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RESEARCH

Antagonistic Effect of Zinc Oxide Nanoparticles Dietary Supplementation Against Chronic Copper Waterborne Exposure on Growth, Behavioral, Biochemical, and Gene Expression Alterations of African Catfish, *Clarias gariepinus* (Burchell, 1822)

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Abstract

The harmful impact of waterborne copper (Cu) as a common abiotic stressor in aquatic environments has gained much more interest. The present study aimed to investigate the utilization of zinc oxide nanoparticles (ZnONPs) dietary supplementation to mitigate the chronic toxicity of Cu in African catfish (*Clarias gariepinus)*. Two hundred and forty fish (92.94 ± 0.13 g) were assigned into six groups for 60 days. Control (C), ZnONPs20, and ZnONPs30 groups were fed on basal diets fortified with 0, 20, and 30 mg kg^{-1} ZnONPs without Cu exposure. Cu, Cu + ZnONPs20, and Cu + ZnONPs30 groups were exposed to Cu at a dose of 10 mg L^{-1} and fed on basal diets fortified with 0, 20, and 30 mg kg^{-1} ZnONPs, respectively. The results revealed that the Cu-exposed fish experienced abnormal clinical signs and behavioral changes. The growth indices and acetylcholine esterase activity were significantly decreased ($P < 0.05$) in the Cu group. Meanwhile, hepatorenal and serum stress indices ($P < 0.05$) were significantly elevated with chronic Cu exposure. In addition, a higher expression of stress ($P < 0.05$) (heat shock protein 60 and hypoxia-inducible factor-1 alpha) and apoptotic-related genes (C/EBP homologous protein, caspase-3, and Bcl-2 Associated X-protein) with down-regulation ($P < 0.05$) of the anti-apoptotic-related genes (B-cell lymphoma 2 and proliferating cell nuclear antigen) was noticed in the Cu-exposed fish. Histopathological alterations in the gills, liver, kidney, and spleen were markedly reported in the Cu-exposed group. The dietary supplementation with ZnONPs significantly alleviated the negative impacts of chronic waterborne-Cu exposure on growth performance, physiological changes, gene expression, and tissue architecture, especially at 30 mg kg^{-1} diet level. In particular, the inclusion of ZnONPs at the 30 mg kg^{-1} diet level produced better outcomes than the 20 mg kg^{-1} diet. Overall, ZnONPs could be added as a feed supplement in the *C. gariepinus* diet to boost the fish's health and productivity and alleviate the stress condition brought on by Cu exposure.

Keywords African catfish · Copper toxicity · Zinc oxide nanoparticles · Chronic stress · Apoptosis

Introduction

The aquaculture sector is challenged by various biotic and abiotic stressors that affect fish productivity and health [1–3]. Pollution is abiotic stressor that gains access to aquatic bodies through industrial processes employing water, as well as the discharge of industrial and urban development effluents; aquatic bodies, particularly freshwater bodies, are more susceptible to contamination than other settings [4, 5]. Among the most prevalent pollutants in aquatic bodies are heavy metals. Several natural and human processes can lead to the buildup of heavy metals [6–8]. Pollution with heavy metals in aquatic ecosystems occurs as a direct consequence of atmospheric depositing, as an outcome of geological weathering, or as a consequence of the agricultural and industrial waste materials released through wastewater facilities [9, 10].

Copper (Cu) is a crucial component of metabolic enzymes and a necessary trace element and micronutrient for the metabolism of cells in living organisms [11]. Meanwhile, high concentrations of Cu can be exceedingly hazardous to aquatic creatures' intracellular processes

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[12]. Furthermore, the extensive use of pesticides and garbage disposal contributes to increasing Cu contamination [13]. Because Cu has been used as a plant protection product for over 50 different diseases in viticulture, hops, arable crops, and horticulture, it is currently one of the most common contaminants in natural waterways. Cu has detrimental effects on the aquatic environment [14]. Cu is used in many industrial applications, electrical equipment, building materials, and antibacterial agents [15]. Cu residues were detected in Mudfish (*Clarias anguillaris* L), Nile tilapia (*Oreochromis niloticus* L) [16, 17], rainbow trout (*Oncorhynchus mykiss*) [18], some crustacean spp. (barnacles, amphipods, and caridean decapods) [19], marine bivalve (*Mytilus galloprovincialis*), and echinoderm (*Strongylocentrotus purpuratus*) [20]. In addition, the Cu residues were detected in the different water bodies, sediment, and tissues of *O. niloticus,* African catfish (*Clarias gariepinus),* and catfish (*Bagrus bayad*) in Sharkia Province, Egypt [21].

Overcoming the problem of heavy metal pollution is a must for maintaining sustainable aquaculture development [22]. Using feed additives for mitigating heavy metal toxicity exposure in fish has been investigated [23]. Recently, the aquaculture and fish feed industries have given various metal-based nanoparticles substantial consideration as feed additives [24, 25]. The oral addition of zinc oxide nanoparticles (ZnONPs) is recommended as a practical and affordable solution in fish feed [26]. Nanotechnology may be used in feed formulation and open the door to safely adding ZnONPs to aquatic animals' diets [27]. Because of their low toxicity, chemical stability, and biocompatibility, ZnONPs have garnered a lot of attention in biological research recently. These nanoparticles are environmentally safe and satisfy all of the body's needs [28]. ZnONPs improved the hematological, growth, and biochemical indices in *Labeo rohita* fish [29] and *C. gariepinus* [30] when added to the fish feed. In addition, ZnONPs were proven to have adsorbent potential against Cu [31].

African Catfish, *C. gariepinus* is becoming a widely produced fish species in many nations owing to its capability to survive in poor water quality, grow using a variety of production methods, and have a fast growth rate [32–34]. Controlling water pollution and prohibiting the use of Cu-based agrochemicals in agriculture practices are difficult tasks. Thus, there is an urgent need for safe alternative protocols. Therefore, the current work was the first to look into the potential mitigating effects of ZnONPs diets against chronic Cu exposure in *C. gariepinus*. This study's primary objective is to assess the protective impact of ZnONPs-supplemented diets on productivity, well-being, behaviors, and biochemical stress indicators, as well as modulation of the stress and apoptotic/anti-apoptotic gene expression in the Cu-exposed fish.

Materials and Methods

Synthesis and Characterization of ZnONPs

ZnONPs were synthesized by the precipitation method, according to Kumar et al. [35]. 100 ml of deionized water dissolved 28.75 g of zinc sulfate heptahydrate (Milli-Q, Millipore, USA). Next, 8 g of sodium hydroxide was added dropwise during magnetic stirring for 30 min. Multiple rounds of clean water were used to filter and wash the precipitates. The resulting precipitates were dried at 60 °C for 24 h and calcined at 400 °C for two hours. High-resolution transmission electron microscopy (HR-TEM, Tecnai G20, FEI, Netherlands) was used to evaluate the shape and size. An X-ray diffractometer (XRD, X'Pert Pro, PanAlytical, Netherlands) was used to examine the crystalline and phase structure of the synthesized ZnONPs.

Diet Preparation

Three isoenergetic and isonitrogenous experimental diets were prepared to address the dietary requirements of *C. gariepinus* [36] in the Fish Research Unit at the Faculty of Veterinary Medicine, Zagazig University, Egypt. The control diet was a basal diet (Table 1); ZnONPs20 and ZnONPs30 were basal diets with ZnONPs added by 20 and 30 mg kg^{-1} level, according to Mahboub et al. [37]. Briefly, the diet ingredients were ground, and water was added and blended to create a homogenous dough. The mixture was pelletized using an electric meat mincer (Bosch, Gerlingen, Germany) afterward air-dried at room temperature, and finally maintained in dry plastic bags at 4 °C till required.

Fish Rearing Condition

Fingerlings of *C. gariepinus* (92.94 ± 0.13 g) were obtained from a Private Fish Farm (Abbassa, EL-Sharkia Providence, Egypt). The fish were transferred to the Aquatic Animal Medicine Department laboratory at Zagazig University's Faculty of Veterinary Medicine, Egypt, and acclimatized for two weeks in glass aquaria (100 L). During this period, the fish were fed the basal diet to apparent satiation and examined clinically following CCoA [38] and had no history of disease outbreak. The water quality parameters were maintained during the adaptation and experimental period as follows: dissolved oxygen 5.9 ± 0.40 mg L^{-1} (portable dissolved oxygen meter, HI-9142, HANNA Instruments, US), pH 6.90 ± 0.2, temperature 25 ± 1.9 ◦C, (Digital pH Meter, Research

Table 1 Ingredients and chemical composition of the basal diet content (g kg^{-1} on dry matter basis)

Ingredients	basal diet
Soybean meal 49% CP	310
Wheat bran	60
Fish meal 70.7% CP	150
Ground yellow corn	186.5
Corn gluten 67% CP	100
Fish oil	60
Wheat flour	100
Premix[#]	30
methionine	3.5
Proximate chemical composition (g kg^{-1})	
NFE [*]	431.3
Crude protein	371.5
Fat	96.4
Ash	61.6
Crude fiber	39.2
GE (KJ kg^{-1}) [**]	207.2
Methionine	10.8
Lysine	20.04

[#] Premix (1 kg) contain: B1 58 mg; vitamin B2 34 mg; vitamin B6 34 mg; vitamin B12 58 mg; vitamin C 0.1 mg; vitamin E 720 mg; vitamin K3 142 mg; vitamin D3 8600 IU; vitamin A 580,000 IU; calcium iodide 25 mg; sodium selenite 25 mg; cobalt sulfate 572 mg; copper sulfate 3400 mg; iron sulfate 2000 mg; zinc methionine 3000 mg; manganese sulfate 65 mg; pantothenic acid 8 mg; folic acid 86 mg; biotin 50 mg; calcium carbonate as carrier up to till 1 kg

[*]NFE "Nitrogen free extract

[**]Gross energy (GE) was computed according to NRC [36]

Model 211, HANNA Instruments, US) and photoperiod was adjusted automatically using fluorescent tubes at 12 h light: 12 h darkness according to APHA [39].

Determination of the 96-h-lethal Concentration 50 (96 h LC_{50}) of Cu

For the determination of 96 h LC_{50} of Cu, 60 Nile tilapia were assigned equally into six groups and exposed to different levels of $CuCl_2$ (Alpha Chemika, India) (0, 50, 100, 150, 200, and 250 mg L^{-1}). There was no water exchange and the fish were not fed during the exposure time (96 h). In addition, the fish deaths and visible signs were recorded. Finney's Probit analysis was used to calculate the 96 h LC_{50} value [40] and found to be 100 mg L^{-1}.

Experimental Design

Two hundred and forty fish were divided into six groups in four replicates of 10 fish each and 40 fish per group. The control (C), ZnONPs 20, and ZnONPs 30 groups were fed the basal diets with supplements containing 0, 20, and 30 mg kg^{-1} ZnONPs, respectively, and were maintained in clean water. Cu, Cu + ZnONPs20, and Cu + ZnONPs30 groups were exposed to 1/10 of the 96 h LC_{50} of $CuCl_2$ (10 mg L^{-1}) and were fed the basal diets supplemented with 0, 20, and 30 mg kg^{-1} ZnONPs, respectively. During the experimental duration, the fish were fed three times daily (09:00, 12:00, and 15:00), till apparent satiation daily. The daily water exchange rate was 30% while maintaining the respect Cu level.

Clinical Signs, Behavior, and Survival

Fish were checked daily during the study period to collect data on clinical signs, behavior, and survival. Using an adjustable timer camera, clinically documented symptoms and behavioral changes may be immediately observed between 10:00 and 17:00 [41]. The following behaviors were noted; breath gasping (Surfacing): according to Noga [42], it monitored how frequently fish would occasionally come to the water's surface to breathe. According to Chen et al. [43], abnormal swimming behavior is either rapid or slow. According to De Boer [44], aggressive behavior occurs when fish constantly swim wildly after one another and fish move directly toward one another. Daily recording of the dead fish was done during the experimental period to determine the fish survival using the Kaplan–Meier curves.

Growth Metrics

The weighting of the fish at the beginning and after 60 days of the exposure trial was conducted to record their initial weight (IW) and final weight (FW), respectively. The total amount of feed consumed during the experiment was determined (feed intake, FI). The total weight gain (TWG). Feed conversion ratio (FCR), specific growth rate (SGR), and average daily weight gain (ADWG) were determined according to Amer et al. [45] as follows.

$$\mathrm{TWG}\left(\mathrm{g\ fish^{-1}}\right) = (\mathrm{FW} - \mathrm{IW}).$$
$$\mathrm{FCR} = \mathrm{FI\ (g)}/\mathrm{TWG\ (g)}.$$
$$\mathrm{SGR}\left(\%\ \mathrm{day^{-1}}\right) = 100 \times (\ln\ \mathrm{FW} - \ln\ \mathrm{IW})/\mathrm{days}.$$
$$\mathrm{ADWG}\left(\mathrm{g\ fish^{-1}\ day^{-1}}\right) = \mathrm{TWG}/\mathrm{days}.$$

Sampling

After termination of the exposure trial, fish (12 fish/ group; 3 fish/replicate) were tranquilized (100 mg L^{-1} benzocaine solution). Without using an anticoagulant, blood samples from fish caudal blood vessels were taken to separate the serum after 15 min of centrifugation at 3000 rpm to evaluate the biochemical parameters. In addition, fresh tissue samples

were taken from euthanized fish after killing using an over dose of anesthesia (250 mg benzocaine/L) for evaluation of the brain neurotransmitter (brain), expression of stress and apoptotic/anti-apoptotic genes (gills), histological analysis (gills, liver, kidney, and spleen), and Cu residues (gills, liver, kidney, and muscles) [46].

Biochemical Indices and Stress Biomarkers

The acetylcholine esterase enzyme (AChE) level in brain samples was spectrophotometrically evaluated following the Ellman and Courtney [47]. Reitman and Frankel [48] and Burtis and Ashwood [49] protocols were followed to assess the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). According to Bartles et al. [50] and Fawcett and Scott [51], creatinine and urea concentrations in the serum were assessed, respectively. The technique used to evaluate serum glucose levels was developed by Nikkila [52]. The estimation of serum triglycerides and cholesterol was conducted according to Allain et al. [53] and McGowan et al. [54], respectively.

Transcriptional Analysis

Total RNA was recovered from the *C. gariepinus* gills tissues (12 sample/group) using Quiazol (Qiagen, Germany) following the manufacturer's instructions. The recovered RNA was put through agarose gel electrophoresis and a spectrophotometer (BioRad, CA, USA) to determine its concentration and purity. The obtained RNA was handled with DNase (Takara, Shiga, Japan) to eliminate any contaminating DNA. The complementary DNA (cDNA) was obtained using the reverse transcriptase kit (Applied Biosystem, California, United States) following the manufacturer's instructions. Real-time quantitative PCR (RT-qPCR) investigation of heat shock protein 60 *(HSP60),* hypoxia-inducible factor-1 alpha *(HIF1a),* *C/EBP* homologous protein *(CHOP),* caspase-3, Bcl-2 Associated X-protein *(BAX),* B-cell lymphoma 2 *(BCL2*), Proliferating cell nuclear antigen *(PCNA)* were analyzed with specific primers (Sangon Biotech, Beijing, China) using a reference gene (beta-actin, *β-actin)*) (Table 2). The RT-qPCR conditions for the initial denaturation temperature were 95 °C for 10 min, then 40 cycles of 95 °C for 10 s and 60 °C for 15 s, with a dissociation analysis step in between. To ensure that each amplicon was accurate after amplification, a melting curve analysis was performed. After assuring that the primers were almost entirely efficient, the results of gene expression were evaluated using the $2^{-\Delta\Delta CT}$ approach. Following Schmittgen and Livak [55] method, the expression of these genes was normalized against *β-actin* and shown relative to the control.

Histopathological Analysis

Tissue samples (gills, liver, kidneys, and spleen) (12 samples/group) were fixed in 10% formalin for 48 h, dehydrated in ascending grading of ethyl alcohol, cleared in xylene, and then embedded in paraffin wax for obtaining paraffin blocks and cutting using a microtome (5 µm thickness) then stained with hematoxylin–eosin for histopathological examination [58]. The microscopic examination was carried out using an AmScope CMOS C-Mount microscope digital camera (United Scope LLC., CA., USA) attached to a Nikon light microscope (Nikon Inc., NY, USA) according to Bernet et al. [59]

Residues Assay

Gills, liver, and kidney samples (12 samples/group) were tested for Cu residues by the atomic absorption techniques using the Atomic Absorption Spectrophotometer (Buck Scientific 210VGP) following the method of Dalman et al. [60].

Table 2 Primers of stress and apoptotic/anti-apoptotic genes for real-time quantitative PCR amplification

gene	Forward primer	Reverse primer	Reference
β-actin	GCGTGACATCAAGGAGAAG	CAAGACTCCATACCCAAGAAAG	[56]
HIF-1a	TGACCTTGAGATGCTCGCTC	AAGTGCTGGATGTTGGCGA	[57]
CHOP	GTTGGAGGCGTGGTATGAAG	GAAACTCCGGCTCTTTCTCG	[57]
HSP60	GGTTCTCATGGAAAAGCAGCA	GGCAGATTTCAACCCTTGTGT	[57]
BAX	AACTTGGTAGACTGGCTCGC	CCAATCTGGCCATCTGGTGT	XM_053515421.1
caspase-2	GCACTGCTGCAATGAGAAAC	GATGGTGTGCTGAACCTGGA	XM_053495329.1
BCL2	AGCATGTCCCCTCATCCTCT	ATCATGCTCAGGTGTGTGGG	XM_053504284.1
PCNA	CTGAGACGCGCTGAACCTAT	CGAACATTTTTGCGGGGGTT	XM_053515615.1

β-actin beta actin, *HIF-1a* hypoxia-inducible factor 1 alpha, *CHOP* C/EBP homologous protein, *HSP60* heat shock protein 60, *BAX* Bcl-2 Associated X-protein, *BCL2* B-cell lymphoma 2, *PCNA* Proliferating cell nuclear antigen

Data Analysis

Finney's Probit analysis was used to calculate the 96 h LC_{50} value of Cu. Shapiro–Wilk test was used to check the data's normality. The Kaplan–Meier model was applied to compute the fish survival in each group. The log-rank test was performed in pairwise comparisons to seek any differences among groups. The outcomes of the behaviors, growth, biochemical indices, and gene expression were examined using a one-way analysis of variance (ANOVA) by SPSS version 18 (SPSS, Chicago, IL, USA). Duncan's multiple range tests were used ($n = 12$/group) to detect differences in means at a significance level 0.05.

Results

Characterization of ZnONPs

ZnONPs were nearly spherical, and their size range was 42–53.7 nm. The peaks at $2\theta = 31.77°$, 34.42°, 36.25°, 56.59°, 62.85°, 67.94°, and 69.08° were assigned to (100), (002), (101), (110), (103), (112), and (201) of ZnONPs, demonstrating that the crystalline structure of synthesized ZnONPs appeared a hexagonal phase structure of the wurtzite (Zincite, JCPDS 04–004-2776).

Clinical Signs, Behavior, and Survival Rate

The C, ZnONPs20, and ZnONPs30 groups showed normal clinical signs. The Cu group suffered from dark body coloration, skin ulcers, and fin rot. Moreover, the Cu + ZnONPs20 and Cu + ZnONPs30 groups retrieved previous signs except for a slight tail rot. There was no noticeable change between the C, ZnONPs20, and ZnONPs30 groups in the behaviors and brain AChE level (Table 3). The Cu group showed a pronounced increase ($P < 0.05$) in breath gasping, abnormal swimming, and aggressive behaviors with a significant decrease in the AChE level relative to the C group. At the same time, they were modulating previously mentioned behaviors and retrieved AChE levels in the Cu + ZnONPs20 and Cu + ZnONPs30 groups compared to the Cu group. Based on the Kaplan- Meier curves, the survival rate was 100% for the C, ZnONPs20, and ZnoNPs30 groups. While it was 62.5%, 75%, and 87.5% in the Cu, Cu + ZnoNPs20, and Cu + ZnoNPs30 groups, respectively (Fig. 1).

Growth Performance

The growth metrics (FW, TWG, and ADWG) were significantly higher ($P < 0.05$) in the ZnONPs30 group, then ZnONPs20 group relative to the C group (Table 4). While these metrics were noticeably lowered ($P < 0.05$) in the Cu, Cu + ZnONPs20, and Cu + ZnONPs30, respectively, relative to the C group. A noticeable decline ($P < 0.05$) in the FI and SGR with higher FCR was noticed in the Cu group, followed by the Cu + ZnONPs20 group and, eventually, the Cu + ZnONPs30 group relative to the C group. While these metrics did not significantly differ between the C, ZnONPs20, and ZnONPs30 groups.

Serum Stress Indicators and Hepato-Renal Function Indices

The hepato-renal function indices (ALT, AST, creatinine, and urea) were noticeably increased ($P < 0.05$) in the Cu group followed by the Cu + ZnONPs20 and Cu + ZnONPs30 groups, respectively. On the other hand, there was no noticeable difference between the C, ZnONPs20, and ZnONPs30 groups in the level of hepato-renal function indices (Table 5). The serum stress indicators (glucose, cholesterol, and triglyceride) were noticeably increased ($P < 0.05$) in the Cu group, followed by the Cu + ZnONPs20 group and the Cu + ZnONPs30 group compared to the C group. There was

Table 3 Effect of Cu exposure on behavior changes and brain AchE activity of *Clarias gariepinus* fed on ZnONPs supplemented diets for 60 days

Behaviors	Breath gasping (surfacing) behavior	Abnormal swimming behavior	Aggressive behavior	AChE (pg mg^{-1})
C	0.30 ± 0.02^{c}	0.13 ± 0.03^{c}	0.23 ± 0.01^{c}	61.00 ± 0.57^{a}
ZnONPs20	0.29 ± 0.03^{c}	0.14 ± 0.05^{c}	0.31 ± 0.13^{c}	63.33 ± 0.88^{a}
ZnONPs30	0.28 ± 0.04^{c}	0.16 ± 0.02^{c}	0.29 ± 0.09^{c}	61.66 ± 0.82^{a}
Cu	1.42 ± 0.07^{a}	1.89 ± 0.19^{a}	2.98 ± 0.19^{a}	25.22 ± 0.35^{d}
Cu + ZnONPs20	0.73 ± 0.06^{b}	0.82 ± 0.10^{b}	1.75 ± 0.14^{b}	33.33 ± 1.20^{c}
Cu + ZnONPs30	0.82 ± 0.05^{b}	0.89 ± 0.11^{b}	1.69 ± 0.12^{b}	39.66 ± 0.28^{b}
P-value	0.02	0.01	0.04	0.02

Values not sharing the same superscript letter in the same column are significantly different ($P < 0.05$; one-way ANOVA). *AChE* acetylcholine esterase enzyme. C, ZnONPs20, and ZnONPs30 groups were fed on a basal diet supplemented with 0, 20, and 30 mg kg^{-1} zinc oxide nanoparticles (ZnONPs), respectively. Cu, Cu + ZnONPs20, and Cu + ZnONPs30 groups were fed on a basal diet supplemented with 0, 20, and 30 mg kg^{-1} ZnONPs, respectively and exposed to10 mg L^{-1} of $CuCL_2$

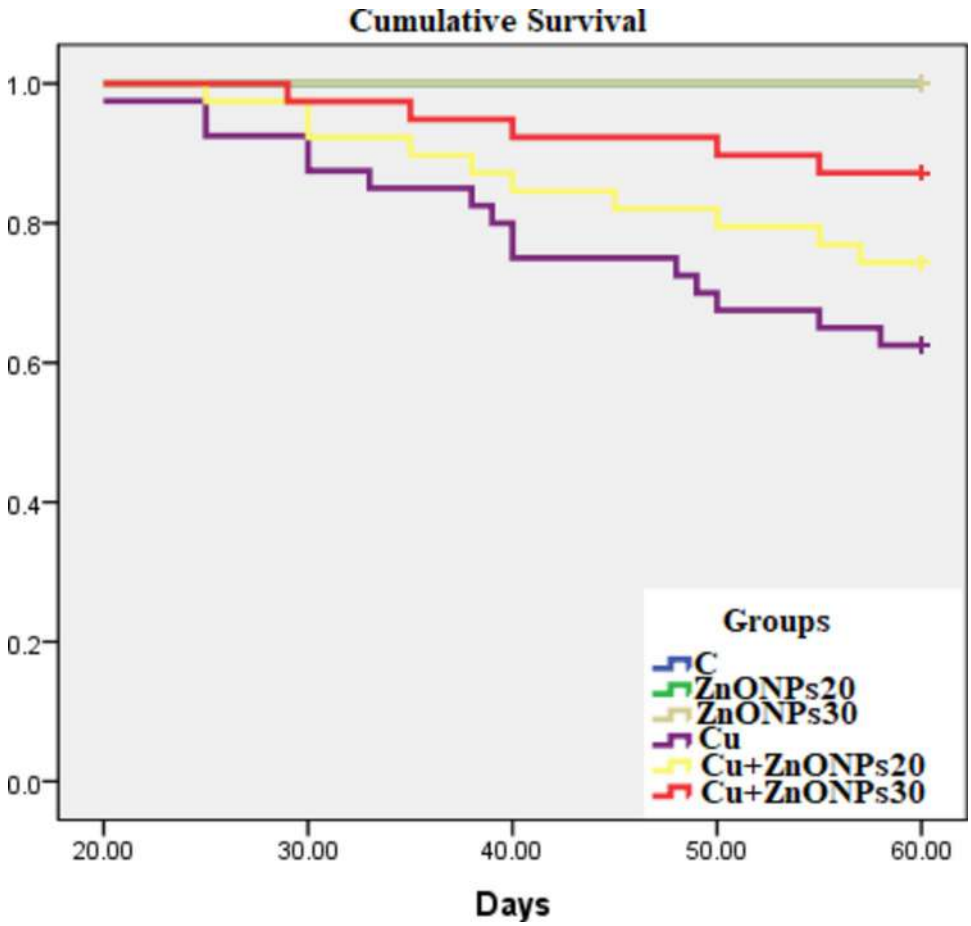

Fig. 1 Survival curves (Kaplan–Meier) of *C. gariepinus* exposed to 10 mg L^{-1} of $CuCL_2$ and fed ZnONPs supplemented diets for 60 days. Survival of C, ZnONPs20, and ZnONPs30 are all 100%

Table 4 Effect of Cu exposure on growth performance of *Clarias gariepinus* fed on ZnONPs supplemented diets for 60 days

Groups	IW (g/Fish)	FW (g/Fish)	TWG (g/Fish)	FI (g/Fish)	FCR	SGR (%/day)	ADWG (g/Fish)
C	93.33 ± 0.29	196.67 ± 3.84^{c}	103.34 ± 1.45^{c}	110.33 ± 3.99 ^{a}	1.06 ± 0.03^{c}	1.25 ± 0.05^{c}	1.72 ± 0.03^{c}
ZnONPs20	92.92 ± 0.48	215.67 ± 3.48^{b}	122.75 ± 0.88^{b}	114.67 ± 4.23^{a}	0.93 ± 0.02^{d}	1.40 ± 0.05^{b}	2.04 ± 0.02^{b}
ZnONPs30	93.33 ± 0.10	226.00 ± 2.30^{a}	132.67 ± 0.66^{a}	113.64 ± 4.17^{a}	0.85 ± 0.01^{e}	1.48 ± 0.03^{a}	2.21 ± 0.01^{a}
Cu	92.40 ± 0.11	140.67 ± 6.00^{f}	48.27 ± 3.17^{f}	69.84 ± 1.68^{d}	1.44 ± 0.28^{a}	0.70 ± 0.08^{f}	0.80 ± 0.07^{f}
Cu + ZnONPs20	92.80 ± 0.34	155.00 ± 4.58^{e}	62.20 ± 1.15^{e}	71.19 ± 4.25^{c}	1.14 ± 0.28^{b}	0.85 ± 0.06^{e}	1.03 ± 0.04^{e}
Cu + ZnONPs30	92.85 ± 0.34	163.33 ± 7.68^{d}	70.48 ± 3.05^{d}	79.47 ± 7.92^{b}	1.12 ± 0.23^{b}	0.93 ± 0.11^{d}	1.17 ± 0.07^{d}
P-value	0.85	0.01	< 0.01	0.03	< 0.01	< 0.01	< 0.01

Values not sharing the same superscript letter in the same column are significantly different ($p < 0.05$; one-way ANOVA). *IW* initial weight, *FW* final weight, *TWG* total weight gain, *FI* feed intake, *FCR* feed conversion ratio, *SGR* specific growth rate, *ADWG* average daily weight gain. C, ZnONPs20, and ZnONPs30 groups were fed on a basal diet supplemented with 0, 20, and 30 mg kg^{-1} zinc oxide nanoparticles (ZnONPs), respectively. Cu, Cu + ZnONPs20 and Cu + ZnONPs30 groups were fed on a basal diet supplemented with 0, 20, and 30 mg kg^{-1} ZnONPs, respectively and exposed to10 mg L^{-1} of $CuCL_2$

no noticeable difference between the C, ZNONPs20, and ZNONPs30 groups in the levels of serum stress indicators (Table 5).

Expression of Stress-Related Genes

The gills expression of stress-related genes (*HSP60, HIF-1a*, and *CHOP)* was up-regulation ($P < 0.05$) in the Cu group followed by the Cu + ZnONPs20 and Cu + ZnONPs30 groups. Nevertheless, the expression of these genes was not significantly differed in the C, ZnONPs20, and ZnONPs30 groups (Fig. 2). Relative to the C group, the fold change was 0.88-, 0.72-, 6.3-, 4.13-, and 2.85- for *HSP60,* 0.80-,0.65-, 9.63-, 6.32-, and 4.73- for *HIF-1a,* and 0.93-, 0.82-, 8.11-, 5.44-, and 3.44- for *CHOP*

Table 5 Effect of Cu exposure on hepatorenal functions, glucose, and some serum lipids indicators of *Clarias gariepinus* fed on ZnONPs supplemented diets for 60 days

Groups	ALT (U L^{-1})	AST (U L^{-1})	creatinine (mg dL^{-1})	urea (mg dL^{-1})	Glucose (mg dL^{-1})	Cholesterol (mg dL^{-1})	Triglycerides (mg dL^{-1})
C	10.30 ± 0.07^{d}	17.10 ± 0.05^{d}	0.51 ± 0.01^{d}	9.13 ± 0.31^{d}	47.88 ± 0.22^{d}	102.22 ± 2.12^{c}	195.56 ± 2.89^{d}
ZnONPs20	10.51 ± 0.26^{d}	16.12 ± 0.33^{d}	0.50 ± 0.01^{d}	8.31 ± 0.08^{d}	45.33 ± 0.88^{d}	100.35 ± 1.52^{c}	192.56 ± 1.58^{d}
ZnONPs30	9.08 ± 0.20^{d}	15.85 ± 0.42^{d}	0.49 ± 0.01^{d}	8.98 ± 0.06^{d}	49.32 ± 1.68^{d}	98.28 ± 2.25^{c}	189.88 ± 2.39^{d}
Cu	24.23 ± 0.49^{a}	29.00 ± 0.57^{a}	0.88 ± 0.05^{a}	19.50 ± 0.30^{a}	97.33 ± 0.88^{a}	170.15 ± 25^{a}	258 ± 2.55^{a}
Cu + ZnONPs20	20.84 ± 0.47^{b}	24.30 ± 0.71^{b}	0.64 ± 0.03^{b}	15.71 ± 0.24^{b}	65.10 ± 2.16^{b}	140.85 ± 2.58^{b}	219.66 ± 1.59^{b}
Cu + ZnONPs30	16.89 ± 0.44^{c}	20.32 ± 0.70^{c}	0.56 ± 0.02^{c}	11.10 ± 0.40^{c}	55.29 ± 1.23^{c}	129.58 ± 0.23^{c}	210.24 ± 2.21^{c}
P-value	0.01	< 0.01	< 0.01	0.03	0.01	0.03	< 0.01

Values not sharing the same superscript letter in the same column are significantly different ($P < 0.05$; one-way ANOVA). *ALT* alanine aminotransferase, *AST* aspartate aminotransferase. C, ZnONPs20, and ZnONPs30 groups were fed on a basal diet supplemented with 0, 20, and 30 mg kg^{-1} zinc oxide nanoparticles (ZnONPs), respectively. Cu, Cu + ZnONPs20, and Cu + ZnONPs30 groups were fed on a basal diet supplemented with 0, 20, and 30 mg kg^{-1} ZnONPs, respectively and exposed to 10 mg L^{-1} of $CuCL_2$

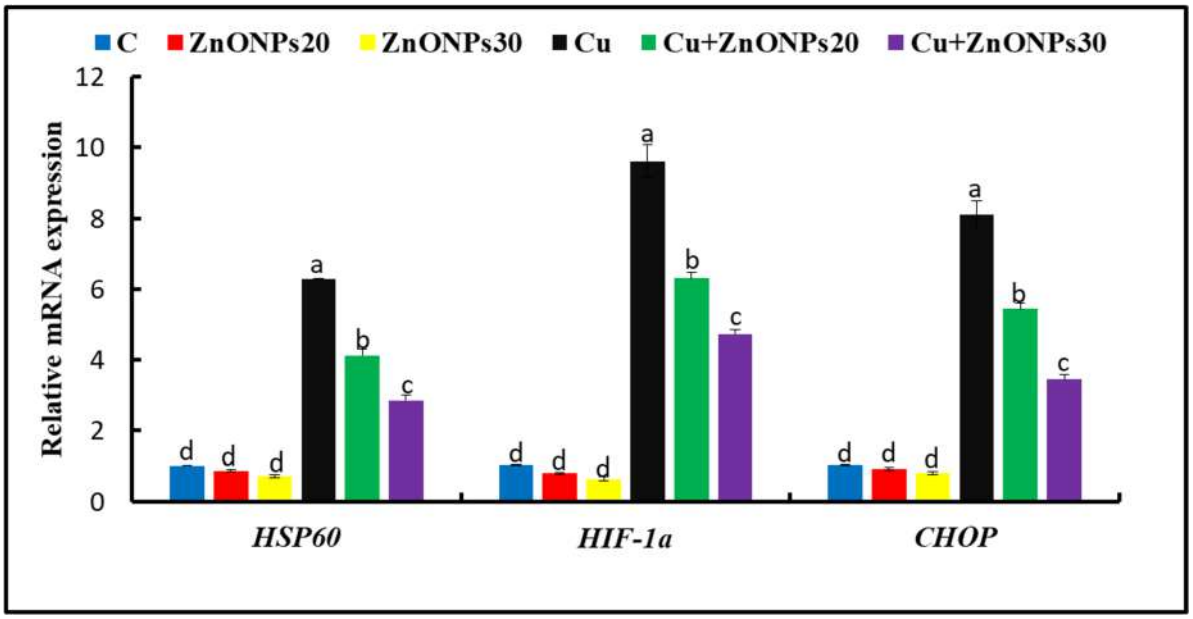

Fig. 2 The expression of anti-apoptotic/apoptotic related genes in gills of *C. gariepinus* exposed to 10 mg L^{-1} of $CuCL_2$ and fed ZnONPs supplemented diets for 60 days. *HSP60, heat shock protein 60; HIF-1a, hypoxia-inducible factor 1 alpha; CHOP, C/EBP homologous protein.* C, ZnONPs20, and ZnONPs30 groups were fed on a basal diet supplemented with 0, 20 and 30 mg kg^{-1} zinc oxide nanoparticles (ZnONPs), respectively. Cu, Cu + ZnONPs20, and Cu + ZnONPs30 groups were fed on a basal diet supplemented with 0, 20 and 30 mg kg^{-1} ZnONPs, respectively and exposed to 10 mg L^{-1} of $CuCL_2$. Values are expressed as mean ± SE ($n = 12$) (one-way aNOVa). Values are not sharing same superscripts letter in the same bar are significantly different ($P < 0.05$; one-way ANOVA)

in the ZnONPs20, ZnONPs30, Cu, Cu + ZnONPs20, and Cu + ZnONPs30 groups, respectively.

Expression of Apoptotic/ Anti-Apoptotic Genes

The gills expression of apoptotic genes (*caspase-3* and *BAX*) was noticeably up-turned ($P < 0.05$) in the Cu group followed by the Cu + ZnONPs20 group and finally, the Cu + ZnONPs30 group. The apoptotic-related genes expression was not significantly differed in the C, ZnONPs20, and ZnONPs30 groups (Fig. 3A). Compared to the C group, the fold change was 1.12-, 1.2-, 4.35-, 3.2-, and 2.11- for *caspase-3,* 1.1-, 1.15-, 5.22-, 3.95-, 2.45- for *BAX* in the ZnONPs20, ZnONPs30, Cu, Cu + ZnONPs20, and Cu + ZnONPs30 groups, respectively. The gills expression of anti-apoptotic genes was demonstrated in Fig. 3B. The *BCL-2* and *PCNA* expression was significantly down-regulated ($P < 0.05$) in the Cu-exposed groups (Cu, Cu + ZnONPs20 and Cu + ZnONPs30 groups, respectively) relative to the C group. Nonetheless, these gene expressions did not

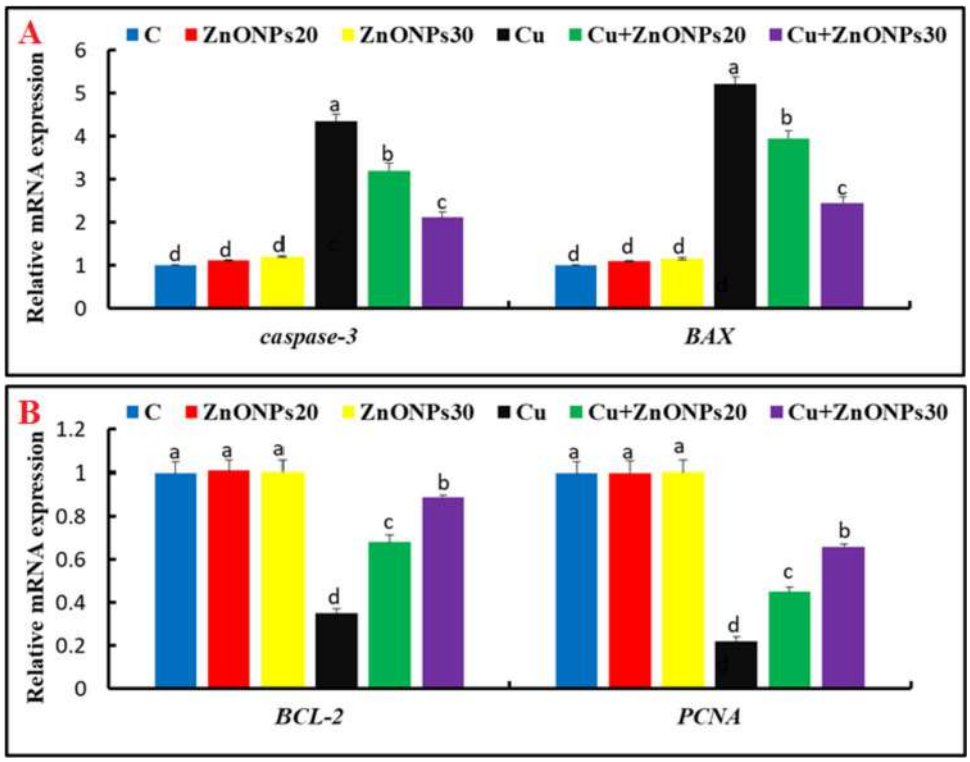

Fig. 3 The expression of anti-apoptotic/apoptotic related genes in gills of *C. gariepinus* exposed to 10 mg L^{-1} of $CuCL_2$ and fed ZnONPs supplemented diets for 60 days. *BAX, Bcl-2 Associated X-protein; BCL2, B-cell lymphoma 2; PCNA, Proliferating cell nuclear antigen.* C, ZnONPs20, and ZnONPs30 groups were fed on a basal diet supplemented with 0, 20 and 30 mg kg^{-1} zinc oxide nanoparticles (ZnONPs), respectively. Cu, Cu + ZnONPs20, and ZnONPs30 groups were fed on a basal diet supplemented with 0, 20 and 30 mg kg^{-1} ZnONPs, respectively and exposed to 10 mg L^{-1} of $CuCL_2$. Values are expressed as mean ± SE ($n = 12$). Values are not sharing same superscripts letter in the same bar are significantly different ($P < 0.05$; one-way ANOVA)

significantly differ in the C, ZnONPs20, and ZnONPs30 groups. Relative to the C group, the fold change was 1.008-, 1.006-, 0.35-, 0.68-, and 0.89- for *BCL-2* and 1.003-, 1.005-, 0.22-, 0.45-, and 0.66- for *PCNA* in the ZnONPs20, ZnONPs30, Cu, Cu + ZnONPs20, and Cu + ZnONPs30 groups, respectively.

Histopathological Findings

Normal primary and secondary gill lamellae and gill arch structures were evident in the C (Fig. 4A), ZnONPs20 (Fig. 4B), and ZnONPs30 (Fig. 4C) groups. However, the Cu group (Fig. 5D) revealed denuded most secondary lamellae with mucous exudate, inflammatory cells, and intense lymphocytic infiltration were detected within primary lamellae. Cu + ZnONPs20 (Fig. 4E) displayed edema and thickened tips of some primary lamellae by inflammatory cells. Cu + ZnONPs30 (Fig. 4F) showed normal gill architectures, except for mildly engorged capillaries within some primary lamellae.

Liver sections of the C, ZnONPs20, and ZnONPs30 groups (Fig. 5A, 5B, and 5C, respectively) maintained normal hepatic parenchymal structure and normal vasculatures. On the other hand, the Cu group exhibited obvious, degenerative changes within most of the hepatic cells in addition to granuloma formation (Fig. 5D). The latter was formed from centrally caseated and calcified material surrounded by aggregations of macrophages followed by round cells infiltrate and encircled finally by fibrous connective tissue capsule (Fig. 5E). In addition, some portal triads showed chronic cholangitis with the presence of melanomacrophages aggregates (Fig. 5F). Cu + ZnONPs20 group (Fig. 5G) showed vacuolization in most hepatic cytoplasm in addition to presence interstitial round cells infiltrations. Meanwhile, the Cu + ZnONPs30 group (Fig. 5H) showed vacuolated hepatocytes and congestion of some hepatic blood vessels.

Normal configurations of renal tubules, glomerular corpuscles, hematopoietic cells, and melanomacrophages were observed in the C (Fig. 6A), ZnONPs20 (Fig. 6B), and ZnONPs30 (Fig. 6C) groups. While the Cu group (Fig. 6D) showed degenerative and necrotic changes in most of the epithelium lining renal tubules beside shrinked some glomerular tufts. Moreover, the Cu + ZnONPs20 group (Fig. 6E) showed degenerative changes in a moderate number of tubular epitheliums besides normal glomeruli, hematopoietic elements, and melanomacrophages. Further, hydropic degeneration in a few renal tubules and apparent normal structures of most glomeruli and renal hematopoietic tissue were seen in the Cu + ZnONPs30 group (Fig. 6F).

Normal architectures of white pulp with ellipsoids arterioles and normal red pulp beside melanomacropahge centers were observed in the C (Fig. 7A), ZnONPs20 (Fig. 7B), and ZnONPs30 (Fig. 7C). While the Cu group (Fig. 7D) demonstrated depletion of the white pulp around ellipsoids arterioles and expanded red pulp area with presence of melanomacrophage centers. Cu + ZnONPs20 group (Fig. 7E)

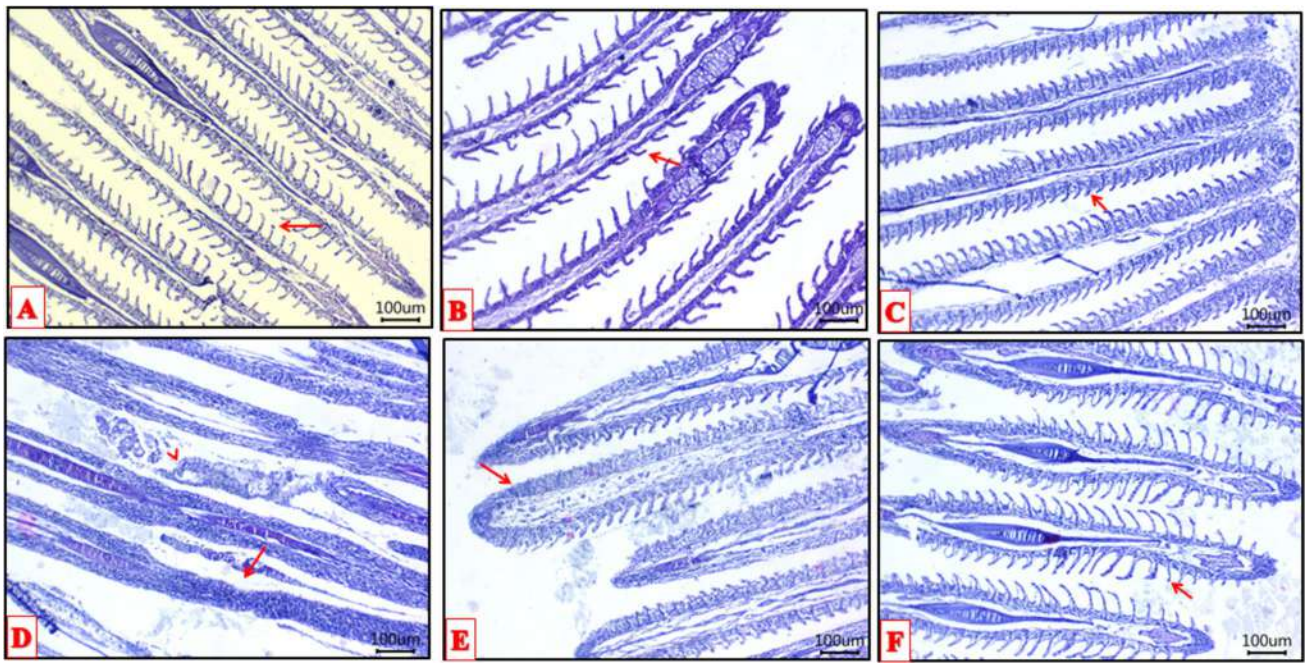

Fig. 4 Photomicrograph of H&E stained sections from gills (Scale bar 100 µm) of *C. gariepinus* exposed to 10 mg L^{-1} of $CuCL_2$ and fed ZnONPs supplemented diets for 60 days. C (**A**), ZnONPs20 (**B**), and ZnONPs30 (**C**) groups were fed on a basal diet supplemented with 0, 20 and 30 mg kg^{-1} zinc oxide nanoparticles (ZnONPs), respectively. Cu (**D**), Cu + ZnONPs20 (**E**), and ZnONPs30 (**E**) groups were fed on a basal diet supplemented with 0, 20 and 30 mg kg^{-1} ZnONPs, respectively and exposed 10 mg L^{-1} of $CuCL_2$

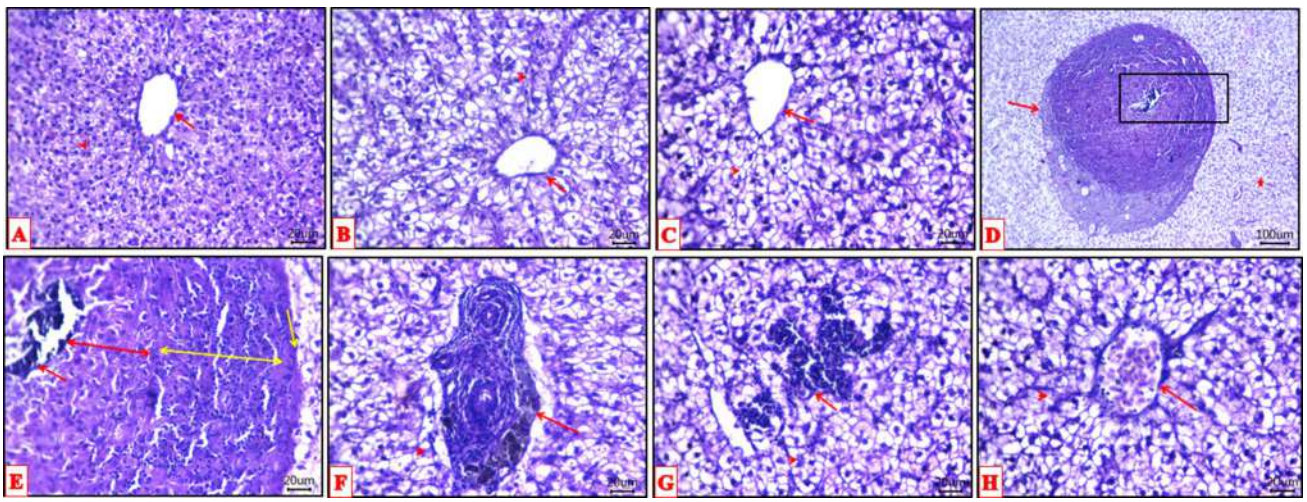

Fig. 5 Photomicrograph of H&E stained sections from liver (Scale bar 20 µm) of *C. gariepinus* exposed to 10 mg L^{-1} of $CuCL_2$ and fed ZnONPs supplemented diets for 60 days. C (**A**), ZnONPs20 (**B**), and ZnONPs30 (**C**) groups were fed on a basal diet supplemented with 0, 20 and 30 mg kg^{-1} zinc oxide nanoparticles (ZnONPs), respectively. Cu (**D**), Cu + ZnONPs20 (**E**), and ZnONPs30 (**E**) groups were fed on a basal diet supplemented with 0, 20 and 30 mg kg^{-1} ZnONPs, respectively and exposed 10 mg L^{-1} of $CuCL_2$

showed central arterioles surrounded by white zone and red zone, reticular fibers, and moderate density of melanomacrophges centers. Cu + ZnONPs30 group (Fig. 7F) showed mildly dilated splenic sinusoids with abundant phagocytic cells with clearly visible red zones and melanomacrophges centers.

Cu Residues in Fish Tissues

Cu residues were detected in the experimental fish's liver, gills, kidneys, and muscles. In trace amounts, the residues were detected in C, ZnONPs20, and ZnONPs30 groups without a significant difference between these groups.

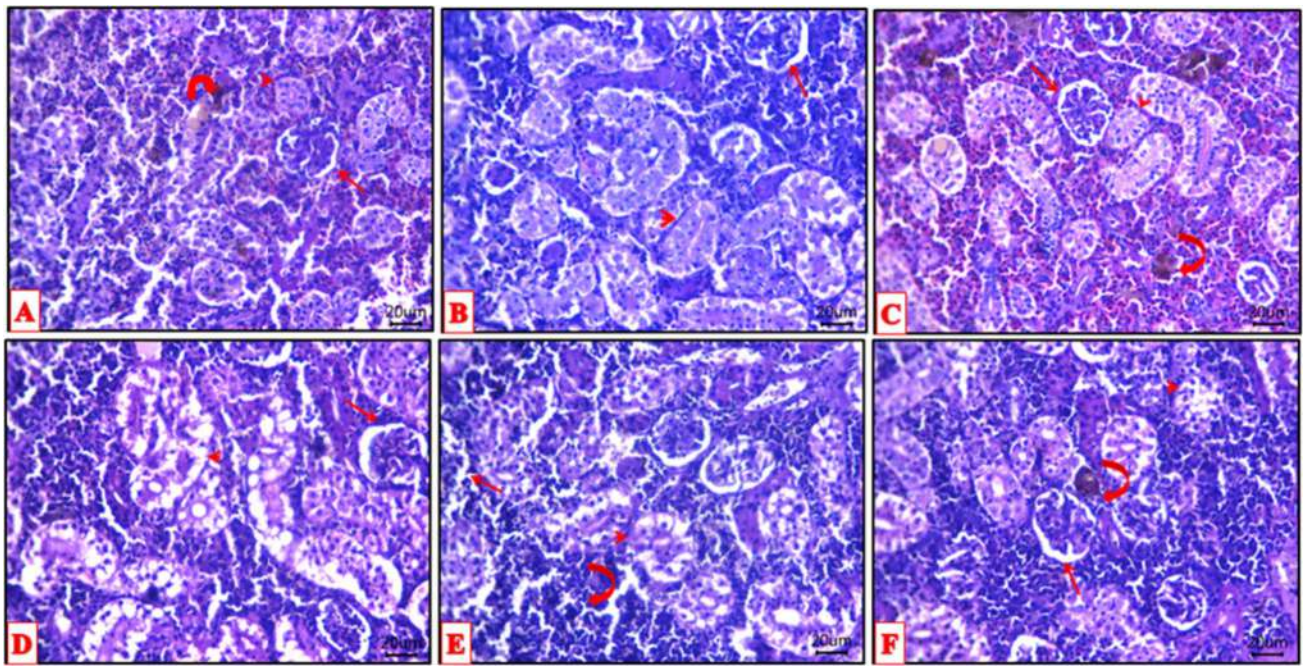

Fig. 6 Photomicrograph of H & E stained sections from kidney (Scale bar 20 µm) of *C. gariepinus* exposed to 10 mg L^{-1} of $CuCL_2$ and fed ZnONPs supplemented diets for 60 days. C (**A**), ZnONPs20 (**B**), and ZnONPs30 (**C**) groups were fed on a basal diet supplemented with 0, 20 and 30 mg kg^{-1} zinc oxide nanoparticles (ZnONPs), respectively. Cu (**D**), Cu + ZnONPs20 (**E**), and ZnONPs30 (**E**) groups were fed on a basal diet supplemented with 0, 20 and 30 mg kg^{-1} ZnONPs, respectively and exposed 10 mg L^{-1} of $CuCL_2$

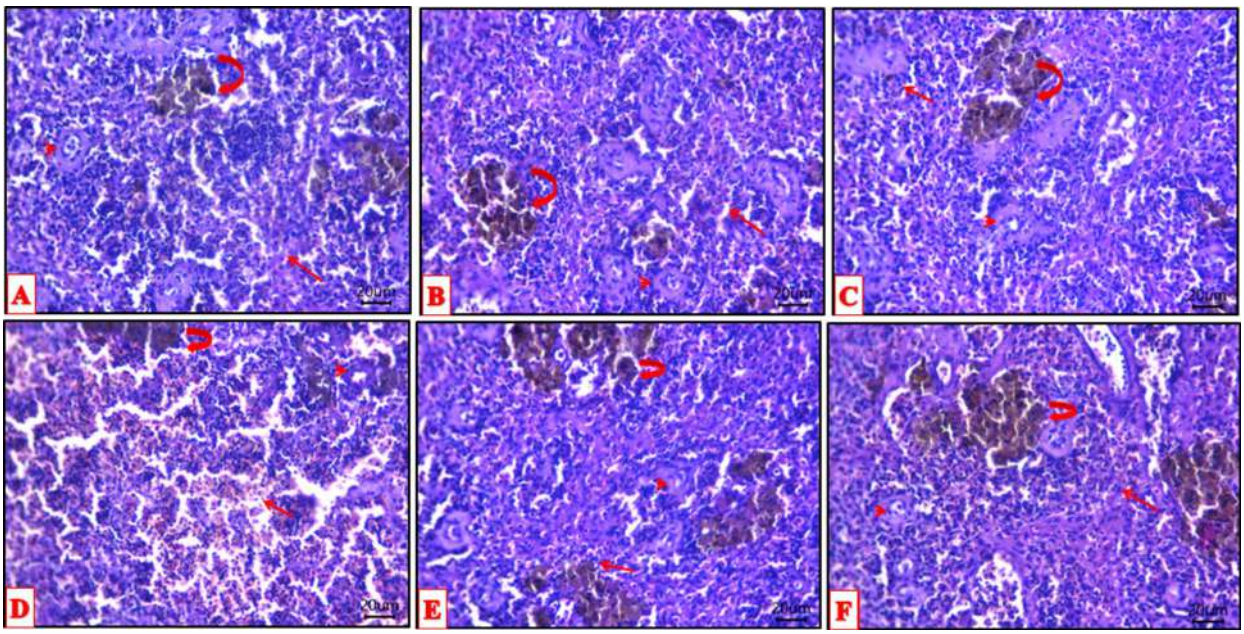

Fig. 7 Photomicrograph of H & E stained sections from spleen (Scale bar 20 µm) of *C. gariepinus* exposed to 10 mg L^{-1} of $CuCL_2$ and fed ZnONPs supplemented diets for 60 days. C (**A**), ZnONPs20 (**B**), and ZnONPs30 (**C**) groups were fed on a basal diet supplemented with 0, 20 and 30 mg kg^{-1} zinc oxide nanoparticles (ZnONPs), respectively. Cu (**D**), Cu + ZnONPs20 (**E**), and ZnONPs30 (**E**) groups were fed on a basal diet supplemented with 0, 20 and 30 mg kg^{-1} ZnONPs, respectively and exposed 10 mg L^{-1} of $CuCL_2$

While the Cu residues were significantly higher ($P < 0.05$) in the Cu group, followed by the Cu + ZnONPs30 and Cu + ZnONPs20, respectively. The Cu residues were higher in gills tissues followed by liver, kidney, and muscles, respectively (Fig. 8).

Discussion

Aquatic pollution is an up-to-date and critical problem affecting fish health and productivity. The negative impact of water pollution not only affects the fish but also could accumulate in the fish tissue parts and be transferred through the food chain to the human, causing health problems [61, 62]. In the present study, the chronic exposure of *C. gariepinus* resulted in decreased survivability and neuro-ethology alteration. The Cu-exposed group showed the lowest survivability (62.5%) and exhibited an increase in breath gasping, abnormal swimming, and aggressive behaviors. The behavioral alteration could be related to decreased AChE activity in the brain of the exposed fish, since AChE is a particularly sensitive enzyme suppressed by prolonged exposure to toxins like metals [63]. The catalytic triad of AChE has been disrupted by the presence of Cu ions, which inhibit the enzyme activity, consequently, increased accumulation of the acetylcholine resulted in behavioral alterations [64]. Even in low doses, Cu pollution in the aquatic environment has been seen to elicit early signs of behavioral and morphological changes in fish [65]. Modulating the behaviors in the Cu-exposed fish fed on ZnONPs-supplemented diets could be related to modulating the AChE level in the ZnONPs-fed fish. According to Xie et al. [66], ZnONPs can lower the degree of behavioral impairment by enhancing neuronal synaptic plasticity. In addition, ZnONPs had an adsorbent effect against Cu ions, reducing their levels and finally reducing the toxic impact of the metal on the brain AChE level [31].

Growth retardation was markedly accompanied by chronic Cu exposure in this study. Decreasing growth performance could be attributed to decreased FI. As previously reported, Cu toxicity reduced growth and increased FCR in rainbow trout (*Salmo gairdneri Richardson*) [67], *Channa punctatus* [68], and *O. niloticus* [69]. Modulation of the growth performance of the Cu-exposed fish, which fed on ZnONPs-supplemented diets, could be attributed to

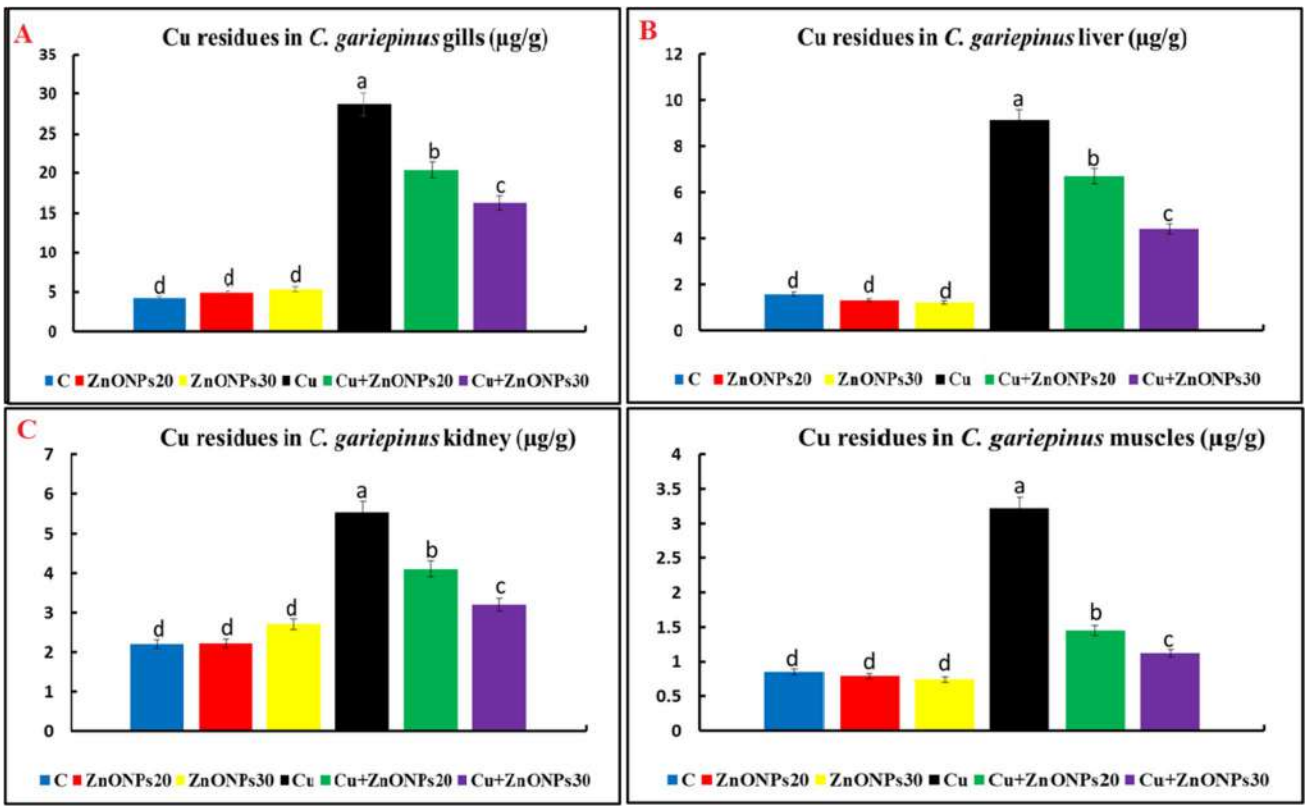

Fig. 8 Cu residues in *C. gariepinus* gills (**A**), liver (**B**), kidney (**C**), and muscles (**D**). C, ZnONPs20, and ZnONPs30 groups were fed on a basal diet supplemented with 0, 20 and 30 mg kg^{-1} zinc oxide nanoparticles (ZnONPs), respectively. Cu, Cu + ZnONPs20, and Cu + ZnONPs30 groups were fed on a basal diet supplemented with 0, 20 and 30 mg kg^{-1} ZnONPs, respectively and exposed to 10 mg L^{-1} of $CuCL_2$. Values are expressed as mean ± SE ($n = 12$). Values are not sharing same superscripts letter in the same bar are significantly different ($P < 0.05$; one-way ANOVA)

the critical role of zinc (Zn) in the physiological process in the fish body. Zn ensures the availability and functions of various metalloenzymes, including carbonic anhydrase, alkaline phosphatase, alcohol dehydrogenase, and others, which are crucial for promoting nutrition metabolism and digestion [70]. Additionally, Zn controls functions of protein synthesis, anti-oxidative enzymes, and nucleic acid metabolism [71]. Dietary Zn supplementation significantly increases fish growth, by improving muscle development [72]. Dietary Zn improved the growth performance of *Pangasianodon hypophthalmus* during lead and temperature stress exposure [73]. In addition, ZnONPs supplements may enter cells faster when supplied as nanoparticles than when they are organic [74]. Previous reports documented the growth-promoting effect of ZnONPs in rohu [27] and *C. gariepinus* [75].

Fish under stress respond in various biochemical, physiological, and behavioral ways to deal with the stressor and, if feasible, return to equilibrium [76]. In this study, the serum stress biomarkers (glucose, cholesterol, and triglyceride) were increased in the Cu-exposed fish. Blood glucose levels are utilized as a stress marker and increase throughout a waterborne heavy metal exposure period [77]. The increase in glucose level to deal with the rise in energy consumption brought on by Cu-induced stress [78]. Furthermore, cholesterol concentrations may rise due to hepatic and renal failure that causes the leakage of cholesterol into the blood. Because heavy metals harm cell structure, particularly the cell membranes, it appears that exposure to them raises blood cholesterol levels [79]. Triglyceride can be utilized as a nutritional status indicator and primarily serves to provide cellular energy and could increase due to renal tissue damage [80]. In similar, Cu exposure in *P. hypophthalmus* fish induced elevated glucose levels [81].

Along with increasing the serum biochemical stress indicators, the expression of the stress genes (*HSP60, HIF-1a*, and *CHOP)* was up-regulated in the Cu-exposed fish. According to Farcy et al. [82], Bhargav et al. [83], and Zhang and Zhang [84], heat shock proteins *(HSPs)* are indicators for exposure to a wide range of chemical, physical, and biological stressors, including temperature change, tissue damage, metal toxicity, radiation, and infection. *HSP60* expression was documented to up-turn due to cellular disruption [85]. Chronic durations of stress can modify *HSP60* [86]. In this study, higher expression of *HSP60* in the gill tissue of the Cu-exposed fish indicated the cellular damage caused by Cu on the gill tissue, which was evident by our histopathological findings. Vertebrates' hypoxic responses are coordinated by *hypoxia-inducible factors (HIF)*, and *HIF-1a* seems to play a more significant role in this process [87]. *HIF1a* expression level has been reported to be over-expressed under a hypoxic state [88]. In this study, increasing *HIF-1a* expression in the Cu-exposed fish indicates that these fish suffered hypoxia, which could contribute to the lowering oxygen-carrying capacity of the gills due to the histopathological lesions. *CHOP* expression was a significantly stress-inducible gene [89, 90]. Also, *CHOP* is considered a master pro-apoptotic stress inducer [91]. *CHOP* expression was previously up-regulated in loach (*Misgurnus anguillicaudatus*) as a response to hydrogen peroxide (H_2O_2) exposure [92] and ammonia exposure [93]. Modulation of the serum biochemical stress indicators and stress-related gene expression in the Cu-exposed fish by feeding on ZnONPs-supplemented diets indicating the anti-stress properties of the ZnONPs [94]. Similar results were obtained by [95] who stated that dietary ZnONPs alleviated the stress (lowered blood glucose and regulated the expression of the heat shock protein 70) in *P. hypophthalmus* exposed to combined stressors (arsenic, ammonia, and temperature).

In addition, Cu exposure increased the liver function enzymes, which could be attributed to the role of the liver in removing circulating Cu from plasma [96], where it is coupled to ceruloplasmin and either eliminated through the bile or stored in protein complexes [97]. According to de Souza Machado et al. [98], when Cu accumulates in the hepatic tissues an oxidative stress condition produces and affects the hepatic cell functions, increasing their permeability, and consequently elevating hepatic enzyme levels in the blood [99]. In the same line, chronic Cu exposure elevated the renal tissue function indicators (creatinine and urea) in this study. Elevated creatinine and urea indicated that the excretion of these nitrogenous compounds from the gills and kidneys was lowered due to tissue damage and dysfunction leading to their accumulation in the blood. This hypothesis was evident in this study by the histopathological alterations observed in these tissues. A previous study reported that Cu accumulation in the renal tissue induced function disturbance [99]. In this study, dietary ZnONPs improved the hepatorenal tissue function and their architecture in the Cu-exposed fish. According to Hosui et al. [100], ZnONPs play a significant role in preserving liver function, as shown by a progressive drop in AST and ALT levels after Zn supplementation. This could be explained by ZnONPs' capacity to enter liver cells and increase the production of cytokines that shield the liver from damage [101]. According to Mohammad et al. [102], ZnONPs increase the production of cytokines and prevent the majority of liver damage mechanisms, such as oxidative stress, endotoxemia, and hepatocyte apoptosis, by attenuating the variables that cause apoptosis.

The apoptotic genes (*caspase-3* and *BAX*) were over-expressed with lower anti-apoptotic (*BCL-2* and *PCNA*) markers in the Cu-exposed fish. Higher expression of the apoptotic genes indicates the apoptotic changes in the tissues of Cu-exposed fish. A class of cysteine proteases known as *caspases* is active when apoptosis is initiated, and they are essential for generating, conveying, and amplifying

intracellular apoptotic signals [103]. One of them, *caspase-3* is a death protease often activated and catalyzes the cleavage of several vital cellular proteins [104]. Through intricate protein interaction networks, members of the *Bcl-2* family that are anti-apoptotic regulate apoptosis [105]. Cell death is caused by the pro-apoptotic protein *BAX*, which activates *caspase-3*. The anti-apoptotic factor *BCL-2* blocks this route [106]. *PCNA* interacts with DNA polymerase to facilitate DNA replication [107]. In addition, *PCNA* analysis can be used to investigate cell proliferation [108]. In this study, Cu-exposure induces apoptotic changes in *C. gariepinus* tissues. Cu exposure was previously reported to produce apoptotic changes in *O. niloticus* tissue [69]. Herein, ZnONPs diets lowered the expression of the apoptotic genes and enhanced the expression of the anti-apoptotic genes. These results could be related to the ZnONPs' role in DNA repair processes, faster cell cycle development, and decreasing apoptosis. Additionally, the antioxidant action of ZnONPs may inhibit apoptosis and encourage cell growth [109]. In similar, Zn-diet regulated the expression of the stress-related genes and enhanced the mitigating ability of *P. hypophthalmus* fish against multiple stressors [110, 111].

The histopathological investigation has been frequently employed as a biomarker in the assessment of fish health because histopathology has been deemed a good technique for evaluating the impacts of heavy metals in fish tissues, such as Cu [112]. In this study, the gill tissue of Cu-exposed fish revealed denuded most secondary lamellae with mucous exudate; inflammatory cells and intense lymphocytic infiltration were also detected within primary lamellae. Similar findings were reported in *O. mykiss* [113], *Clarias batrachus* [114] due to Cu exposure. The hepatic tissues of the Cu-exposed fish revealed degenerative changes within most hepatic cells and the presence of granuloma. The latter was formed from centrally caseated and calcified material surrounded by aggregations of macrophages followed by round cell infiltrates and encircled finally by fibrous connective tissue capsule. In addition, some portal triads showed chronic cholangitis with the presence of melanomacrophages aggregates. Similar pathological alterations were reported in *C. gariepinus* [115] and *Cyprinus carpio* [116] due to Cu exposure. Renal tissue of Cu-exposed fish showed degenerative and necrotic changes in most of the epithelium lining renal tubules besides shrinked some glomerular tufts. Similar findings were reported in *Rutilus rutilus caspicus* [117], *Heterobranchus bidorsalis* [118], and *O. niloticus* [119] as a result of Cu exposure. The splenic tissue of Cu-exposed fish demonstrated depletion of white pulp around ellipsoids arterioles and expanded red pulp area with the presence of melanomacrophage centers. Similar findings were reported in *Carassius gibelio* [120] and *C. carpio* [121] due to Cu exposure. Fish exposed to chronic Cu toxicity have harm to their gills, liver, kidney, and spleen [122]. In this study, improving the tissue integrity in the Cu-exposed fish due to feeding on ZnONPs-supplemented diets could return to ZnONPs's antioxidant properties that were previously reported to have tissue-protective activity [94]. The Cu tissue levels were significantly lowered by feeding the Cu-exposed fish on ZnONPs diets. These results could be attributed to the adsorbent potential of ZnONPs against Cu ions [31]. ZnONPs diets could help *C. gariepinus* overcome the stress caused by chronic Cu exposure. The outcomes of this study introduced a protective strategy for mitigating the problem of metal exposure in aquatic bodies affecting the fish's health and productivity.

Conclusion

We can conclude that chronic Cu exposure affected C. *gariepinus* health and productivity by inducing growth retardation, neuro-ethology disorders, serum biochemical alterations, and reduced survivability of the exposed fish. The stress and apoptotic gene were over-expressed with lower expression of the anti-apoptotic gene in the Cu-exposed fish. Upon feeding the Cu-exposed fish on ZnONPs enriched diets, the growth, health, and survivability were enhanced. In addition, modulation of the stress and apoptotic markers was evident upon feeding the ZnONPs-supplemented diets. Interestingly, feeding on ZnONPs-diets, especially 30 mg kg^{-1} level was helpful for mitigating chronic Cu-exposure in *C. gariepinus.* These outcomes will be beneficial for the aquaculture producers to overcome the persisting problem of heavy metal pollution in the aquatic water bodies, which negatively impact the aquaculture species' health and productivity.

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Author Contribution Conceptualization, Methodology, Formal analysis, and Writing—review and editing: S.A.A, R.E.I. and M.E. Writing—original draft preparation: R.E.I. Methodology, Investigation, Data curation, writing—review and editing: E.M.Y, A.A.A, K.Y.F., S.A.E., S.B., T.K., A.T.M., and S.J.D. All authors read the final manuscript and approved the submission.

Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethical Approval The study protocol was authorized by the Egyptian Laws on animal experimentation committee (ZU-IACUC/2/F/223/2023). All experimental procedures were carried out in accordance with international guidelines for the care and use of laboratory animals.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Competing Interests The authors declare no competing interests.

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