45 \text{ }^\circ\text{C}$  by air circulation and stored at  $4 \text{ }^\circ\text{C}$  until use.

### Experimental conditions

Three hundred and sixty healthy juvenile belugas were obtained from Zahak of propagation center (Sistan & Blouchestan, Iran) with mean weight  $287 \pm 46 \text{ g}$  and stocked in two circle fiberglass tanks (each volume, 6000 l) for 2 weeks before starting of the trial regime in order to adapt fish to experimental conditions and handle procedure.

Then, fish were randomly allocated into 24 circular (200 cm diameter, 40 cm height, 1600 l volume) fiberglass tanks and 15 fish in each tank with three replicate per diet. Fish were fed twice daily (06:00 and 18:00) with experimental diet (feeding rate  $3\% \text{ BW day}^{-1}$ ) for 28 days in rate of 3% of body weight per day. The control group was fed on manufactured basal diets. The 2nd group (T1) was fed on basal diets supplemented chitosan. Groups 3rd, 4th, and 5th (T2, T3, and T4) were fed basal diet supplemented with ZnO at levels 10, 20, and  $40 \text{ mg kg}^{-1}$ , respectively. Groups 6th, 7th, and 8th (T5, T6, and T7) were fed basal diet supplemented with chitosan–ZnO NPs at levels 10, 20, and  $40 \text{ mg kg}^{-1}$ , respectively. Each tank was aired with compressed air via two air stones connected to central air pump. During

**Fig. 1** SEM image of chitosan–zinc nanoparticles (Khajeh et al. 2014)



the experimental period, oxygen content, water temperature, and pH were recorded  $7 \pm 0.5 \text{ mg l}^{-1}$ ,  $24 \pm 2.1 \text{ }^\circ\text{C}$ , and  $7.4 \pm 0.35$ , respectively.

#### Sample collection and blood analysis

At the end of the trial period, 6 fish from each treated and control group were anesthetized with 150 ppm clove oil and about 4 ml of blood was collected from the caudal vein of beluga juveniles by a non-heparinized syringe. For serum isolation, samples were introduced into non-heparinized microtubes. After clotting, blood samples were centrifuged at  $3000 \times g$  for approximately 10 min. Serum filled tubes were stored at  $-20 \text{ }^\circ\text{C}$  for further analysis.

Preparation and assessment of all serological factors were accomplished at the Hamoun International Wetland Research Institute (University of Zabol, Iran) and Viomed laboratory (Rasht, Iran). The total protein concentration of blood sera was measured by biuret colorimetric method (Pavlidis et al. 1997) using a commercial kit (Bionik Reagent Packs, Tehran, Iran) and an automatic biochemical analyzer (BT 1500, Italy). Albumin concentration of serum samples was assessed using the bromocresol green albumin assay method by manufactured kit (Pars Azmoon Inc., Tehran, Iran). Serum glucose level was measured colorimetrically according to the glucose/GOD-PAP technique with a commercial testing kit (Pars Azmoon Inc., Tehran, Iran). Lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) activities in serum were determined using DGKC method with commercial testing kits (Pars Azmoon Inc., Tehran, Iran). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in serum were evaluated by IFCC (without pyridoxal phosphate activation) method with commercial testing kits (Pars Azmoon Inc., Tehran, Iran). Urea concentration in serum was assessed according to the urease-GLDH method with a commercial testing kit (Pars Azmoon Inc., Tehran, Iran). Creatinine level in serum samples was measured by modified Jaffe's method (Junge et al. 2004) with DIALAB (GmbH, Austria) reagent kit. Malondialdehyde (MDA) concentration in serum samples was determined with a commercial chemical colorimetric assay kit (ZellBio GmbH, Ulm, Germany) based on its reaction with thiobarbituric acid (TBA) under acidic condition and high temperature, and the color complex was assayed using spectrophotometer at 535 nm. The sensitivity of MDA kit was  $0.1 \text{ } \mu\text{M}$ , and

intra- and inter-assay coefficients of variation were 5.8% and 7.6%, respectively. Total antioxidant capacity (TAC) amount in serum samples was determined with a colorimetric assay kit (ZellBio GmbH, Germany) at 490 nm. TAC level was considered as the amount of antioxidant in the serum that was compared with ascorbic acid action as a standard. The sensitivity of TAC kit was  $0.1 \text{ mM}$  ( $100 \text{ } \mu\text{mol l}^{-1}$ ). Superoxide dismutase (SOD) activity in serum samples was assayed with a calorimetric enzyme assay kit (ZellBio GmbH, Germany) at 420 nm. The SOD activity unit was defined as the amount of enzyme in serum that catalyzed decomposition of  $1 \text{ } \mu\text{mol}$  of superoxide anion into oxygen and hydrogen peroxide per 1 min. The sensitivity of SOD kit was  $1 \text{ U ml}^{-1}$ . Catalase (CAT) activity in serum samples was assayed with a calorimetric enzyme assay kit (ZellBio GmbH, Germany) at 405 nm. The CAT activity unit was defined as the amount of enzyme in serum that catalyzed decomposition of  $1 \text{ } \mu\text{mol}$  of hydrogen peroxide into water and oxygen per 1 min. The sensitivity of CAT kit was  $0.5 \text{ U ml}^{-1}$ . The glutathione (GSH) activity in serum samples was determined with a commercial chemical colorimetric assay kit (ZellBio GmbH, Germany). The GSH activity measured colorimetrically at a wavelength of 412 nm. The sensitivity of GSH kit was  $0.01 \text{ mM}$  ( $100 \text{ } \mu\text{mol l}^{-1}$ ). Glutathione peroxidase (GPX) activity in serum samples was assayed with a manufactured chemical colorimetric assay kit (ZellBio GmbH, Germany) at 412 nm. The GPX activity unit was defined as the amount of enzyme in serum that catalyzed decomposition of  $1 \text{ } \mu\text{mol}$  of GSH into glutathione disulfide (GSSG) per 1 min and the sensitivity of GPX kit was  $5 \text{ U ml}^{-1}$ . Evaluation of serum glutathione *S*-transferases (GST) activity was done with a commercial ELISA kit (ZellBio GmbH, Ulm, Germany) based on the Biotin double antibody sandwich technology at 450 nm. The assay sensitivity is  $0.27 \text{ ng ml}^{-1}$  and intra- and inter-assay coefficient of variation 10% and 12%, respectively. Total soluble protein was assessed according to the Bradford (1976) using bovine serum albumin as a standard. The activity of enzymes was expressed as specific activity ( $\text{U mg}^{-1} \text{ protein}$ ).

The lysozyme activity in serum samples was determined by turbidimetric assay based on Ellis (1990) method using hen egg white lysozyme ( $1 \text{ mg}$  lysozyme per ml) as standard with partial. Serum samples ( $25 \text{ } \mu\text{l}$  per well) were allocated in triplicate into 96-well plate; then,  $175 \text{ } \mu\text{l}$  of bacteria suspension (contain  $0.2 \text{ mg}$  of *Micrococcus lysodeikticus* at  $1 \text{ ml}$  of  $0.5 \text{ M}$  phosphate



buffer saline (PBS) with pH 6.2) was added. PBS replaced serum was considered as a negative control. The decrease in optical density (OD) was measured after one and five minutes at a wavelength of 513 nm under room temperature condition. The lysozyme activity unit was defined as a reduction in absorbance of  $0.001 \text{ min}^{-1}$ . The total immunoglobulin (IgM) level in serum samples was evaluated based on Biuret method (Teppo 1982): serum (0.1 ml) was added to a solution of 12% polyethylene glycol (0.1 ml, Sigma), and the combination was incubated for 2 h to precipitating down IgM molecules. Precipitation of IgM was extracted using centrifuge,  $5000 \times g$  at  $4 \text{ }^\circ\text{C}$ . The total protein in the supernatant was measured as mentioned above. The total IgM level was determined based on the following formula: Total IgM ( $\text{mg ml}^{-1}$ ) = Total protein in serum – Total protein treated with PEG (Siwicki and Anderson 1993; Amar et al. 2000). Alternative complement activity (ACH50) in serum samples was determined by Yano (1992) method with rabbit red blood cells (RaRBC). The serum samples were serially attenuated (0.1 to 0.25 ml), and different volumes were distributed in test microtubes. The volume of each tube was raised to 0.25 ml by adding barbitone buffer along with EGTA (ethylene glycol-bis (2-aminoethylether)- $N,N,N',N'$ -tetraacetic acid) and  $\text{Mg}^{2+}$ ; thereafter, RaRBC (0.1) was appended to microtubes. The prepared samples were incubated for 2 h at  $22 \text{ }^\circ\text{C}$ . Thereafter, 3.15 ml from 0.9% NaCl was appended. Centrifuging was done to remove unlysed RaRBC (for 5 min at  $836 \times g$ ). The supernatant optical density recorded using a spectrophotometer at 414 nm. The degree of hemolysis was used to determine ACH50 ( $\text{unit ml}^{-1}$ ) for each sample.

### Statistical analysis

Statistical analysis was done by Statistical Package for the Social Sciences (SPSS, version 25, IBM Corp., Armonk, NY, USA). At first, the normality of data was examined using the Kolmogorov–Smirnov test. Levene's test was performed to verify the homogeneity of variance. The data were analyzed by one-way analysis of variance (ANOVA) to test the effects of the dietary treatments. The differences between means were delineated using Tukey's multiple range test that was done as a post-hoc test to compare differences between means at  $p \leq 0.05$ . All data in the text are reported as mean  $\pm$  SD.

## Results

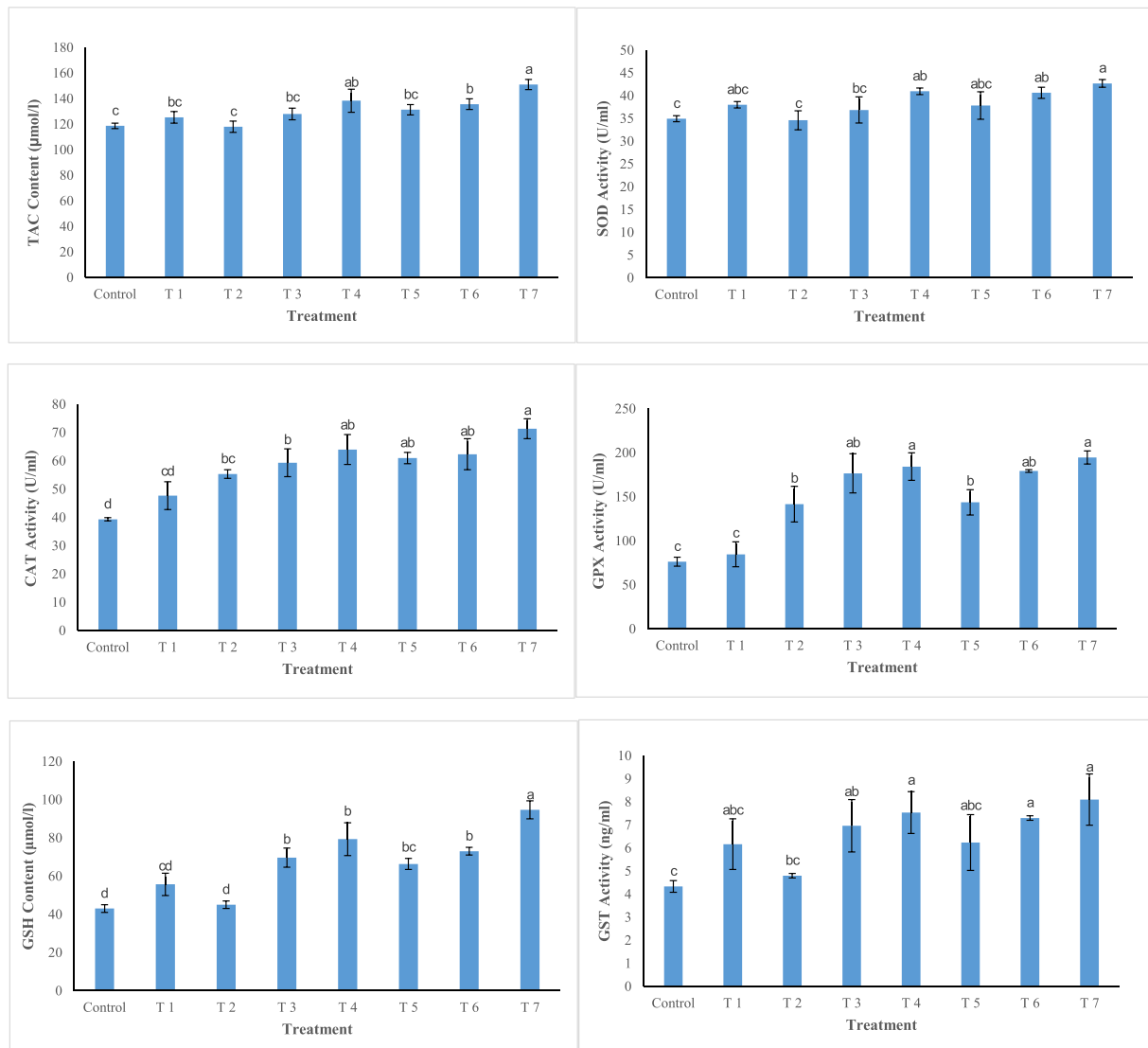
### Fluctuation of antioxidant parameters

The activity of enzymes of TAC, SOD, CAT, GPX, GSH, and GST was assayed in the blood serum of the beluga juveniles under effect of dietary ZnO and chitosan–ZnO NPs, and the results are shown in Fig. 2. The TAC and SOD activities exhibited the highest level significantly in beluga fed with  $40 \text{ mg kg}^{-1}$  ZnO and chitosan–ZnO NP-supplemented diet comparing the control group ( $p < 0.05$ ). Diets supplemented with ZnO and chitosan–ZnO NPs increased significantly CAT activity enzyme compared to control diets ( $p < 0.05$ ). The highest level of CAT recorded in fish fed with  $40 \text{ mg kg}^{-1}$  chitosan–ZnO NP-supplemented diet ( $p < 0.05$ ; Fig. 2). The GPX, GSH, and GST activity enzymes generally increased in beluga fed with ZnO and chitosan–ZnO NP-supplemented diet significantly than the other groups ( $p < 0.05$ ; Fig. 2). The highest level of GPX, GSH, and GST was recorded in fish fed with  $40 \text{ mg kg}^{-1}$  chitosan–ZnO NP-supplemented diet.

### Immunological and biochemical characters

Twenty-eight days after the feeding trial, the effects of different levels of dietary ZnO and chitosan–ZnO NPs on immunological (MDA, ACH50 and total immunoglobulin) and biochemical (total protein, albumin, lysozyme, hepatic marker enzymes, glucose, urea, creatinine) parameters are presented in Figs. 3 and 4. There were no significant differences ( $p > 0.05$ ) in the serum MDA and glucose level of beluga fed with ZnO and chitosan–ZnO NP-supplemented diet compared to the control group ( $p > 0.05$ ). Other data showed that with increasing levels of ZnO and chitosan–ZnO NPs, biochemical parameters (exception in case of creatinine and urea) had a significant increase compared to the control group ( $p < 0.05$ ). The maximum value of ACH50, total immunoglobulin, total protein, albumin, and lysozyme was recorded in beluga fed with  $40 \text{ mg}$  chitosan–ZnO NP-supplemented diet ( $114.6 \pm 1.5\%$ U,  $6.8 \pm 0.1 \text{ mg ml}^{-1}$ ,  $979.3 \pm 8.5 \text{ mg dl}^{-1}$ ,  $294.3 \pm 9.07 \text{ mg dl}^{-1}$ , and  $16.3 \pm 1.5 \text{ U ml}^{-1} \text{ min}^{-1}$ ).

During 28 days of the feeding trial, urea and creatinine levels decreased with increasing both ZnO and chitosan–ZnO NPs concentrations. At the end of the trial, LDH, ALT, and AST levels decreased significantly ( $p > 0.05$ ) compared to control group, whereas the levels



**Fig. 2** Antioxidant capacity parameters and metabolic enzyme in beluga fed with different diets (T1 (chitosan), T2 (10 mg ZnO kg<sup>-1</sup> diet), T3 (20 mg ZnO kg<sup>-1</sup> diet), T4 (40 mg ZnO kg<sup>-1</sup> diet), T5 (10 mg chitosan–ZnO NPs kg<sup>-1</sup> diet), T6 (20 mg chitosan–ZnO

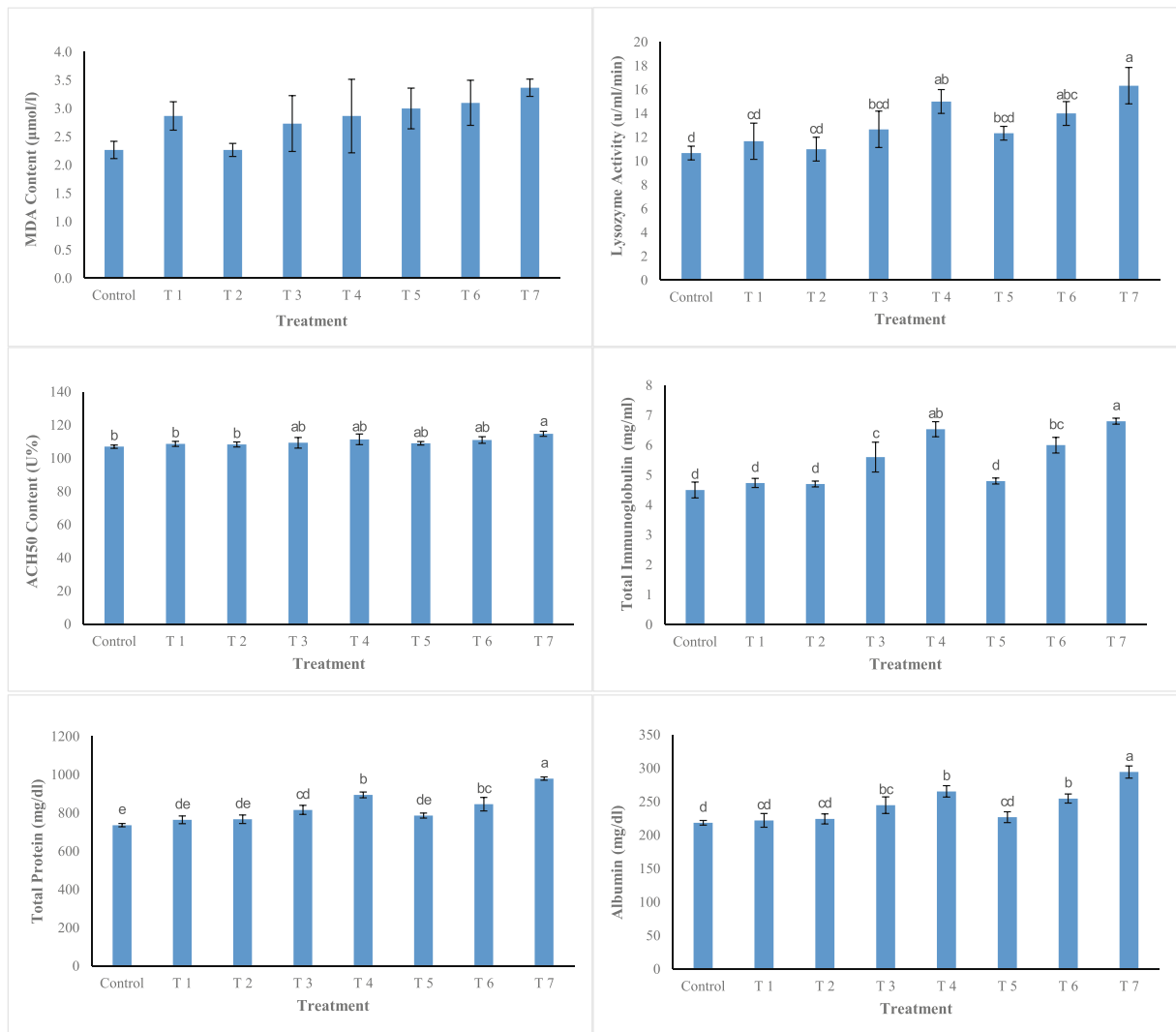
NPs kg<sup>-1</sup> diet), and T7 (40 mg chitosan–ZnO NPs kg<sup>-1</sup> diet) for 28-day feeding trial. Values are means ± SD (*n* = 5). Different letters represent significant differences between bars (*P* < 0.05)

of ALP showed significant increase in the fish fed with ZnO and chitosan–ZnO NP-supplemented diets.

## Discussion

Under normal physiological conditions, ~ 2% of the total oxygen consumed by mitochondria in mitochondrial electron transport chain has released as reactive oxygen species (ROS), which, it can lead to DNA damage, inactivate enzymes, oxidative damage to

structural proteins, and peroxidation of membrane lipids (Halliwell and Gutteridge 2007; Jafarnejad et al. 2018). The ROS are also formed when fish are exposed to microbial infections, pollutants, toxins, and pesticides (Faggio et al. 2018; Sehonova et al. 2019). Oxidative stress can be defined as a disturbance in homeostasis states between ROS and antioxidant defense mechanisms (Birben et al. 2012). The biological effects of ROS are controlled in fish by a broad range of antioxidants, such as antioxidant enzymes (e.g., GPX, GST, SOD, CAT), nutrient-derived antioxidants (e.g.,



**Fig. 3** Immunological and biochemical parameters of blood in beluga fed with different diets (T1 (chitosan), T2 (10 mg ZnO kg<sup>-1</sup> diet), T3 (20 mg ZnO kg<sup>-1</sup> diet), T4 (40 mg ZnO kg<sup>-1</sup> diet), T5 (10 mg chitosan–ZnO NPs kg<sup>-1</sup> diet), T6 (20 mg chitosan–ZnO

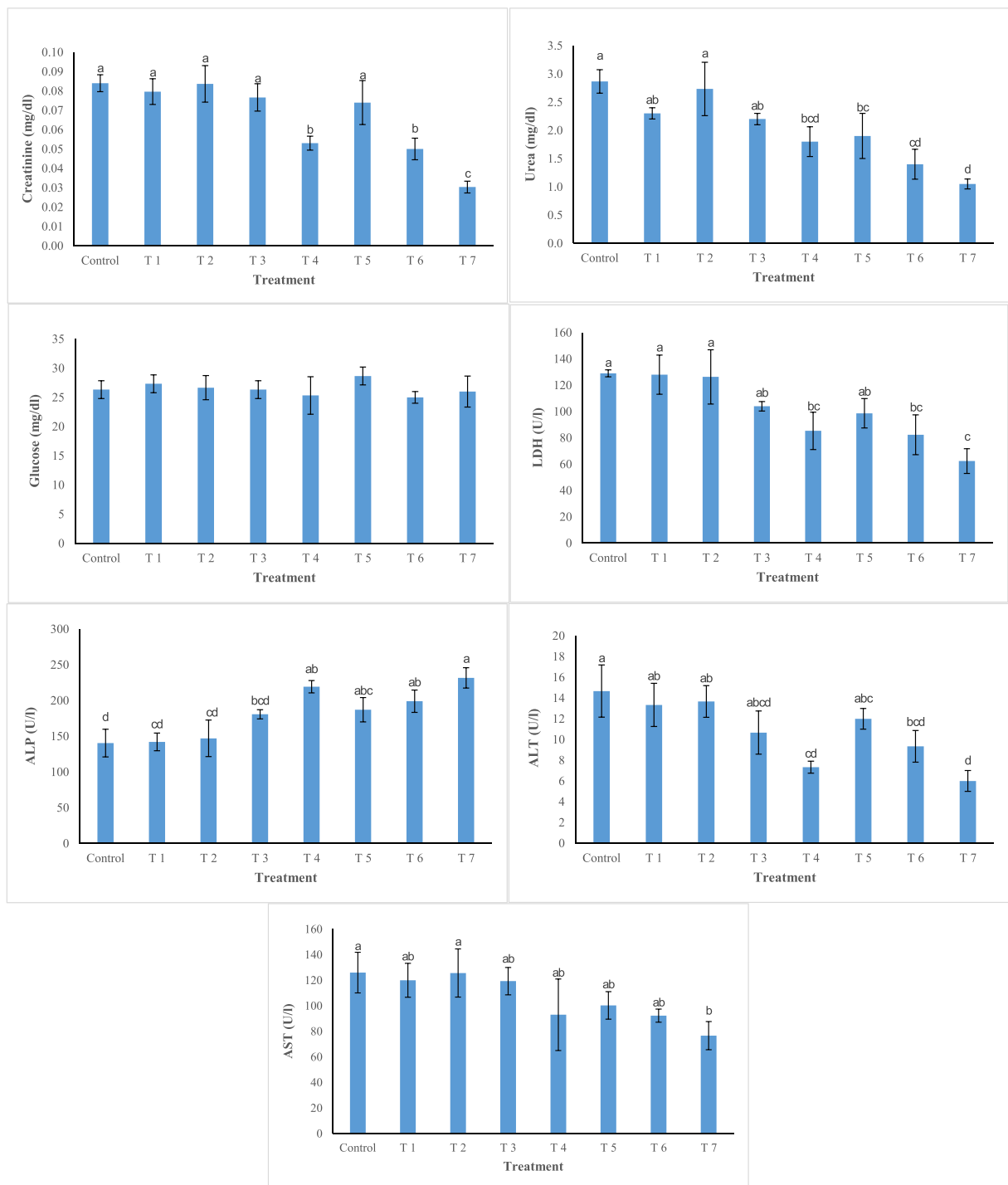
NPs kg<sup>-1</sup> diet), and T7 (40 mg chitosan–ZnO NPs kg<sup>-1</sup> diet) for 28-day feeding trial. Values are means ± SD (*n* = 5). Different letters represent significant differences between bars (*P* < 0.05)

vitamins C, E, and A; glutathione; uric acid; GSH; and lipoic acid), minerals (e.g., zinc, selenium, copper, manganese, and iron), and metal chelating proteins (e.g., ferritin, lactoferrin, albumin, and ceruloplasmin) (Krishnamurthy and Wadhvani 2012).

The zinc physiological concentration restrains the generation of ROS, i.e., superoxide anion radical, hydroxyl radical, and hydrogen peroxide (Ogawa et al. 2011). The antioxidant effect of zinc may be mediated through the direct action of zinc ion, its structural role in antioxidant proteins, and modulation metallothionein induction. Direct antioxidant activity of Zn ions is

associated with its binding to thiol groups and thus protects them from oxidation (Olechnowicz et al. 2018). SOD as an antioxidant enzyme can accelerate decomposition O<sub>2</sub><sup>-</sup> to H<sub>2</sub>O<sub>2</sub><sup>-</sup> (Ruas et al. 2008). Zn plays a cofactor role for copper-zinc superoxide dismutase enzyme, which is an important antioxidant enzyme. Zinc deficiency suppresses expression and activity Cu-ZnSOD (Li et al. 2010).

In this research, the activity of SOD increased significantly in serum of the fish fed with ZnO and ZnO–chitosan NP-supplemented diets compared with control group (*p* < 0.05). A similar increment of SOD level was



**Fig. 4** Activities of ALT, AST, ALP, LDH, and contents of glucose, urea, and creatinine in blood serum of beluga fed with different diets T1 (chitosan), T2 (10 mg ZnO kg<sup>-1</sup> diet), T3 (20 mg ZnO kg<sup>-1</sup> diet), T4 (40 mg ZnO kg<sup>-1</sup> diet), T5 (10 mg chitosan–

ZnO NPs kg<sup>-1</sup> diet), T6 (20 mg chitosan–ZnO NPs kg<sup>-1</sup> diet), and T7 (40 mg chitosan–ZnO NPs kg<sup>-1</sup> diet). Values are means ± SD ( $n = 5$ ). Different letters represent significant differences between bars ( $P < 0.05$ )

previously published by Jiang et al. (2016) in the liver of blunt snout bream (*Megalobrama amblycephala*) with

addition level of zinc in diet. Similar results were found for the common carp (*Cyprinus carpio*) exposed to



0.5 mg l<sup>-1</sup> ZnO NPs (Hao and Chen 2012). Hidalgo et al. (2002) showed that zinc deficiency decreased the activity of SOD in the liver of rainbow trout (*Onchorhynchus mykiss*). Feng et al. (2011) evidenced that the SOD levels in the intestine, muscle tissue, serum, and liver of common carp (*Cyprinus carpio* var. Jian) increased with increase in Zn concentration in diet. Huang et al. (2015) indicated that the SOD concentration in serum of Nile tilapia (*Oreochromis niloticus*) increased significantly followed by increasing dietary zinc level. Luo et al. (2011) demonstrated that the SOD activity of yellow catfish (*Pelteobagrus fulvidraco*) increased with increase in zinc content of diet up to the ideal level. Wu et al. (2011) showed that the expression of mRNA for copper/zinc-SOD and manganese-SOD in abalone (*Haliotis discus hannai*) increased and attained the peak at the 33.8 mg kg<sup>-1</sup> Zn in diet. Wu et al. (2015) reported that the SOD level in muscle of grass carp (*Ctenopharyngodon idella*) increased with increasing levels of Zn up to a point. The result of this study about SOD is in line with those of previous studies.

The performance of SODs as antioxidant enzymes depends on their collaboration with the other antioxidant agents, such as CAT, GPXs, and glutathione reductase (GR) (Faggio et al. 2016; Burgos Aceves et al. 2018). H<sub>2</sub>O<sub>2</sub> that is produced by the action of SODs or the action of oxidases is reduced to water by CAT and GSH-Px (Birben et al. 2012). CAT is an omnipresent tetrameric heme-containing antioxidant enzyme that accelerates the conversion of 2 molecules of H<sub>2</sub>O<sub>2</sub> into H<sub>2</sub>O and O<sub>2</sub> (Sharma et al. 2012). GPX catalyzes the transformation of H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O or organic peroxides into their analogous stable alcohols by oxidation the reduced GSH to GSSG (Manduzio et al. 2004). GST can detoxify xenobiotics through attachment of their electrophilic groups to the GSH the sulfhydryl (-SH) group to enhance their elimination from cells (Elia et al. 2006). In this study, the CAT and GPX activities increased in serum of beluga with increasing dietary Zn levels (ZnO and chitosan-ZnO NPs) and reached to the maximum at the dietary chitosan-ZnO NPs at the level of 40 mg kg<sup>-1</sup>. Also, it has been shown that the simultaneous increase in the activity of SOD and GPX enzymes enhances the activity of NADPH oxidase, which is responsible for scavenging of superoxide anion (Sheikh Asadi et al. 2018).

Another important finding was that the activity of GSH and GST significantly increased in fish that were

fed with ZnO/chitosan-ZnO NP-supplemented diets. The maximum level of TAC was found in serum of beluga fed with 40 mg kg<sup>-1</sup> chitosan-ZnO NP-supplemented diet. This finding is consistent with that of Hidalgo et al. (2002) who evidenced that zinc shortage decreased CAT level in the liver of rainbow trout. In other study, Feng et al. (2011) demonstrated that the GSH-Px, GST, and CAT enzyme activities in intestine, muscle tissue, serum, and the liver of common carp increased with increase in Zn content of diet. Huang et al. (2015) indicated that the level of GSH-Px in serum of adult Nile tilapia significantly augmented with increasing of Zn content of diet, but CAT activity was decreased. Wu et al. (2015) reported significant increases in CAT and GSH levels in muscle of grass carp with increasing levels of Zn up to a point. Jiang et al. (2016) expressed that increasing zinc content of diet, the GSH-Px, CAT, and TAC levels in the liver blunt snout bream significantly increased, and Yuan et al. (2016) indicated that activity of antioxidant enzymes developed in yellow croaker (*Larimichthys croceus*) with raising dietary Zn levels in groups fed with high levels of copper. They showed that a high level of zinc in diet can decrease toxicity effects of copper in fish. Wu et al. (2011) reported that the mRNA level of catalase and mu glutathione S-transferase (GST mu) in abalone increased and attained the peak at the 33.8 mg kg<sup>-1</sup> Zn in diet. The results of this work match those observed in earlier studies.

MDA is an important non-enzymatic antioxidant as a biomarker use for lipid peroxidation and health condition of biological membranes (which rich unsaturated fatty acids) (Khosravi-Katuli et al. 2018). Mostly, the level of oxidative stress in an organism is determined by the production of reactive aldehyde as a biomarker. Besides, ROS destruct polyunsaturated lipids by producing malondialdehyde (MDA) (Jafarnejad et al. 2018). In present study, MDA level showed no significant differences ( $p < 0.05$ ) in fish that were fed with ZnO or chitosan-ZnO NP-supplemented diets in compared with the control. However, unlike this study, Saddick et al. (2017) were reported increased level of MDA in *Oreochromis niloticus* exposed to ZnO NPs. Although it is demonstrated that MDA level was increased significantly in *Rutilus rutilus caspicus* exposed to Zn NPs in acute and sub-acute condition (4 and 14 days, respectively) (Khosravi-Katuli et al. 2018), based on our observation the increasing trend of MDA level in treatment

groups, if the trial period was longer, the same results would be probably achieved.

Our findings showed that the fish fed with ZnO and chitosan–ZnO NP–supplemented diets enhanced the total protein and albumin, which is probably due to the augmentation of protein synthesis in the liver (Sakr et al. 2005; Akrami et al. 2015). The liver plays an important role in maintaining the equilibrium of osmotic pressure between blood and tissue spaces. In addition, these proteins are highly susceptible to metallic toxicity. Simultaneous increase in total protein and albumin under the influence of dietary ZnO and chitosan–ZnO NPs indicates the important role of protein during Zn transportation (Sakr et al. 2005). Albumin in fish blood is closely related to total protein (Akrami et al. 2015) which is supposed to be associated with stronger innate immunity response (Wiegertjes et al. 1996). The positive and negative effects of Zn concentration on the diet in fish (*Oreochromis niloticus*, *Gadus morhua*, *Carassius auratus*) and shrimp (*Penaeus monodon*, *Penaeus vannamei*, *Macrobrachium rosenbergii*) have been reported (Davis et al. 1993; Shiau and Jiang 2006; Herland et al. 2011; Hasnat et al. 2012; Muralisankar et al. 2014; Adel Abdel-Khalek et al. 2015). Adel Abdel-Khalek et al. (2015) addressed that serum albumin in *O. niloticus* exposures to ZnO bulk and ZnO NPs is increased under acute and sub-acute condition. Similarly, the increasing serum albumin level was recorded in *O. niloticus* in response to exposure to Zn and its composition with Cd (Firat and Kargın 2010).

Unlike this study, Halliwell (2007) and Wang et al. (2007a, 2007b) showed that total protein decreases after exposure to NPs which may be caused by the excessive production of ROS in the tissue that can damage macromolecules such as DNA, protein, lipids, and carbohydrates.

Unfortunately, there is lack of information about the effect of nanoparticles on the lysozyme activity enzyme and ACH50 level in chondrosteian fish. Lysozyme and the alternative complement activity (classical and alternative pathway) are exceptionally a widespread as humoral components associate in the innate immune system that is important for protecting against fish disease (Kaya et al. 2016). In our study, lysozyme and ACH50 levels were increased significantly ( $p > 0.05$ ) in fish were fed with ZnO and chitosan–ZnO NP–supplemented diets (Fig. 3). This incremental change in the present study is probably because of the immunosuppressive effects of the nanoparticles (Kaya et al. 2016). IgM

produced by the plasma cells of the spleen and lymph nodes and secreted into serum. In fish, in terms of structural and physiological features, IgM is considered as an effective immune molecule (Akrami et al. 2015). Previous studies demonstrated that IgM fluctuations are related to fish size, environmental conditions, and fish health status (Klesius 1990; Picchitti et al. 2001). According to the results, IgM level in serum of fish was fed with 20 and 40 mg ZnO and chitosan–ZnO NP–supplemented diets increased significantly ( $p < 0.05$ ) compared to the control group. It can be due to the effect of Zn on the immune system, including the natural development and function of the mediate cells of non-specific receptors such as neutrophils. This result coincides with the investigation of Tawfik et al. (2017) who reported increasing IgM level in *O. niloticus* fed with ZnO and ZnO NPs.

After 4 weeks, changes in the glucose levels were not significantly different compared to control group. Similarly, Lee et al. (2014) reported, at 12 weeks, the glucose level in highest level of ZnO NPs significantly increased (without significant increase in glucose levels at 4 and 8 weeks). They believe that the increase in glucose levels was related to liver injury under long-term exposure to ZnO NPs. High concentration of glucose in the blood indicates that a fish is in stress and is intensively using its energy reserve (Vosylienė 1999; Burgos Aceves et al. 2019).

Unfortunately, there are not many studies on effects of ZnO and ZnO NPs on urea and creatinine in sturgeon fishes. Most urea in fish is produced by the liver and excreted mainly by the gills (Alkaladi et al. 2015), while creatinine extracted mainly by the kidneys. The results of this study showed that the amount of urea and creatinine decreased with increasing ZnO and chitosan–ZnO NP levels. Contrary to these results, Llobet et al. (1988) reported that the concentrations of urea and creatinine in plasma of rat significantly increased after high-dose exposure to zinc acetate dihydrate in drinking water.

ALPs are a group of zinc-dependent enzymes and present in most tissues of the body whose induce transfer activity, catalytic activity, and generally leakage from the liver (Gharaei et al. 2011; Estaki et al. 2014). Zn and magnesium are two important cofactors of this enzyme (Ray et al. 2017). ALP is associated with calcification process in bone tissue and fat transfer in the intestine. For this reason, the level of the enzyme in the blood is higher in the periods of animal life cycle that calcification process develops (Coleman 1992; Ray et al. 2017).

Some researches demonstrated that Zn and magnesium deficiency in the body of animals reduce ALP enzyme activity (Ray et al. 2017). In the present study, we found that with the increase of Zn level in fish diet, ALP level increased in fish serum (Fig. 4). Similar results were reported by Liang et al. (2012) and Li and Huang (2016) who conducted study on the effect of Zn on fish which could enhance the level of ALP activity enzyme. The relationship between Zn and ALP in this study suggests that increased levels of Zn in the diet may have an effect on the calcification process (Sarker and Satoh 2009; Jiang et al. 2016), which may increase the level of ALP in the serum. On the other hand, it has been proven that Zn is also a major contributor to insulin-like effects and the regulation of carbohydrate metabolism in the activity of many digestive enzymes in the gut (Tang and Shay 2001; Ilouz et al. 2002). Therefore, it is thought that increased Zn absorption in the intestinal cells triggers the mechanisms involved in absorbing glucose. Glucose acts as a substrate in the biosynthesis process of some macromolecules. Therefore, the presence of available glucose may be the starting point for increasing the activity of ALP, which is structurally a glycoprotein macromolecule (Dong et al. 2013). In addition, it is a part of the catalytic structure of the ALP enzyme, and it is reasonable to assume that increased Zn absorption would increase the activity of this enzyme.

LDH is the non-specific enzyme responsible for catalyzing lactate to pyruvate and considered an important enzyme for energy generation in the cells (Gharaei et al. 2011). LDH is a zinc-containing metalloenzyme (Low and Ikram 1976). The muscle, liver, and red blood cells (hemolysis) are the major sources of serum LDH activity (Smith et al. 2013). Zinc deficiency in rats increased the osmotic fragility of erythrocytes, due to structural defect in the plasma membrane (Roozbeh et al. 2009). It is widely accepted that total serum LDH principally raises because of hemolytic anemias (Cohen et al. 1998). Luo et al. (2011) showed that dietary Zn decreased concentration of serum LDH with increasing dose in yellow catfish, *Pelteobagrus fulvidraco*. Fathi (2016) found that in Broiler chicken fed by Zn NPs (0, 10, 20, 40 mg kg<sup>-1</sup>) levels of LDH activity significantly decreased compared to the control groups. Reducing LDH in the blood of fish fed with ZnO/chitosan–ZnO NP-supplemented diets is probably attributable to the improvement in the cellular activity of this enzyme, because it has been proven that LDH secretion

increases in blood along with damage in many tissues (El-Demerdash and Elagamy 1999; Gharaei et al. 2011).

AST and ALT are both non-specific enzymes of blood that exist in many organs including the liver, heart, kidney, gills, and muscle (Akrami et al. 2015). The results of this study revealed that a significant decrease in AST and ALT activity level depends on Zn dose in all treatment groups (Fig. 4). It is proven that Zn could prevent lipid peroxidation process in the cell membrane and the increase of foresaid of above mentioned enzymes in blood, due to its antioxidant and antiradical characteristics (Taheri et al. 2017). The decrease in liver enzymes in the blood is probably due to a decrease in production, excretion or change in their half-life (Balistrei and Rej 1994). The decrease in the activity of ALT and AST enzymes in fish indicates that transaminase is inactivated and reduced amino acid catabolism (Bibiano Melo et al. 2006). Zinc is involved in the structure of some amino acids such as tryptophan. Similar results were reported for Nile tilapia, common carp, and broiler chicken fed by Zn and Zn NP-supplemented diets (Huang et al. 2015; Fathi 2016; Taheri et al. 2017).

## Conclusion

In conclusion, we emphasized to some changes in the physiological and biochemical parameters of blood under effect dietary ZnO and chitosan–ZnO NPs. Based on the data, the level of MDA and glucose in the blood serum of the beluga juveniles has been not affected by ZnO/chitosan–ZnO NP supplementation. Our result also suggested that diets supplemented with ZnO and chitosan–ZnO NPs increased significantly TAC, SOD, GPX, GSH, GST, and CAT activity enzymes. Other data showed that with increasing levels of ZnO and chitosan–ZnO NPs, ACH50, total immunoglobulin, total protein, albumin, lysozyme, ALP, and glucose (exception in case of ALT, LDH, AST, creatinine, and urea) had a significant increase. Taken together, the level of chitosan–ZnO NPs at 40 mg kg<sup>-1</sup> supplementation demonstrated positive effect on digestive performance, antioxidant system, and health status of beluga. Finally, we hope that the findings of the current study will be of help to aquaculture officials for future decisions in development

of fish farms and be considered in restoration programs of sturgeon populations.

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