The effects of silver nanoparticles (Ag-NPs) sublethal concentrations on common carp (Cyprinus carpio): Bioaccumulation, hematology, serum biochemistry and immunology, antioxidant enzymes, and skin mucosal responses

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ABSTRACT

The present study aimed to evaluate the effects of different waterborne sublethal concentrations of Ag-NPs LC50 (96h) on common carp Cyprinus carpio using a multi-biomarker approach. Fish (9.22 ± 0.12 g) were stocked in fiberglass tanks and exposed to concentrations of 0 (control), 12.5%, 25% and 50% of Ag-NPs LC50 (96h) or Ag–NO3 LC50 (96h), as the source of Ag⁺ ion, for a period of 21 days. At the end of study, tissue Ag contents were significantly (P < 0.05) higher and different in fish exposed to concentrations of 25% and 50% compared to the control. The numbers of RBCs, hematocrit, and MCHC values at these concentrations differed significantly in respect to the control. No significant effects were observed for hemoglobin, MCH, and MCV values. The number of WBCs was significantly higher at concentrations of 12.5% and 25% compared to the control. Meanwhile, the percentage of neutrophils significantly elevated at concentrations of 25% and 50%. Serum total protein at concentration of 50% detected significantly lower than that of 12.5% or the control. The serum albumin and globulin levels significantly declined in Ag-NPs-exposed groups versus the control. The serum ACH50 and total immunoglobulins showed significantly lower values in the treatments of 25% and 50% compared to the control. The exposure to the concentrations of 25% and 50% significantly dwindled the lysozyme activity and total immunoglobulin levels in skin mucus. In conclusion, sublethal concentrations of Ag-NPs LC50 (96h) impaired fish health status at higher concentrations and 12.5% of Ag-NPs LC50 (96h) was presumably safe for common carp aquaculture.

1. Introduction

Nano-technology is commonly defined as the understanding, control, and application of matter on the nano-scale, approximately between 1 and 100 nm. Nano-particles have different properties from the corresponding bulk materials and are used in various industries and consumers goods (Jeevanandam et al., 2018). In recent years, especially the production and application of silver nanoparticles (Ag-NPs) has increased, accounting for over 50% of the global nanomaterial products in 2015, with expectation of increase nearly 13% until 2024 (Inshakova and Inshakov, 2017). These nano-particles are commonly used in cosmetics, textiles, toothpastes, shampoos, paints, washing machines, food supplements, water treatment, and more (Hedayati et al., 2019; Inshakova and Inshakov, 2017; Mao et al., 2018). A large volume of industrial and municipal wastewater is accidentally or deliberately discharged into natural waters resources including rivers, lakes, and coastal waters. The concentrations for Ag-NPs was predicted 1.3–4.4 mg/kg (Europe and USA) in sewage sludge, 9 μg/L in sewage effluent (European region) (Kwok et al., 2012), and 10⁻¹¹, 10⁻⁷ μg/L in surface waters of different regions (Barros et al., 2020; Peters et al., 2018); thus, raising concerns about the end up of Ag-NPs...
nanoparticles into the environment.

In this regard, aquatic nano-toxicology is an relatively emerging field of research that has attracted the attentions. In general, aquatic animals may be exposed to nanoparticles through food, water, and sediments. The available information demonstrate that Ag-NPs exposure cause toxicity in some fish species and other animals (Ale et al., 2018a; Bacchetta et al., 2017; Guilherme et al., 2016; He et al., 2012; Mao et al., 2018; Otsazewskas et al., 2018; Rajkumar et al., 2016; Shi et al., 2013). The median lethal concentration (LC50) tests are run to determine the susceptibility and survival potential of animals to particular toxic materials (Shah and Altindag, 2005). Previously, 96th LC50 of Ag-NPs has been determined for common carp (Cyprinus carpio) through waterborne exposure (Khaleghi et al., 2018), but the information on the effects of its sub-lethal concentrations on this species are scarce. Inhibition of Na+ K+ ATPase enzyme activity in gills; alterations in concentrations of plasma biochemicals (electrolytes, proteins, lipids), oxidative stress/cell damage markers (glucose, cortisol, glycogen, lactate dehydrogenase, aspartate aminotransferases, alanine aminotransferases, antioxidant enzymes, lipid peroxidation), and histopathological changes are among other deleterious effects of exposure to different sublethal concentrations of Ag-NPs LC50 (96h) in fish (Ale et al., 2018b; Govindasamy and Rahuman, 2012; Grosell et al., 2000; Sathy et al., 2012; Shobana et al., 2018; Wu and Zhou, 2013).

In common carp, exposure to LC50 (96h) concentration of Ag-NPs and Ag–NO3 caused increased tissue Ag content, concomitantly with higher antioxidant and tissue damage enzymes activities as well as elevated stress genes and histological lesions (Khosrovari-Katuli et al., 2018). This information helps us to understand what physiological alterations this important freshwater aquaculture species (Rahman, 2015) experience upon encountering sub-lethal concentration of Ag-NPs, finally recommending a safe level.

The hematology is one of the most important processes in assessing the toxicity of nanoparticles for aquatic animals (Hedayati et al., 2019). Besides, the mucosal surface of fish acts as the first line of defense against harmful agents present in the aquatic environment (Guardiola et al., 2015; Lazado and Caipang, 2014; Mohammadi et al., 2020), and it is considered a useful tool to assess the effects of toxicants. Available literature show fish skin mucus immune indices are significantly affected by the exposure to crude oil (Dzul-Caamal et al., 2016b), different heavy metals and pollutants (Ale et al., 2018b; Dzul-Caamal et al., 2016a, 2013; Guardiola et al., 2015; Stabili and Pagliara, 2009) as well as in experimentally ulcerated fish (Tapia-Paniagua et al., 2018). These changes are mainly attributed to oxidative stress, thereby proteins and enzymes damage, and histological injuries (Ale et al., 2018a; Dzul-Caamal et al., 2016a, 2016b; Govindasamy and Rahuman, 2012).

The toxicity of silver nitrate (AgNO3) is related to the release of Ag+ in the water (Morgan et al., 1997). Hence, the silver nitrate (AgNO3) is often used as an Ag+ ion source to compare the effects of Ag-NPs to those caused by free Ag+ ions (Cambier et al., 2018). Therefore, the present study aimed to evaluate the effects of different sublethal concentrations of Ag-NPs LC50 (96h) or Ag–NO3 LC50 (96h) on hematology, serum biochemicals and immune parameters, antioxidant enzymes, and skin mucus immune responses of common carp (Cyprinus carpio).

2. Materials and methods

2.1. Experimental fish, design and conditions

All common carps (Cyprinus carpio) were purchased from Gilan Aquaculture Center (Gilan, Iran) and transferred to the laboratory. After 10 days of adaptation, fish were visually examined to ensure normal body structure. Then, 480 healthy fish with an initial weight of 9.22 ± 0.12 g were stocked in 24 fiberglass tanks (each 100-L) randomly (20 fish per tank) in triplicate. In the current study, sub-lethal concentrations of 12.5%, 25%, and 50% of waterborne Ag-NPs LC50 (96h) were tested for 21 days against a control without Ag-NPs. The LC50 (96h) of waterborne silver nanoparticles (Ag-NPs) for common carp has been reported 2 mg/L (Khaleghi et al., 2018). Also, fish were separately exposed to different sub-lethal concentrations of 0 (control), 12.5%, 25%, and 50% of waterborne silver nitrate (Ag–NO3) LC50 (96h) to decipher and determine the differences in responses between Ag-NPs and soluble Ag+ ions, knowing that LC50 (96h) of waterborne Ag–NO3 for common carp is 0.9 mg/L. Therefore, sufficient amounts of Ag-NPs or Ag–NO3 were separately added to water in tanks to make concentrations of 0 (control) of 12.5%, 25%, and 50% of Ag-NPs LC50 (96h) or Ag–NO3 LC50 (96h), as described in section 2.2.

During the experiment, all fish were fed 4% of their body weight three times a day at 7 a.m., 2 p.m., and 6 p.m. with a commercial common carp pellet diet containing 5–11% moisture, 35–38% crude protein, 4–8% crude lipid, 4–7% crude fiber, 7–11% ash, and 1–1.5 phosphorus (Faradaneh Co. aquatic animals feed producer, Shahrakord, Iran). The water temperature, dissolved oxygen, pH, and salinity were adjusted at 28 ± 0.5 °C, 6 ± 0.8 mg/L, 7.6 ± 0.2 and 4 ± 0.1 g/L, respectively. The experiment was conducted in a greenhouse structure and tanks were exposed to natural photoperiod.

2.2. Ag-NPs suspension and AgNO3 stock solution

The powders of Ag-NPs (average particle size 20 nm, morphology spherical, surface area 18–22 m²/g, density 10.5 g/cm³, and purity of 99.99%; TECNAN Co, Spain) were coated with 0.2 wt% polyvinylpyrrolidone (PVP). The representative scanning electron microscopy (SEM) image of the Ag-NPs powder and the X-ray diffraction curves of Ag-NPs powders are presented as a supplementary file. Ag-NPs stock solution was prepared using sterile deionized water as vehicle and ultrasonicated (UP100H, Hielscher Co., Germany) for 30 min, following the manufacturer’s instructions. In addition, Ag–NO3 (≥ 99.99% purity; TECNAN Co, Spain) was used as source of Ag+ ion. Ag–NO3 stock solution (1000 mg/L) was freshly prepared before the exposure using mixing with sterile deionized water by vortexing.

Then, the adequate amounts of stock solutions were separately added to the tanks to acquire the concentrations of 12.5%, 25%, and 50% of Ag-NPs LC50 (96h) or Ag–NO3 LC50 (96h) for common carp (Khaleghi et al., 2018). The concentrations of the Ag-NPs or Ag–NO3 solutions were confirmed using an inductively coupled plasma optical emission spectroscopy (ICP-OES) (Carrazco-Quevedo et al., 2019), before and after each exposure time. Finally, fish were transferred to each tank and exposed to sub-lethal concentrations of waterborne Ag-NPs or Ag–NO3 for 21 days. Water exchange was performed once a day to remove excess feed and avoid trapping of Ag-NPs by organic matter (Salari Joo et al., 2013) as well as maintaining water quality. Then, Ag-NPs or Ag–NO3 solutions were redosed in each tank. Water was well mixed by aeration to prevent NPs from aggregation or precipitation (Wang et al., 2011).

2.3. Blood and serum collection

At the end of the study (day 21), 21 fish (seven fish per tank) were anesthetized using clove powder (500 mg/L, 20 min) for blood sampling. Then, blood was drawn from caudal tail vessels using 23-gauge needles and collected in heparinized Eppendorf tubes for hematological assays. Also, aliquots of blood were transferred into Eppendorf tubes without anticoagulant and stored for 6 h at 4 °C. After blood clotting, samples were centrifuged (at 3000g for 5 min, 4 °C) and plasma was separated and stored at −80 °C until analysis.

2.4. Hematology

White blood cell (WBC) and red blood cell (RBC) counts were made with a Neubauer crystalline counting chamber. Hemoglobin content (Hb) was measured by the cyanmethemoglobin method at 546 nm on a
spectrophotometer (Unico 1100RS, USA) (Blaxhall and Daigle, 1973; Klontz, 1994). The hemocrit value (Hct) was determined using microhemocrit capillary tubes centrifuged at 1680 × g for 5 min (Cyrja et al., 1989). The mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were calculated using standard formulas provided by (Benfey and Sutterlin, 1984). Differential white cell counts (monocytes, lymphocytes, and neutrophils) were performed following Giemsa staining protocols and then examining blood smears under a light microscope (Ghiasi et al., 2010).

2.5. Serum biochemicals and immunology

Serum samples were analyzed using automatic biochemical analyzer (Hitachi 911, Tokyo, Japan) and attached kits (Pars Azmoun, Tehran, Iran) for determination of total protein (TP), glucose (GLU), Albumin (ALB), cholesterol (CHO), and triglyceride (TRG) levels (Mohammadi et al., 2020; Sabzi et al., 2017). Serum cortisol levels were measured by using a commercial ELISA kit based on manufacturer instructions (IBL, Germany). The difference between serum total protein and serum albumin concentration was considered as serum globulin (GLB) concentration (Bayunova et al., 2002).

The immunoglobulin (Ig) molecules were precipitated down using a 12.5% solution of polyethylene glycol (Sigma) and the difference in the protein contents prior and after immunoglobulin molecules precipitation was considered as the total Ig content (Siwicki and Anderson, 1993). Lysozyme activities were determined using a turbidimetric method at 450 nm in a spectrophotometer (Ellis, 1993). Lysozyme activities were determined using Micrococcus lysodeikiticus suspension (75 μg/mL, Sigma, USA) was added to 20 μL of serum samples and the changes in absorbance were recorded continuously for 1 h at 450 nm using a spectrophotometer. The complement (ACH50) activity was estimated by hemolytic assay of rabbit erythrocytes. Briefly, after developing the lysis curve of rabbit red blood cells, a volume of serum that causes 50% hemolysis is considered as serum ACH50 activity (Yano, 1992).

The determination of aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) activities using commercial kits (Pars Azmoun, Iran). All samples were analyzed in triplicate by this scientist. The skin mucus protease activities were measured using the azo-casein hydrolysis assay (Palaksha et al., 2008). Skin mucus TP contents were estimated as described in Section 2.5.

2.6. Bioaccumulations of Ag-NPs and Ag–NO3

At the end of study (day 21), 21 fish were randomly sampled from each tank (21 fish per treatment), anesthetized with clove powder (500 mg/L) and then 10 mL of 50 mmol NaCl was sprayed on their skin and after 2 min of gentle shaking of fish in a polyethylene bag, mucus samples were collected in test tubes (Subramanian et al., 2007). Then, mucus samples were immediately centrifuged (1500g, 10 min, 4 °C) and stored at −80 until analysis.

Skin mucus lysozyme, ALP, and ACH50, as well as Ig and TP contents were measured using similar methods described in Section 2.5. The skin mucus protease activities were measured using the azo-casein hydrolysis assay (Palaksha et al., 2008). Skin mucus TP contents were estimated as described in Section 2.5.

2.8. Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal’s author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The proposal and protocols of the present work was reviewed and approved by this scientific committee.

2.9. Statistical analysis

The data normality was checked using Kolmogorov-Smirnov test. And then, one-way analysis of variance (ANOVA) was performed followed by Duncan test to find significant differences among treatments at a probability level of P < 0.05. All the data are presented as mean ± standard deviation (S.D.), and Windows SPSS software (Version 24) was used for all statistical analysis.

3. Results

3.1. Bioaccumulations of Ag-NPs and Ag–NO3

Effects of common carp waterborne exposure to Ag-NPs and Ag–NO3 on tissue Ag contents are presented in Table 1. After 21 days of exposure, the pattern of Ag accumulation in tissues was as follow: 50% of LC50 (96h) > 25% of LC50 (96h) > 12.5% of LC50 (96h) > control. The gills and liver showed the highest Ag accumulation. Also, the bioaccumulation pattern of Ag in the intestines differed between Ag-NPs and Ag-NPs exposed groups, since there were not any significant dose-dependent elevations of Ag in the intestines upon Ag–NO3.

Table 1: Bioaccumulations of Ag (μg/g) released from Ag-NPs and Ag–NO3 in particular organs of Cyprinus carpio exposed to different sub-lethal concentrations of Ag-NPs LC50 (96h) or Ag–NO3 LC50 (96h) for 21 days.

<table>
<thead>
<tr>
<th>Organ (μg/g tissue)</th>
<th>Treatments</th>
<th>control</th>
<th>12.5% S.D.</th>
<th>25% S.D.</th>
<th>50% S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gill (μg/g)</td>
<td>Ag-NPs</td>
<td>0.82 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.66 ± 0.94&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.43 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.66 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Ag–NO3</td>
<td>0.37 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.60 ± 0.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.06 ± 0.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.33 ± 0.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver (μg/g)</td>
<td>Ag-NPs</td>
<td>0.63 ± 0.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.30 ± 0.79&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.06 ± 0.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.33 ± 0.66&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Ag–NO3</td>
<td>0.40 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.03 ± 0.65&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.40 ± 0.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.56 ± 0.45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Intestine (μg/g)</td>
<td>Ag-NPs</td>
<td>0.41 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.03 ± 0.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.13 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.10 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Ag–NO3</td>
<td>0.11 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.40 ± 0.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.73 ± 0.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.76 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are represented as means ± SD (n = 3). Means in the same row with different superscripts show significant differences (P < 0.05).
Hematological parameters of *Cyprinus carpio* exposed to different sub-lethal concentrations of Ag-NPs LC₅₀ (96h) or Ag–NO₃ LC₅₀ (96h) for 21 days.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatments</th>
<th>control</th>
<th>12%</th>
<th>25%</th>
<th>50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (× 10⁶/μl)</td>
<td>Ag-NPs</td>
<td>1.34 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.33 ± 0.03&lt;sup&gt;*a&lt;/sup&gt;</td>
<td>1.24 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.26 ± 0.04&lt;sup&gt;*b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Htc (%)</td>
<td>Ag-NPs</td>
<td>23.12 ± 1.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.47 ± 1.75&lt;sup&gt;*a,b&lt;/sup&gt;</td>
<td>20.02 ± 0.16&lt;sup&gt;*b&lt;/sup&gt;</td>
<td>19.47 ± 1.75&lt;sup&gt;*b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HB (g/dl)</td>
<td>Ag-NPs</td>
<td>7.50 ± 0.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.33 ± 0.25&lt;sup&gt;*a&lt;/sup&gt;</td>
<td>7.46 ± 0.15&lt;sup&gt;*a&lt;/sup&gt;</td>
<td>7.40 ± 0.26&lt;sup&gt;*a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>Ag-NPs</td>
<td>32.46 ± 2.77&lt;sup&gt;*a&lt;/sup&gt;</td>
<td>30.05 ± 1.68&lt;sup&gt;*b&lt;/sup&gt;</td>
<td>34.23 ± 1.22&lt;sup&gt;*a&lt;/sup&gt;</td>
<td>35.66 ± 2.15&lt;sup&gt;*a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>Ag-NPs</td>
<td>56.66 ± 4.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>54.90 ± 4.16&lt;sup&gt;*a&lt;/sup&gt;</td>
<td>60.09 ± 2.73&lt;sup&gt;*a&lt;/sup&gt;</td>
<td>58.62 ± 2.93&lt;sup&gt;*a&lt;/sup&gt;</td>
</tr>
<tr>
<td>WBC (× 10³/μl)</td>
<td>Ag-NPs</td>
<td>171.89 ± 15.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>182.10 ± 13.30&lt;sup&gt;*a&lt;/sup&gt;</td>
<td>165.06 ± 13.29&lt;sup&gt;*a&lt;/sup&gt;</td>
<td>169.58 ± 9.61&lt;sup&gt;*a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are represented as means ± SD (n = 3). Means in the same row with different superscripts show significant differences (P < 0.05).

<sup>a</sup> Red blood cell.
<sup>b</sup> Hematocrit value.
<sup>c</sup> Hemoglobin.
<sup>d</sup> Mean corpuscular hemoglobin concentration.
<sup*e</sup> Mean cell hemoglobin.
<sup>f</sup> Mean cell volume.
<sup>g</sup> White blood cells.
<sup>h</sup> Monocytes.
<sup>i</sup> Lymphocytes.
<sup>j</sup> Neutrophils.

**3.2. Hematology**

Effects of Ag-NPs on hematological parameters of common carp are presented in Table 2. After 21-day of waterborne exposure, the number of red blood cells (RBCs) in fish exposed to 25% and 50% of Ag-NPs LC₅₀ (96h) were significantly (P < 0.05) lower than the treatment of 12.5% and the control. The exposure of carps to Ag-NPs resulted in significantly lower hematocrit values in treatments of 25% and 50% of LC₅₀ (96h) versus the control. However, hemoglobin levels of all tested groups were statistically constant and showed no significant differences (P > 0.05). The lowest and the highest MCHC values were recorded for the control and the treatment of 50% of LC₅₀ (96h), respectively, and they significantly differed when compared to each other. The different concentrations of Ag-NPs LC₅₀ (96h) tested in this study had no remarkable effect on mean cell hemoglobin (MCH) and mean cell volume (MCV) values.

Effects of Ag-NPs on white blood cells (WBCs) of common carp are presented in Table 2. The number of WBCs were significantly higher in treatments of 12.5% and 25% versus the treatment of 50% and the control. The differential count of leucocytes demonstrated that Ag-NPs exposure has reduced monocytes percentage in the treatments of 25% and 50% of LC₅₀ (96h) approximately by two-folds compared to the control, yet there were not any significant differences to report. The mean levels of lymphocytes did not differ significantly among experimental groups. However, neutrophils percentage significantly diminished in fish exposed to 25% and 50% of Ag-NPs LC₅₀ (96h) in comparison to fish in the control.

**3.3. Serum immunology**

Effects of different levels of Ag-NPs LC₅₀ (96h) on serum immune parameters of common carp are shown in Fig. 1. Fish exposed to Ag-NPs showed decreased lysozyme (Lys) activity compared to the control, but no significant differences were observed. However, alternative complement (ACH50) activity and total immunoglobulins (Ig) content were significantly lowered in the treatments of 25% and 50% of Ag-NPs LC₅₀ (96h) compared to the control.

The serum analysis showed that 12.5% of Ag-NPs LC₅₀ (96h) insignificantly (P > 0.05) increased the serum TP compared to the control. Meanwhile, serum TPs in 25% and 50% of Ag-NPs LC₅₀ (96h) were significantly (P < 0.05) lower than 12.5% of Ag-NPs LC₅₀ (96h) and they fell to a level similar to the control. The serum ALB and GLO levels decreased and their contents were significantly lower in all exposed groups compared to the control.

**3.4. Serum biochemicals**

Effects of different levels of Ag-NPs LC₅₀ (96h) on serum stress biomarkers of common carp are shown in Table 3. Serum cortisol (COR) and glucose (GLU) levels significantly increased upon Ag-NPs exposure. COR levels were especially significantly higher in the treatment of 25% of Ag-NPs LC₅₀ (96h) when compared to the control. Meanwhile, significantly higher levels of GLU were record LC₅₀ ed for fish in 25% and 50% of Ag-NPs LC₅₀ (96h) compared to the control or 12.5% of Ag-NPs LC₅₀ (96h).

Table 4 represents the effects of different concentrations of Ag-NPs LC₅₀ (96h) on liver damage and antioxidant biomarkers of common carp after 21 days of waterborne exposure. The highest activities of ALT and ALP were similarly observed in the treatments of 25% and 50% of Ag-NPs LC₅₀ (96h) which were significantly different relative to the control (P < 0.05). The highest activity of superoxide dismutase (SOD) was recorded for 12.5% of Ag-NPs LC₅₀ (96h) and was significantly different from other tested groups. However, SOD activity in treatment of 25% of Ag-NPs LC₅₀ (96h) diminished to a level similar to the control and then fell to significantly lower level in the treatment of 50% of Ag-NPs LC₅₀ (96h) relative to the control. Catalase (CAT) activity had a
Fig. 1. Serum proteins and immunological parameters of Cyprinus carpio exposed to different sub-lethal concentrations of Ag-NPs LC50 (96h) or Ag-NO3 LC50 (96h) for 21 days. Values are represented as means ± SD (n = 3). The different superscripts show significant differences (P < 0.05).
similar trend, with the exception that there were no significant differences between exposed groups and the control. Yet, CAT activities in treatments of 25% and 50% of Ag-NPs LC50 (96h) demonstrated significantly lower levels compared to the treatment of 12.5% of LC50 (96h).

3.5. Skin mucus immune parameters

Effects of different concentrations of Ag-NPs LC50 (96h) on skin mucus parameters of common carp are given in Fig. 2. Ag-NPs exposure at 25% and 50% of LC50 (96h) significantly lowered both the lysozyme activities and total Ig contents of mucus samples compared to the control (P < 0.05). The mucus ACH50 and protease activities of ex-mucus samples compared to the control in the treatments of 25% and 50% of Ag-NPs LC50 (96h) demonstrated comparable to the control, while Hb values did not significantly differ among study groups. RBCs in combination with Hb play an important role in supplying oxygen to the body’s cells, and hematocrit (Htc) is defined as the volumetric percentage of RBCs in the blood which is affected by the number and size of RBCs. Hence, any increase in the number and size of RBCs or Hb may result in Htc index change. The small Ag-NPs enter the bloodstream by passing gastrointestinal tract or gills membrane and accumulate in RBCs where they may cause deleterious effects (Ale et al., 2018a). The main mechanism of Ag-NPs toxicity is the generation of ROS and oxidative stress (Choi et al., 2010).

Therefore, the adverse effects of Ag-NPs on hematological parameters include the lyses of RBCs due to high concentration of polysaturated fatty acid in their cell membrane and thus susceptibility to peroxidation (Massarsky et al., 2014); damage to hematopoietic tissues and thus disorder in erythropoiesis and RBCs formation (Shaluei et al., 2013); morphological alterations in RBCs and appearance of abnormal and small cells (Sayed et al., 2018); inhibition of erythrogenesis (Cui et al., 2016); down-regulation in expressions of hemoglobin genes (Chae et al., 2019); and inhibition of aerobic glycolysis and thus lack of sufficient energy to Hb synthesis (Imani et al., 2015). The findings on the effects of NPs on hematological parameters are interesting. In Labeo rohita exposed to Fe2O3-NPs, an incremental count of RBCs was observed through time but Htc and Hb levels increased up to 10th day and then significantly declined afterwards compared to the control (Remya et al., 2015). On the contrary, Vignesh et al. reported significantly higher Hb and Htc vice versa significantly lower RBC in L. rohita on 7th and 21st day of exposure to Ag-NPs (Vignesh et al., 2013).

4. Discussion

The current study demonstrated significantly lowered RBCs and Htc in fish exposed to 25% and 50% of Ag-NPs LC50 (96h) compared to the control, while Hb values did not significantly differ among study groups. RBCs in combination with Hb play an important role in supplying oxygen to the body’s cells, and hematocrit (Htc) is defined as the volumetric percentage of RBCs in the blood which is affected by the number and size of RBCs. Hence, any increase in the number and size of RBCs or Hb may result in Htc index change. The small Ag-NPs enter the bloodstream by passing gastrointestinal tract or gills membrane and accumulate in RBCs where they may cause deleterious effects (Ale et al., 2018a). The main mechanism of Ag-NPs toxicity is the generation of ROS and oxidative stress (Choi et al., 2010).

Therefore, the adverse effects of Ag-NPs on hematological parameters include the lyses of RBCs due to high concentration of polysaturated fatty acid in their cell membrane and thus susceptibility to peroxidation (Massarsky et al., 2014); damage to hematopoietic tissues and thus disorder in erythropoiesis and RBCs formation (Shaluei et al., 2013); morphological alterations in RBCs and appearance of abnormal and small cells (Sayed et al., 2018); inhibition of erythrogenesis (Cui et al., 2016); down-regulation in expressions of hemoglobin genes (Chae et al., 2019); and inhibition of aerobic glycolysis and thus lack of sufficient energy to Hb synthesis (Imani et al., 2015).

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Table 3

Serum stress biomarkers of Cyprinus carpio exposed to different sub-lethal concentrations of Ag-NPs LC50 (96h) or Ag–NO3 LC50 (96h) for 21 days.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
</tr>
<tr>
<td>GLU (g/dL)</td>
<td>Ag-NPs</td>
</tr>
<tr>
<td></td>
<td>Ag–NO3</td>
</tr>
<tr>
<td>CHO (g/dL)</td>
<td>Ag-NPs</td>
</tr>
<tr>
<td></td>
<td>Ag–NO3</td>
</tr>
<tr>
<td>COX (ng/mL)</td>
<td>Ag-NPs</td>
</tr>
<tr>
<td></td>
<td>Ag–NO3</td>
</tr>
<tr>
<td>TRX (mg/dL)</td>
<td>Ag-NPs</td>
</tr>
<tr>
<td></td>
<td>Ag–NO3</td>
</tr>
</tbody>
</table>

Values are represented as means ± SD (n = 3). Means in the same row with different superscripts show significant differences (P < 0.05).

a Alanine transaminase.
b Aspartate transaminase.
c Alkaline phosphatases.
d Superoxide dismutase.
e Catalase.

Table 4

Liver damage and antioxidant biomarkers of Cyprinus carpio exposed to different sub-lethal concentrations of Ag-NPs LC50 (96h) or Ag–NO3 LC50 (96h) for 21 days.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
</tr>
<tr>
<td>ALT (U/ml)</td>
<td>Ag-NPs</td>
</tr>
<tr>
<td></td>
<td>Ag–NO3</td>
</tr>
<tr>
<td>AST (U/ml)</td>
<td>Ag-NPs</td>
</tr>
<tr>
<td></td>
<td>Ag–NO3</td>
</tr>
<tr>
<td>ALP (U/ml)</td>
<td>Ag-NPs</td>
</tr>
<tr>
<td></td>
<td>Ag–NO3</td>
</tr>
<tr>
<td>SOD (U/mg prot)</td>
<td>Ag-NPs</td>
</tr>
<tr>
<td></td>
<td>Ag–NO3</td>
</tr>
<tr>
<td>CAT (U/mg prot)</td>
<td>Ag-NPs</td>
</tr>
<tr>
<td></td>
<td>Ag–NO3</td>
</tr>
</tbody>
</table>

Values are represented as means ± SD (n = 3). Means in the same row with different superscripts show significant differences (P < 0.05).
Fig. 2. Skin mucus layer parameters of *Cyprinus carpio* exposed to different sub-lethal concentrations of Ag-NPs LC<sub>50</sub> (96h) or Ag–NO<sub>3</sub> LC<sub>50</sub> (96h) for 21 days. Values are represented as means ± SD (n = 3). Different superscript shows significant differences at P < 0.05.
Krishna Priya et al. observed similar fluctuations in L. rohita after 3 days of exposure to SiO₂-NPs; while on 4th day, RBCs significantly increased at high NPs concentration compared to the control but Hb and Htc showed decreased values relative to previous day yet did not significantly differed compared to the control (Krishna Priya et al., 2015). However, Rajkumar et al. recorded significantly lower RBC, Htc, and Hb in L. rohita treated orally with the lethal concentration (LC₅₀) of Ag-NPs (Rajkumar et al., 2016), and Hajirezaee et al. showed significantly reduced RBCs in Common carp exposed to TiO₂-NPs, without any significant changes in Htc or Hb values (Hajirezaee et al., 2019). Therefore, in the current study, the statistically constant levels of Hb among study groups vice versa significantly lower RBCs in the treatments of 25% and 50% of Ag-NPs LC₅₀ (96h) compared to fish exposed to concentration of 12.5% or the control could be caused by a moderate damage to RBCs and endeavor of fish to cope with diminished number of RBCs at higher Ag-NPs concentrations.

Following these alterations, MCV, MCH, and MCHC levels might change because they are calculated based on Hct, Hb and RBCs values. The different concentrations of Ag-NPs LC₅₀ (96h) tested in this study had no significant effect on mean cell hemoglobin (MCH) and mean cell volume (MCV) values. Our results are in agreement with findings of other studies on NPs, metals, and pesticides stress (Imani et al., 2015; Khabbazi et al., 2015a; Laban et al., 2010; Shaluei et al., 2013; Soltani et al., 2016; Srivastava and Punia, 2011).

There are five types of white blood cells (WBCs) including basophils, eosinophils, neutrophils, lymphocytes, and monocytes which all are fighters of the innate immune system and any decrease in the number or activity of these cells indicates a decrease in the body’s defensive capacity. Our data demonstrated that Ag-NPs exposure at sublethal concentrations initially elevated and then lowered the WBCs at lower and higher NPs concentrations, respectively (Table 2). A similar trend was observed in WBCs count when fish were exposed to various metals and pollutants, including Ag-NPs (Dau et al., 1987; Khabbazi et al., 2015b; Kori-Siakpere and Ubogu, 2008; Mirghaed et al., 2018; Shaluei et al., 2013). The higher number of leucocytes at lower NPs concentration is considered as a normal reaction of the immune system to foreign substances or disease condition. It has been shown that Ag-NPs exposure exerted immunogenic impacts, activated the phagocytosis and promoted the engulfment of NPs (Bruneau et al., 2016; Imani et al., 2015). Kettler and co-workers found that Ag-NPs can be taken up by leucocytes and induce immunosuppression and leucocytes cytotoxicity intracellularly through the release of highly toxic Ag⁺ into cells which is called “Trogen horse mechanism” (Kettler et al., 2016). Accordingly, Ag⁺ ions induce the depolarization of the mitochondrial membrane, inactivation of enzymes involved in several cellular pathways and damage to lysosomes. As a consequence, ROS production increases in an Ag-NPs concentration or degree of ionization dependent-manner, leading to lesions in the cell membrane, necrosis, DNA damage, autophagy, and apoptosis (Jorge de Souza et al., 2019).

Therefore, we suggest that Ag-NPs at high sublethal concentrations may cause cytotoxicity in leucocytes through a ROS-dependent pathway, thereby lowering immune status of fish. This is similar to the findings of other researchers (Greulich et al., 2011; Massarsky et al., 2014; Pratsinis et al., 2013; Shin et al., 2007; Soares et al., 2016).

The serum TP, ALB and GLO of serum have important immunological and nutritional implications. Since these proteins are mainly produced and stored by the liver tissue, any reduction in the level of these component can be a sign of liver impairment, reduced feed intake, and weakened immune responses (Alkaladi et al., 2015; Bunglavan et al., 2014; Tsipotis et al., 2015; Yang and Chen, 2003). Ag-NPs could lower TP, ALB, and GLO in different ways, including use of proteins as a source of energy during stress, damages to intestine and kidney tissues and thus discharge of proteins, disruption in absorption of amino acid and lower protein synthesis by the liver (Imani et al., 2015), and structural alterations due to protein corona formation which disrupts their interaction with cells (Jorge de Souza et al., 2019).

Similar observations are reported upon exposure to pollutants such as pesticides, heavy metals, and NPs including Ag-NPs (Gopal et al., 1997; Imani et al., 2015; Ramesh et al., 2009; Srivastava and Punia, 2011). Lysozyme is a remarkable antibacterial enzyme produced and secreted by leukocytes and is an essential part of innate immunity in fish (Mohammadi et al., 2020; Saurabh and Saboo, 2008). Complement proteins are important immune globulins produced by the liver and cooperates with lysozyme to destroy the bacterial cell and also increase the phagocytic activity of leukocytes (Holland and Lambris, 2002; Zhou et al., 2001). Immunoglobulins are mainly excreted by B cells and function as pathogen and toxin neutralizing components of the fish immune system (Kaattari, 1992; Masoff and Criscitiello, 2016). In systemic circulation, NPs can bind to immune proteins or enzymes and lower their activities by the formation of protein corona. Moreover, as shown earlier in the case of WBCs, Ag-NPs can exhaust immune cells through continuous stimulation at lower concentrations and degenerate them through oxidative damage at higher concentrations (Jovanovic et al., 2012). Therefore, the lower activity of LYZ and ACH50 as well as Ig content following Ag-NPs exposure in the current study is suggestive of impaired liver function, weakened immune responses and fish sensitivity to pathogens and diseases.

COR and GLU are well-known and extensively applied biomarkers in determination of stressful conditions and intensity of stress in toxicological studies on fish (Akbary et al., 2016;Pottinger and Carrick, 1999). Serum cortisol (COR) and glucose (GLU) levels significantly increased upon Ag-NPs exposure. Our findings are consistent with other studies on NPs exposure, including Ag-NPs (Abarghoie et al., 2015; Canli et al., 2018; Clark et al., 2018; Ghafari Farsani et al., 2017; Hedayati et al., 2019). Typically, stress conditions increase the secretion of cortisol hormone from adrenocortical cells, leading to an acceleration in glucose production from liver glycogen (glycogenolysis) or protein (gluconeogenesis) reservoirs by the act of hepatocytes (Hongeta et al., 1993;Sheridan, 1986; Zhang et al., 2015). Consequently, glucose concentration in blood elevates and more energy is provided for stressed cells to neutralize the stress conditions.

Therefore, high levels of serum COR and GLU following Ag-NPs exposure are indicative of stress condition and fish endeavor to cope with those conditions. The high levels of COR may lead to liver malfunction, immunosuppression, weakened immune responses, and fish disease (Hongeta et al., 1992; Shaluei et al., 2013; Tort, 2011). In the present study, lower concentrations of serum TP, ALB, and GLB in the treatment of 50% of Ag-NPs LC₅₀ (96h) compared to 25% of Ag-NPs LC₅₀ (96h) (Fig. 1) may be related to an excessive utilization of stored carbohydrates and proteins to produce GLU and provide more energy for the increased metabolic demands at higher NPs concentrations. Our findings are consistent with other studies on NPs exposure, including Ag-NPs (Canli et al., 2018; Clark et al., 2018; Ghafari Farsani et al., 2017; Hedayati et al., 2019). Meanwhile, fish exposed to Ag-NPs showed incremental and dose-dependent levels of cholesterol (CHO) and triglycerides (Tri), yet no significant differences were detected among treatments (Table 3). The higher levels of serum triglycerides in fish exposed to Ag-NPs may be attributed to the use of this compound to meet the increased energy demands of stressed fish (Dave et al., 1979; Lupatsch et al., 2010). This is in agreement with results obtained in other studies (Canli et al., 2018; Haghj and Banaee, 2017).

The waterborne exposure of fish to NPs contribute to entrance of these very small particles into respiratory and digestive organs (Handy et al., 2008; Shi et al., 2013). Then, NPs are disturbed in the body by systemic circulation and finally deposited in the liver (Chen et al., 2011; Jang et al., 2014; Nemmar et al., 2002; Olmedo et al., 2002), as liver tissue is considered as an important metabolic organ that takes part in many physiological processes, including detoxification of toxins (Evans et al., 1993; van der Oost et al., 2003). These are supported by significantly higher levels of Ag detected in gills, intestines, and liver of fish exposed to Ag-NPs sublethal concentration in the current study (Table 1).
The main mechanism of Ag-NPs toxicity is the generation of ROS and oxidative damage to the cells (Choi et al., 2010; Hedayati et al., 2019). As discussed earlier, Ag-NPs can infiltrate the cell membrane by diffusion or endocytosis and produce ROS into the cell, damaging mitochondria, proteins, acids, and DNA (Ale et al., 2018a; Ketterl et al., 2016). Ag-NPs also attack the cell membrane and release Ag⁺ ions near the cell surface, still more toxicity (Farkas et al., 2010). These damages to the hepatocytes are supported by higher activities of alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatases (ALP) in Ag-NPs exposed fish of the current study (Table 4). ALT and AST are intracellular enzymes involved in amino acid metabolism and gluconeogenesis. These enzymes are leaked into plasma upon liver cells destruction (Banaee et al., 2011; Sookoian and Pirola, 2012). ALP is a membrane-associated enzyme and any damage to liver cells membrane could change the ALP activity in serum (Molina et al., 2005).

Ag-NPs oxidative stress is further supported by higher activities of SOD and CAT in fish exposed to 12.5% of LC50 (96h) and their subsequent lower activities imposed by 25% and 50% of LC50 (96h). SOD and CAT enzymes are essential components of the antioxidant defense system of fish, and thus their activities change upon ROS stress. The result of collaborative action of these two enzymes is the conversion of highly reactive O₂⁻ to harmless H₂O₂ and O₂ (Kanak et al., 2014; Ling et al., 2011). In the current study, at higher Ag-NPs concentrations, the antioxidant system of serum showed weakened activity and possibly couldn't effectively detoxify or control the free oxygen radicals. That is because damages to fish were more severe at higher NPs concentrations, as shown by higher serum ALT, AST, and ALP in these concentrations. Our findings are in good agreement with the results of other researchers (Alkaladi et al., 2015; Bruneau et al., 2016; Hedayati et al., 2019; Massarsky et al., 2014; Rajkumar et al., 2016).

The mucus layer present on the surface of the skin, intestines, and gills is the first line of fish innate immune system, separation fish from the external environment (Jovanović et al., 2012; Mohammadi et al., 2020). This layer acts as a chemical barrier protecting the fish against bacterial invasion by excretion of innate immunity components including Lys, ACH50, Ig, Pro, and ALP that are basically produced by immune cells residing in systemic circulation and mucosa-associated lymphoid tissues (Lazado and Caipang, 2014; Parra et al., 2015). Among these, ALP acts as an important antimicrobial enzyme during stress and infection (Lallès, 2019); and Pro enzymes are activators of other components of mucus layer defense system including ACH50 and Ig (Hjelmeland et al., 1983).

The mucus layer can also act as a physical barrier and trap NPs, modify their surface charge properties by the formation of protein corona and decrease their penetration rate due to formation of NPs aggregates (Handy et al., 2008; Jovanović et al., 2012). However, Ag-NPs exposure at higher concentrations could result in mucus layer cells damage, formation of a thickened mucus layer and decreased sloughing and renewal of mucus, leading to accumulation of chemicals, metals, and pathogens on the fish body surface (Ale et al., 2018a; Garcia-Reyero et al., 2015). Therefore, Ag-NPs exposure could lower the activity of immune enzymes by structural alterations, weaken skin immune responses by degeneration of immune cells in lymphoid tissues through intracellularly ROS production or membrane damage, and thus making fish susceptible to secondary infections (Bordas et al., 1996).

Our results showed that Ag-NPs waterborne exposure decreased the skin mucus layer immune responses, especially at higher Ag-NPs concentrations (Fig. 2). The reduced mucosal immunity in the current study is supported by our previous research that indicated TiO₂-NPs waterborne exposure at 0.125 mg/L for 21 days significantly decreased Ig values and Lys activity of skin mucus in Cyprinus carpio (Hajirezaee et al., 2019). Besides, the presence of a variety of pollutants elicited serious oxidative stress and increased protein oxidation in skin mucus layer, and other organs of fish (Dziul-Caamal et al., 2016a, 2016b, 2013), which comprises immune parameters analyzed in the current study. Similarly, the aqueous arsenic, cadmium, and mercury induced significant alterations in the profile of mucus proteins in Sparus aurata, and depending on exposure duration and type of metal, the activities of protease, antiprotease, alkaline phosphatase, and IgM values escalated or diminished (Guardiola et al., 2015). Also, Stabili and Pagliara reported inhibited activity of lysozyme-like enzyme in mucus of Martiásterias glacialis exposed to zinc (Stabili and Pagliara, 2009). In addition, scientists observed increased mucus secretion caused by Ag-NPs (Bilberg et al., 2012; Wu and Zhou, 2013) to get rid of excess NPs and lower oxidative stress (Garcia-Reyero et al., 2015; Hawkins et al., 2014), which could result in diluted mucus in terms of immune proteins due to mucus hypersecretion.

5. Conclusion

In the current study, based on a multi-biomarker approach, we showed that different sublethal concentrations of aqueous Ag-NPs LC50 (96h) for 21 days induce toxicity toward common carp. Ag-NO₃-induced toxicity was comparable to that of Ag-NPs. Fish exposed to higher concentrations (25% and 50%) of Ag-NPs demonstrated deteriorated hematometry, metabolism, immunity, antioxidant system, and skin mucus layer immune responses. Interestingly, the highest tissue Ag content was observed at concentrations of 25% and 50%. The main reason for Ag-NPs toxicity could be the generation of ROS and oxidative stress. The supportive biomarkers included escalated activities of liver-damage biomarkers (ALT, AST, and ALP) and diminished activity of antioxidant enzymes (e.g. SOD) at higher concentrations of Ag-NPs. It is worthwhile to mention that the initial enhancement of some biomarker upon Ag-NPs exposure at 12.5% of LC50 (96h) could be the counteract of fish to cope with the low-level stress conditions imposed by Ag-NPs, a response which was not observed at higher Ag-NPs concentrations possibly due to higher oxidative damage and weekend immune responses. Therefore, the result showed that during exposure to different sublethal concentrations of Ag-NPs LC50 (96h), fish health status was remarkably impaired at higher concentrations, while Ag-NPs injuries were controlled to some extends at lower concentration (12.5%), and thus 12.5% of Ag-NPs LC50 (96h) and lower concentrations are recommended for safe common carp aquaculture.


