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Original Research Article

Dietary copper improves intestinal structural integrity in juvenile grass carp (*Ctenopharyngodon idella*) probably related to its increased intestinal antioxidant capacity and apical junction complex

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ABSTRACT

This research evaluated the effects of copper (Cu) on intestinal antioxidant capacity and apical junctional complex (AJC) in juvenile grass carp. A total of 1080 healthy juvenile grass carp (11.16 ± 0.01 g) were fed six diets including different dosages of Cu, namely 0, 2, 4, 6, 8 mg/kg (Cu citrate [CuCit] as Cu source) and 3 mg/kg ($CuSO_4 \cdot 5H_2O$ as Cu source). The trial lasted for 9 weeks. The findings revealed that dietary optimal Cu supplementation (2.2 to 4.1 mg/kg) promoted intestinal growth, including intestinal length, intestinal length index, intestinal weight, and intestinal somatic index ($P < 0.05$). Furthermore, optimal Cu boosted the intestinal mucosal barrier in juvenile grass carp. On the one hand, optimal Cu reduced diamine oxidase and D-lactate levels in serum ($P < 0.05$), reduced levels of the oxidative damage indicators malondialdehyde, reactive oxygen species (ROS), protein carbonyl, superoxide dismutase ($P < 0.05$), and catalase mRNA levels were elevated ($P < 0.05$), thus boosting intestinal antioxidant capacity, the binding protein Keap1a/1b/Nrf2 signaling pathway might be involved. Optimal Cu had no impact on glutathione peroxidase 1b (*GPx1b*) gene expression ($P > 0.05$). On the other hand, optimal Cu increased intestinal tight junction (TJ) proteins (except for claudin 15b) and adherens junction (AJ) proteins (E-cadherin, α-catenin, β-catenin, nectin and afadin) mRNA levels ($P < 0.05$), which could be connected to the signaling pathway formed by the Ras homolog gene family, member A (RhoA), Rho-associated kinase (ROCK), and myosin light chain kinase (MLCK). Finally, based on serum indicator D-lactate and intestinal oxidative damage index (ROS), Cu requirement (CuCit as Cu source) for juvenile grass carp from initial weight to final weight (from 11 to 173 g) was determined to be 4.14 and 4.12 mg/kg diet, respectively. This work may provide a theoretical foundation for identifying putative Cu regulation pathways on fish intestinal health.

1. Introduction

As an essential micronutrient for animals, copper (Cu) participates in physiological and biochemical reactions in the body in various enzyme forms, and promotes animal growth (Barry et al., 2010). The previous studies observed that optimal Cu boosted growth performance in fish, for example, in juvenile (Ma et al., 2023) and on-growing grass carp (Tang et al., 2013), juvenile Russian sturgeon (Wang et al., 2016), juvenile grouper (Lin et al., 2010), and Nile tilapia (Luo et al., 2020). Fish growth is tightly

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linked to intestinal structural integrity (Wei et al., 2018). Yet, research into the structural integrity of grass carp intestine by Cu has not been reported. Therefore, it is necessary to conduct research on whether Cu improves intestinal structural integrity.

In general, animal intestinal structural integrity is closely linked to intestinal antioxidant capacity (Liu et al., 2021). Reactive oxygen radicals are the most important type of free radicals, which can damage cells when they are in excess (Prasad et al., 2017). Malondialdehyde (MDA) and protein carbonyl (PC) reflect a state of oxidative damage to lipids (Burcham and Kuhan, 1996) and proteins (Van Montfort et al., 2003), respectively, resulting in oxidative stress in the organism. Oxidative stress causes damage to cellular integrity and decreases the antioxidant capacity in the organism (Tie et al., 2022). Superoxide dismutase (SOD) (Harris, 1992) and glutathione peroxidase (GPx) are antioxidant enzymes that play a vital role in antioxidation (Gu and Zhao, 2015). Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that regulates the transcription of antioxidant systems, and is essential for maintaining cellular redox homeostasis; it is negatively controlled by Kelch-like ECH-associated protein 1 (Keap1) (Tonelli et al., 2018). At present, there are few reports on the antioxidant capacity of Cu in fish intestine. Some research conducted revealed that Cu increased antioxidant enzyme activities such as copper-zinc superoxide dismutase (CuZnSOD) of on-growing grass carp (Tang et al., 2013), juvenile Russian sturgeon (Wang et al., 2016), juvenile grouper (Lin et al., 2010), and Nile tilapia (Luo et al., 2020). Moreover, a study indicated that dietary Cu increased selenium levels in rat hepatocytes (Schwarz et al., 2023), and optimal selenium levels upregulated *Nrf2* mRNA abundance in spleen of grass carp (Zheng et al., 2018). Thus, Cu-enhanced antioxidant damage capacity of fish intestine may be attached to the Nrf2 signaling mechanism; however relevant study needs to be conducted.

Small intestinal mucosal villous epithelial cells and intestinal mucosal barrier damage will release large amounts of D-lactate and diamine oxidase (DAO) in serum (Fukudome et al., 2014; Soreng and Levy, 2011). Intestinal epithelial monolayers form trans- and para-cellular barriers, and the paracellular barrier consists of an intercellular apical junctional complex (AJC), regulated mainly by apical junctional proteins and adherens junction (AJ) related proteins. The intestinal barrier's homeostasis is disrupted by tight junction (TJ) proteins, which are mostly cytoplasmic proteins like zonula occludens (ZO) and transmembrane proteins like claudin and occludin (Slifer and Blikslager, 2020). AJ consist of transmembrane adhesion molecules whose extracellular structural domains form intercellular junctions with neighboring cells (Mack and Georgiou, 2014). AJ consist of clusters of calmodulin (cadherin) and adhesin (nectin), linked together and coordinated by actin filaments (Indra et al., 2013). The AJC barrier can be regulated by a signal transduction cascade, which requires activation of phosphorylated myosin light chain kinase (MLCK) and nonmuscle myosin II (NMII) through the Ras homolog gene family, member A/Rho-associated kinase (RhoA/ROCK) signaling pathway leading to actin contraction (Benais-Pont et al., 2003; Wang et al., 2005a). There are no published studies on the effect of Cu on fish intestine AJC. One study found that optimal Cu level reduced zinc levels in patients (Duncan et al., 2015), and zinc deficiency decreased mRNA abundance of intestinal TJ related genes in grass carp (Song et al., 2017). Furthermore, It has been proved that Cu supplementation increased E-cadherin protein levels in oral cancer cells (Lee et al., 2016). Another study indicated that E-cadherin expression was significantly upregulated after the addition of Cu cysteamine compared to the control group (Chen et al., 2022). In addition, Cu may be indispensable for iron transfer and metabolism (Sharp, 2004); previous research demonstrated that iron decreased *MLCK* gene expression in the head kidney, spleen, and skin of on-growing grass carp (Guo et al., 2017). In summary, we hypothesize that Cu may be related to intercellular AJC in juvenile grass carp intestine; this should be explored.

Typically, both inorganic (like $CuSO_4 \cdot 5H_2O$) and organic sources of Cu are used. However, inorganic trace elements affect the palatability of animals, have an unstable structure, are not easily absorbed, affect the acid–base balance of the organism, and in excess can cause toxicity in the organism. Additionally, inorganic trace elements can result in low absorption rate and high emissions, leading to environmental pollution (Dozier Iii et al., 2003). Cu citrate (CuCit), in which citric acid and Cu ions can be combined to form a stable biologically effective compound, is one of the organic Cu sources approved for food fortification. Therefore, the development of feed grade CuCit provides a new safe, effective, and environmentally friendly Cu source for the feed industry (Yan et al., 2015). CuCit with low Cu content was more effective in promoting broiler growth than $CuSO_4 \cdot 5H_2O$ with high Cu content (Pesti and Bakalli, 1996). As a result, the bioavailability of CuCit may be greater than that of $CuSO_4$. There is no study on the comparative effect of CuCit and $CuSO_4 \cdot 5H_2O$ in fish, which is an important guide in the practical production of grass carp culture.

This study used a growth experiment from our early research (Ma et al., 2023) and was part of a larger test that involved determining the effects of Cu on the growth and health of fish. In this work, we built on our team's previous research on the digestion and absorption capacity of grass carp (Tang et al., 2013); we investigated the effects of dietary Cu on the ability of fish intestine to act as an antioxidant, and the impact of Cu on the AJC of the intestine and its potential mechanism. This offers some theoretical support for research into how Cu affects fish intestinal health. For the first time, the optimal Cu level when CuCit as a Cu source in juvenile grass carp was established in this study, and the application effects of $CuSO_4 \cdot 5H_2O$ and CuCit were compared. This has the potential to serve as a crucial foundation for the manufacture and use of CuCit in fish.

2. Materials and methods

2.1. Animal ethics statement

Compilation of all animal care protocols were in line with the Sichuan Agricultural University's Animal Care Advisory Committee (No. MR-2020314085).

2.2. Diet preparation

The experimental diet was the same as in our early research (Ma et al., 2023). The components of the basal diet are listed in Table 1. Casein, gelatin, and wheat gluten constituted protein sources, while fish oil and soybean oil made up fat sources. The Soxhlet exhaustive extraction technique and Kjeldahl method, respectively, were used to assess the crude fat and protein contents of the basal diet. These measurements were done following the accepted methodology (AOAC, 1995). And the calcium and total phosphorus levels were analyzed according to China National Standard (GB/T 6436-2018 and GB/T 6437-2018a, b, respectively). The crude fiber and gross energy were analyzed by a fiber analyzer (A2000I, ANKOM Technology, New York, USA) and oxygen bomb calorimeter (6400, Parr Instrument Company, Moline, USA), respectively, following the method reported by China National Standard (GB/T 6434-2022) and Zhang (2022), respectively. We used two Cu sources: CuCit (34.5%) and $CuSO_4 \cdot 5H_2O$ (24.5%), both from Sichuan Animal Feed Co., Ltd. One Cu-free control group, 4 CuCit groups and 1 $CuSO_4 \cdot 5H_2O$ group were employed in the test. Cu contents in all raw material components of the control group (Cu-free) were

Table 1
Composition and nutrient contents of the basal diet (air-dried basis, g/kg).

Ingredients	Content	Nutrient content	Content
Fish meal (CP, 67.10%)	80.00	n-3 PUFA[6]	10.40
Wheat gluten (CP, 71.53%)	80.00	n-6 PUFA[6]	9.60
Gelatin (CP, 90.18%)	100.00	Available phosphorus[7]	8.40
Amino acid mix[1]	123.30	Total phosphorus[8]	15.48
Fish oil	21.80	Crude protein[8]	324.34
Soybean oil	17.60	Crude lipid[8]	47.87
Corn starch	209.15	Calcium[8]	5.27
α-Starch	240.00	Crude fiber[8]	67.72
Microcrystalline cellulose	50.00	Gross energy, MJ/kg[8]	17.10
NaH_2PO_4	33.00		
Vitamin premix[2]	10.00		
Mineral premix (Cu-free)[3]	20.00		
Choline chloride[4]	10.00		
CuCit/$CuSO_4 \cdot 5H_2O$ premix[5]	5.00		
Butylated hydroxyanisole	0.15		

CP = crude protein; CuCit = Cu citrate; PUFA = polyunsaturated fatty acids.
[1] Provided the following (g/kg of amino acid mix): Lys, 1.22; Met, 0.55; Trp, 0.23; Thr, 0.88; Arg, 0.52; His, 0.54; Leu, 1.33; Ile, 0.80; Phe, 0.62; Tyr, 0.51; Val, 0.91, respectively.
[2] Provided the following (g/kg of vitamin premix): retinyl acetate (1,000,000 IU/g), 0.40; cholecalciferol (500,000 IU/g), 0.32; DL-α-tocopherol acetate (50%), 40.00; menadione (50%), 0.38; cyanocobalamin (1%), 0.94; D-biotin (2%), 1.55; folic acid (95%), 0.38; thiamine nitrate (98%), 0.13; ascorbic acid (95%), 16.32; niacin (99%), 2.58; inositol (97%), 22.06; calcium-D-pantothenate (98%), 3.85; riboflavin (80%), 0.78; pyridoxine hydrochloride (98%), 0.62. All ingredients were diluted with corn starch to 1 kg.
[3] Provided the following (g/kg of mineral premix): $MnSO_4 \cdot H_2O$ (31.8% Mn), 3.07; $MgSO_4 \cdot H_2O$ (15.0% Mg), 237.83; $FeSO_4 \cdot H_2O$ (30.0% Fe), 12.25; $ZnSO_4 \cdot H_2O$ (34.5% Zn), 7.68; selenium yeast (0.2% Se) 13.65; $Ca(IO_3)_2$ (3.2% I), 1.56. All ingredients were diluted with corn starch to 1 kg.
[4] Provided the following (g/kg of choline chloride premix): choline chloride (50%), 306.71, and the rest was diluted with corn starch to 1 kg.
[5] CuCit/$CuSO_4 \cdot 5H_2O$ premix provided the following Cu per kilogram diet for the treatments: 1.0 (Cu-free) and 2.2, 4.1, 6.2, 8.1 and 3.2 mg/kg, and the rest was diluted with microcrystalline cellulose.
[6] n-3 PUFA, and n-6 PUFA were calculated by NRC (2011) and their contents were referenced to Zeng et al. (2016).
[7] Available phosphorus was calculated according to NRC (2011).
[8] Crude protein, crude lipid, total phosphorus, calcium, crude fiber and gross energy were measured values.

measured using atomic absorption spectrometry (CONTAA700, Analytik Jena AG, Germany) and calculated to be 0.96 mg/kg diet. Graded contents of Cu were added to the basal diet to obtain 2.00, 4.00, 6.00, 8.00 mg/kg CuCit and 3.00 mg/kg $CuSO_4 \cdot 5H_2O$ diet. The Cu contents were 1.0 (Cu-free), 2.2, 4.1, 6.2, 8.1 and 3.2 mg/kg in the six treatments, respectively, which were measured using atomic absorption spectrometry. The diets were prepared using the procedure outlined by Mai et al. (2009). Simply said, the raw materials were crushed into a fine powder and passed through a 300-μm sieve. The pre-mixed powder was thoroughly mixed with water, then extruded with an extruder, and dried naturally. In accordance with earlier procedures, the feeds (diameter: 2 mm) were divided into smaller pieces, sieved into pellets, and kept at −20 °C (Tang et al., 2013).

2.3. Feeding management

Trial juvenile fish (1 to 11 g) were purchased from a nearby farm (Tongwei, Chengdu, China). Microscopic examination showed that the gills, hepatopancreas, and intestines were normal. They were fed 6 times a day at 06:00, 08:00, 11:00, 13:00, 15:00, and 19:00 in 3 outdoor freshwater ponds. They received a commercial feed (crude protein content of commodity feed ≥36%, crude fat content ≥5%) before the trial to acclimate to the experimental environment. In the early stage of domestication, we slowed down the feeding speed to allow the grass carp to get used to artificial feeding, followed by a 2-week Cu-free diet to reduce Cu concentration (Tang et al., 2013). The initial mean weight of 1080 fish was 11.16 g/fish; these fish were then randomly placed into 3 ponds (18 nets, 150 cm × 150 cm × 150 cm, 60 fish each net) and separated into 6 treatments with 3 replicates per treatment. In order to collect any leftover feed, we attached an 80 cm-diameter disc with 1 mm-thick gauze to the bottom of each cage, as stated by our early research (Wu et al., 2017). The fish were fed matching experimental meal at 4% of their initial body weight (08:00, 11:00, 13:00, 15:00, and 19:00) (Wang et al., 2005b). Following a 30-min feeding, the remaining feed was removed, dried, and weighed to determine feed intake on a dry matter basis by the technique outlined by Cai et al. (2005). The trial lasted for 9 weeks. In each pool, water purified through a sand filter was filled to remove contaminants from the culture water and lower the ammonia concentration (Wu et al., 2017). Microporous aeration was used throughout the test period, and water quality was assessed and modified daily. The pH was found to be 7.5 ± 0.4, water temperature was 27.8 ± 3 °C, and dissolved oxygen was ≥6.0 mg/L. Atomic absorption was used to calculate Cu content of culture water, which was found to be 5 μg/L.

2.4. Sample collection and analysis

Following the feeding trial, all fish were starved for 24 h before being sedated with a benzocaine water bath (50.0 mg/L). Fish tail venous blood was isolated based on Wang et al. (2015), and supernatant was then removed after centrifugation and kept at −80 °C. Fish were euthanized to get intestinal samples. After the visceral mass was removed, the intestinal tract was isolated, intestinal contents were extruded with tweezers, fat around the mesentery was cleaned as much as possible, the intestinal tract was weighed, intestinal length (IL) was measured, and the fish intestines were divided into proximal intestine (PI), mid intestine (MI) and distal intestine (DI). The length from sphincter to the first turn was used to define the PI segment, and distance from the last turn to the anus was used to define the DI segment according to the measurement described by Ni and Wang (1999). For section examination, the intestinal samples were kept in 4% paraformaldehyde. Intestinal tissue was frozen with liquid N_2, and then kept at −80 °C to analyze protein, mRNA, and enzyme levels.

2.5. Hematoxylin and eosin (H&E) staining

The 4% paraformaldehyde-treated intestinal tissue was embedded in paraffin wax, cut into 4-μm slices, stained with H&E (Lilai Biotechnology Co., Ltd., Chengdu, China), examined and captured on camera using a Nikon TS100 optical microscope (Nikon, Tokyo, Japan).

2.6. Biochemical analysis

The quantities of D-lactic acid and DAO in the serum were measured using ELISA kits provided by Beijing Qisong Biotechnology Co., Ltd. A total of 100 mg intestinal tissue was homogenized in 0.9% sterile saline (1:10, wt/vol), then the samples were centrifuged at 6000 × *g* for 20 min at 4 °C. The supernatant was collected to acquire intestinal tissue homogenates for assessing intestinal oxidative damage indicators (reactive oxygen species [ROS], MDA, PC) and antioxidant-related enzymes (SOD and GPx) (Zhang et al., 2022). All the kits for antioxidant indices were purchased from Nanjing Jiancheng Bioengineering Institute.

2.7. Real-time quantitative PCR analysis

The real-time PCR experiment was carried out utilizing the method outlined by Ma et al. (2020). Intestinal tissue was processed to isolate total RNA using a RNAiso Plus kit (Takara, Dalian, China). After that, DNAse I was applied to total RNA. Electrophoresis on 1% agarose gels and Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) were used to measure the quality and quantity of RNA (A260/280 ratio). Reverse RNA to cDNA transcription was also carried out using the PrimeScript RT kit (TaKaRa, Dalian, China). The Sus-Scrofa sequences in the NCBI database were utilized to produce the primers, which were made by Bioengineering (Shanghai) Co., Ltd. using Primer 5.0 software. Table S1 contains a list of the primers. Based on selection, the endogenous control was β-actin. The amplification efficiencies of target gene and housekeeping gene were measured according to the melting curves. After confirming amplification efficiency of the primers was about 100%, effects on mRNA abundance were quantified using $2^{-\Delta\Delta Ct}$ technology.

2.8. Western blotting

To analyze Western blotting, an earlier technique was used (Dong et al., 2018). Total intestinal protein was extracted using RIPA lysis buffer (Beyotime, Shanghai, China), and protein concentrations were determined using a BCA assay kit (5000001, Bio-Rad, Hercules, CA, USA) (Zhao et al., 2022). The target proteins (40 g each sample) were then separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes by a wet Trans-Blot system (Bio-Rad, Hercules, USA). The PVDF membranes were blocked for 2 h at room temperature, and primary antibodies (nuclear *Nrf2*, *MLCK*, *ROCK* and β-actin) were incubated on them overnight at 4 °C. Nuclear Nrf2 was referenced using the Lamin B antibody, and MLCK and ROCK were referenced using β-actin. ABclonal Technology Co., Ltd supplied these antibodies. The antibodies significantly cross-reacted with grass carp protein after testing. Subsequently, the blots were treated with an enzyme-labeled secondary antibody (goat anti-rabbit, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:8000 dilution) for 2 h after being washed 3 times in Tris—HCl buffer salt solution with Tween (TBST). Finally, enhanced chemiluminescence kit (Beyotime, Shanghai, China) was used for imaging, and Image Lab 6.1 was used to visualize the immunological complex.

2.9. Immunohistochemistry (IHC)

For IHC staining, intestinal samples were fixed in 4% paraformaldehyde buffer. The procedure described previously by the research using immunohistochemistry technique was used (Zhao et al., 2022). In short, sectioned samples were made, the antigen was microwave-repaired, and then the primary antibodies (ZO-1 and occludin; Ebtek Biotechnology Co., Ltd.) were incubated overnight. Following secondary antibody incubation, DAB and hematoxylin staining were performed on the sections. Ultimately, photos were obtained and studied under a light microscope (TS100, Nikon, Tokyo, Japan) after all slices had been dehydrated, made transparent, and sealed with neutral glue. The expressions of the important antibodies ZO-1 and occludin were assessed using integrated optical density (IOD) in Image Pro Plus (Version 5.0, Rockville, MD, USA).

2.10. Statistical analysis

All data results were expressed as mean ± standard deviation, and univariate analysis of variance (ANOVA) was performed using IBM SPSS Statistics 21.0 (SPSS Inc., Chicago, IL, USA). Combined with Duncan method, Cu-free and CuCit groups were compared for multiple comparisons. The Cu-free group vs. $CuSO_4 \cdot 5H_2O$ group, and CuCit optimal group vs. $CuSO_4 \cdot 5H_2O$ group were tested by independent sample *t*-test. $P < 0.05$ was used as the significance level. According to different indices, the dietary Cu requirement of juvenile grass carp was determined by quadratic regression model. The following are the formulas for determining the intestinal length index (ILI) and the intestinal somatic index (ISI):

$$\text{ILI}\ (\%) = 100 \times \text{intestine length (cm)/body length (cm)},$$

$$\text{ISI}\ (\%) = 100 \times \text{intestine weight (g)/body weight (g)}.$$

3. Results

3.1. Intestinal growth

Table 2 reveals the effect of Cu on the intestinal growth of juvenile grass carp. The IL and ISI increased significantly ($P < 0.05$) with rising Cu content and reached a plateau in the 4.1 mg/kg CuCit group. Compared to the Cu-free group, ILI raised sharply ($P < 0.05$) in the 2.2 mg/kg CuCit group, however, among the other groups, the difference was not statistically significant ($P > 0.05$). Intestinal weight (IW) increased markedly ($P < 0.001$) with rising Cu content and reached a maximum in the 4.1 mg/kg CuCit group, and then declined markedly with higher content. When compared to the $CuSO_4 \cdot 5H_2O$ group, the IW was considerably higher in the 4.1 mg/kg CuCit group ($P < 0.05$).

3.2. Serum D-lactate, DAO, and apparent morphology

According to Fig. 1, in juvenile grass carp serum, DAO activity and D-lactate content dramatically dropped ($P < 0.001$) as Cu content rose up to 2.2 mg/kg CuCit, and then increased. Compared with the $CuSO_4 \cdot 5H_2O$ group, D-lactate concentration in the 2.2 mg/kg CuCit group was considerably lower ($P < 0.05$). Effect of Cu addition on the apparent intestinal morphology is shown in Fig. 2, where intestinal fold height was dramatically increased by dietary Cu supplementation when compared to the Cu-free group ($P < 0.001$).

3.3. Intestinal antioxidants

3.3.1. Oxidative damage indicators and antioxidant-related enzymes

Figure 3 indicates effects of dietary Cu on intestinal antioxidant indices. The outcomes demonstrated that compared to the Cu-free group, ROS, MDA, and PC contents were notably lowered ($P < 0.001$) with rising Cu content. Compared with the $CuSO_4 \cdot 5H_2O$ group, ROS and MDA levels were significantly decreased in the 2.2 and 4.1 mg/kg CuCit groups ($P < 0.001$). When Cu contents were raised to 2.2 and 4.1 mg/kg diet, SOD activity dramatically increased ($P < 0.001$), and then stabilized at higher levels. Compared to the Cu-free group, GPx activity increased significantly with rising Cu content ($P < 0.001$); the highest GPx activity was observed when Cu content reached 2.2 mg/kg diet.

Table 2
Effects of dietary copper (Cu) on intestinal growth and development of juvenile grass carp (*Ctenopharyngodon idella*).

Item	Dietary Cu levels, mg/kg diet						*P*-value	
	Cu-free	CuCit				$CuSO_4·5H_2O$		
	1.0	2.2	4.1	6.2	8.1	3.2	P_L	P_Q
IL, cm	27.34 ± 1.92[a]	28.26 ± 0.55[a]	34.66 ± 2.73[b]	29.08 ± 0.54[a]	26.68 ± 0.50[a]	31.64 ± 1.39[*]	0.936	0.014
ILI, %	142.75 ± 7.43[a]	167.09 ± 14.98[b]	157.73 ± 9.56[ab]	142.23 ± 6.31[a]	142.26 ± 3.83[a]	161.92 ± 9.33	0.299	0.077
IW, g/fish	1.24 ± 0.23[a]	2.14 ± 0.06[b]	3.68 ± 0.13[d]	2.69 ± 0.19[c]	1.87 ± 0.09[b]	2.34 ± 0.12[*#]	0.266	<0.001
ISI, %	1.45 ± 0.08[a]	1.65 ± 0.07[b]	1.85 ± 0.10[c]	1.73 ± 0.11[bc]	1.62 ± 0.06[b]	1.69 ± 0.08	0.145	<0.001

CuCit = Cu citrate; IL = intestinal length; ILI = intestinal length index; IW = intestinal weight; ISI = intestinal somatic index; P_L = *P*-value of linear analysis; P_Q = *P*-value of quadratic analysis.
Values are means ± SD, $n = 3$ (for 3 replicate groups, 6 fish per replicate).
[a–d]Values within the same row with different letters are significantly different ($P < 0.05$). The asterisk (*) indicates a significant difference between Cu-free and $CuSO_4·5H_2O$ groups ($P < 0.05$); the number sign (#) indicates a significant difference between CuCit optimal and $CuSO_4·5H_2O$ groups ($P < 0.05$).

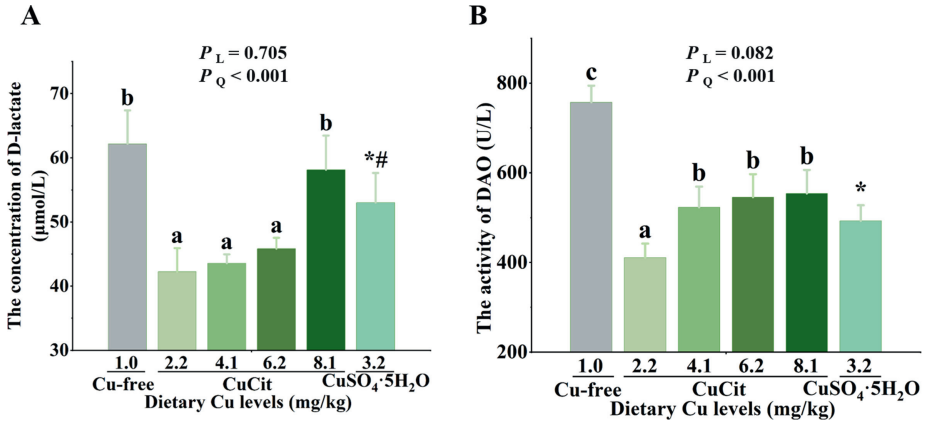

Fig. 1. Effects of dietary copper (Cu) on intestinal mucosal permeability in juvenile grass carp. CuCit = Cu citrate; DAO = diamine oxidase; P_L = *P*-value of linear analysis; P_Q = *P*-value of quadratic analysis. Values are means ± SD, $n = 3$ (for 3 replicate groups, 2 fish per replicate). [a–c]Bars with different letters are significantly different ($P < 0.05$). The asterisk (*) indicates a significant difference between Cu-free and $CuSO_4·5H_2O$ groups ($P < 0.05$); the number sign (#) indicates a significant difference between CuCit optimal and $CuSO_4·5H_2O$ groups ($P < 0.05$).

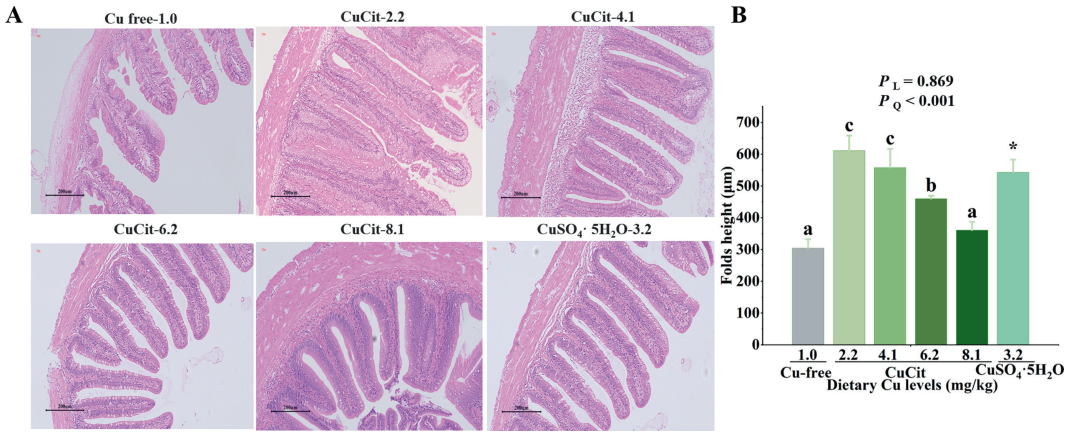

Fig. 2. The intestinal histology of juvenile grass carp fed diets containing graded levels of copper (Cu) for 9 weeks. (A) The morphology and structure of the mid intestine (MI). The sections were stained with hematoxylin–eosin (H&E) and observed at 10× original magnification. Scale bar = 200 μm. (B) The quantitative analysis of the fold height of the intestine. Fold height is expressed in micron. CuCit = Cu citrate; P_L = *P*-value of linear analysis; P_Q = *P*-value of quadratic analysis. Data represent means ($n = 3$), and error bars indicate SD. [a–d]Bars with different letters are significantly different ($P < 0.05$). The asterisk (*) indicates a significant difference between Cu-free and $CuSO_4·5H_2O$ groups ($P < 0.05$).

3.3.2. Antioxidant-related mRNA expression levels and protein abundance

Figure 4 indicates that *CuZnSOD*, manganese superoxide dismutase (*MnSOD*), catalase (*CAT*), *GPx1a*, *GPx4a*, *GPx4b* and *Nrf2* mRNA abundances were significantly upregulated ($P < 0.05$) in fish intestine and then gradually decreased. *CuZnSOD* and *GPx4a* mRNA abundances were significantly higher in the 4.1 mg/kg CuCit group compared to the $CuSO_4·5H_2O$ group ($P < 0.05$). With rising Cu contents, *Keap1a* and *Keap1b* mRNA levels in the intestine were significantly downregulated ($P < 0.05$), reaching the lowest value in the 4.1 mg/kg CuCit group. Compared to the $CuSO_4·5H_2O$ group, *Keap1b* mRNA abundance was significantly lower at 4.1 mg/kg Cu ($P < 0.05$). However, there was no effect of dietary Cu content on *GPx1b* mRNA abundance. In addition, the protein level of nuclear

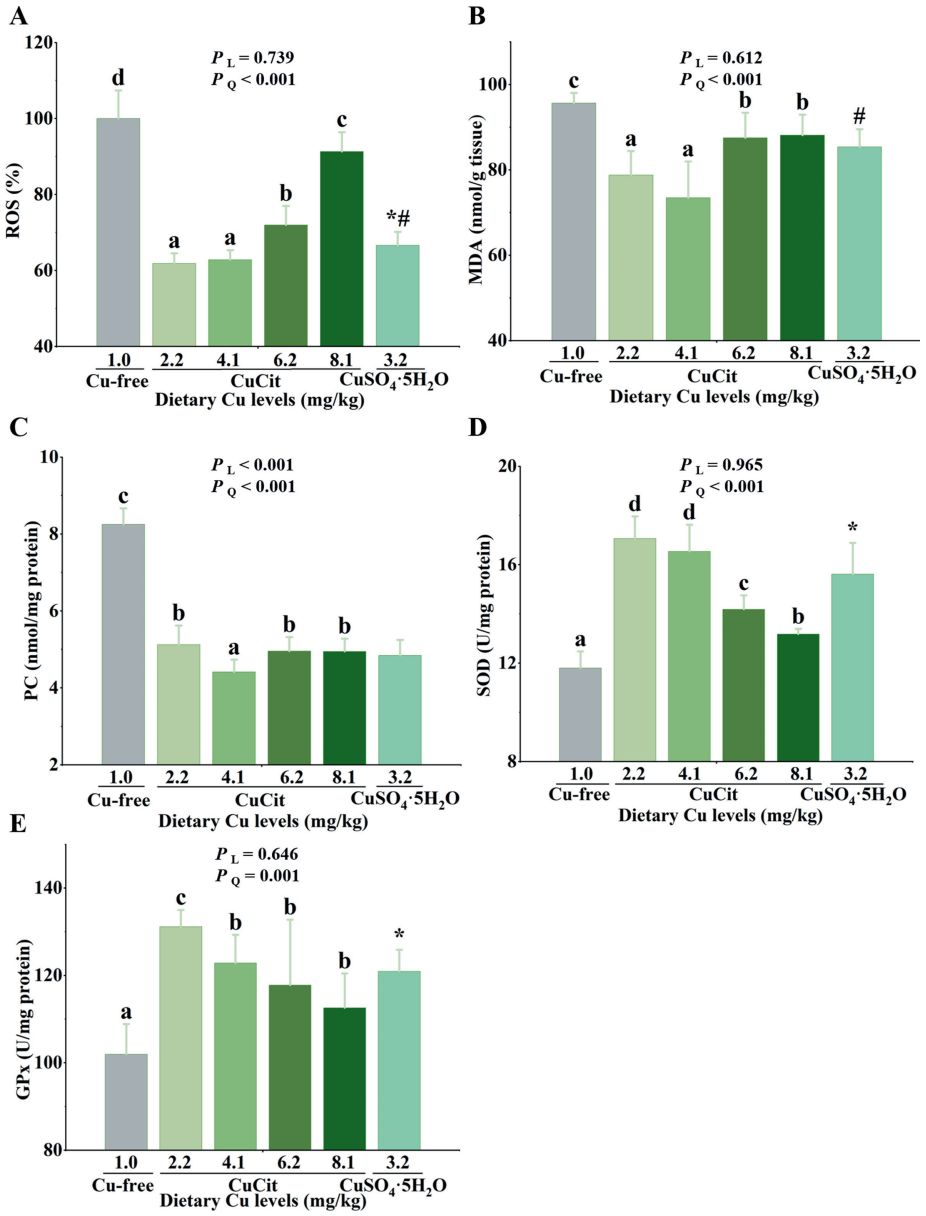

Fig. 3. Effects of dietary copper (Cu) on intestinal antioxidant-related indices in juvenile grass carp. CuCit = Cu citrate; ROS = reactive oxygen species; MDA = malondialdehyde; PC = protein carbonyl; SOD = superoxide dismutase; GPx = glutathione peroxidase; P_L = *P*-value of linear analysis; P_Q = *P*-value of quadratic analysis. Values are means ± SD, $n = 3$ (for 3 replicate groups, 2 fish per replicate). $^{a-d}$Bars with different letters are significantly different ($P < 0.05$). The asterisk (*) indicates a significant difference between Cu-free and $CuSO_4 \cdot 5H_2O$ groups ($P < 0.05$); the number sign (#) indicates a significant difference between CuCit optimal and $CuSO_4 \cdot 5H_2O$ groups ($P < 0.05$).

Nrf2 increased markedly ($P < 0.05$) with rising Cu content up to 4.1 mg/kg and decreased thereafter, and was significantly higher ($P < 0.05$) at 4.1 mg/kg CuCit group compared to the $CuSO_4 \cdot 5H_2O$ group.

3.4. Intestinal AJC-related parameters

3.4.1. TJ and AJ mRNA expression levels and IHC analysis

Figure 5 shows the effects of Cu on intestinal TJ-related proteins in juvenile grass carp. Compared with the Cu-free group, *ZO-1*, occludin, and claudin-b, -c, -7a, -7b, and -11 mRNA abundance increased significantly before reaching 4.1 mg/kg dietary Cu content ($P < 0.05$), and then gradually decreased. Claudin-12 mRNA levels gradually decreased and reached a minimum at 4.1 mg/kg Cu content ($P < 0.05$), and the value at this time was notably lower than in the $CuSO_4 \cdot 5H_2O$ group ($P < 0.05$). Claudin-f mRNA level tended to increase as Cu content increased to 4.1 mg/kg and gradually lowered at higher contents. Claudin-15a mRNA abundance tended to decrease as Cu content increased to 4.1 mg/kg and were gradually up-regulated at higher levels. However, there was no significant effect of dietary Cu on claudin-15b mRNA abundance ($P > 0.05$). The mRNA abundances of intestinal AJ-associated protein in juvenile grass carp are also shown in Fig. 5A. Compared to the Cu-free group, E-cadherin and β-catenin mRNA abundance were significantly higher ($P < 0.05$) as dietary Cu content reached 4.1 mg/kg. The gene expression of α-catenin and nectin increased with dietary Cu addition from 2.2 mg/kg to 4.1 mg/kg, showing an increasing trend, and then gradually decreased at higher contents. Compared to the Cu-free group, afadin mRNA abundance was significantly up-regulated ($P < 0.05$) as Cu content increased from

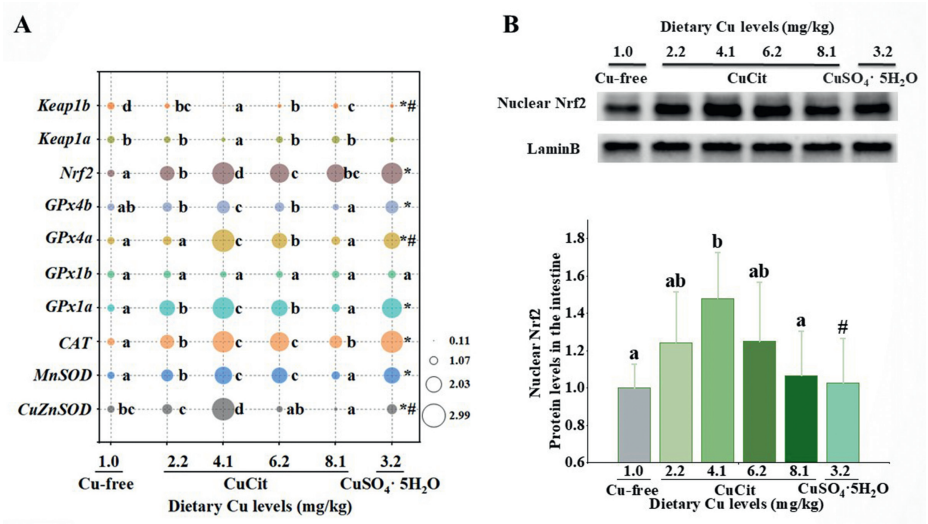

Fig. 4. Effects of dietary copper (Cu) on intestinal antioxidant-related parameters of juvenile grass carp. (A) The relative mRNA expression levels of genes; (B) the relative protein levels of nuclear Nrf2. CuCit = Cu citrate; Nrf2 = nuclear factor erythroid 2-related factor 2; Keap1 = Kelch-like ECH-associated protein 1; *GPx* = glutathione peroxidase; *CAT* = catalase; *MnSOD* = manganese superoxide dismutase; *CuZnSOD* = copper-zinc superoxide dismutase. Data represent means, $n = 3$ (for 3 replicate groups, 2 fish per replicate), and error bars indicate SD. $^{a-d}$Dots or bars with different letters are significantly different ($P < 0.05$). The asterisk (*) indicates a significant difference between Cu-free and $CuSO_4 \cdot 5H_2O$ groups ($P < 0.05$); the number sign ($^{\#}$) indicates a significant difference between CuCit optimal and $CuSO_4 \cdot 5H_2O$ groups ($P < 0.05$).

2.2 mg/kg to 4.1 mg/kg. In addition, compared to the $CuSO_4 \cdot 5H_2O$ group, mRNA level of β-catenin was notably higher ($P < 0.05$) at 4.1 mg/kg dietary Cu content.

Figure 5 shows the effects of Cu on TJ (ZO-1 and occludin) of fish intestinal epithelial cells detected by immunohistochemistry. Compared to the Cu-free group, the IOD value of ZO-1 and occludin were significantly increased in the 4.1 mg/kg CuCit group ($P < 0.05$), respectively, and both were significantly higher than in the $CuSO_4 \cdot 5H_2O$ group at this time ($P < 0.05$).

3.4.2. The mRNA expression levels and protein abundances for intestinal AJC-related signal molecules

As can be seen from Fig. 6, compared to the Cu-free group, *NMII*, *RhoA*, *ROCK* and *MLCK* mRNA abundance were significantly declined ($P < 0.05$) as dietary Cu content increased to 4.1 mg/kg in fish intestine, respectively. When Cu content reached 2.2 mg/kg diet, ROCK protein expression started to decline ($P < 0.05$). In addition, at 4.1 mg/kg Cu content, MLCK protein expression was strongly down-regulated ($P < 0.05$) and then up-regulated.

4. Discussion

4.1. Dietary Cu promoted growth and development in fish intestine

A growth trial was used in this investigation as part of our earlier work, which found that Cu increased muscle growth in juvenile grass carp (Ma et al., 2023). The IL, IW, ILI, and ISI can reflect the growth and development of intestine well (Tang et al., 2013). Our findings indicated that optimal Cu content (2.2 to 4.1 mg/kg) enhanced IL, ILI, IW, and ISI of juvenile grass carp, indicating that Cu boosted growth and development of fish intestine. The same results were obtained for on-growing grass carp (Tang et al., 2013). We hypothesized this might be due to its ability to promote structural integrity of the intestine, so we further verified this.

4.2. Dietary Cu boosted physical barrier function in fish intestine

Serum D-lactate and DAO can reflect intestinal mucosal barrier function (Karabulut et al., 2013; Qing et al., 2019). Intestinal fold height is one of the important indicators of intestinal health in fish, and the higher fold height, the better the ability to absorb nutrients (Kotze and Soley, 1995). Our results found that compared to Cu-free treatment, serum D-lactate content and DAO activity were significantly lower and intestinal fold height was significantly higher at 2.2 mg/kg Cu level, and both were superior to the $CuSO_4 \cdot 5H_2O$ group at this time. This indicates that Cu could enhance physical barrier function in fish intestine. As previously stated, cellular, and intercellular structural integrity are key factors in intestinal physical barrier function. Therefore, the effects of Cu on intestine cellular structural integrity and intercellular structural integrity in fish and its possible mechanisms were the subject of our next investigation.

4.3. Dietary Cu enhanced antioxidant ability in fish intestine

Antioxidant capacity to prevent damage is largely responsible for intestinal cell structural integrity, which is closely related to their antioxidant defense system (Dai et al., 2023). Low levels of ROS are essential for biological functions like cell development, proliferation, differentiation, and survival (Gupta et al., 2020). MDA content can reflect lipid oxidation degree (Lee et al., 2019). PC can be converted to disulfide bonds during oxidation, resulting in a decrease in sulfhydryl content and leading to a decrease in antioxidant activity (Van Montfort et al., 2003). Our findings indicated that optimal Cu content reduced PC and MDA in fish intestine, indicating that Cu reduced oxidative damage in fish intestine. The diminished oxidative damage is partly due to the increased antioxidant capacity (Wang et al., 2015). Previous research in our laboratory showed that Cu increased antioxidant enzymatic activities like SOD, CAT, and GPx in intestine of on-growing grass carp (Tang et al., 2013). Our findings suggested that optimal Cu level increased SOD and GPx enzymatic activities in the fish intestine, further confirming the prior results. However, enzyme activity is closely related to its mRNA level (Jiang et al., 2016). As a result, we further investigated the effect of Cu on mRNA abundance of antioxidant enzymes in juvenile grass carp. The results revealed that optimal Cu level raised *MnSOD*, *CuZnSOD*, *GPx4a*, *GPx4b*, and *CAT* mRNA abundance in the fish intestine, further confirming that Cu could

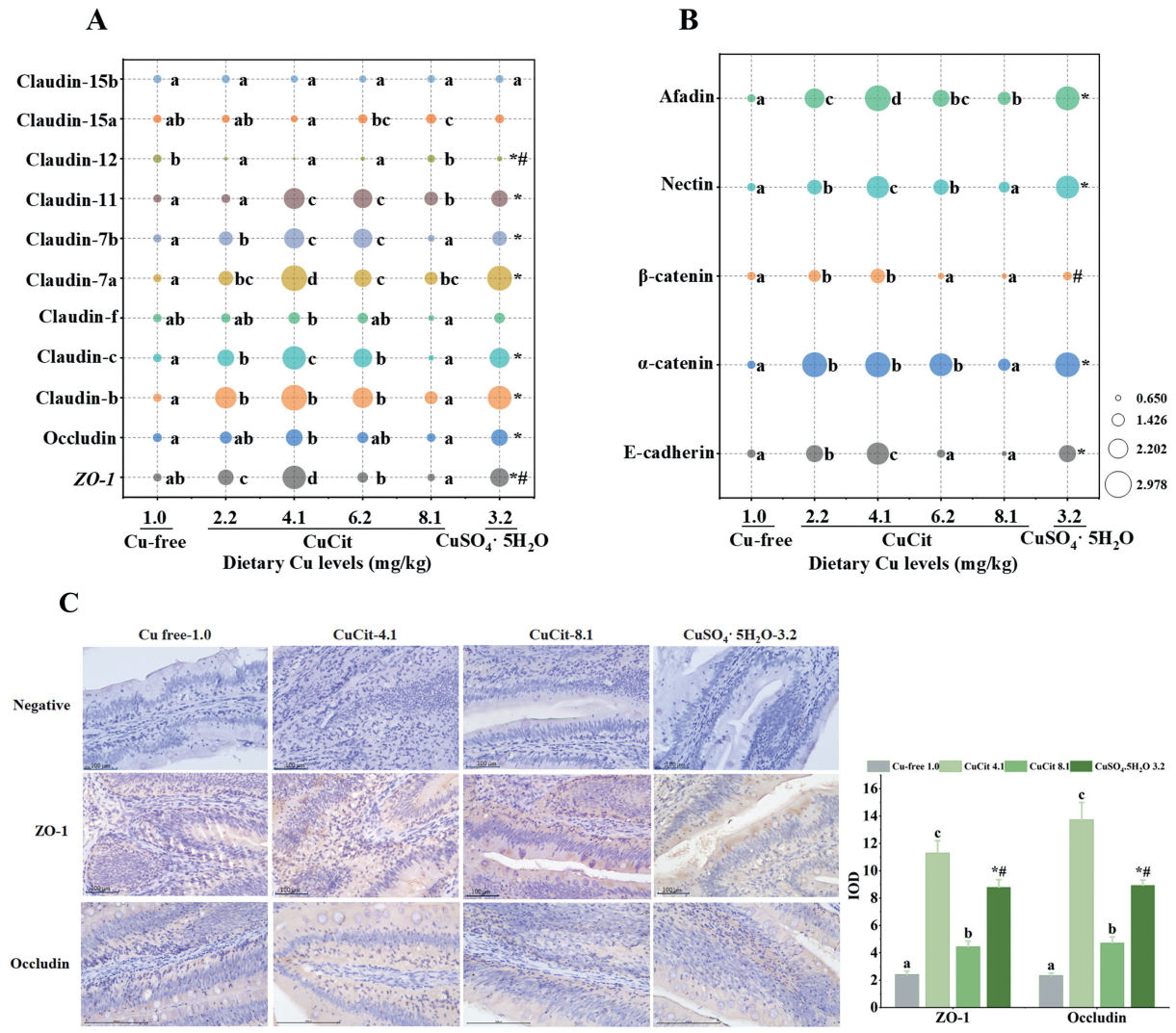

Fig. 5. Effects of dietary copper (Cu) on intestinal tight junction (TJ) and adherens junction (AJ) related parameters of juvenile grass carp. (A and B) The relative mRNA expression levels of genes. Data represent means, $n = 3$ (for 3 replicate groups, 2 fish per replicate), and error bars indicate SD. (C) ZO-1 and occludin protein levels of the mid intestine (MI) in the CuCit 1.0, 4.1, 8.1 mg/kg groups and $CuSO_4 \cdot 5H_2O$ 3.2 mg/kg group. The sections were observed at 400× original magnification. Scale bar = 100 μm. Quantification of the positive area as revealed by Image Pro Plus 5.0. Data represent means ($n = 3$), and error bars indicate SD. CuCit = Cu citrate; ZO-1 = zonula occludens protein 1; IOD = integrated optical density. $^{a-d}$Dots or Bars with different letters are significantly different ($P < 0.05$). The asterisk (*) indicates a significant difference between Cu-free and $CuSO_4 \cdot 5H_2O$ groups ($P < 0.05$); the number sign ($^{\#}$) indicates a significant difference between CuCit optimal and $CuSO_4 \cdot 5H_2O$ groups ($P < 0.05$).

increase intestinal antioxidant enzymatic activities through their mRNA levels, thereby improving the antioxidant capacity of intestine of juvenile grass carp. An evolutionary conserved system that guards against oxidative stress in cells is the Keap1-Nrf2 pathway. Keap1 interacts with Nrf2 in steady-state circumstances and causes its rapid proteasomal degradation, and Keap1 is regarded as a "stress sensor" and an inhibitor of Nrf2 activation (Nguyen et al., 2020). Our findings indicated that optimal Cu content upregulated *Nrf2* mRNA abundance, downregulated *Keap1a* and *Keap1b* mRNA abundance, and increased nuclear Nrf2 protein levels in juvenile grass carp intestine. These findings suggested that Cu could enhance antioxidant enzyme-related gene expression and enzyme activity, which might be due to the Keap1/Nrf2 signaling pathway (Slifer and Blikslager, 2020).

Interestingly, we found no effect of dietary Cu on mRNA abundance of intestinal *GPx1b* in juvenile grass carp, which might be due to threonine nutrient interactions in the organism. It was shown that dietary Cu promoted threonine biosynthesis in Pacific white shrimp hepatopancreas (Shi et al., 2021). In contrast, threonine had no effect on the gene expression of *Gpx1b* in the gill of juvenile grass carp (Dong et al., 2018). Therefore, we speculate that Cu might increase intestinal threonine level in fish, while threonine resulted in no effect on the *GPx1b* mRNA abundance, but this conjecture needs further study.

4.4. Dietary Cu enhanced the structure of fish intestinal AJC

The paracellular barrier consists of a highly organized intercellular junctional complex regulated mainly by apical TJ proteins and AJ-related proteins, while dysregulation of tight junctional proteins such as ZO-1, occludin, and claudins disrupts homeostasis of the intestinal barrier (Slifer and Blikslager, 2020). Our findings stated that optimal Cu content up-regulated claudin-b, -c, -7a, -7b, and -11 mRNA abundance and down-regulated claudin-12 mRNA abundance in juvenile grass carp intestine. AJ is located below the TJ and can link cells with nearby cells, mainly acting on intercellular adhesion, and acting together with TJ to promote AJC structural integrity (Ohta et al., 2014). Previous study in grass carp showed

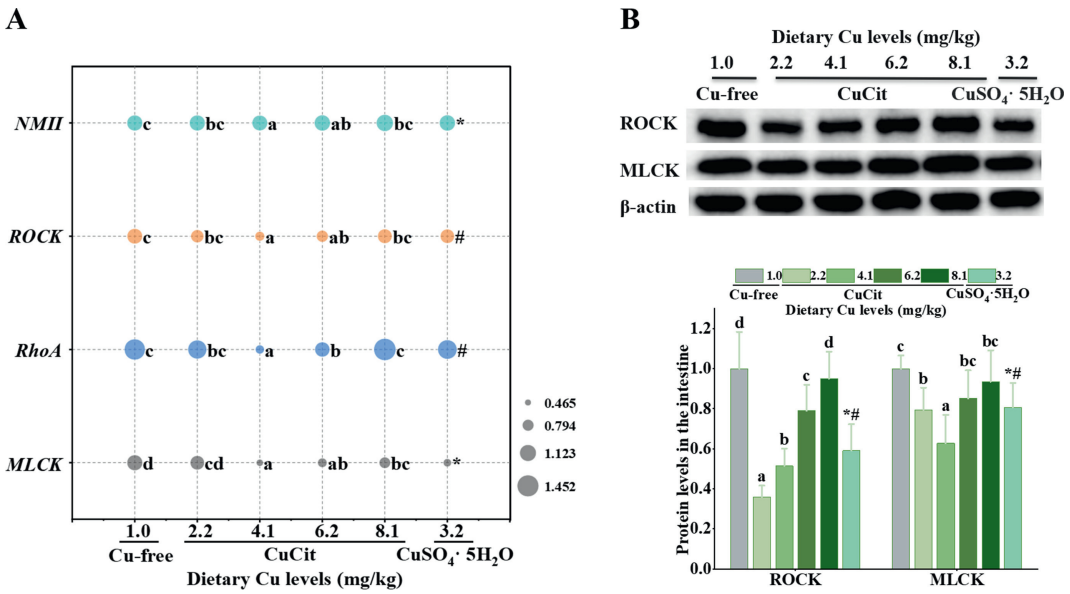

Fig. 6. Effects of dietary copper (Cu) on intestinal apical junctional complex (AJC) related parameters of juvenile grass carp. (A) The relative mRNA expression levels of genes; (B) the relative protein levels of Rho-associated protein kinase (ROCK) and myosin light chain kinase (MLCK). CuCit = Cu citrate; *NMII* = nonmuscle myosin II; *RhoA* = Ras homolog gene family member A. Data represent means, $n = 3$ (for 3 replicate groups, 2 fish per replicate); and error bars indicate SD. $^{a-d}$Dots or bars with different letters are significantly different ($P < 0.05$). The asterisk (*) indicates a significant difference between Cu-free and $CuSO_4 \cdot 5H_2O$ groups ($P < 0.05$); the number sign ($^{\#}$) indicates a significant difference between CuCit optimal and $CuSO_4 \cdot 5H_2O$ groups ($P < 0.05$).

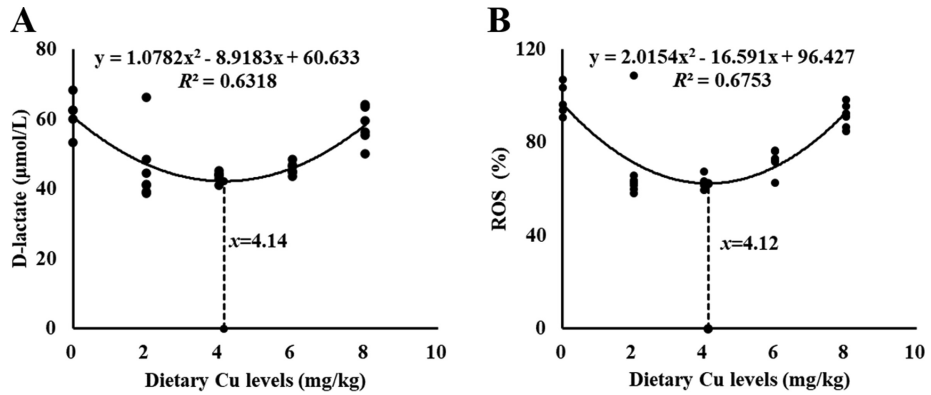

Fig. 7. Quadratic regression analyses of (A) serum D-lactate and (B) intestinal reactive oxygen species (ROS) for juvenile grass carp fed diets containing graded levels of copper (Cu).

that upregulation of gene levels of intestinal AJ (like E-cadherin and β-catenin) increased AJ structural compactness (Liu et al., 2021). Our findings stated that optimal Cu content upregulated α-catenin, β-catenin, E-cadherin, nectin, and afadin mRNA abundance in fish intestine compared with the Cu-free group, indicating that Cu enhanced the integrity of the fish intestinal AJ, which might be related to NAD (P) H: quinone oxidoreductase 1 (NQO1). Previous study showed that Cu upregulated NQO1 protein levels in duck kidney tubular epithelial cells (Fang et al., 2021), whereas AJ-related protein (E-cadherin) was upregulated by NQO1 in human breast cancer cells (Yang et al., 2017). Therefore, we speculate that Cu improvement in AJ integrity may be due to NQO1, but this needs to be explored further.

Interestingly, we discovered individual variations in the effect of Cu on the abundance of the mRNA of several TJ-related proteins. There was no marked difference in claudin-15b mRNA abundance by Cu; this might be connected to the interaction between Cu and phosphorus in vivo. It has been shown that Cu and phosphorus interact in vivo after supplementing the rations of cows with Cu and phosphorus (Saxena et al., 2010). Moreover, previous study in our laboratory suggested that phosphorus had no impact on claudin-5b mRNA abundance in grass carp intestine (Song et al., 2017). However, this speculation has still to be verified.

MLCK was very important in the process of intestinal mucosal barrier damage and repair (Miao et al., 2016). Previous research showed that inhibition of MLCK could downregulate its phosphorylation levels, repair the intestinal mucosal barrier and restore its function (Feng et al., 2016). This research suggested that optimal Cu content down-regulated *MLCK* mRNA and protein levels. Our current study on AJC-related signaling molecules suggested that 4.1 mg/kg Cu content reduced the abundance of signaling molecule mRNA such as *RhoA*, *ROCK* and *NMII* in juvenile grass carp intestine, and *ROCK* and *RhoA* mRNA abundance were superior to the $CuSO_4 \cdot 5H_2O$ group at this time. Further study showed that 4.1 mg/kg Cu content reduced ROCK protein levels in the fish. These results suggest that optimal Cu content improves intestinal AJC integrity by boosting TJ and AJ, which could be partially connected to inhibition of the RhoA/ROCK signaling pathway.

4.5. Cu requirements of juvenile grass carp according to serum D-lactate and intestinal ROS levels

Based on quadratic regression analysis, Cu requirements (using CuCit as Cu source) were determined to be 4.14 and 4.12 mg/kg for serum D-lactate and antioxidant-related index ROS, respectively (Fig. 7). Apparently, Cu requirements for growth performance and intestinal structural integrity are similar, which might be due to the fact that Cu acts as a coenzyme or cofactor and participates in physiological activities and metabolism of animals. Thus, Cu may maintain a nutritional balance in similar amounts in fish. When Cu nutrition is out of tune, the lack or excess of Cu in fish will lead to related diseases.

5. Conclusion

In summary, our findings showed that dietary Cu stimulated grass carp intestinal growth. Cu increased the activity and mRNA abundance of intestinal antioxidant enzymes in fish, which boosted intestinal antioxidant capacity and preserved the structural integrity of intestinal cells and this could be associated with the signaling pathway Keap1a/1b/Nrf2 being activated by Cu. Cu increased AJC associated protein mRNA and protein levels, therefore enhancing intestinal cell structural integrity, which might be connected to the RhoA/ROCK signaling pathway being inhibited by Cu. Finally, based on serum D-lactate and intestinal ROS levels, Cu requirements for the diets of juvenile grass carp from initial weight to final weight (from 11 to 173 g) under the current experimental conditions were calculated to be 4.14 and 4.12 mg/kg, respectively.

Author contributions

Rui Ma: Manuscript writing, Formal analysis. **Lin Feng:** Methodology, Supervision. **Pei Wu**, **Yang Liu:** Methodology. **Shu-Wei Li, Ling Tang:** Resources. **Hong-Mei Ren, Xiao-Wan Jin:** Management. **Xiao-Qiu Zhou:** Writing - review & editing, Funding acquisition, Project administration, Supervision. **Wei-Dan Jiang:** Conceptualization, Supervision. **Wei-Dan Jiang** had primary responsibility for the final content of the manuscript. All authors carefully read and approved the final revision of the manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.02.005.

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