Copper-induced tight junction mRNA expression changes, apoptosis and antioxidant responses via NF-κB, TOR and Nrf2 signaling molecules in the gills of fish: Preventive role of arginine

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A B S T R A C T

This study explored the possible preventive effects of dietary arginine on copper (Cu)-induced tight junction mRNA expression changes, apoptosis and antioxidant responses in the gills of young grass carp (Ctenopharyngodon idella). The results indicated that exposure to 0.7 mg/L (11.01 μmol/L) Cu for 96 h induced the production of reactive oxygen species (ROS), thereby increasing protein oxidation, lipid peroxidation and DNA damage in the gills of fish. However, these oxidative effects were prevented by arginine supplementation. Arginine also prevented the toxic effects of Cu on the activities of copper/zinc superoxide dismutase (SOD1), glutathione-S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR) and the glutathione (GSH) content (P < 0.05). However, Cu induced an adaptive increase in the activity of catalase (CAT), and arginine supplementation further increased CAT activity (P < 0.05). Moreover, Cu induced increases in the relative mRNA expressions of SOD1, CAT, GPx, GST, caspase-3, caspase-9, NF-E2-related factor 2 (Nrf2), Kelch-like-ECH-associated protein 1a (Keap1a), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-8 (IL-8), transforming growth factor-β (TGF-β) and nuclear transcription factor-κB p65 (NF-κB p65) in the gills of grass carp (P < 0.05). In contrast, the relative mRNA expression levels of occludin, zonula occludens-1 (ZO-1), claudin b, claudin 3, claudin 12, target of rapamycin (TOR) and inhibitor factor κBα (IκBα) in the gills were decreased by Cu (P < 0.05). However, pre-treatment of fish with arginine prevented Cu-induced relative mRNA expression decrease. Interestingly, Cu exposure resulted in increases in claudin 15a mRNA expression (P < 0.05) but could not induce claudin c, caspase-8 and interleukin-10 (IL-10) mRNA expression changes in the gill of fish (P > 0.05). These results indicated that Cu exposure induced apoptosis and antioxidant system and tight junction mRNA changes in the fish gills, which could be completely blocked by dietary arginine pre-supplementation.

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1. Introduction

Trace metal contamination in freshwater ecosystems is of concern due to the bioaccumulation and toxicity of these elements (Jain, 2004). Copper (Cu), which is released into the aquatic environment through agriculture and industry activities, is considered one of the major pollution-causing metals (Frias-Esparciueta et al., 2011). Elevated aquatic Cu concentrations induce the overproduction of reactive oxygen species (ROS), which cause oxidative damage to aquatic organisms (Upadhyay and Panda, 2010). In fish, the gill is the dominant site of gas exchange, osmoregulation,

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acid–base balance and nitrogenous waste excretion, which plays a central role in the physiology of fish (Jönsson et al., 2006). The fish gill is the first organ that directly contacts the waterborne contaminants and the main place for waterborne copper uptake due to its large surface area and permeability (Pandey et al., 2008). Therefore, it becomes the primary target of Cu toxicity. In addition, evidence shows that Cu exposure disrupts the structural and functional integrity of the gill (Pandey et al., 2008), thereby decreasing the fish growth rate (Sutherland and Meyer, 2007). Given the potentially harmful effects of Cu on biota, it is important to expand our knowledge of the toxicity mechanism of Cu and to determine how to efficiently protect against Cu toxicity in the gills of fish.

Nutritional deficiency can aggravate trace metal toxicity and decrease growth performance. A previous study showed that dietary methionine deficiency can aggravate Cu-induced growth depression in chicks (Jensen and Maurice, 1978). Arginine (Arg) is an essential nutrient for fish (Singh and Khan, 2007). Our previous study showed that dietary Arg increases grass carp growth performance and flesh quality (Wang et al., 2015). In general, fish growth is positively correlated with the structural integrity of the gills (Sutherland and Meyer, 2007). A fish gill is mainly composed of epithelial cells (Sardet et al., 1979; Bopp et al. (2008) reported that Cu exposure causes structural damage to the epithelial cells in the gills of rainbow trout. Previous studies have shown that the gill cell structure integrity is associated with oxidative damage and antioxidant status in fish (Pandey et al., 2008). Some available evidence indicates that dietary myoinositol deficiency can aggravate Cu-induced Jian carp enterocytes (Jiang et al., 2013) and brain (Jiang et al., 2014) oxidative damage. It has been reported that the Arg guanidine group possesses the appropriate chemical properties of a metal ligand (Broach and Jarrett, 2006). A previous study showed that a metal ligand can chelate Cu²⁺ to decrease its toxicity in channel catfish (Straus, 1993). In addition, a previous study indicated that Arg deficiency enhances the plasma ammonia level in grass carp (Wang et al., 2015). James and Sampath (1995) reported that a high level of plasma ammonia can aggravate Cu toxicity through forming very stable complex cations in catfish. These results indicate that Arg deficiency may aggravate Cu toxicity in fish, but this finding needs further investigation. However, no reports have shown the relationship between Arg- and Cu-induced oxidative damage in fish gills. In addition, trace metal–induced oxidative stress causes apoptosis in fish (Xiang et al., 2000), which could be regulated by target of rapamycin (TOR) (Barbone et al., 2008). However, it is unclear whether Arg can regulate Cu-induced apoptosis through TOR in the fish gill. Moreover, a previous study showed that antioxidant enzymes such as superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR) play an important role in oxidative stress–induced apoptosis in HepG2 cells (Martín et al., 2010). Furthermore, it has been reported that the antioxidant enzyme activities are partly related to gene transcription, which is regulated by the NF-E2-related nuclear factor 2 (Nrf2) and Kelch-like-ECH–associated protein 1 (Keap1) signaling molecules in fish (Jiang et al., 2014). Chen et al. (2013) reported that the up-regulation of Nrf2 expression could elevate the antioxidant gene (including SOD, CAT, GPx, GR and GST) expression levels in the mice liver. However, little information is available regarding the effects of Arg on the Nrf2 signaling pathway in fish gills. Our previous study showed that dietary Arg deficiency decreased the nitric oxide (NO) content which can regulate Nrf2 mRNA expression in grass carp muscle (Wang et al., 2015). These results indicated that there may be a relationship between the Arg and Nrf2 signaling molecules in fish gills and it is worthwhile to reveal the mechanisms underlying the effects of Arg on Cu-induced gill oxidative damage and apoptosis in fish. Moreover, Cu-induced oxidative stress could result in disruption of the gill barrier function (Jönsson et al., 2006), which was regulated by the tight junction (TJ) proteins between rainbow trout gill epithelial cells (Chasiotis et al., 2012). The TJ proteins are composed of a set of transmembrane TJ proteins, such as occludin and members of the claudin superfamily, and cytosolic TJ protein, such as ZO-1 (Kim et al., 2012). Previous studies have characterized ZO-1, occludin, claudin b, claudin c and claudin 3 ortholog as barrier-forming TJ proteins (Chasiotis and Kelly, 2011; Kwong and Perry, 2013), whereas claudin 12 and claudin 15 act as pore-forming TJ proteins (Günzel and Alan, 2013). In rats, Wang et al. (2007) reported that iron deprivation aggravates trace metal toxicity through increases in the level of disruption of lead-induced brain TJ barrier function. However, information regarding the effects of Arg deficiency on Cu-induced TJ complex damage in fish remains scarce. Furthermore, Coyne et al. (2002) reported that pro-inflammatory cytokines, such as interleukin–1β (IL–1β), interleukin–8 (IL–8) and tumor necrosis factor–α (TNF–α), are involved in the regulation of the TJ structure in human bronchial epithelial cells. However, no previous study investigated whether Arg can affect Cu-induced TJ mRNA expression through the regulation of pro-inflammatory cytokines in fish. Study showed that anti-inflammatory cytokines such as IL-10 and TGF-β are thought to counteract the production of pro-inflammatory cytokines (Opal and Depalo, 2000). In mammals, the expression of pro-inflammatory cytokines could be regulated by nuclear transcription factor–κB (NF–κB) (Correa et al., 2004). In addition, our laboratory cloned the cDNA of NF–κB p65 (GenBank accession number KJ526214) and inhibitor of κBα (iκBα) (GenBank accession number KJ125069) of grass carp for the first time. In Atlantic salmon, our study showed that dietary Arg deficiency reduces the liver polyamine level (Andersen et al., 2013). Additionally, it has been reported that polyamine can regulate the NF–κB transcriptional activity in rat intestinal epithelial IEC–6 cells (Pfeffer et al., 2001). These results indicated that Arg may modulate trace metal-induced TJ mRNA expression change through NF–κB signaling molecules in fish, but these findings warrant further investigation.

This study is in line with our previous investigations, which were part of a larger research study on how dietary Arg improves fish growth and flesh quality (Wang et al., 2015). Dietary Arg requirement for the optimal growth of young grass carp was determined to be 13.45 g/kg diet (corresponding to 43.64 g/kg dietary protein) in our previous (Wang et al., 2015). Fish growth is partly related to the structural and functional integrity of the gill. Hence, the aim of the present study was to investigate the preventive effects of dietary Arg on Cu-induced tight junction mRNA expression, apoptosis and antioxidant responses in the gills of fish. After the 8-week feeding trial, grass carp from each treatment group were exposed to Cu-free water (control group) and a high Cu²⁺ concentration (0.7 mg/mL) (treated group) water during 96 h to investigate Cu toxicity and arginine prevention role. In addition, we further investigated the effects of Arg on Cu-induced the relative mRNA expression of the TOR, NF–κB p65, iκBα, Nrf2 and Keap1 mRNA signaling molecules in the gills of young grass carp, which may provide evidence for the mechanism through which optimum Arg can prevent trace metal toxicity, thereby improving growth performance.

2. Materials and methods

2.1. Diets and feeding management

To investigate the preventive effects of Arg against Cu toxicity, a growth experiment was conducted prior to the Cu exposure trial. The experimental diets were the same as those used in our previous study (Wang et al., 2015). Fishmeal, casein and gelatin
were used as the main protein sources. Six isonitrogenous diets (308.18 g crude protein kg⁻¹ diet) were formulated by the supplementation of appropriate amounts of crystalline glycine. The final Arg concentrations of the six experimental diets were measured through high-performance liquid chromatography (Agilent Technologies, Palo Alto, CA, USA) to be 6.9 (was equal to 39.38 mmol/kg diet), 10.4 (was equal to 59.36 mmol/kg diet), 14.1 (was equal to 80.47 mmol/kg diet), 17.6 (was equal to 100.45 mmol/kg diet), 21.4 (was equal to 122.14 mmol/kg diet) and 24.5 (was equal to 139.83 mmol/kg diet) g Arg/kg diets, respectively. The basal diet was the arginine-unsupplemented group or Arg deficiency group (6.9 g/kg diet). Arginine concentration for optimal growth is 13.45 g/kg diet (Normal), which was established by our previous study (Wang et al., 2015). Meanwhile, excess group arginine concentration was more than 21.4 g/kg diet. All of the ingredients were mixed, pelleted, and stored at −20 °C until use, as described by Le and Fotedar (2014).

All experimental procedures used in this study were approved by the Animal Care Advisory Committee of Sichuan Agricultural University. A total of 540 fish (initial average weight of 278.82 ± 0.68 g) from the aclimatzation cages were randomly distributed into six groups of three replicates each. Each experimental diet was randomly assigned to cages in triplicate. The fish were fed their respective experimental diets to apparent satiation four times daily for 8 weeks. During the experimental period, the dissolved oxygen concentration was greater than 6 mg/L, and the pH and water temperature were maintained at 7.0 ± 0.5 and 26 ± 2 °C, respectively. The feeding trial was conducted under a natural light and dark cycle.

2.2. Fish exposure to copper

After the 8-week feeding trial, 30 fish with a similar body weight from each treatment group were moved to labeled cages in triplicate. In this study, the fish from six treatments were exposed to CuSO₄ for 96 h, and the final Cu²⁺ concentration in water was found to be 0.7 mg Cu/L water (was equal to 11.01 μmol Cu/L water), which has been proved to induce oxidative stress in grass carp according to our preliminary study data. In addition, the Deficiency/Ctrl (pre-treatment/exposure) treatment was performed by exposing the fish from the Arg deficiency group (6.9 g Arg/kg diet) to Cu-free water. Therefore, there were seven different pre-treatment/exposure groups, i.e., Deficiency/Ctrl, Deficiency/Cu, 10.4 g kg⁻¹ Arg/Cu, 14.1 g kg⁻¹ Arg/Cu, 17.6 g kg⁻¹ Arg/Cu, 21.4 g kg⁻¹ Arg/Cu and 24.5 g kg⁻¹ Arg/Cu, with three replicates per group and 10 fish per replicate. During the Cu exposure period, the experimental conditions were the same as those in the feeding trial. At the end of the challenge trial, all of the living fish from each cage were anesthetized in a benzocaine bath according to Basic et al. (2013). In addition, the gills of fish were quickly removed, frozen in liquid nitrogen and stored at −80 °C for later analysis.

2.3. Antioxidant parameters and DNA fragmentation analysis

The malondialdehyde (MDA) and protein carbonyl (PC) contents were measured according to Tokur and Korkmaz (2007). The activities of SOD1, CAT and GPX were determined by the method described by Chen et al. (2013). The glutathione-S-transferase (GST) and glutathione reductase (GR) activities were measured by the method described by Pandey et al. (2008) with minor modification. GR activity was measured by following the oxidation of 0.25 mM NADPH at 340 nm in medium containing 0.1 M phosphate buffer (pH 7.4), 0.5 mM EDTA, 1 mM GSSG and 50 μL of supernatant in a total volume of 2.0 mL. Glutathione-S-transferase (GST) activity was measured by monitoring the formation of an adduct between 5 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene (at 340 nm) in a reaction mixture containing 0.1 M phosphate buffer and 50 μL of desalted supernatant. One unit of CAT, GPx, GST and GR activity is defined as the amount of the enzyme that consumes 1 μmol/L of substrate or generates 1 μmol/L of product per min. Activity was expressed in international units (or millilitres) per mg of protein. The reduced GSH content was measured according to the method described by Rhee et al. (2013). The anti-superoxide radical anion (ASA) (O₂⁻ scavenging ability) and anti-hydroxyl radical (AHr) (OH scavenging ability) capacities were determined by the method described by Jiang et al. (2009). The gill ROS production was measured according to Rhee et al. (2013). Protein concentration was determined spectrophotometrically by the method of Bradford (1976). The fragmented DNA of the gill cells was isolated by the method of Kawakami et al. (2008). DNA fragmentation was analyzed by electrophoresis for 5 h at 40 V using 1% agarose gel. The gel was examined and photographed using a Gene Genius Bio-Imaging system (Syngene, Frederick, MD, USA).

2.4. Gene expression analysis

The total RNA of the gills was isolated using an RNAiso Plus Kit (Takara, Dalian, China) followed by DNase I treatment. The RNA purity of each sample was determined by calculating the 260/280 ratio. The RNA integrity was assessed by inspection of the 28S and 18S ribosomal RNA bands in a 1% agarose gel. Subsequently, the RNA was reverse transcribed to cDNA using a PrimeScript™ RT reagent kit (Takara, Dalian, China). Specific primers for the TOR, ZO-1, claudin 12, CR, Nrf2, Keap1, NF-κB p65, IκBα and caspase-8 gene were designed according to sequences of young grass carp cloned in our laboratory (Table 1), and the primers for SOD1, CAT, GPX, GST, occludin, claudin b, claudin c, claudin 3, claudin 15a, IL-1β, IL-8, TNF-α, TGF-β, IL-10, caspase-3, caspase-9 and β-actin were designed using the published sequences of young grass carp (Table 1). All of the real-time PCR analyses were performed using a CFX96™ Real-Time PCR Detection System (Bio-Rad, Laboratories, Inc.) with a SYBR® Prime Script RT-PCR Kit II (Takara, Dalian, China). The mRNA levels of these genes were normalized to the mRNA level of the grass carp β-actin, which was used as a housekeeping gene. The amount of the target gene was based on the threshold cycle number (CT), and the CT for each sample was determined using the CFX Manager™ software. All of the primer amplification efficiencies were approximately 100%. The gene expression results were analyzed using the 2⁻ΔΔCT method according to Luzzio et al. (2013).

2.5. Calculations and statistical analysis

The data were presented as the means ± standard error (SE). All data were subjected to one-way analysis of variance followed by Duncan’s multiple range test to determine significant differences among treatment groups using the software SPSS 18.0 (SPSS Inc., Chicago, IL, USA). A difference was considered significant at the level of P<0.05.

3. Results

3.1. Antioxidant indicators, ROS and NO levels in the gill of fish

As shown in Table 2, the Deficiency/Cu treatment significantly increased MDA and PC contents compared with the unexposed control group (Deficiency/Ctrl) (P<0.05). However, Arg pre-treatment completely decreased MDA and PC formation (P<0.05). ASA did not differ between all groups (P>0.05). The activity of AHR in the gills of grass carp was significantly increased by Cu exposure (Deficiency/Cu) (P<0.05), while dietary Arg pre-supplementation (Arg/Cu) could not change AHR activity (P>0.05).
### Table 1

Primer sequences and optimal annealing temperatures of genes selected for analysis by real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences of primers</th>
<th>Annealing temperature</th>
<th>Amplification products</th>
<th>Accession number</th>
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<td>5'-GGTGTCTCTCAGGCG-3'</td>
<td>59.4</td>
<td>154</td>
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<tr>
<td>ZO-1 Reverse</td>
<td>5'-TTATGTCATCGGTT-3'</td>
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<tr>
<td>ZO-1 Occludin</td>
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<td>59.4</td>
<td>154</td>
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<tr>
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<tr>
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<td>154</td>
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B. Wang et al. / Aquatic Toxicology 158 (2015) 125–137
As shown in Table 3, compared with the Deficiency/Ctrl group, Deficiency/Cu exposure caused significant decrease in SOD1 activity and increase in CAT activity (P < 0.05). Interestingly, dietary Arg pre-supplementation (Arg/Cu) increased the activities of SOD1 and CAT (P < 0.05). Compared with the results obtained for the Deficiency/Ctrl group, the activities of Gpx, GST and GR were all significantly decreased by the Deficiency/Cu treatment (P < 0.05). Arg pre-supplementation (Arg/Cu) significantly prevented the toxic effects of Cu on these enzyme activities (P < 0.05). Deficiency/Cu treatment caused decreases in the gill GST content (P < 0.05). However, pre-treatment with 14.1–24.5 g kg⁻¹ of Arg prior to exposure to Cu significantly increased GST content (P < 0.05). As shown in Fig. 1, the NO content in grass carp gill was the highest in the 17.6/Cu, 21.4/Cu and 24.5/Cu groups and lowest in the Deficiency/Ctrl group (P < 0.05) (Fig. 1A). Compared with control group, ROS generation significantly increased in Cu exposure group (Deficiency/Cu). However, Arg pre-treatment decreased ROS production compared with Deficiency/Cu (P < 0.05) (Fig. 1B). Meanwhile, the dietary Arg requirement of young grass carp estimated by the quadratic regression analysis based on MDA estimated to be 17.26 g kg⁻¹ diet (57.38 g kg⁻¹ protein) (Fig. 2), which was higher than the requirement estimated based on the percent weight gain (13.45 g kg⁻¹ diet or 43.64 g kg⁻¹ protein), suggesting that more Arg is required for the prevention of trace metal Cu toxicity in the fish gill.

3.2. Antioxidant-related and apoptosis-related gene expression in the gills of fish

As shown in Fig. 3, compared with the control, Cu exposure significantly increased a ladder-like pattern of DNA fragments on the gel (P < 0.05). However, pre-treatment with Arg decreased DNA fragmentation in the gills of grass carp (P < 0.05). As shown in Figs. 4 and 5, the Deficiency/Cu treatment increased the relative mRNA expression levels of SOD1, CAT, Gpx, GST and Nrf2 in the gills of grass carp compared with the Deficiency/Ctrl group (P < 0.05). In addition, dietary Arg pre-supplementation (Arg/Cu) further increased SOD1, CAT, Gpx, GST and Nrf2 mRNA expression (P < 0.05). The relative mRNA expression of GR was higher in the 10.4/Cu and 14.1/Cu groups and lower in the Deficiency/Ctrl.
group \((P < 0.05)\). Compared with the results obtained for the Deficiency/Ctrl group, the relative mRNA expression of Keap1a was significantly increased by the Deficiency/Cu treatment \((P < 0.05)\). Arg pre-supplementation \((\text{Arg/Cu})\) significantly decreased Keap1a mRNA expression \((P < 0.05)\). As shown in Figs. 6 and 7, exposure to Cu significantly increased the caspase-3 and caspase-9 transcript abundances in the gills of grass carp compared with the untreated control \((P < 0.05)\). However, pre-treatment with Arg effectively prevented the increase in caspase-3 and caspase-9 mRNA expression in the gill \((P < 0.05)\). Cu exposure has no effect on caspase-8 mRNA expression \((P > 0.05)\), but appropriate Arg pre-treatment significantly decreased caspase-8 mRNA level compared with Deficiency/Cu group \((P < 0.05)\). Compared with the results obtained from the Deficiency/Ctrl group, the relative mRNA expression of TOR was significantly decreased by the Deficiency/Cu treatment \((P < 0.05)\). Arg pre-supplementation \((\text{Arg/Cu})\) significantly increased TOR mRNA expression \((P < 0.05)\).

3.3. Tight junction protein gene expression in the gills of fish

As shown in Fig. 8, the Deficiency/Cu treatment decreased the relative mRNA expression levels of ZO-1, occludin, claudin b, claudin 3 and claudin 12 in the gill of grass carp compared with the Deficiency/Ctrl group \((P < 0.05)\). However, dietary Arg pre-supplementation \((\text{Arg/Cu})\) increased the ZO-1, occludin, claudin b, claudin 3 and claudin 12 mRNA expression levels \((P < 0.05)\). Compared with the results obtained from the Deficiency/Ctrl group, the relative mRNA expression of claudin 15a was significantly increased by the Deficiency/Cu treatment \((P < 0.05)\). Arg pre-supplementation \((\text{Arg/Cu})\) significantly decreased the claudin 15a mRNA level \((P < 0.05)\). No statistically significant changes were observed in the gill claudin c mRNA expression among the treatments \((P > 0.05)\).

3.4. Cytokine gene expression in the gill of fish

As shown in Figs. 9 and 10, the Deficiency/Cu treatment increased the relative mRNA expression levels of IL-1β, IL-8, TNF-α and NF-κB p65 in the gill of grass carp compared with the Deficiency/Ctrl group \((P < 0.05)\). However, Arg pre-supplementation \((\text{Arg/Cu})\) significantly decreased the IL-1β, IL-8, TNF-α and NF-κB p65 mRNA expression levels \((P < 0.05)\). The highest relative mRNA expression levels of IL-10 and TGF-β were found in the Arg/Cu groups, and the lowest were observed in the Deficiency/Ctrl group \((P < 0.05)\). Compared with the results obtained for the Deficiency/Ctrl group, the relative mRNA expression of IkBα was significantly decreased by the Deficiency/Cu treatment \((P < 0.05)\). Arg pre-supplementation \((\text{Arg/Cu})\) significantly increased the IkBα mRNA level \((P < 0.05)\).

4. Discussion

4.1. Cu toxicity in the gills of fish under arginine deficiency

4.1.1. Cu induces oxidative damage in fish gills under arginine deficiency

The fish gill is the first organ that directly contacts the waterborne contaminants and is the main place for waterborne copper uptake (Pandey et al., 2008). Therefore, it is highly vulnerable to Cu toxicity attack. The acute toxicity of Cu is suggested to be mainly caused by the formation of ROS in fish (Bopp et al., 2008). In the present study, exposure to 0.7 mg L\(^{-1}\) Cu for 96 h significantly induces ROS production (Fig. 1) in the gills of grass carp fed arginine deficiency diet. Bopp et al. (2008) reported that excessive ROS production leads to oxidative damage to lipid and protein in rainbow trout gill cells. In fish, the MDA and PC contents were widely used as biochemical indicators of oxidative damage to lipids and proteins, respectively (Jiang et al., 2014). The present study showed that Cu exposure significantly increases MDA and PC formation (Table 2) in the gills of young grass carp fed arginine deficiency diet, suggesting that Cu induces fish gill oxidative damage. The Cu-induced fish gill oxidative damage may be partly related to disturbance of the antioxidant system. In this study, Cu exposure significantly decreased the activities of antioxidant enzymes, such as SOD1, GPx, GST and GR, and the GSH content (Table 3) in the gills of grass carp fed arginine deficiency diet, which showed the opposite pattern compared with MDA and PC, suggesting that Cu induces fish oxidative damage partly through disturbance of the antioxidant system.
This effect may be due to inhibition of the enzymes by the binding of 
Cu to their active site thiol groups, which cause disturbances in 
enzyme function (Letelier et al., 2005). However, Cu exposure sig-
nificantly elevated CAT activity in the gills of grass carp fed arginine 
deficiency diet. The enhancement of CAT activity could compen-
sate for the decreased activity of other antioxidant enzymes. A 
previous study showed that the changes in the activities of antioxi-
dant enzymes occurred due to changes in mRNA expression in fish 
(Jiang et al., 2014). Thus, we then detected the influence of Cu on 
antioxidant gene mRNA expression in the gill of fish.

The results of our current study demonstrated that Cu expo-
sure significantly increased mRNA levels of SOD1, CAT, GPx, GST 
and GR in the gills of grass carp fed arginine deficiency diet (Fig. 4), 
which exhibited an opposite pattern with their respective enzyme 
changes. Different patterns between the relative mRNA levels and 
enzymatic activities were also reported in zebrafish exposed to Cu, 
suggesting that the Cu-induced oxidative stress inhibits the antioxi-
dant enzyme activity and in turn stimulates transcript expression 
(Craig et al., 2007). The up-regulation of antioxidant gene mRNA 
levels may result from activating the antioxidant-related signaling 
molecules. To date, the molecular mechanism underlying the 
effects of Cu on antioxidant gene mRNA expression has not been 
reported in fish. Nrf2 has been demonstrated to be a critical tran-
scription factor that promotes the transcription of antioxidant gene, 
including SOD, CAT, GPx, GR and GST, in fish (Ma, 2013). Therefore, 
we then investigated the effects of Cu on the mRNA expression 
of these antioxidant-related signaling molecules in the fish gill.

Chen et al. (2013) reported that the up-regulation of Nrf2 
expression could elevate the antioxidant gene mRNA expression 
levels in the mouse liver. Our results showed that a significant 
up-regulation of Nrf2 mRNA level was observed in the gills of grass 
carp fed arginine deficiency diet after exposure to Cu (Fig. 5A). 
This effect may be due to the Cu-induced production of H2O2 
which can up-regulate Nrf2 mRNA expression in the European eel 
(Anguilla anguilla) liver (Giullani & Regoli, 2014). Additionally, 
the positive effects of Cu-induced antioxidant gene mRNA expression 
may be partly ascribed to an increase in Nrf2 nuclear translocation. 
Keap1 is identified as an Nrf2-binding protein that prevents Nrf2
translocation to the nucleus and promotes the ubiquitination-proteasomal degradation of Nrf2 (Ma, 2013). Interestingly, our study showed that Cu induces the up-regulation of Keap1a mRNA level in the gill of young grass carp fed arginine deficiency diet (Fig. 5B). The Cu-induced Keap1a mRNA expression may be partly related to Nrf2 feedback regulation. Lee et al. (2007) reported that the Keap1 gene promoter contains a functional antioxidant response element sequence, which could be up-regulated by Nrf2 in mouse hepatoma cells. Accordingly, Cu may induce antioxidant gene mRNA expression through activating the Nrf2 and Keap1a signaling molecules, and the detailed mechanism needs to be further investigated. Moreover, it has been reported that trace metal-induced oxidative stress causes apoptosis in fish (Xiang et al., 2000). Thus, we next investigated the effects of Cu on fish gill cell apoptosis.

4.1.2. Cu induces fish gill cell apoptosis under arginine deficiency

Apoptosis is a highly genetically controlled type of cell death that is accompanied by the activation of a large number of intracellular proteases and endonucleases in fish (Takle and Andersen, 2007). Kawakami et al. (2008) reported that DNA fragmentation has been utilized as a characteristic feature of apoptosis. The results of our current study demonstrated that Cu exposure induces DNA fragmentation in the gills of grass carp fed arginine deficiency diet (Fig. 3), suggesting that Cu induces fish gill cell apoptosis. Luzio et al. (2013) reported that Cu induces apoptosis partly via the caspase-dependent pathway. In general, caspase-3 is the major executioner caspase in the caspase-dependent pathway.

Fig. 5. NF-E2-related factor-2 (Nrf2) (A) and Kelch-like-ECH-associated protein 1a (Keap1a) (B) mRNA levels in the gill of young grass carp (C. idella) fed diets containing graded levels of arginine for 8 weeks, followed by exposure to 0.7 mg Cu/L (11.01 μmol Cu/L) water for 96 h. Data represent means of six replicates, error bars indicate S.E. Values having different letters are significantly different (P < 0.05).

Fig. 6. Caspase-3 (A), caspase-8 (B) and caspase-9 (C) mRNA levels in the gill of young grass carp (C. idella) fed diets containing graded levels of arginine for 8 weeks, followed by exposure to 0.7 mg Cu/L (11.01 μmol Cu/L) water for 96 h. Data represent means of six replicates, error bars indicate S.E. Values having different letters are significantly different (P < 0.05).
signaling molecule TOR (Barbone et al., 2008). The results of our current study demonstrated that Cu exposure significantly decreased the relative mRNA expression of TOR in the grass carp gill (Fig. 7), which showed opposite patterns compared with caspase-9 mRNA levels, suggesting that Cu may induce caspase-9 mRNA expression by down-regulating TOR mRNA expression in fish. Together, the results show that the Cu-induced apoptosis may be partly attributed to the intrinsic pathway (involving caspase-9 and caspase-3) through down-regulation of TOR mRNA expression in fish. However, the underlying mechanism in fish is unclear, which warrants further investigation. In addition, Chin et al. (2006) reported that caspase-dependent apoptosis result in disruption of the intestinal epithelial cell barrier function. In fish, TJ proteins play an appreciable role in maintaining the physical barrier function (Chasiotis et al., 2012). Thus, we then investigated the effects of Cu on TJ proteins in the gill of fish.

4.1.3. Cu induces tight junction protein mRNA changes in fish gills under arginine deficiency

The TJ proteins play an important role in the gill barrier function (Chasiotis et al., 2012). In this study, Cu exposure significantly decreased the gene mRNA expression of ZO-1, occludin, claudin b, claudin 3 and claudin 12 in the gills of grass carp fed arginine deficiency diet (Fig. 8), suggesting that Cu disrupts the tight junction barrier function and ion channel function in fish. The negative effects of Cu on TJ protein mRNA expression may be partly explained by the pro-inflammatory cytokines. In vitro, it has been reported that TNF-α and IL-8 down-regulate the expression of ZO-1 and claudin 5 in human vascular endothelial cells (Avelar et al., 2010) and bovine retinal endothelial cells (Yu et al., 2013), respectively. In addition, IL-1β decreases the expression of occludin in human retinal epithelial cells (Abe et al., 2003). Therefore, we then investigated the effect of Cu on cytokine mRNA expression in the gill of young grass carp. In this study, Cu exposure significantly increases the relative mRNA expression of the pro-inflammatory cytokines IL-1β, IL-8 and TNF-α in young grass carp gills (Fig. 9), which showed opposite patterns compared with the mRNA levels of ZO-1, occludin, claudin b, claudin 3 and claudin 12, suggesting that Cu can disrupt the tight junction barrier function partly through inducing the production of pro-inflammatory cytokines in fish. However, no previous study has examined the effects of Cu on cytokine mRNA expression in fish gills. In terrestrial animals, the gene expression of pro-inflammatory cytokines could be regulated by the signaling molecule NF-κB (Correa et al., 2004). The current study showed that the mRNA expression of NF-κB p65 in the gills of young grass carp exposed to Cu was significantly increased compared with the unexposed control (Fig. 10A), and the increasing pattern was similar to that found for changes in the TNF-α, IL-1β and IL-8 mRNA levels, suggesting that Cu exposure up-regulates pro-inflammatory cytokine mRNA expression partly through up-regulating NF-κB signaling molecule mRNA expression in fish. Additionally, the positive effects of Cu on pro-inflammatory cytokine mRNA levels may be partly ascribed the promotion of NF-κB nuclear translocation. It has been reported that IκBα, an inhibitory cytoplasmic protein, is well known to sequester NF-κB in the cytoplasm and thereby prevent its nuclear translocation (Correa et al., 2004). In this study, Cu exposure significantly decreased IκBα mRNA expression in grass carp gills (Fig. 10B), implying that Cu-induced mRNA expression of pro-inflammatory cytokines may be partly attributed to the promotion of NF-κB translocation to the nucleus by the down-regulation of IκBα mRNA expression in fish. Together, the results show that the Cu-induced TJ mRNA expression change may be partly attributed to the up-regulation of pro-inflammatory cytokine mRNA expression through the up-regulation of NF-κB p65 and the down-regulation of IκBα mRNA expression in fish. However, the underlying mechanism in fish is unclear, which warrants further investigation.

In this study, Cu exposure significantly increased claudin 15a mRNA expression in the gill of grass carp (Fig. 8). This effect may be related to ion homeostasis in gill epithelial cells, which is an important guarantee of their function (Zambrano and Canelo, 1996). It has been reported that Na⁺, K⁺-ATPase-mediated transport of Na⁺ out of gill cells can be inhibited by Cu exposure in Nile tilapia (Sola et al., 1995) and that the up-regulation of claudin 15 mRNA expression increased the extracellular Na⁺ concentrations in zebrafish epithelial cells (Bagnat et al., 2007). Thus, the Cu-induced claudin 15a mRNA expression in the gill observed in this study may be an adaptation to the gill osmoregulation function, but the mechanism is still unknown and needs to be further investigated.

In summary, the present study showed arginine deficiency aggravated trace metal Cu toxicity on gill oxidative damage, apoptosis and TJ mRNA changes. Hence, it is necessary to expand our knowledge of how to prevent trace metal Cu toxicity in fish gills.

4.2. Preventive effects of Arg in Cu-induced fish gill damage

Arg is an essential nutrient for fish (Singh and Khan, 2007). In mammals, Arg has been recognized as a scavenger of ROS (Lass et al., 2002). Additionally, Broach and Jarrett (2006) reported that the Arg guanidine group possesses the appropriate chemical properties of a metal ligand. A previous study showed that metal ligands can chelate trace metals to decrease their toxicity in channel catfish (Straus, 1993). These results indicate the potential preventive effects of Arg on Cu-induced fish gill damage.

In the present study, dietary Arg deficiency and excess significantly increased Cu-induced MDA and PC increases in grass carp gill and completely blocked by optimum Arg pre-treatment, suggesting that Arg could protect against Cu-induced fish gill protein oxidation and lipid peroxidation. This effect may be related to ROS scavenging capacity which could be reflected by non-enzymatic antioxidant and antioxidant enzymes. In the present study, Arg deficiency significantly decreased antioxidant enzyme SOD1, CAT, GPX, GR and GST activities and GSH content in Cu-exposed grass carp gill and completely restored with some concentrations of dietary Arg, which showed opposite patterns compared with MDA and PC, suggesting that the preventive role of Arg against Cu-induced oxidative damage may be attributed to its ability to maintain the activities of radical scavenging enzyme. The increment of antioxidant enzyme activities by optimum Arg may be due to up-regulation of antioxidant gene mRNA expression. Results of our current study demonstrated that Arg deficiency and excess significantly
Fig. 8. Claudin c (A), Claudin b (B), Claudin 3 (C), Claudin 12 (D), Claudin 15a (F), Occludin (F) and Zonula occludens-1 (ZO-1) (G) mRNA levels in the gill of young grass carp (C. idella) fed diets containing graded levels of arginine for 8 weeks, followed by exposure to 0.7 mg Cu/L (11.01 μmol Cu/L) water for 96 h. Data represent means of six replicates, error bars indicate S.E. Values having different letters are significantly different (P<0.05).

Reduced antioxidant gene mRNA expression in gill of grass carp and increased with optimal Arg treatment, which showed similar patterns compared with antioxidant enzyme activities, suggesting that the enhancement of antioxidant enzyme activities partly attributed to the fact that dietary Arg up-regulated the gene transcription of antioxidant enzyme in fish. Up-regulation of the antioxidant gene mRNA expression may result from activating Nrf2 signaling molecules in fish. Our results showed that pre-treatment with Arg up-regulated the Nrf2 gene mRNA expression of Cu-treated gill to that of Deficiency/Cu group, which showed similar patterns compared with antioxidant gene mRNA expression, suggesting that Arg elevated fish gill antioxidant gene mRNA expression may be through up-regulating Nrf2 gene transcription. However, no information is available to date about the effect of Arg on Nrf2 gene mRNA expression in fish gill. The positive influence of Arg on the regulation of grass carp gill Nrf2 mRNA expression may be in part related to its metabolite NO. Our previous study showed that the increased NO content up-regulated Nrf2 mRNA expression in grass carp muscle (Wang et al., 2015). Result of our current study demonstrated that Arg deficiency reduced grass carp gill NO content and
increased optimum Arg supplementation. Correlation analysis indicated that grass carp gill Nrf2 mRNA levels was positively correlated to NO (see in Table 4), suggesting that Arg up-regulated Nrf2 mRNA expression may be party via enhancement of NO content in fish gill. In addition, the positive effects of Arg on antioxidant gene mRNA expression may be partly ascribed to the increase of Nrf2 nuclear translocation. In present study, Arg supplementation significantly down-regulated Cu-induced grass carp gill Keap1 mRNA expression, suggesting that Arg increased antioxidant enzymes gene mRNA expression may be partly attributed to promote Nrf2 translocation to the nucleus by down-regulating Keap1 mRNA expression. However, the underlying mechanism needs to be further investigated. Moreover, it has been reported that oxidative stress causes apoptosis in fish (Xiang et al., 2000). Thus, we next investigated the preventive effects of Arg on fish gill cell apoptosis.

The results of our current study demonstrated that dietary Arg deficiency significantly increased Cu-induced DNA fragmentation increases in grass carp gill and completely blocked by 17.6 g/kg Arg pre-treatment, suggesting that optimum Arg could protect against Cu-induced fish gill cell apoptosis. This effect may be partly due to caspase mRNA expression in the fish gill. The present study indicated that Arg deficiency increased Cu-induced caspase-3, caspase-8 and caspase-9 mRNA levels increases in the grass carp gill. Arg decreased Cu-induced caspase mRNA expression partly through inactivation of upstream factors (such as TOR) that mediate apoptosis in fish. The results of our current study demonstrated that Arg pre-treatment significantly increased TOR mRNA expression in the grass carp gill, which showed opposite patterns compared with caspase (3, 8 and 9) mRNA expression, suggesting that Arg decreased Cu-induce caspase mRNA expression by up-regulating TOR mRNA expression in fish.

To our knowledge, TJ proteins play an crucial role in maintaining the barrier integrity (Chasiotis et al., 2012). In present study, dietary Arg deficiency significantly decreased claudin b, claudin 3, occludin and ZO-1 mRNA expression in grass carp gill and completely restored with Arg supplementation, implying that optimum
Arg could protect against Cu-induced epithelial TJ barrier function damage through up-regulation of ZO-1, occludin, claudin b and claudin 3 in fish. However, no information is available about the relationship between Arg and TJ protein in fish. The effects of dietary Arg on regulation of TJ protein mRNA expression may be partly due to cytokines in the gill. In this study, Arg deficiency up-regulated pro-inflammatory cytokines IL-1β, TNF-α and IL-8 mRNA expression in young grass carp gill. Correlation analysis indicated that ZO-1, occludin, claudin b and claudin 3 were negatively correlated to IL-1β, IL-8 and TNF-α (see in Table 4), suggesting that up-regulation of TJ proteins mRNA expression partly through regulation of Cu-induced pro-inflammatory cytokines mRNA expression by Arg in fish. The effects of dietary Arg on regulation of pro-inflammatory cytokines mRNA expression may be partly due to signaling molecules of NF-κB p65 in fish. The current study showed that the relative mRNA level of NF-κB p65 was down-regulated with dietary optimum Arg supplementation in Cu exposure grass carp. Correlation analysis indicated that grass carp gill IL-1, IL-8 and TNF-α mRNA levels were positively correlated to NF-κB p65 (see in Table 4), suggesting that the benefits of Arg on pro-inflammatory cytokines mRNA expression may be partly explained by Arg down-regulated NF-κB p65 mRNA expression in fish. In addition, the positive effects of Arg on pro-inflammatory cytokines mRNA expression may be partly ascribed to inhibit NF-κB nuclear translocation by IκBα. In the present study, significantly increase in mRNA levels of IκBα was observed in the grass carp gill with appropriate arginine supplementation, suggesting that Arg-decreased the relative mRNA expression of pro-inflammatory cytokines may be partly attributed to inhibit NF-κB nuclear translocation by up-regulating IκBα mRNA expression.

In other words, this report provides the first demonstration that appropriate Arg plays a preventive role against Cu-induced gill damage in fish and thus could provide effective measures to address trace metal Cu toxicity in fish.

5. Conclusions

In summary, the results presented in this manuscript demonstrate that exposure to Cu can stimulate oxidative damage and caspase-dependent apoptosis in fish gills and these effects were partly due to disruptions of the antioxidant system and the induction of apoptosis-related gene mRNA expression (caspase-3 and caspase-9). In addition, this study reported that Cu exposure induces gill tight junction mRNA expression change partly through the up-regulation of the mRNA expression of pro-inflammatory cytokines in the fish. Furthermore, the changes in the mRNA expression of antioxidant gene and pro-inflammatory cytokines may be partly related to Nrf2, Keap1a, NF-κB P65 and IκBα signaling molecules in fish. Moreover, this study provides the first demonstration that Arg deficiency can aggravate Cu toxicity and that appropriate Arg pre-supplementation completely blocks Cu toxicity in fish gills. However, the detailed toxicity mechanism through which Arg decreases Cu toxicity needs to be further investigated in fish. In addition, the dietary Arg requirement of young grass carp for the prevention of Cu toxicity estimated based on the MDA content was proven to be 17.26 g kg⁻¹ diet (57.38 g kg⁻¹ protein).

Table 4

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