Effect of Iron Nanoparticles on the Intestinal Bacterial Flora and Histology of the Intestine and Kidney in Stellate Sturgeon (*Acipenser stellatus* Pallas, 1771)

Pouya Ebrahimi[1], Reza Changizi[1], Rashid Alijani Ardeshir[2*] and Poulin Shohreh[3**]

ABSTRACT

Regarding the important role of iron in the physiological process in fish bodies and the presence of iron nanoparticles (Fe-NPS) in the aquatic environment, this study was designed to evaluate the effects of Fe-NPS on the intestinal bacterial flora and histology of the intestine and kidney in a stellate sturgeon (*Acipenser stellatus* Pallas, 1771). Juvenile stellate sturgeon, averaging 182.09±9.05 g, were fed with diets containing varying doses of Fe-NPs: 0 (T0), 25 (T1), 50 (T2), and 100 (T3) mg·kg^{-1} of food for 60 days. Based on the glucose and cortisol levels in different treatments, it seems that the best dose of Fe-NPs for stellate sturgeon was 50 mg·kg^{-1} food (T2) under experimental conditions. The most important histological changes observed in the intestine were the shortening of the intestinal villi, a high number of mucus-secreting cells, and mucosal secretions within the intestinal tract. These changes in the kidney were shrinkage of renal glomeruli, increasing Bowman's capsule space, mild degeneration of renal tubules, and infiltration of white blood cells into the kidney tissue. The most effective dose of Fe-NPs was 50 mg·kg^{-1} Fe-NPs with a less negative effect on fish intestine and kidney histology. Fe-NPs led to a significant increase in the mean total count of aerobic bacteria and lactic acid bacteria. Generally, the fish food supplemented with 50 mg·kg^{-1} Fe-NPs led to the least stress and histological damage in the kidney and intestine and the highest number of intestinal bacterial flora in the stellate sturgeon.

Keywords: Bacterial flora, Cortisol, Fe-Nanoparticles, Histology, Starry sturgeon

INTRODUCTION

Regarding the increase in the human population, the introduction of new strategies for the enhancement of food production is necessary. Aquaculture research has been going on to find the best way to increase stimulators and decrease inhibitors of production, and in this case, nanotechnology can be a good option. Nanotechnology and nanoparticles are increasingly recognized for their potential applications in various aspects of human, animal, and animal welfare (Milanova Sertova, 2020). Nanotechnology helps to deliver useful ingredients and micronutrients into animals' bodies, such as fish (Jafari and McClements, 2017). The potential benefits of nanotechnology in aquaculture are considerable, so that diets supplemented by nanoparticles of elements such as selenium, iron, etc. could improve the growth of fish (Fajardo *et al*., 2022). Araujo *et al*. (2021) found that diets containing Se-Nano were more effective than sodium selenite in preventing oxidative stress and improving antioxidant activity in Nile tilapia (Araujo *et al*., 2021). Nano-Fe is a form of nanoparticles

[1]Department of Aquaculture, Babol Branch, Islamic Azad University, Babol, Iran
[2]Department of Marine Biotechnology, Faculty of Biotechnology, Amol University of Special Modern Technologies, Amol, Iran
[3]Department of Clinical Sciences, Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran
[*]Corresponding author. E-mail address: r.alijani@ausmt.ac.ir
[**]Corresponding author. E-mail address: poulin_shohreh@yahoo.com
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with great interest in being used as a food additive in fish food (Abbas, 2021), but little is known about its effects on fish, especially sturgeons. Fe-NPs have unique properties due to their small size and large surface area, making them efficient for better penetration into tissues and cells, potentially increasing the therapeutic efficacy of drugs. Fe_2O_3 is less commonly used for direct biological applications compared to Fe-NPs. However, it can serve as a source of iron ions essential for biological processes (Yang *et al*., 2023).

Iron (Fe) has important roles in physiological processes such as oxygen transport, cellular respiration, and lipid oxidation reactions and is necessary for the normal function of the organs and tissues of fish (Chandrapalan and Kwong, 2021). Adding Fe-NPs to young carp and sturgeons' diets increased their growth rate to 30% and 24%, respectively (Rather *et al*., 2011). Feeding Indian major carp, *Labeo rohita*, with a diet supplemented with Fe-NPs and ferrous sulfate ($FeSO_4 \cdot 7H_2O$) could improve the final weight and antioxidant enzymatic activities (Behera *et al*., 2014). Nirmalkar *et al*. (2023) reported that supplementation of Fe-NP at the 20 $mg \cdot kg^{-1}$ level to the regular fish diet has a better impact on the growth and health of the fish (Nirmalkar *et al*., 2023).

Despite the advantage of using Fe-NP in fish diets, their high levels can be toxic (Abbas, 2021). Fe-NPs have various applications in different areas, including magnetic and electrical instruments, medicine, and catalytic reactions (Ali *et al*., 2016). This extensive use of this nanoparticle can lead to its high presence in the environment, especially the aquatic environment (Von der Heyden *et al*., 2019). Thus, Fe-NP can be orally ingested by fish and interact with intestinal bacterial flora. Intestinal bacteria have several functions in the host intestine. These bacteria play an important role in the synthesis of vitamins and the regulation of metabolism. In addition, it facilitates the absorption of nutrients, the development of the immune system, and protection against pathogens (Nayak, 2010). Intestinal bacteria are primarily exposed to nanoparticles through eating food containing NPs. Therefore, disruption of the gut microbiome may affect the health of the organism (Khosravi and Mazmanian, 2013). Nevertheless, suitable doses of NPs may have positive effects on this microbiome.

It is necessary to study the potential effects and useful applications of nanotechnology in solving problems in aquaculture. Studies like this can be used in aquaculture and environmental safety by identifying the potential risks and informing guidelines for the safe use of nanoparticles in aquatic environments, developing new treatments for fish diseases, improving water quality, and environmental remediation (Hamed *et al*., 2022). Our previous study focused on the effects of Fe-NPS on the liver, growth, and immune system of stellate sturgeon (Ebrahimi *et al*., 2020). Therefore, this study evaluated the effect of Fe-NPS as a food additive on the bacterial flora and histology of the intestine and kidney in the stellate sturgeon (*Acipenser stellatus* Pallas, 1771).

MATERIALS AND METHODS

Fish and rearing condition

Stellate sturgeon juveniles with an average weight of 182.09±9.05 g were purchased from a local farm and adapted to a new condition for a week. They were fed a basal diet during acclimation. Twelve fiberglass tanks (2,000 L) were used to randomly distribute 12 fish per tank density (3 tanks per treatment). The average water temperature, dissolved oxygen, pH, and salinity were 25.19±0.71 ºC, 5.98± 0.57 $mg \cdot L^{-1}$, 7.49±0.06, and 2.7 ppt, respectively (Hosseini *et al*., 2019).

Experimental diet and feeding

Fe-NPS was purchased from the US Research Nanomaterials Company and characterized using SEM analysis. A commercial pellet diet (BioMar, France) containing 47% protein, 14% lipid, 8.4% ash, and 20.40 $MJ \cdot kg^{-1}$ GE was grounded and mixed with this NP at various doses. The approximate chemical composition of the formulated diet was determined according to standard methodology (AOAC, 2005). The control group (T0) was fed

the basal diet without Fe-NPs, while fish in T1, T2, and T3 were fed the diets supplemented with 25, 50, and 100 mg Fe-NPS·kg^{-1} feed, respectively. The feeding trial lasted for 60 days. Fish were fed three times a day (8:00, 13:00, and 18:00).

Blood sampling and measurements

Fish have not been fed a day before bloodshed to prevent stress and are anesthetized with clove oil (100 mg·L^{-1}) (Chebanov and Galich, 2011). Blood was collected from the caudal vein and transferred into non-heparinized tubes. Samples were placed in the refrigerator for 2 h and then centrifuged at 4 °C with 1,500 ×g for 15 min. The serum was stored at -20 °C until analysis. The amount of glucose and cortisol was measured using commercial kits (Yekta Tajhiz Azma, Tehran, Iran).

Tissue sampling and processing

After obtaining blood, fish were sacrificed and dissected, and kidneys and intestines were fixed in Bouin's solution for 48 h. The tissue was treated with a series of ethanol, then cleared with xylene, embedded in paraffin, cut into 5 µm sections, and stained with hematoxylin and eosin (H & E) (Slaoui *et al*., 2017).

Intestinal bacterial flora examination

At the end of the experiment, 12 fish were randomly sampled from all treatments, and the predominant bacterial flora in their intestines was examined. Initially, the fish were anesthetized and then sacrificed. Their surface was sterilized with sterile iodine and 70% alcohol, and the abdominal was cut with a sterile scalpel. Intestinal contents were collected from the beginning to 1 cm from the end of the intestine and subsequently weighed. One gram of intestinal contents was then isolated, and serial dilution were prepared using sterile physiological serum. Dilutions of 10^{-3} and 10^{-4} were cultured in BHI agar (Merck, Germany) and MRS agar (Difco Detroit, MI, USA) to determine the total count of aerobic bacteria and lactic acid bacteria, respectively. The plates were then incubated for 48 h at 35 °C, after which the number of colonies grown on each plate was then counted (Rajan and Arockiaselvi, 2014).

Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze data. Initially, the data underwent distribution and normalization checks through the Kolmogorov-Smirnov test. Mean comparisons among treatments were conducted using Duncan's Multiple Range Tests at a significant level of 5%. The statistical analysis was performed using SPSS version 18 software.

RESULTS

The results of SEM analysis of Fe-NPs are shown in Figure 1. This NP, with a purity of 99.5 % trace metals basis, had an average size of 35–45 nm. The highest levels of glucose and cortisol were observed in T0, showing a significant difference compared to T2 ($p<0.05$). Glucose levels showed no significant difference between T1, T2 and T3 ($p>0.05$), as well as between T1, T3 and T0 ($p>0.05$). Lower cortisol levels were observed in T2 with significant differences compared to T0 and T1 ($p<0.05$). The highest cortisol levels were observed in T0 with significant differences compared to T2 and T3 ($p<0.05$) (Table 1).

The results of counting the intestinal bacterial flora of stellate sturgeon fed with diets supplemented with different doses of Fe-NPs for 60 days are presented in Table 2. The highest total count of bacteria was observed in T3, showing significant differences compared to the other groups ($p<0.05$). However, the highest number of lactic acid bacteria was observed in T1 and T2, with significant differences from the control group ($p>0.05$).

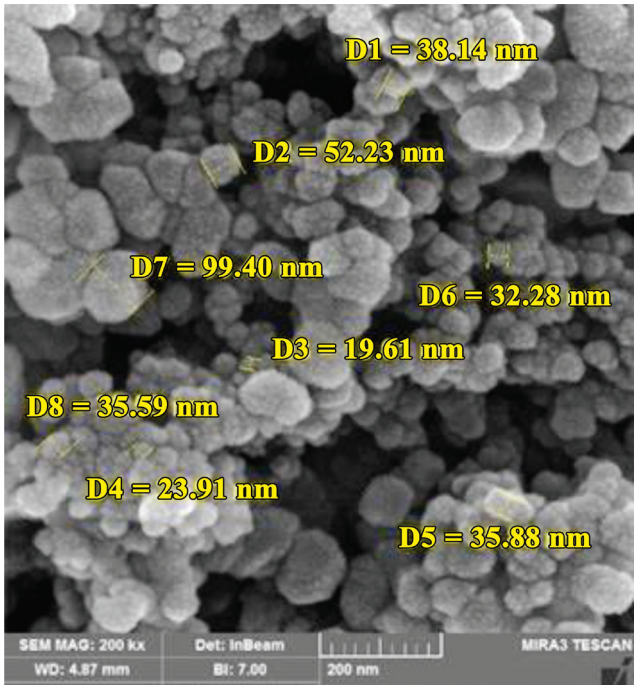

Figure 1. SEM micrograph showing morphology of Fe-NP used in this study.

Table 1. Mean±SD of serum glucose and cortisol of stellate sturgeon fed diets supplemented with different doses of Fe-NPs after 60 days.

Doses of Fe-NPs	Glucose (g·dL^{-1})	Cortisol (µg·dL^{-1})
0 mg Fe-NPS·kg^{-1} feed (T0)	75.08±5.91^{b}	7.85±0.35^{c}
50 mg Fe-NPS·kg^{-1} feed (T1)	67.48±10.27^{ab}	6.23±1.10^{bc}
50 mg Fe-NPS·kg^{-1} feed (T2)	49.06±6.08^{a}	3.93±0.41^{a}
100 mg Fe-NPS·kg^{-1} feed (T3)	70.55±4.87^{ab}	5.06±0.47^{ab}

Note: Mean±SD in the same column superscripted with different lowercase letters are significantly different ($p<0.05$).

Table 2. Mean±SD of total aerobic and lactic acid bacteria count in the intestine of stellate sturgeon fed with diets supplemented with different doses of Fe-NPs after 60 days.

Doses of Fe-NPs	Total count of aerobic bacteria (CFU·g^{-1})×10^{6}	Lactic acid bacteria count (CFU·g^{-1})×10^{6}
0 mg Fe-NPS·kg^{-1} feed (T0)	1.59±0.60^{a}	0.051±0.008^{a}
50 mg Fe-NPS·kg^{-1} feed (T1)	2.03±0.42^{a}	0.188±0.02^{b}
50 mg Fe-NPS·kg^{-1} feed (T2)	2.44±0.26^{a}	0.181±0.09^{b}
100 mg Fe-NPS·kg^{-1} feed (T3)	4.80±0.30^{b}	0.098±0.006^{ab}

Note: Mean±SD in the same column superscripted with different lowercase letters are significantly different ($p<0.05$).

The histology of intestine tissue from stellate sturgeon fed diets supplemented with various doses of Fe-NPs for 60 days is presented in Figure 2. The intestine tissue exhibited structural integrity, with no observable changes in its overall structure. However, a reduction in the length of intestinal villi and an increase in the number of mucus-secreting cells, along with mucosal secretions within the intestinal tract, were observed with increasing doses of Fe-NPS (Figure 2).

Kidney histology results showed that 100 mg·kg^{-1} Fe-NP (T3) causes changes in kidney tissue, including shrinkage of renal glomeruli, increasing Bowman's capsule space, mild degeneration of renal tubules, and infiltration of white blood cells into the kidney tissue. Although these symptoms were also observed in other treatments, these changes were greater in treatment 3. However, the glomerular capsule membrane was intact and no hyperemia or bleeding was observed in the treated and control fish (Figure 3).

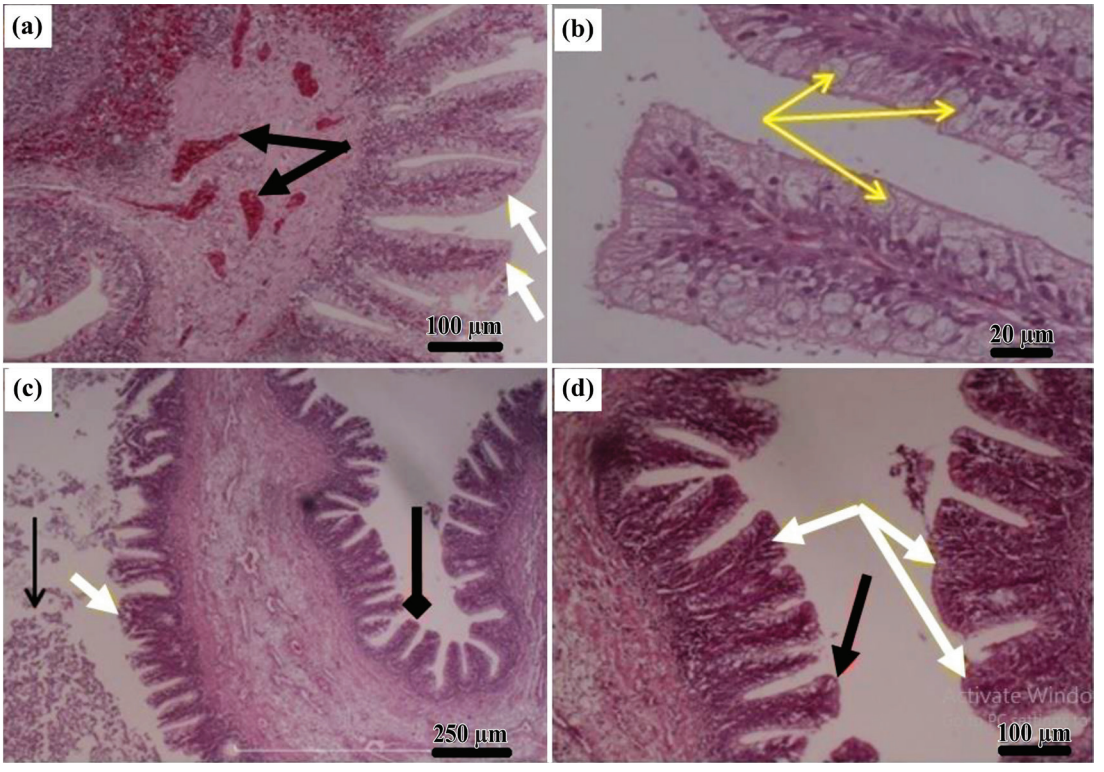

Figure 2. The intestine of stellate sturgeon fed with diets supplemented with various doses of Fe-NPs for 60 days: (a) Healthy structure of intestinal villi and its epithelium (white arrows), sub-mucosal muscle tissue (black arrows) (×10) of fish fed a basal diet (T0); b) Healthy structure of mucosa-secreting cells in intestinal villi (arrows) (×40) of fish fed a basal diet (T0); (c) Intestine of the fish fed with a diet supplemented with 50 mg·kg^{-1} Fe-NPs (T2), note the attached intestinal villi (white arrows), normal villi (arrow with cubic tip) and increase mucous secretions (black arrow) (×10); d) Intestine of a fish fed with a diet supplemented with 100 mg·kg^{-1} Fe-NPs (T2), note the attached intestinal villi (white arrows) and normal villi (black arrow) (×40).

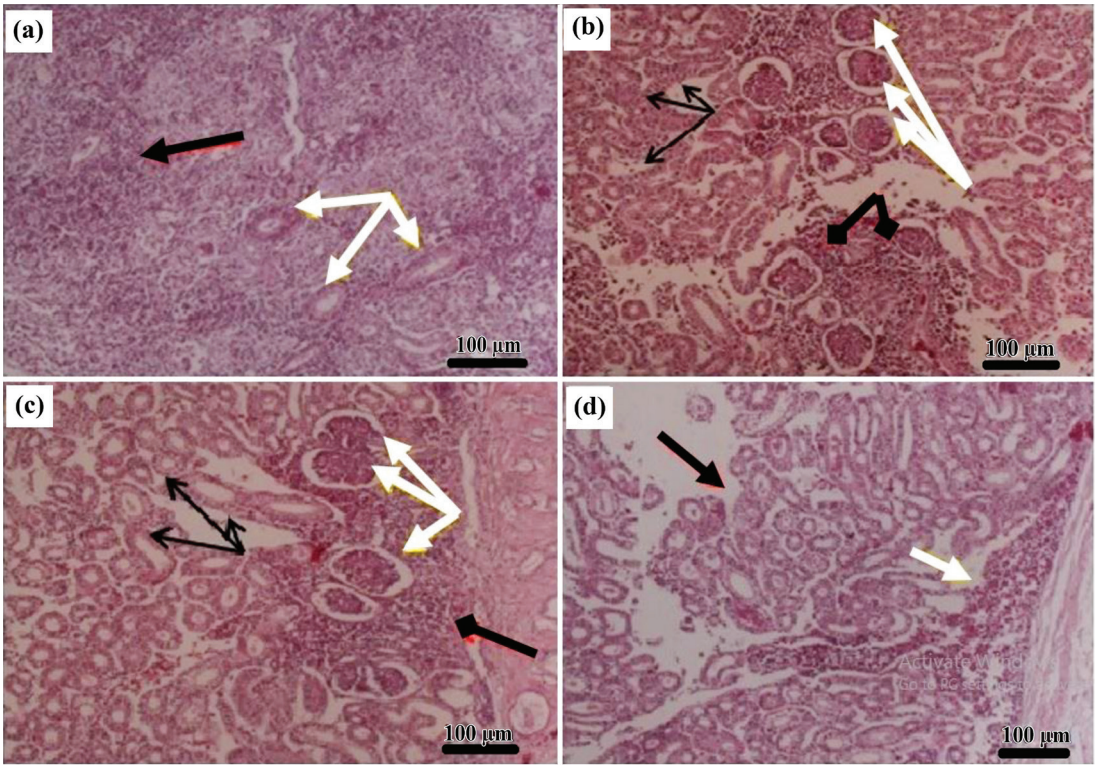

Figure 3. The kidney of stellate sturgeon fed with diets supplemented with various doses of Fe-NPs for 60 days: (a) Healthy renal tubules (white arrows) and hematopoietic tissue (black arrows) (×10) of the fish fed with the control diet; (b) Kidney of stellate sturgeon fed with a diet supplemented with 25 mg·kg^{-1} Fe-NPs (T1), note the shrinkage of renal glomeruli and increasing the Bowman's capsule space (white arrows), infiltration of lymphocytes into kidney tissue (black arrows with cubic tip), and healthy tubule structure in kidney tissue (black arrow) (×10); (c) Kidney of stellate sturgeon fed with a diet supplemented with 50 mg·kg^{-1} Fe-NPs (T2), note the shrinkage of renal glomeruli and increasing the Bowman's capsule space (white arrows), infiltration of lymphocytes into kidney tissue (black arrow with cubic tip), and healthy tubule structure in kidney tissue (black arrow) (×10); (d) Kidney glomeruli, renal tubules, Bowman's capsule of stellate sturgeon fed with a diet supplemented with 100 mg·kg^{-1} Fe-NPs (T3), note the white blood cell infiltration (white arrow) and healthy renal tubules (black arrow) (×4).

DISCUSSION

Iron oxide nanoparticles (Fe_2O_3 NPS) are popular because of their interesting physicochemical properties. They can be used in various areas such as food additives, antimicrobial additives, drug carriers, etc., due to their superparamagnetic properties and potential biocompatibility (Hooshmand *et al*., 2021). However, little is known about iron metabolism in fish, especially in sturgeon fish.

In the present study, considering glucose and cortisol levels in different treatments, it seems that the best dose of Fe-NPs for stellate sturgeon was 50 mg·kg^{-1} food (T2) under experimental conditions. Iron deficiency (in T0 and T1) or a high dose of iron (in T3) do not meet the fish's needs and can cause stress and possible toxicity. Therefore, in fish treated with the appropriate dose of Fe-NPs (T2), the lowest cortisol and glucose levels were observed, but in other treatments, an increase in cortisol and glucose was seen.

Total aerobic bacteria and lactic acid bacteria are two important types of bacteria commonly found in the digestive tract, playing significant roles in gut health and fermentation processes. These bacteria are often used as indicators of the general microbial ecology and balance in the gut. Changes in their abundance or function can provide important insights into alterations in the intestinal microflora and potential impacts on host health and metabolism (Florou-Paneri *et al*., 2013). The results of the present study showed that Fe-NPs generally led to a significant increase in the mean total count of aerobic bacteria and lactic acid bacteria isolated from intestinal microbial flora in stellate sturgeon. A few studies have been conducted about the effect of metallic nanoparticles added to fish fed on intestinal bacteria flora. A previous study on rainbow trout (*Oncorhynchus mykiss*) showed that intestine bacterial counts increased with the use of Fe-NPS (50 and 100 $\mu g \cdot kg^{-1}$ Iron nanoparticles) in combination with *Lactobacillus casei*, and no significant differences were observed in lactic acid bacteria count between the control group and the fish intestine that received Fe-NPs in feed. Probably, no significant effect on lactic acid bacteria count is due to their gram-positive characteristic and having thick peptidoglycan (Shrivastava *et al*., 2007).

The intestine tissue maintained its structural integrity, with no observable changes in its overall structure. However, exposure to increasing doses of Fe-NPS resulted in notable alterations. These included the shortening of intestinal villi, an increase in the number of mucus-secreting cells, and heightened mucosal secretions within the intestinal tract. This rise in mucus-secreting cells is likely attributed to their role in facilitating iron absorption from the environment through mucin secretion (Omidzahir *et al*., 2019). Sayedi *et al*. (2020) exposed blackfish (*Capoeta fusca*) to iron oxide nanoparticles at various concentrations (ranging from 1 to 100 $mg \cdot L^{-1}$). Their findings revealed an increase in goblet cell count, swelling of these goblet cells, higher blood cell count, and expansion of villi at their surface (Sayadi *et al*., 2020). Probably, the low toxicity of iron nanoparticles is related to their removal from the blood by the immune system (Shubayev *et al*., 2009).

After absorption of iron from the intestine, it can be stored in certain organs such as the kidney (Bury and Grosell, 2003). In the present study, shrinkage of renal glomeruli, an increase in Bowman's capsule space, mild degeneration of renal tubules, and infiltration of white blood cells into the kidney tissue were observed in the kidney of fish fed with a diet supplemented with 100 $mg \cdot kg^{-1}$ Fe-NP (T3). The severity of abnormality in T3 was higher than in other groups, indicating that Fe-NPs less than 100 $mg \cdot kg^{-1}$ had a less harmful effect on kidney tissue health in stellate sturgeon. There is limited information about the effect of Fe-NPs on fish kidney histology. Gürkan *et al*. (2021) exposed rainbow trout to alpha and gamma iron oxide nanoparticles (0, 1, 10, and 25 $mg \cdot L^{-1}$ for 10 days) and found histological damages in the kidney, including an increase in melanomacrophage aggregation, deformations, cytoplasmic vacuolizations and hypertrophies in kidney tubular epithelium, and necrosis (Gürkan *et al*., 2021). Kidneys are important organs that play a major role in the removal of toxic substances. The initial stage of tubule degeneration involves the formation of hyaline eosinophilic granules in kidney cells, resulting from the re-absorption of plasma protein (Aghamirkarimi *et al*., 2017).

CONCLUSION

It can be concluded that adding 50 $mg \cdot kg^{-1}$ of dietary Fe-NPs to stellate sturgeon food had no toxic effects on the fish. However, no significant differences were observed in some positive effects in T2 in comparison to other groups. Additionally, no abnormalities were detected in the intestinal structure, and there was no increase in glucose and cortisol levels, indicating that e-NPs did not induce toxic or stressful effects in fish fed a diet supplemented with 50 $mg \cdot kg^{-1}$ Fe-NPs. It is recommended that similar next studies with this treatment (50 $mg \cdot kg^{-1}$ Fe-NPs) be repeated for longer exposure time (e.g., 12 months).

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