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Copper nanoparticles induced oxidation stress, cell apoptosis and immune response in the liver of juvenile *Takifugu fasciatus*

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ABSTRACT

Copper nanoparticles (Cu NPs) are a new pollutant in aquaculture, representing a hazard to aquatic organisms. We investigated the effects of Cu NPs exposure on oxidative stress, apoptosis and immune response in an economically important model species, *Takifugu fasciatus*. The juvenile fish were exposed to control, 20 or 100 μg Cu NPs/L for 30 days. The growth of *T. fasciatus* was inhibited after Cu NPs exposure. Copper accumulation in liver increased with increasing Cu NPs dose. Oxidative stress indicators [malondialdehyde (MDA), total superoxide dismutase (T-SOD), catalase (CAT) and glutathione (GSH)], apoptosis index and activities of caspases (caspase-3, caspase-9) were all increased with the increase of Cu NPs concentration in liver. With an increase in Cu NPs dose, the activities of succinate dehydrogenase (SDH) and Na^+-K^+-ATPase as well as cytochrome c (Cyt-c) concentration in mitochondria decreased, accompanied by increased Cyt-c concentration in cytosol. Apoptosis-related gene expressions of *p53*, *caspase-3*, *caspase-9* and *Bax* were increased with the increase of Cu NPs dose. However, the opposite result was found in *Bcl2* expression. The physiological indicators of immune response [heat shock protein 70 (HSP70), heat shock protein 90 (HSP90), immunoglobulin M (IgM) and lysozyme (LZM)] as well as the mRNA levels of *HSP70*, *HSP90*, *IgM* and *C-LZM* were all increased after Cu NPs exposure. Our results will be helpful in understanding the mechanism of Cu NPs toxicity in *T. fasciatus*.

1. Introduction

Nanoparticles (NPs) are materials with at least one dimension below 100 nm. NPs exhibit unique properties that are desirable in lots of applications [1,2]. A dramatic increase in the production and use of NPs in industrial and consumer products has caused an increase in the input of NPs into aquatic systems [3]. Copper nanoparticles (Cu NPs) are one of the most used NPs materials, for example in textiles, wood preservation, bioactive coatings, air and liquid filtration, skin products and coatings on integrated circuits due to their novel physical and chemical properties [1,4]. They are also widely used in boat antifouling paints, and their release into the water can induce toxicity to aquatic organisms [2]. Moreover, given that Cu NPs are promising antimicrobial and antifouling agents, their introduction in aquaculture has been supposed to ensure advantages in the near future [2,5]. Unfortunately, the knowledge about the risks that come with use of Cu NPs is limited.

Many studies reported that Cu NPs could cross cell membranes through endocytosis or diffusion, and can accumulate in intracellular organelles (e.g. mitochondria, nucleus) [6,7]. Recently, it was recognized that their frequent use can create compatibility challenges for the recipient ecosystems [8]. Although the body of scientific evidence regarding adverse effects of Cu NPs is increasing, information on mechanisms of toxicity is still limited [8] in aquatic ecosystems. Therefore, it is important to examine toxicity of Cu NPs and understand the molecular mechanisms underlying possible effects. Many studies, including our own, have proved fish liver as the target organ for the Cu NPs accumulation [9,10]. Also, the liver is a significant organ for detoxification and metabolizing of xenobiotics, which reflected a fish body status under stress [11].

Takifugu fasciatus (formerly known as *Takifugu obscurus*), as a delicacy and commercially farmed puffer fish, is widely distributed in Sea of Japan, East China Sea, South China Sea, and inland waters in China and Korean Peninsula [12]. *T. fasciatus* migrates to freshwater rivers to reproduce in spring then travels back to the sea. Given that Gomes et al. [1] and Buffet et al. [13] reported large amounts of Cu NPs in the river and sea water, *T. fasciatus* is likely to be affected by Cu NPs during their whole lifecycle. However, to our knowledge, no study has reported the effects of new environment pollutant Cu NPs on *T. fasciatus*. Importantly, some studies asserted *T. fasciatus* could be used as a good model to study the effects of environmental stressors [14–18].

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The aim of this study was to reveal the toxic mechanisms of Cu NPs in the liver of *T. fasciatus*. Oxidative stress (malondialdehyde MDA and activities of total superoxide dismutase T-SOD, catalase CAT and glutathione GSH), cell apoptosis and immune responses in the liver of *T. fasciatus* were used as biomarkers of Cu NPs toxicity. The results of this study provided novel insights into the toxicity mechanisms of Cu NPs, contributing to safety and profitability of *T. fasciatus* aquaculture. Also, this study provided specific toxicity data regarding Cu NPs in aquaculture water, contributing to improved use of Cu NPs in fish production.

2. Materials and methods

2.1. Cu NPs preparation and particle characterization

Stock solutions of Cu NPs were prepared as well as characterized as described in detail in our previous study [6,10]. Briefly, powder form of Cu NPs (particles 10–30 nm; purity 99.9%) was purchased from Aladdin Chemistry Co., Ltd (Shanghai, China). Cu NPs suspension containing 1.0 g Cu/L was prepared daily by dispersing NPs in ultrapure water, sonicated for 30 min and then stirred for 1 h at room temperature to increase dispersion. The particle size of Cu NPs was characterized using transmission electron microscopy (TEM, JEOL JEM-2100, Japan) and nanoparticle tracking analysis (NTA, NanoSight LM10). For TEM analysis, the primary range of Cu NPs was measured manually from micrographs by analyzing 60 randomly selected NPs. In addition, particle size distribution in aquaculture water of *T. fasciatus* was measured by NTA at 20 mg/L [19]. It was not possible to confirm NPs size distribution in the exposure water directly because current methods (including NTA) are not sufficiently accurate at μg/L concentrations [4].

Total Cu in each treatment was measured by acid digestion of water samples. According to our previous study [20], dissolved Cu was defined as Cu present in the supernatant of samples centrifuged at 10,000 $\times g$ for 30 min. The Cu concentration was measured by inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer NexION®2000). Six repetitions were made per sample, and the average was used to calculate Cu concentration from a seven-point standard curve.

2.2. Fish toxicity assessment

Juvenile *T. fasciatus* were obtained from Zhongyang Group Co., Ltd (Nantong, Jiangsu Province, China). The initial body weight and length of the puffer fish were 8.7 ± 0.6 g and 6.1 ± 0.3 cm, respectively. The fish were acclimated for 15 days in laboratory conditions before formal experiment. After acclimation, fish were randomly divided into three treatments: control (no added Cu), 20 μg Cu NPs/L and 100 μg Cu NPs/L. In this study, the effects of Cu^{2+} released from Cu NPs to *T. fasciatus* were excluded (see discussion part 3.1). Every treatment was repeated thrice, with 20 fish in each replicate tank containing 100 L of water. The dose of 20 μg Cu NPs/L was chosen because it reflected the measured environmental concentration [4]. High Cu dose (100 μg Cu NPs/L) was chosen because this dose might be found in areas with intensive manufacturing industries, agricultural and mining activities, and municipal waste depositions [4,6]. Analogous to our previous work (e.g. Wang et al. [6]; Wang et al. [10]) on the effects of Cu NPs on fish, the dose of 20 or 100 μg Cu NPs/L could disrupt the fish body balance associated with osmoregulation, oxidative stress and cell apoptosis.

The treatment exposure lasted for 30 days. All water was changed every 24 h with treatment re-dosing after each change. The fish were fed with a commercial pellet diet (Jiaji Feed Co. Ltd., Zhenjiang, China, 42.0% protein) to apparent satiation twice daily. To minimize the risk of ingestion of Cu NPs during feeding, the fish were fed after each water change, but prior to Cu re-dosing. During the experiment, the water quality parameters were controlled as follows: water temperature 25 ± 0.5 °C, pH 7.1 ± 0.1 and total ammonium below 1.0 mg/L. Continuous aeration was used to ensure dissolved oxygen (DO) above 5 mg/L. The photoperiod was 12 h light: 12 h dark.

2.3. Fish sampling

At the end of 30-day exposure, 24 h after the last feeding, all fish were euthanized by 10 mg/L MS-222 to conduct measurements. After obtaining the final weight and length of each fish, nine fish per tank were randomly selected, then dissected on ice. The liver and viscera were removed and weighed. Then, three fish liver samples were flushed by normal saline solution (salinity 8.6 g/L, 4 °C), placed into a centrifuge tube and stored at −80 °C for analyzing the oxidative stress, immune response and mitochondria parameters. Another three fish liver samples (the left lobe of liver was selected) were fixed in 10% v/v neutral buffered formalin for apoptosis assessment. The remaining three fish liver samples were used to analyze Cu accumulation.

2.4. Tissue ion analyses

Tissue ions were analyzed according to our previous study [20] with minor variations. The protocol details and results were shown in the *Supplementary Material*, Fig.S1.

2.5. Oxidative stress analyses

The livers were defrosted and washed in ice-cold 0.8% w/v saline, and were then weighed and homogenized with 10 vol of 0.8% w/v saline. The livers were centrifuged at 2000 $\times g$ for 10 min at 4 °C, and the supernatants were used for analyzing MDA and GSH concentrations and T-SOD and CAT activities using kits purchased from Nanjing Jiangcheng Bioengineering Institute (Nanjing, China) according to Xu et al. [11] and Cheng et al. [14]. Total soluble protein concentration was measured by the Bradford (1976) method [21].

2.6. Cell apoptosis analyses

The left lobes of liver were taken out from 10% v/v neutral buffered formalin, embedded in paraffin wax, and the 6–7 μm sections were cut. The sections were further treated using the terminal deoxynucleotidyltransferase-mediated dUPT nick end labeling (TUNEL) assay following the protocol of Apoptosis Detection Kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China), and the results were examined under a light microscope. The percentage of TUNEL-positive liver cells (Brown-yellow granules) was calculated manually following observation. The apoptosis index is the percentage of positive cells counted per total number of cells in five non-overlapping fields-of-view selected randomly under a 20 × objective.

2.7. Caspase-3 and caspase-9 activities

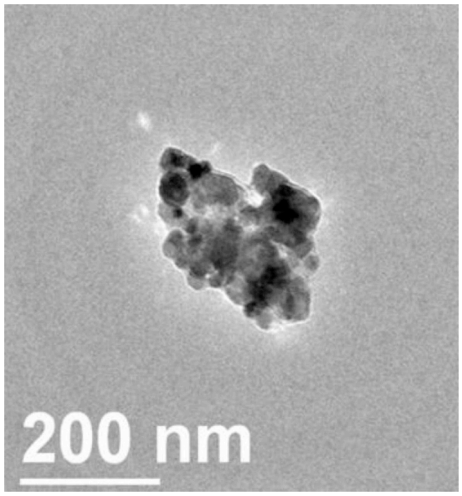
Caspase-3 and caspase-9 activities were measured spectrophotometrically using a CaspACE™ Assay System, Colorimetric (Promega, Madison WI, USA). The liver samples were homogenized in lysis buffer, lysates were centrifuged at 4 °C for 15 min at 10,000 $\times g$, and the supernatants were incubated in the dark at 37 °C for 4 h with 5 μL Ac-DEVD-pNA (for caspase-3) and Ac-LEHD-pNA (for caspase-9) as the substrates. The optical density at 405 nm was measured using a microplate reader (Model VERSAmax, Molecular Devices, Sunnyvale, CA, USA). The incubation with lysis buffer instead of test samples was used as the negative control. The caspase-3 or caspase-9 activities were calculated as optical density (test)/optical density (negative control).

2.8. Mitochondrial status

2.8.1. The isolation of liver mitochondria

The isolation of liver mitochondria was performed by differential

Table 1
Characteristics of Cu NPs used in this study.

Particle characterization	Method	Cu NPs
Particle image	TEM	
Primary particle size diameter (nm)	TEM	80 ± 15[a]
Mean particle diameter in aquaculture water (nm)	NTA	141 ± 68[b]

[a] Data are means ± SD (n = 60 particles).
[b] Data are means ± SD (n = 3).

centrifugation according to our published methods [6]. The mitochondrial pellet and the cytosolic supernatant were stored at −80 °C for further analysis.

2.8.2. Mitochondrial parameters

The activity of succinate dehydrogenase (SDH) and Na^+-K^+-ATPase in mitochondrial pellet was measured using the commercial kit (Biovision, Milpitas, California, USA). Mitochondrial protein concentration was determined according to the method of Lowry et al. [22]. Enzymatic activities were expressed as units (U) per milligram of mitochondrial protein. Cytochrome c (Cyt-c) concentration in the mitochondrial pellet and the cytosolic fraction were measured using an enzyme-linked immunosorbent kit (ELISA) (ADL Co-Lab, USA) according to Xu et al. [11].

2.9. Activities of immune response enzymes

The livers were homogenized as described above in section 2.5. The concentrations of heat shock proteins 70 (HSP 70) and 90 (HSP 90) were determined following the ELISA Detection Kit instructions (EKS-700, Stressgen Biotechnologies Corporation, Victoria, BC, Canada) [6,23]. Immunoglobulin M (IgM) concentration was determined using the ELISA method according to the protocol of Cuesta et al. [24]. The lysozyme (LZM) activity was measured by the method of Esteban et al. [25] based on lysis of lysozyme-sensitive bacterium (*Micrococcus luteus*) using a turbidimetric assay.

2.10. Apoptosis and immune-response-related gene expression

The protocol details were included in the *Supplementary Material*.

2.11. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using SPSS (20.0; SPSS Inc., Chicago, IL, USA) for Windows software. Tukey's test was used to compare differences among treatments. The level of significance was set at $p \leq 0.05$. All data were expressed as means ± SD. (standard deviation).

3. Results and discussion

3.1. Cu NPs characteristics

The results of this study showed that Cu NPs were hazardous to *T. fasciatus*, inducing oxidative stress, apoptosis and immune responses in the liver. The primary and secondary particle sizes are important parameters of Cu NPs toxicity [1,26]. In this study, the average particle size of Cu NPs in aquaculture water of *T. fasciatus* was larger than the primary particle diameter (Table 1), suggesting aggregation of NPs. This aggregation is likely driven by the low zeta potential of Cu NPs and the presence of divalent ions in aquaculture waters [27]. The present study also found Cu ions were released from Cu NPs into aquaculture water, more so at the high-dose (100 μg Cu NPs/L) than the low-dose treatment (20 μg Cu NPs/L) after 24-h exposure (*Supplementary Material*, Table S2). These results were similar to our previous study in seawater [20]. It is worth noting that only 0.16 ± 0.02 and 1.42 ± 0.06 μg Cu^{2+}/L were released from, respectively, 20 and 100 μg Cu NPs/L after 24-h exposure in the present study (*Supplementary Material*, Table S2). Given the concentration of Cu^{2+} in the control was 2.3 ± 0.1 μg/L, the effects of Cu^{2+} released from Cu NPs were considered unimportant.

3.2. Growth and morphological parameters

In the present study, Cu NPs exposure inhibited growth (weight and length) of juvenile *T. fasciatus* (Table 2, see also Zhao et al. [9]). The reduction in growth might have been caused by the increased expenditure of energy for metal detoxification and homeostasis, resulting in less energy available for growth, as suggested by Kim and Kang [28]. Cu NPs did not significantly influence viscerosomatic index (VSI) and condition factor (CF), whereas hepatosomatic index (HSI) significantly increased with an increase in Cu NPs dose (Table 2). Berntssen et al. [29] suggested that CF was less responsive than growth to elevated Cu exposure. The increased HSI suggested the liver damage (such as inflammation and fat accumulation) in the high-dose Cu NPs treatment (cf. Huang et al. [30]). However, our previous study found HSI of fish *Epinephelus coioides* decreased with increasing Cu NPs dose in seawater

Table 2
Effect of Cu NPs exposure on growth performance and morphological parameters of *T. fasciatus* after 30 days.

Cu NPs treatments	Weight (g)	Length (cm)	HSI (%)	VSI (%)	CF (%)	S (%)
Control	14.26 ± 1.11^{a}	8.23 ± 0.44^{a}	11.96 ± 0.66^{c}	19.12 ± 1.5^{a}	2.57 ± 0.21^{a}	100 ± 0.0^{a}
20 μg/L	12.21 ± 1.08^{ab}	7.72 ± 0.39^{ab}	13.73 ± 0.61^{b}	18.96 ± 1.2^{a}	2.66 ± 0.17^{a}	100 ± 0.0^{a}
100 μg/L	10.17 ± 1.21^{b}	7.01 ± 0.35^{b}	15.32 ± 0.59^{a}	18.65 ± 1.4^{a}	2.55 ± 0.09^{a}	93.33 ± 2.89^{b}

Values are means ± SD (n = 3). The different letters in each column indicate significant differences among treatments ($p \leq 0.05$). HSI (hepatosomatic index) = 100 × (liver weight)/(body weight); VSI (viscerosomatic index) = 100 × (viscera weight)/(body weight); CF (condition factor) = 100 × (weight)/(body length)^{3}; S (survival rate) = 100 × (final fish number/initial number).

Table 3
Effects of 30-day exposure to Cu NPs on oxidative stress and mitochondrial parameters in the liver of *T. fasciatus*.

Cu NPs treatments	Oxidative stress parameters in liver				Mitochondrial parameters			
	MDA (nmol/mg protein)	T-SOD (U/mg protein)	CAT (U/g protein)	GSH (mg/g protein)	SDH (U/mg protein)	Na^{+}-K^{+}-ATPase (U/mg protein)	Cyt-c (ng/mL)	Cytosolic Cyt-c (ng/mL)
Control	4.86 ± 0.30^{b}	222.91 ± 9.92^{c}	4.92 ± 0.67^{b}	8.92 ± 0.33^{c}	1.97 ± 0.12^{a}	1.33 ± 0.11^{a}	21.21 ± 0.48^{a}	6.91 ± 0.46^{c}
20 μg/L	5.29 ± 0.15^{b}	261.85 ± 6.81^{b}	5.23 ± 0.08^{b}	14.56 ± 1.22^{b}	1.42 ± 0.14^{b}	1.14 ± 0.13^{a}	16.51 ± 1.25^{b}	9.38 ± 1.12^{b}
100 μg/L	6.68 ± 0.52^{a}	336.21 ± 5.16^{a}	7.91 ± 0.77^{a}	17.91 ± 0.77^{a}	0.98 ± 0.08^{c}	0.71 ± 0.03^{b}	13.83 ± 0.35^{c}	11.81 ± 0.56^{a}

Data are means ± SD (n = 3). Significant differences ($p \leq 0.05$) among treatments in each column were indicated by different letters. MDA: malondialdehyde; T-SOD: total superoxide dismutase; CAT: catalase; GSH: glutathione; SDH: succinate dehydrogenase; Cyt-c: cytochrome c.

[10]. The difference between two studies might have been caused by the different fish species. In the present study, only 6.7% of fish died at 100 μg Cu NPs/L during the 30-day experiment (Table 2), but in other studies rainbow trout (*Oncorhynchus mykiss*) showed 14% mortality in the 100 μg Cu NPs/L treatment at day 4 [4]. These results indicate differential sensitivity of different species to Cu NPs toxicity, but further work is necessary to elucidate relevant relationships.

3.3. Copper accumulation in liver

Fish liver is the central organ for Cu accumulation after Cu^{2+} exposure [2,4]. In the present study, Cu accumulated in the liver of *T. fasciatus* exposed to Cu NPs (*Supplementary Material*, Fig. S1). So, exposure to either Cu^{2+} or Cu NPs caused Cu accumulation in the fish liver. In the present study, the amount of Cu^{2+} released from Cu NPs was small, suggesting that Cu accumulation in the liver was completely due to Cu NPs. Indeed, Cu NPs could pass through the cell membrane into the cell [1]. Other studies found Cu NPs were internalized via the mouth and gills [4,9]. In particular, Cu NPs in the water could be transferred directly from gills to bloodstream and then to other organs (such as liver) [9].

3.4. Oxidative stress

Oxidative stress is emerging as a potential mechanism of NPs toxicity [4]. Copper has the capacity to cause formation of excess ROS through the Fenton-type reactions, leading to production of oxyradicals that activate antioxidant systems [31], inducing enzymes and other compounds involved in antioxidant defense (e.g. SOD, CAT and GSH) [1,4]. In the present study, the MAD concentration (as an indicator of lipid peroxidation), the T-SOD and CAT activities and the GSH concentration in the liver were all significantly increased with an increase in the Cu NPs dose compared to the control (Table 3). These findings indicate that: (i) the exposure to Cu NPs can exacerbate ROS generation in the liver of *T. fasciatus*, causing the liver damage (as judged from cell apoptosis); and (ii) the antioxidant defense system in *T. fasciatus* was activated to counteract the toxic effects of ROS after Cu NPs exposure. Some studies reported lipid peroxidation occurred due to the antioxidant enzymes being unable to completely remove excessive ROS [32,33]. Thus, we also infer the defense system was not sufficiently effective to protect completely *T. fasciatus* under intense oxidative stress.

3.5. Cell apoptosis

To clarify the mechanism of toxicity of Cu NPs in the liver of juvenile *T. fasciatus*, the influence of Cu NPs on apoptosis in the liver cells was monitored. Apoptosis plays a very important role in maintaining environmental stability of fish body, but severe apoptosis would have a negative effect on fish [34]. The TUNEL method in the present study found that increasing Cu NPs dose exacerbated liver cell apoptosis, but only a few apoptosis cells were found in the liver of control fish (Fig. 1). Similar results were found in other species after the Cu ions exposure as reported by Zhai et al. [35] and Rhee et al. [36]. The current study also found that the environmentally-relevant (20 μg/L) Cu NPs concentration could induce apoptosis in the liver of juvenile *T. fasciatus* (Fig. 1); hence, Cu NPs should be strictly monitored in the aquatic environment used in fish production.

ROS are important for apoptosis [32]. Generation of excess ROS can impair mitochondrial membrane permeability and the respiratory chain. SDH (a constitutive molecule of complex II of the mitochondrial respiratory chain located in the inner mitochondrial membranes) has been reported to play an important role at high respiration rates; thus, the activity of this enzyme has been considered a good indicator of the mitochondrial oxidative metabolic capacity and the status of mitochondrial membranes [37,38]. Na^{+}-K^{+}-ATPase (a membrane-bound enzyme) is responsible for transporting ions through the cell membranes and thus contributing to regulation of osmotic pressure and membrane permeability [39]. In the present study, the activities of SDH and Na^{+}-K^{+}-ATPase were decreased with increasing Cu NPs dose (Table 3), suggesting mitochondrial membranes were damaged. Consequently, Cyt-c was released from mitochondria, and the cytosolic concentration of Cyt-c increased [6] (Table 3, see also Xu et al. [11]; Cheng et al. [32]).

Caspase activity is a useful marker for the detection of stress-induced apoptosis in juvenile fish [32]. Caspase plays an essential role in both extrinsic and intrinsic pathways involved in apoptosis [40]. Caspase-9 is the initiator of caspases and activates downstream caspase [41]; in contrast, caspase-3 is the major executioner of caspases, responsible for the proteolytic cleavage of many critical cellular proteins

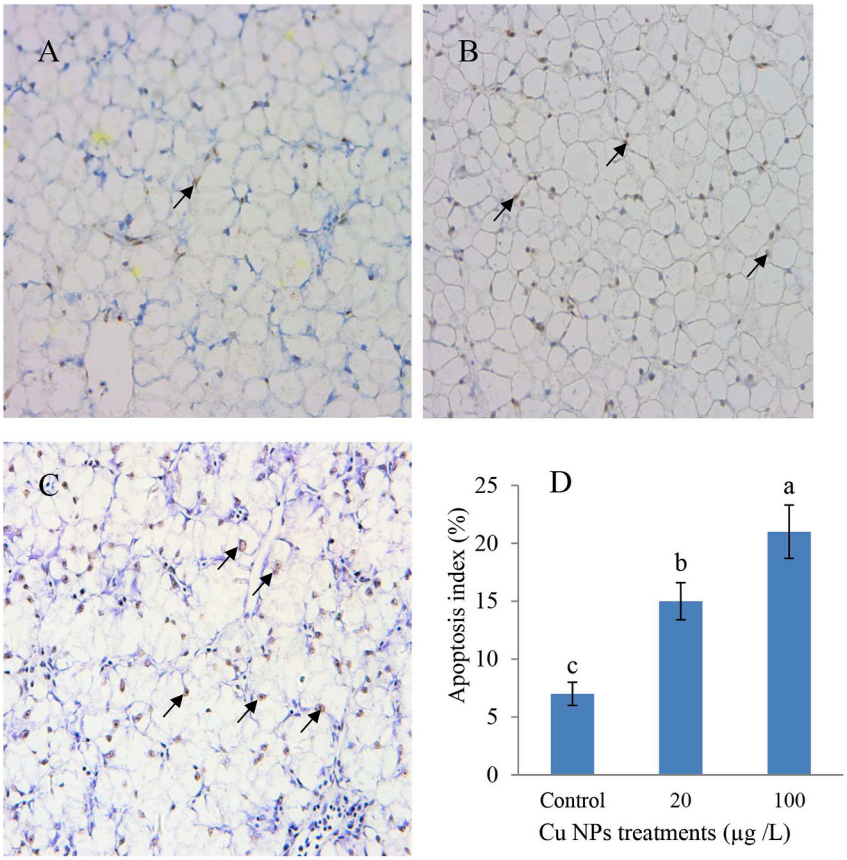


Fig. 1. Cell apoptosis in the liver of *T. fasciatus* after exposure to different doses of Cu NPs for 30 days. (A) Control, (B) 20 μg Cu/L and (C) 100 μg Cu/L. Brown-yellow dots (some examples indicated by arrows) represent the positive (apoptotic) cells. (D) Apoptosis index in liver of juvenile *T. fasciatus* after exposure to different doses of Cu NPs. Data are means ± SD (n = 3). Significant differences ($p \leq 0.05$) among treatments were indicated by different letters. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

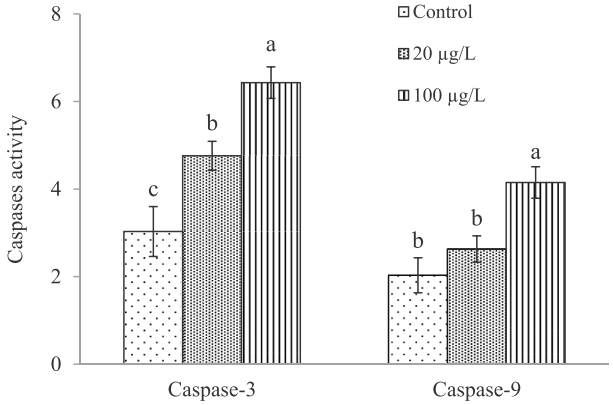


Fig. 2. Caspase-3 and caspase-9 activities in the liver of juvenile *T. fasciatus* after exposure to different doses of Cu NPs for 30 days. Data are means ± SD (n = 3). Significant differences ($p \leq 0.05$) among treatments for each parameter were indicated by different letters.

[42]. A Cyt-c release into cytosol can activate caspase-9 as the downstream effector, subsequently leading to caspase-3 activation and the induction of apoptosis [6,43]. In the present study, both gene expression (Fig. 3 B and C) and enzyme activity (Fig. 2) of caspase-3 and caspase-9 improved in the liver of *T. fasciatus* after Cu NPs exposure, suggesting that the mitochondrial-mediated caspase-dependent pathway was playing an important role in Cu NPs-induced apoptosis in *T. fasciatus*.

The tumor-suppressor protein p53 is a universal sensor of environmental stress and plays a critical role in regulating expression of genes involved in mediating apoptosis [44]. ROS generation induced by stresses can regulate p53 activity [42]. In this study, the p53 expression was significantly elevated after Cu NPs exposure (Fig. 3 A), indicating that p53 could be involved in Cu NPs-induced apoptosis. Moreover, p53 can induce apoptosis by up-regulating the transcription of pro-apoptotic genes (such as *Bax*) and down-regulating that of anti-apoptotic genes (such as *Bcl2*) [32]. Similar results were found in this study, with the expression of *Bax* significantly increased (Fig. 3 D) and that of *Bcl2* significantly decreased (Fig. 3 E) after Cu NPs exposure compared to the control. Bax can induce the release of Cyt-c into the cytosol, whereas the anti-apoptotic Bcl2 can inhibit the release of Cyt-c from mitochondria. Thus, an increase of the Bax-to-Bcl2 ratio can induce cell apoptosis [45,46]. In this study, the Bax-to-Bcl2 ratio was increased after exposure to Cu NPs, suggesting that Cu NPs might trigger apoptosis via the p53-Bax-Bcl2 pathway in the liver of juvenile *T. fasciatus*.

3.6. Immune responses

Oxidative stress and cell apoptosis could activate the immune defense system of fish [6]. As reliable immune response proteins, HSP 70 and HSP 90 play a crucial role in regulation of apoptosis by inhibiting the apoptotic cell signal cascade [32,47]. In the present study, the concentration and expression of HSP 70 and HSP 90 in the liver of *T. fasciatus* were significantly increased after Cu NPs exposure (Fig. 4 A, B, E and F), indicating HSPs were crucial for prevention of apoptosis and cellular signaling in *T. fasciatus* exposed to Cu NPs stress. However, apoptosis still did occur, so HSP 70 and HSP 90 were only partially successful in potentially minimizing the extent of apoptosis.

LZM activity and IgM concentration are reliable indicators of fish health, which are commonly measured in research on fish immunology.

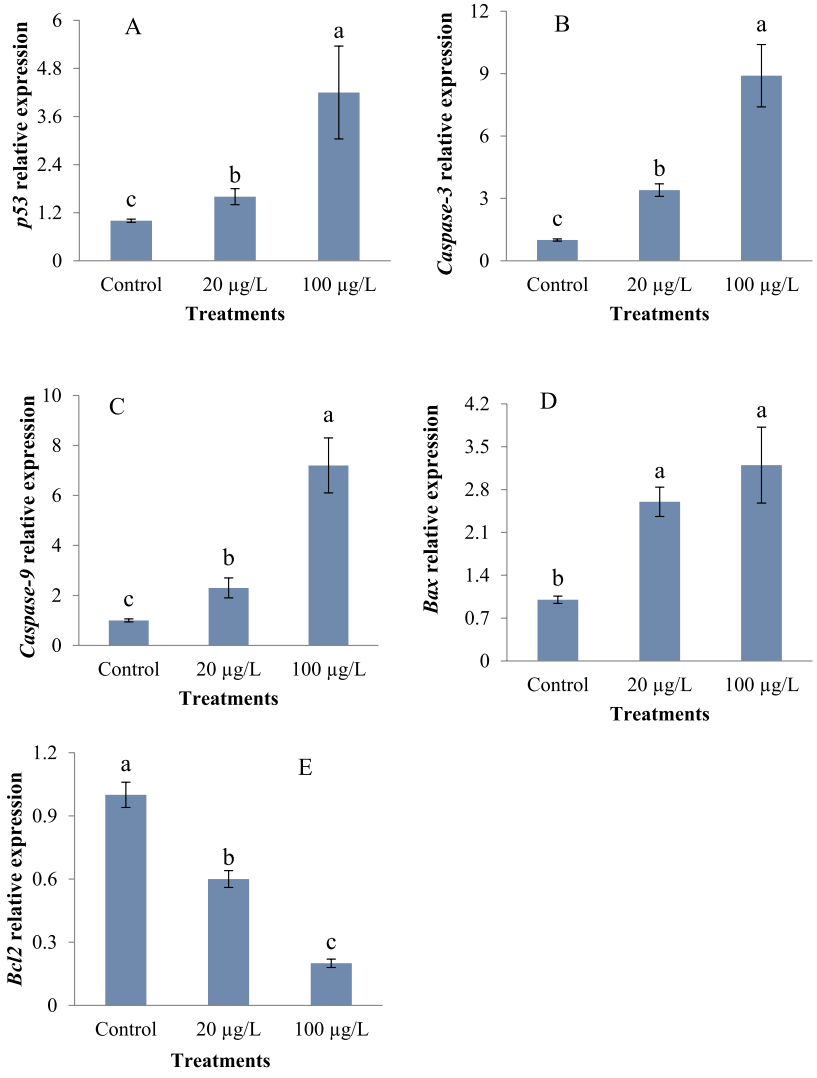


Fig. 3. Apoptosis-related gene expression in the liver of juvenile *T. fasciatus* after exposure to different doses of Cu NPs for 30 days. Data are means ± SD (n = 3). Significant differences ($p \leq 0.05$) among treatments in each graph were indicated by different letters.

IgM is the first antibody produced in the immune system and provides a crucial first line of defense, particularly in bony fish that have no other immunoglobulin class except IgM [48]. LZM (as an important index of innate immunity of fish) are antibacterial enzymes in the immune system by cleaving the β-(1,4)-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan layer of bacterial cell walls [49]. Our results found LZM activity and IgM concentration, and corresponding genes expression were all significantly increased in the liver of *T. fasciatus* after Cu NPs exposure (Fig. 4C, D, G and H), which clearly indicated the immune responses in juvenile *T. fasciatus* were induced by Cu NPs.

4. Conclusions

We demonstrated the effects of Cu NPs exposure on apoptosis, oxidative stress and immune responses in the liver of juvenile *T. fasciatus*. The present findings indicate that Cu NPs induced apoptosis in the liver of juvenile *T. fasciatus* via the mitochondria-mediated caspase-dependent pathway and the p53-Bax-Bcl2 pathway (Fig. 5). The antioxidant and immune defense system in the liver was activated to protect cells from oxidative stress and apoptosis, but it was not completely effective in protecting the fish body under intense oxidative conditions. Further studies are needed to elucidate a relationship between apoptosis and antioxidant or immune response under Cu NPs exposure.

Disclosure statement

The authors declare no competing financial interests.

Funding information

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Data statement

All data included in this study are available upon request by contact with the corresponding author.

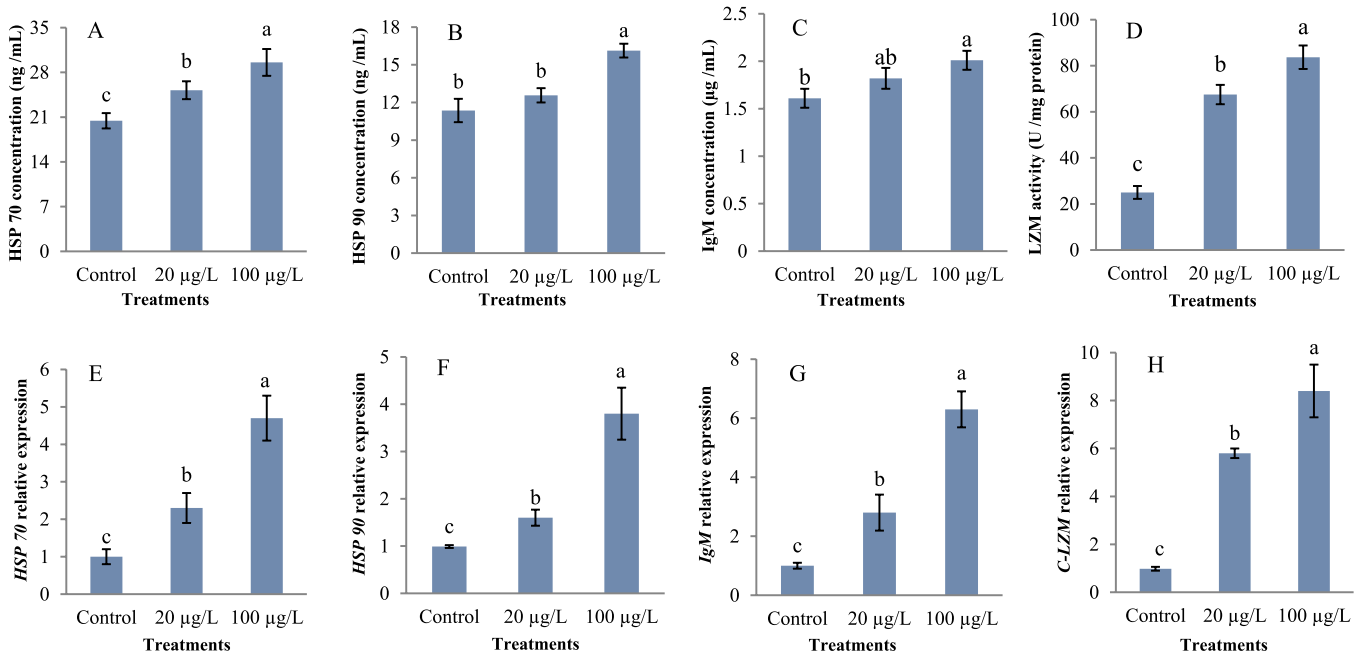

Fig. 4. The immune response indicators (A–D) and corresponding genes expression (E–H) in the liver of juvenile *T. fasciatus* after exposure to different dose of Cu NPs for 30 days. HSP 70: heat shock proteins 70; HSP 90: heat shock proteins 90; IgM: immunoglobulin M; LZM: lysozyme; C-LZM: C type lysozyme. Data are means ± SD (n = 3). Significant differences ($p \leq 0.05$) among treatments in each graph were indicated by different letters.

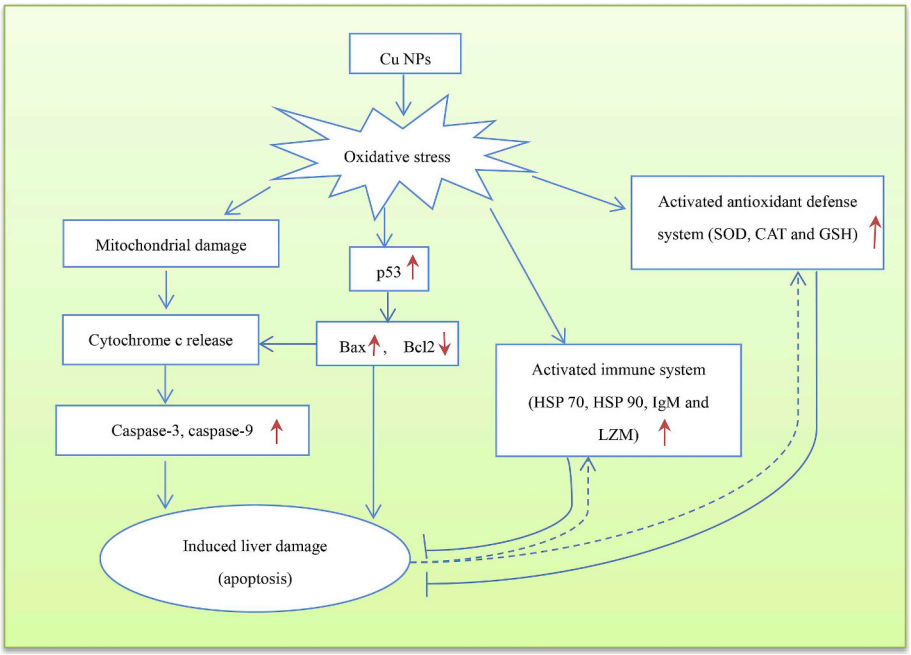

Fig. 5. The putative mechanism of Cu NPs effects on inducing oxidative stress, apoptosis and immune responses in the liver of juvenile *T. fasciatus* after 30-day exposure.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.053.

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