

Glutamate ameliorates copper-induced oxidative injury by regulating antioxidant defences in fish intestine

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Abstract

The objective of this study was to determine the protective effect of glutamate (Glu) in Cu-induced oxidative injury in fish intestine *in vivo* and enterocytes *in vitro*. The results indicated that exposure to 6 mg/l Cu for 72 h induced the production of reactive oxygen species, thereby increasing protein oxidation and lipid peroxidation in enterocytes of grass carp *in vitro*. Cells exposed to Cu alone resulted in a significant increase in lactate dehydrogenase release, which is accompanied by depletions of antioxidants, including total superoxide dismutase (T-SOD), glutathione S-transferase (GST), glutathione reductase (GR), anti-superoxide anion (ASA), anti-hydroxy radical (AHR) activities and GSH content. Pre-treatment with Glu remarkably prevented the toxic effects of Cu on the T-SOD, GST, GR, AHR, and ASA activities and GSH content in enterocytes. However, Cu induced an adaptive increase in the activities of catalase and glutathione peroxidase (GPx). Glu supplementation further increased GPx activity in enterocytes. Interestingly, the experiment *in vivo* showed that Glu pre-supplementation significantly elevated SOD, GPx, GST, GR, ASA and AHR activities, as well as GSH content. Further results showed that pre-treatment with Glu could alleviate Cu-induced oxidative injury by elevating antioxidant enzyme activities through regulating the expression of NF-E2-related nuclear factor 2 (Nrf2) mRNA. Together, these results indicated that Glu could attenuate Cu-induced cellular oxidative damage in fish intestine, likely mediated through Nrf2 signalling pathways regulating mRNA expressions of antioxidant enzyme genes and synthesis of GSH.

Key words: Glutamate: Copper: Oxidative injury: Antioxidants: NF-E2-related nuclear factor 2: Intestine

L-Glutamate (Glu) is a non-essential amino acid with versatile functions in animal physiology and metabolism⁽¹⁾. Glu has an important role in the amino acid metabolism through its conversion to α -ketoglutarate or other amino acids, including alanine, aspartate, ornithine and proline in intestine^(2,3). Thus, Glu can serve both locally inside enterocytes and through the production of other amino acids in an interorgan metabolic perspective⁽²⁾. In addition, Glu is a key transamination partner and is required for the synthesis of GSH, which is an important component in the defence against oxidative stress⁽⁴⁾. Our previous studies demonstrated that dietary Glu supplementation increased intestinal anti-superoxide anion (ASA), anti-hydroxy radical (AHR), glutathione reductase (GR), catalase (CAT) and total superoxide dismutase (T-SOD) activities, and GSH content

in grass carp *Ctenopharyngodon idella*⁽⁵⁾, improved antioxidant capacity and regulated antioxidant-related signalling molecule expression of fish enterocytes⁽⁶⁾. Sivakumar *et al.*⁽⁷⁾ reported that Glu alleviated isoproterenol-induced oxidative stress in rats by increasing antioxidant enzyme activities and GSH content from exogenous Glu. These results suggested that Glu could offer protection during intestinal oxidative stress.

Cu is known for its essentiality for living organisms, including fish, which is required for maintaining cellular function and acting as a cofactor for a number of key metabolic enzymes^(8,9). Nevertheless, Cu is one of the most important pollution-causing metals, because it is commonly released into the environment through industrial wastes and used in the form of copper

Abbreviations: AHR, anti-hydroxy radical; AKP, alkaline phosphatase; ASA, anti-superoxide anion; CAT, catalase; DMEM, Dulbecco's Modified Eagle's Medium; Glu, glutamate; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S-transferase; LDH, lactate dehydrogenase; MDA, malondialdehyde; MTS, 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; Nrf2, NF-E2-related nuclear factor 2; OD, optical density; PC, protein carbonyl; ROS, reactive oxygen species; SOD, superoxide dismutase.

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sulphate as algacide, fungicide, bactericide and herbicide. In fish culture systems, Cu is regularly used in the form of copper sulphate (CuSO_4) to control algal blooms and aquatic macrophyte infestations⁽¹⁶⁾. Recently, aquatic organisms may suffer from exposure to Cu concentrations that might be ten to fifty times higher than the required concentrations⁽¹⁰⁾. The danger of Cu is aggravated by their almost indefinite persistence in water because they cannot be destroyed biologically but are only transformed from one state to another⁽¹¹⁾. Also, high concentrations of waterborne Cu can be toxic, because perturbations in Cu homeostasis result in oxidative stress and increased free-radical production^(12–14). The intestine is very sensitive to a wide range of stressors⁽¹⁵⁾. The recent reports show that the intestine is a major target for waterborne Cu toxicity in both freshwater and seawater fish^(16–18). Although Cu uptake is via gills, Cu exposure highly elevates fish intestinal Cu load⁽¹⁷⁾. In grass carp, accumulation of Cu in intestine is higher than that in gill⁽¹⁹⁾. Our previous study demonstrated that Cu exposure could induce oxidative stress in intestine and the enterocytes of juvenile Jian carp *Cyprinus carpio* var. Jian⁽¹⁶⁾. However, few studies examined how to efficiently protect the intestine against Cu-induced oxidative damage. Given the increasing release of Cu into the environment and its potentially harmful effects on fish, it is important to expand our knowledge of how to protect fish against Cu toxicity. Some nutrients could prevent Cu-induced oxidative damage and change antioxidant capacity in fish enterocytes⁽¹⁶⁾.

Piscine antioxidant capacity can be assessed by the content of non-enzymatic compounds (e.g. GSH) and activities of antioxidant enzymes including superoxide dismutase (SOD), CAT and glutathione peroxidase (GPx), glutathione S-transferase (GST) and GR⁽²⁰⁾. These antioxidant compounds and enzymes have key roles in eliminating the reactive oxygen species (ROS) in fish^(21–23). ROS are generated during normal cellular function, but high doses and/or inadequate removal of ROS results in oxidative stress that may cause severe metabolic malfunctions and impair cell health status⁽²⁴⁾. NF-E2-related nuclear factor 2 (Nrf2) is an important transcription factor that can bind to the antioxidant responsive element (ARE) and induce transcription of antioxidant enzyme genes⁽²⁵⁾. Kelch-like ECH-associated protein 1 (Keap1) was identified as an Nrf2-binding protein, which depresses Nrf2 translocation to the nucleus⁽²⁶⁾. Our previous report showed that antioxidant enzyme activities were regulated by the Nrf2 and Keap1 signalling molecules in fish⁽²⁷⁾. Chen *et al.*⁽²⁸⁾ reported that the up-regulation of *Nrf2* expression could elevate the antioxidant gene (including *SOD*, *CAT*, *GPx*, *GR* and *GST*) expression levels in the mouse liver. However, to date, no study has addressed the effect of Glu on Nrf2 signalling pathway in fish. Our recent study showed that Glu could regulate *Nrf2* and *Keap1a* gene expression, maybe mediating the signal transduction involved in increased gene expressions of antioxidant enzymes in fish enterocytes⁽⁶⁾.

The present study was designed to investigate whether Glu could attenuate Cu-induced cellular oxidative damage, mediating through Nrf2 signalling pathways regulating mRNA expressions of antioxidant enzymes genes and synthesis of GSH in fish intestine.

Methods

In vivo experiments

Animal collection and acclimation conditions. Animal Care advisory Committee of Sichuan Agricultural University specifically approved this study. Yong grass carp were obtained from Tong Wei fisheries and acclimated for 4 weeks. Dissolved O_2 was not <6.0 mg/l. Water temperature and pH were 24 (SEM 3)°C and 7.5 (SEM 0.5), respectively.

Protective effect of glutamate in copper-induced oxidative stress in the intestine. The formulations of the basal and experimental diets (Table 1) were similar as in our previous study⁽⁵⁾. In brief, it contained 280 g of crude protein/kg diet. The basal diet was Glu unsupplemented control (Ctrl). Glu was added to the basal diet to provide 8 g Glu/kg diet, which was the required Glu concentration for optimal growth established by our previous study⁽⁵⁾. Procedures for diet preparation and storage were the same as those described by Shiau & Su⁽²⁹⁾. A total of 180 fish with an average initial weight of 247 (SEM 7.5) g from the acclimatisation tank were randomly assigned into two groups of three replicates each. The groups were fed either the Ctrl diet or the Glu diet for 56 d. The experimental conditions were the same as in our previous study⁽⁵⁾.

At the end of the feeding trial, the fish in each tank were weighed and collected for Cu exposure. Fish with a similar body weight from both the Ctrl and Glu groups were exposed to 0.7 mg Cu/l water for 96 h, which has been proved to induce oxidative stress in grass carp according to our previous study⁽³⁰⁾.

Table 1. Feed formulation and chemical composition of diets

Diets	Ctrl group	Glu group	Ctrl group	Glu group
Formulation (g/kg diet)			Essential amino acids	
Soyabean meal	200	200	Lys	7.2 7.2
Rapeseed meal	220	220	Met	2.7 2.7
Cotton meal	92	92	Thr	9.7 9.8
Wheat flour	424	416	Arg	9.2 9.3
Soya oil	10	10	Leu	15.4 16.1
Monocalcium phosphate	18	18	His	4.8 5.0
Choline chloride	6	6	Ile	8.2 8.8
Vitamin premix*	10	10	Phe	8.4 9.2
Mineral premix†	20	20	Val	11.8 11.6
L-Glu	0	8	Non-essential amino acid	
Total	1000	1000	Ala	14.9 15.3
			Asp	15.2 15.8
Analysed chemical composition (g/kg DM)			Glu	40.5 47.4
Proximate nutrients			Gly	18.5 18.3
Crude protein	266	277	Pro	13.4 13.5
Crude lipid	37.5	37.2	Ser	12.5 12.3
Crude ash	73.1	74.4	Tyr	5.5 5.9

* Vitamin premix (g/kg): retinyl acetate (150 g/kg), 0.80 g; cholecalciferol (12.5 g/kg), 0.48 g; DL- α -tocopheryl acetate (500 g/kg), 2000 g; menadione (230 g/kg), 0.22 g; thiamine hydrochloride (980 g/kg), 0.12 g; riboflavin (800 g/kg), 0.99 g; pyridoxine hydrochloride (980 g/kg), 0.62 g; cyanocobalamin (10 g/kg), 0.10 g; niacin (990 g/kg), 2.58 g; D-biotin (20 g/kg), 5.00 g; meso-inositol (990 g/kg), 52.33 g; folic acid (960 g/kg), 0.52 g; ascorbyl acetate (930 g/kg), 7.16 g; calcium-D-pantothenate (900 g/kg), 2.78 g. All ingredients were diluted with maize starch to 1 kg.

† Mineral premix (g/kg): $\text{FeSO}_4 \cdot \text{H}_2\text{O}$, 25.00 g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.60 g; $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$, 4.35 g; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 2.04 g; KI, 1.10 g; NaSeO_3 , 2.50 g; $\text{MgSO}_4 \cdot \text{H}_2\text{O}$, 230.67 g. All ingredients were diluted with CaCO_3 to 1 kg.

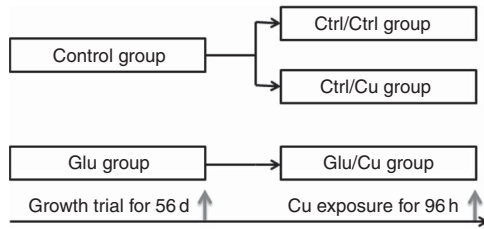


Fig. 1. Overview of experiment design *in vivo*.

In addition, the Ctrl/Ctrl treatment (fish from the Ctrl) was performed by exposing the fish from the Glu unsupplemented group to Cu-free water. Therefore, there were three different pre-treatment/exposure groups, Ctrl/Ctrl, Ctrl/Cu and Glu/Cu, with three replicates per group and twelve fish per replicate (thirty-six fish for each group) (Fig. 1). During the Cu exposure period in replicates, the experimental conditions were the same as those in the growth trial, but no food was provided and the water was not renewed⁽¹⁶⁾. At the end of the challenge trial, all of the living fish from each tank were anaesthetised in a benzocaine bath according to Basic *et al.*⁽³¹⁾. The intestines of fish were quickly removed, frozen in liquid N₂ and stored at -80°C for further analysis. Because the fish were fasted for 96 h during Cu exposure, it was not necessary to empty the intestinal lumen⁽³²⁾.

In vitro experiments

Chemicals. Copper sulphate pentahydrate (CuSO₄·5H₂O), Glu, insulin, collagenase, dispase, D-sorbitol, Triton X-100, transferrin, benzyl penicillin and streptomycin sulphate were purchased from Sigma. Dulbecco's Modified Eagle's Medium (DMEM), Hank's balanced salt solution (HBSS) and fetal bovine serum (FBS) were purchased from Hyclone. Glu-free DMEM was ordered from Beijing Tsing Skywing Bio Tech Co. Ltd. 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega Corporation.

Primary enterocyte culture. The isolation and culture of primary enterocytes from grass carp *C. idella* intestine were performed according to the methods of Jiang *et al.*⁽²²⁾ with minor modifications. In brief, healthy grass carp with an average weight of 48.5 (SEM 1.2) g were food deprived for 24 h before the experiment and killed by decapitation. The intestines were rapidly separated from the carcass, opened and rinsed with HBSS containing antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Enterocytes were isolated by collagenase and dispase digestion. Next, cells were suspended in DMEM (containing 2% D-sorbitol, S-DMEM) and washed with S-DMEM five times to remove any undigested material and single cells according to Booth & O'Shea⁽³³⁾ with slight modifications. Isolated enterocytes were seeded in twenty-four-well culture plates (Falcon) at the density of 2×10^5 cells per well that had been previously coated with collagen I (Sigma), as previously described by us⁽³⁴⁾. The cells were cultured in DMEM supplemented with 5% FBS, 0.02 mg transferrin/ml, 0.01 mg insulin/ml and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 26 (SEM 0.5)°C under a Biochemical

Incubator (Shanghai Boxun Industry & Commerce Co. Ltd) in an air atmosphere. The cells were allowed to attach to plates for 72 h.

Prevention of copper-induced oxidative stress by glutamate in fish enterocytes.

To investigate the effect of Glu on Cu-induced oxidative stress in fish enterocytes, cells were pre-treated with different concentrations of Glu (0–12 mmol/l) for 72 h. Next, cells were exposed to 6 mg Cu/l Glu-free medium for 24 h in a 27°C incubator. The Cu exposure concentration was chosen because previous experiments showed that 6 mg/l Cu of medium could induce oxidative stress in carp enterocytes⁽¹⁶⁾. Thus, there were eight groups (pre-treatment/Cu exposure): Ctrl/Ctrl, Ctrl/Cu, 2mmol/l Glu/Cu, 4mmol/l Glu/Cu, 6mmol/l Glu/Cu, 8mmol/l Glu/Cu, 10mmol/l Glu/Cu and 12mmol/l Glu/Cu. At the end of the exposure, the MTS assay was performed. Cytotoxicity was determined by measuring lactate dehydrogenase (LDH) activity and malondialdehyde (MDA) in the culture media supernatants. Cell lysates were collected to detect protein carbonyl (PC) and GSH contents, alkaline phosphatase (AKP), ASA, AHR, SOD, CAT, GPx, GST and GR activities, and *CAT*, *GPx*, *GST*, *GR*, *Nrf2* and *Keap1a* mRNA expression.

Analysis and measurement

Cell viability and differentiation assays. Cell viability *in vivo* experiments were quantified using the CellTiter 96[®] Aqueous One Solution cell proliferation assay kit (Promega). In brief, at the end of the experiment, 40 µl of MTS working solution was added to each well. After incubation for 2 h, the amount of formazan was determined by measuring the optical density (OD) at 490 nm on a plate reader (Wellsan MK3; Labsystems). AKP activity was assayed according to Krogdahl *et al.*⁽³⁵⁾.

Lactate dehydrogenase, malondialdehyde, protein carbonyl and antioxidant parameter analysis.

Cu-induced cytotoxicity was quantified by measuring the amounts of LDH released into the culture medium from injured cell *in vitro* experiments^(36,37). The amount of LDH released was measured using the method of Mulier *et al.*⁽³⁸⁾. MDA content was analysed as described by Zhang *et al.*⁽³⁹⁾ using the thiobarbituric acid reaction. The content of PC was determined according to the method described by Armenteros *et al.*⁽⁴⁰⁾ using 2, 4-dinitrophenylhydrazine reagent. The activities of SOD, CAT and GPx were determined by the method described by Chen *et al.*⁽²⁸⁾. GST and GR activities were measured by the method described by Pandey *et al.*⁽⁴¹⁾ with minor modification. GSH contents were determined by using a method described by Chen *et al.*⁽²³⁾ with a minor modification. The method is based on the formation of yellow colour when dithio nitrobenzoic acid reacts with compounds containing sulfhydryl groups. The amount of GSH was expressed as nmol of GSH per mg protein. ASA (anti-superoxide anion) and AHR (anti-hydroxy radical) activities were assayed according to the method described by Jiang *et al.*⁽⁴²⁾.

ASA was determined using the Superoxide Anion Free Radical Detection Kit (Nanjing Jiancheng Bioengineer Institute).

Superoxide anion (O_2^-) were generated by the action of xanthine and xanthine oxidase. When the electron acceptor added, a coloration reaction is developed using the nitro blue tetrazolium. The coloration degree is directly proportional to the quantity of superoxide anion in the reaction. If the sample has anti-superoxide anion activity, the superoxide anion in the reaction will decrease, so the coloration will be weak; if the sample can promote the production of superoxide anion, the coloration will be strong. The superoxide anion can be measured by colorimetry, and then ASA of the homogenate was calculated by the following formula: $ASA \text{ (U/mg protein)} = (OD_{\text{control}} - OD_{\text{sample}}) / (OD_{\text{standard}} - OD_{\text{blank}}) \times \text{standard } V_C \text{ concentration (mmol/l) / protein contain (mg)}$. One unit is 1 mg of homogenate scavenged superoxide anion free radical, which equals 1 mg vitamin C scavenging in 40 min at 37°C.

AHR was determined using the Hydroxyl Radical Detection Kit (Nanjing Jiancheng Bioengineer Institute). It was on the basis of Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$). According to the principle, hydroxyl radicals are generated by Fenton reaction. When the electron acceptor added, a coloration reaction is developed using the nitro blue tetrazolium. The coloration degree is directly proportional to the quantity of hydroxyl radicals in the reaction. If the sample has anti-hydroxy radical activity, the hydroxyl radicals in the reaction will decrease, so the coloration will be weak; if the sample can promote the production of hydroxyl radicals, the coloration will be strong. The hydroxyl radicals can be measured by colorimetry, and then AHR of the homogenate was calculated by the following formula: $AHR \text{ (U/mg protein)} = (OD_{\text{Control}} - OD_{\text{Sample}}) / (OD_{\text{standard}} - OD_{\text{blank}}) \times \text{standard } H_2O_2 \text{ concentration (mmol/l) / protein contain (mg)}$. One unit is 1 mg homogenate decreased 1 mmol/l H_2O_2 in 1 min at 37°C. The protein contents were measured using the method of Bradford with bovine serum albumin standards⁽⁴³⁾.

Real-time quantitative PCR. Total RNA was isolated using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. 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 2 μ l total RNA was used to synthesise complementary DNA (cDNA) using the PrimeScript[®] RT reagent Kit with gDNA Eraser (Takara Biotechnology Co. Ltd). Real-time quantitative PCR analysis of *CAT*, *GPx*, *GST*, *GR* and house-keeping gene (β -actin) was performed in a CFX96 Real-Time PCR Detection System (Bio-Rad). The gene-specific primers used in this study were listed in Table 2. The PCR mixture consisted of 1 μ l of the first-strand cDNA sample, 0.5 μ l each of forward and reverse primers from 10 μ M stocks, 3 μ l of RNase-free dH₂O and 5 μ l of 2 \times Ssofast EvaGreen Supermix (Bio-Rad). Cycling conditions were 98°C for 10 s, followed forty cycles of 98°C for 5 s, annealing at a different temperature (Table 2) for each gene for 10 s and 72°C for 15 s. Target gene mRNA levels were normalised to the mRNA levels of the reference gene β -actin. 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 analysed using the $2^{-\Delta\Delta CT}$ method according to Jiang *et al.*⁽⁴⁴⁾.

Statistical analysis

A *t* test was used for comparisons between two groups in the growth trial. The other data were analysed by one-way ANOVA using SPSS 13.0 (SPSS Inc.). Duncan's multiple-range test was used to determine significant differences. Data are presented as means with their standard errors. $P < 0.05$ was considered to be statistically significant.

Results

Glutamate prevented copper-induced oxidative damage of the intestine in vivo

Dietary supplementation with Glu significantly increased the growth of grass carp when compared with the Ctrl group – final weight: 614 (SEM 12.2) *v.* 560 (SEM 5.6) g ($P < 0.05$). The effects of Glu on MDA, PC, SOD, CAT, GPx, GST, GR, ASA, AHR and GSH contents in intestine of grass carp under Cu exposure are

Table 2. The primers and annealing temperatures used in real-time quantitative PCR

Names	Sequence (5'–3')	Annealing temperature (°C)	GenBank ID
<i>CAT</i>	QF-GAAGTTCTACACCGATGAGG	58.7	FJ560431
	QR-CCAGAAATCCCAAACCAT		
<i>GR</i>	QF-GTGTCCAACCTCTCCTGTG	59.4	JX854448
	QR-ACTCTGGGGTCCAAAACG		
<i>GPx</i>	QF-GGGCTGGTTATTCTGGGC	61.5	EU828796
	QR-AGGCGATGTCATTCCCTGTTT		
<i>GST</i>	QF-TCTCAAGGAACCCGTCTG	58.4	EU107283
	QR-CCAAGTATCCGTCCACA		
<i>Nrf2</i>	QF-CTGGACGAGGAGACTGGA	62.5	KF733814
	QR-ATCTGTGGTAGGTGGAAC		
<i>Keap1a</i>	QF-TTCCACGCCCTCCTCAA	63.0	KF811013
	QR-TGTACCCTCCCGCTATG		
β -Actin	QF-GGCTGTGCTGTCCCTGTA	61.4	M25013
	QR-GGGCATAACCCCTCGTAGAT		

CAT, catalase; *GR*, glutathione reductase; *GPx*, glutathione peroxidase; *GST*, glutathione S-transferase; *Nrf2*, NF-E2-related nuclear factor 2; *Keap1*, Kelch-like ECH-associated protein 1.



Table 3. Malondialdehyde (MDA), protein carbonyl (PC) and GSH contents (nmol/mg protein), anti-superoxide anion (ASA), anti-hydroxy radical (AHR), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), glutathione reductase (GR) activities (U/mg protein) in the intestine of grass carp-fed diets containing different glutamate levels for 56 d, followed by exposure to 0.7 mg/l Cu for 96 h (Mean values with their standard errors, *n* 6)

Groups	MDA		PC		ASA		AHR		SOD		CAT		GPx		GST		GR		GSH	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Ctrl/Ctrl	1.50 ^a	0.08	2.76 ^a	0.14	358 ^c	13.4	531 ^c	11.7	62.5 ^c	2.9	50.7 ^a	3.3	93.6 ^c	4.6	191 ^b	15.1	108 ^b	11.5	13.2 ^c	1.4
Ctrl/Cu	2.59 ^c	0.12	3.72 ^b	0.18	261 ^a	13.0	310 ^a	15.2	41.8 ^a	4.0	67.0 ^b	2.7	58.9 ^a	5.5	143 ^a	6.1	65.7 ^a	4.7	5.52 ^a	0.61
8 g/kg Glu/Cu	1.65 ^b	0.14	2.82 ^a	0.13	319 ^b	16.4	406 ^b	13.7	56.4 ^b	3.0	54.1 ^a	4.1	78.7 ^b	6.3	189 ^b	9.2	112 ^b	10.8	11.1 ^b	1.1

^{a,b,c} Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).

Table 4. Effect of different concentrations of glutamate on lactate dehydrogenase (LDH) activity and malondialdehyde (MDA) content in media, 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium optical density (MTS OD), alkaline phosphatase (AKP) activities and protein carbonyl (PC) content in copper-exposed grass carp enterocytes* (Mean values with their standard errors, *n* 6)

Groups	MTS OD		AKP (U/g protein)		LDH (U/g protein)		MDA (nmol/ml)		PC (nmol/mg protein)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Ctrl/Ctrl	0.189 ^d	0.005	2.09 ^c	0.17	37.0 ^b	2.8	1.53 ^a	0.12	0.27 ^a	0.01
Ctrl/Cu	0.157 ^a	0.009	1.71 ^a	0.09	47.3 ^d	2.3	2.86 ^d	0.23	0.40 ^d	0.02
2 mmol/l Glu/Cu	0.152 ^{a,b}	0.005	1.88 ^b	0.10	43.3 ^c	1.9	2.78 ^d	0.18	0.39 ^d	0.02
4 mmol/l Glu/Cu	0.160 ^b	0.006	1.97 ^{b,c}	0.09	39.5 ^b	1.4	2.72 ^d	0.08	0.36 ^c	0.01
6 mmol/l Glu/Cu	0.169 ^c	0.005	2.03 ^c	0.10	37.0 ^b	1.1	2.45 ^c	0.10	0.31 ^b	0.02
8 mmol/l Glu/Cu	0.173 ^c	0.008	2.04 ^c	0.09	31.1 ^a	2.4	2.31 ^c	0.10	0.29 ^{a,b}	0.02
10 mmol/l Glu/Cu	0.175 ^c	0.006	2.07 ^c	0.16	29.6 ^a	1.9	2.13 ^b	0.07	0.29 ^{a,b}	0.02
12 mmol/l Glu/Cu	0.176 ^c	0.007	2.04 ^c	0.13	32.0 ^a	1.9	2.15 ^b	0.09	0.28 ^a	0.03

^{a,b,c,d} Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).

* The cells were pre-treated with different concentrations of Glu for 72 h, followed by exposure to 6 mg/l Cu for 24 h.

displayed in Table 3. Compared with the Ctrl/Ctrl group, Ctrl/Cu exposure caused a significant increase in MDA and PC content in the intestine ($P < 0.05$). However, dietary Glu pre-supplementation (Glu/Cu) significantly decreased MDA and PC formation ($P < 0.05$). The activity of CAT in the intestine of grass carp was significantly increased by Cu exposure (Ctrl/Cu) ($P < 0.05$), whereas dietary Glu pre-supplementation (Glu/Cu) significantly prevented the increase in CAT activity ($P < 0.05$). In contrast, Ctrl/Cu exposure significantly decreased ASA, AHR, SOD, GPx, GST and GR activities in the intestine ($P < 0.05$). Glu pre-supplementation (Glu/Cu) significantly prevented the adverse effects of Cu on these enzyme activities ($P < 0.05$). The intestinal GSH content was the lowest in the Ctrl/Cu treatment, followed by the Glu/Cu treatment, and the GSH content was the highest in the Ctrl/Ctrl group ($P < 0.05$).

Glutamate reduced copper-induced oxidative damage of enterocytes in vitro

The present study has investigated the protective effects of Glu pre-treatment on Cu-induced enterocyte oxidative stress. The Cu exposure (Ctrl/Cu) significantly increased LDH release, MDA content in the medium and PC content in enterocytes as compared with the unexposed Ctrl group (Ctrl/Ctrl) ($P < 0.05$) (Table 4). However, pre-treatment with 6–12 mmol/l before exposure to Cu significantly reduced LDH release, MDA generation and PC formation induced by Cu ($P < 0.05$) (Table 4). Exposure to Cu was shown to cause a significant decrease in

MTS OD values and AKP activity compared with that of the Ctrl ($P < 0.05$) (Table 4). As expected, pre-treatment with Glu partially prevented the decrease in cell viability and AKP activity induced by Cu ($P < 0.05$) (Table 4). ASA and AHR activities of enterocytes are shown in Fig. 2. Cu exposure alone significantly decreased ASA and AHR activities in enterocytes. When cells were pre-treated with increasing doses of Glu, before Cu stress, ASA and AHR activities were increased in a dose-dependent manner ($P < 0.05$).

The effects of Glu on antioxidant parameters in enterocytes under Cu exposure are displayed in Table 5. The T-SOD, GST and GR activities and GSH content were significantly decreased in cells exposed to Cu as compared with the unexposed Ctrl treatment ($P < 0.05$). However, pre-treatment with Glu partially prevented a marked reduction in T-SOD, GST and GR activities and GSH content induced by Cu ($P < 0.05$) (Table 5). In contrast, Cu exposure alone significantly increased the CAT and GPx activities. However, pre-treatment with Glu partially prevented the increase in CAT activity. Interestingly, cells pre-treated with Glu further increased high GPx activity (Table 5).

Effects of glutamate on antioxidant-related and NF-E2-related nuclear factor 2 signalling molecule gene expression in the intestine in vivo

As shown in Fig. 3, the Ctrl/Cu treatment increased the relative mRNA expression levels of *CAT* in the intestine of grass carp

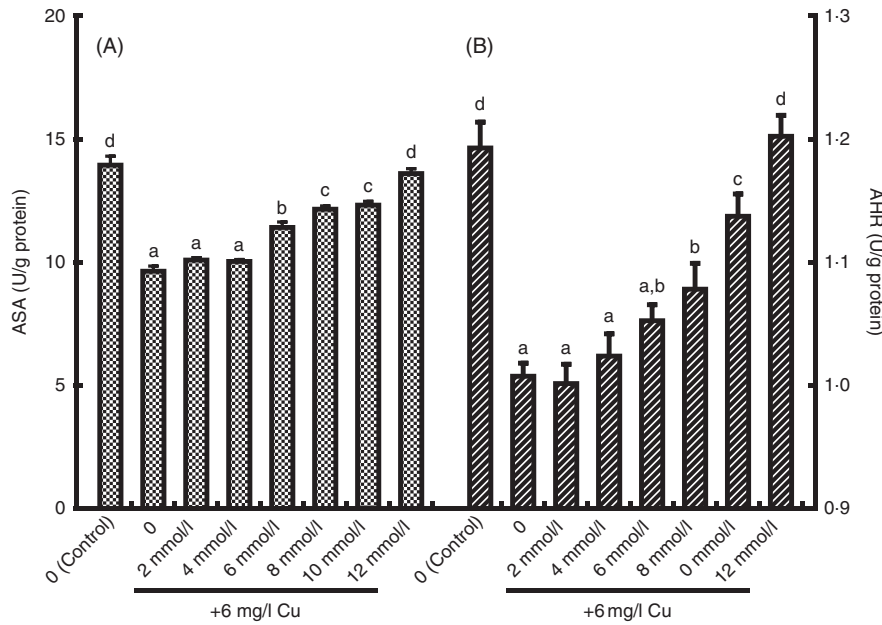


Fig. 2. The anti-superoxide anion (ASA, U/g protein) (A) and anti-hydroxyl radical (AHR, U/g protein), (B) activities in enterocytes cultured with medium containing graded levels of Glu for 72 h, followed by exposure to 6 mg/l Cu for 24 h. Values are means of six replicates, with their standard errors. ^{a,b,c,d} Mean values with unlike letters were significantly different ($P < 0.05$).

Table 5. Effect of different concentrations (U/mg protein) of glutamate on total superoxide dismutase (T-SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), glutathione reductase (GR) activities and GSH content (nmol/mg protein) in copper-exposed grass carp enterocytes* (Mean values with their standard errors, $n = 6$)

Groups	T-SOD		CAT		GPx		GST		GR		GSH	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Ctrl/Ctrl	10.9 ^d	1.0	2.02 ^a	0.12	492.2	13.3 ^a	161.6 ^d	11.6	22.7 ^e	0.9	21.3 ^e	0.5
Ctrl/Cu	3.58 ^a	0.57	7.11 ^e	0.20	571.8 ^b	12.3	96.6 ^a	10.1	13.0 ^a	1.0	14.3 ^a	0.3
2 mmol/l Glu/Cu	3.96 ^a	0.19	7.01 ^{d,e}	0.17	578.3 ^b	8.9	100.9 ^a	12.7	14.0 ^{a,b}	0.8	14.2 ^{a,b}	0.2
4 mmol/l Glu/Cu	4.10 ^{a,b}	0.20	6.76 ^d	0.16	582.5 ^b	13.5	110.4 ^{a,b}	10.3	14.5 ^b	0.9	15.9 ^b	0.4
6 mmol/l Glu/Cu	4.51 ^b	0.20	6.25 ^c	0.28	616.4 ^c	11.6	117.6 ^b	11.6	16.1 ^c	0.8	16.9 ^c	0.3
8 mmol/l Glu/Cu	4.52 ^b	0.23	5.40 ^b	0.18	645.4 ^d	9.2	120.5 ^b	7.8	17.6 ^d	1.3	17.5 ^d	0.3
10 mmol/l Glu/Cu	5.21 ^c	0.17	5.26 ^b	0.26	666.5 ^e	10.4	134.9 ^c	13.1	15.9 ^c	1.0	16.9 ^c	0.4
12 mmol/l Glu/Cu	5.42 ^c	0.13	5.34 ^b	0.42	670.8 ^e	29.5	162.4 ^d	12.8	18.3 ^d	1.0	18.3 ^d	0.3

^{a,b,c,d,e} Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).

* The cells were pre-treated with different concentrations (0, 2, 4, 6, 8, 10, 12 mmol/l) of Glu for 72 h, followed by exposure to 6 mg/l of Cu for 24 h.

compared with the Ctrl/Ctrl group ($P < 0.05$). Dietary Glu treatment (Glu/Cu) significantly prevented the up-regulation of *CAT* mRNA expression ($P < 0.05$). The Ctrl/Cu treatment caused a significant decrease in *GR*, *GPx* and *GST* mRNA expression in the intestine when compared with the Ctrl/Ctrl ($P < 0.05$). Pre-treatment with Glu prevented down-regulation of the *GR*, *GPx* and *GST* mRNA expression ($P < 0.05$). The effects of Glu on *Nrf2* and *Keap1a* mRNA expression in the intestine of fish following Cu exposure are presented in Fig. 3. Fish exposed to Cu showed a decrease in *Nrf2* mRNA expression of intestine as compared with the Ctrl/Ctrl group ($P < 0.05$), and Glu markedly inhibited down-regulation of Cu-induced *Nrf2* mRNA expression ($P < 0.05$). Exposure to Cu significantly increased *Keap1a* transcript abundances in intestine of grass carp compared with the untreated control ($P < 0.05$). Pre-treatment with Glu decreased *Keap1a* mRNA expression ($P < 0.05$).

Effects of glutamate on antioxidant enzyme genes and NF-E2-related nuclear factor 2 signalling molecule in vitro

Relative gene expressions of *CAT*, *GPx*, *GST*, *GR*, *Nrf2* and *Keap1a* in enterocytes were presented in Fig. 4. Cu exposure alone significantly increased *CAT* and *GPx* mRNA expression ($P < 0.05$). Pre-treatment with Glu resulted in a significant increase of levels of *GPx* mRNA as compared with the Cu stress treatment. However, *GST* and *GR* mRNA expression were significantly decreased by Cu stress alone, and Glu markedly inhibited Cu-induced down-regulation of *GST* and *GR* mRNA expression ($P < 0.05$). The relative expression level of *Nrf2* in enterocytes was significantly down-regulated by Cu stress alone ($P < 0.05$). Pre-treatment with Glu before Cu exposure significantly depressed Cu-induced down-regulation of *Nrf2* mRNA ($P < 0.05$). Cu exposure significantly increased the

relative mRNA expression levels of *Keap1a* in enterocytes ($P < 0.05$). Pre-treatment of cells with Glu partially prevented the increase in Cu-induced relative mRNA expression of *Keap1a* ($P < 0.05$).

Discussion

Cu is an essential nutrient, which has numerous functions in cellular biochemistry (such as a cofactor for many different enzymes)⁽⁴⁵⁾. However, Cu at higher concentrations may be toxic. The toxicity of Cu is suggested to be mainly caused by oxidative stress in fish⁽⁴⁶⁾. It was demonstrated that Cu exposure could induce oxidative stress in zebrafish hepatocytes⁽¹⁴⁾, rainbow trout gill cell⁽⁴⁶⁾ and carp enterocytes⁽¹⁶⁾. Oxidative damage in cultured cells has been assessed by LDH release⁽⁴⁷⁾. The extent of cell damage was assessed by measuring the release of the cytosolic enzyme LDH from damaged cells to the bathing medium⁽⁴⁸⁾. The present study demonstrated that Cu

exposure significantly increased LDH levels in the medium, indicating severe enterocyte damage. The colorimetric assay using MTS can rapidly quantify the cell viability of fish enterocyte^(44,49). AKP serves as an enterocyte differentiation marker and is considered to be involved in the absorption of nutrients⁽⁵⁰⁾. In the present study, Cu exposure significantly decreased cell viability and AKP activity. Interestingly, treatment of Cu-exposed enterocytes with some concentrations of Glu depressed LDH release, and elevated cell viability and AKP activity. MDA and PC contents were widely used as biochemical indicators of oxidative damage⁽⁵¹⁾. Exposure of enterocytes to 6 mg/l Cu was shown to cause a significant increase in MDA and PC contents, suggesting that Cu exposure caused lipid and protein oxidative damage in enterocytes, which may be attributed to the increase of ROS production. ROS are produced in response to metals⁽⁵²⁾. Increasing the levels of ROS can lead to severe cell injury or death⁽⁵³⁾. Superoxide anions can cause a wide range of oxidative damage within the cell⁽⁵¹⁾. The present study showed that MDA and PC contents were decreased in cells treated with Glu in a dose-dependent manner. The result suggested that Glu may exert potent protection of the intestine against Cu-induced oxidative damage. To our knowledge, no study has investigated the protective effects of Glu against metal toxicity. This is the first report demonstrating that Glu could attenuate Cu-induced cellular damage.

To investigate the potential protective effects of Glu against Cu-induced oxidative damage, the antioxidant enzymes such as SOD, CAT, GPx, GST, GR and GSH content were determined. The SOD is the first enzyme to respond against O₂ radicals and important endogenous antioxidants for protection against oxidative stress⁽⁵⁴⁾. CAT has been implicated as an essential defence against the potential toxicity of hydroxyl radicals⁽⁵⁵⁾. The GSH-dependent enzymes (GST, GPx and GR) are able to counteract peroxidative damage⁽⁵⁶⁾. GSH is the major endogenous antioxidant scavenger that protects cells from oxidative stress, and GSH/GSSG represents the major cellular redox buffer and therefore is a representative indicator for the redox environment of the cell⁽⁵⁷⁾. In the present study, a significant decrease in SOD and GR activities and GSH content was found

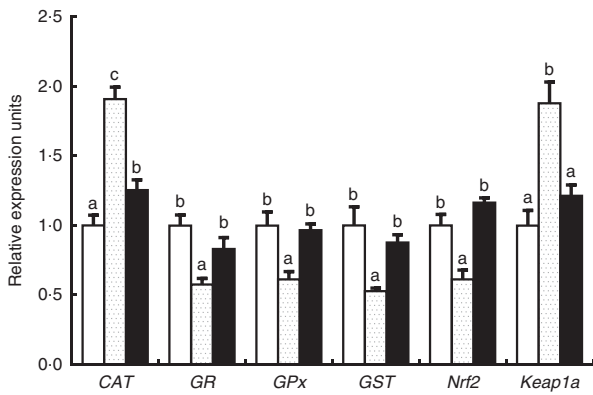


Fig. 3. Relative gene expression (shows to be relative to Ctrl/Ctrl (□), which was set at 1.0, in arbitrary units) of catalase (*CAT*), glutathione reductase (*GR*), glutathione peroxidase (*GPx*), glutathione S-transferase (*GST*), NF-E2-related nuclear factor 2 (*Nrf2*) and Kelch-like ECH-associated protein 1 (*Keap1*) in the intestine of young grass carp (*Ctenopharyngodon idella*) fed diets containing different Glu levels for 56 d, followed by exposure to 0.7 mg/l Cu for 96 h. Values are means of six replicates, with their standard errors. ^{a,b,c} Mean values with unlike letters were significantly different ($P < 0.05$). □, Ctrl/Cu; ■, Glu/Cu.

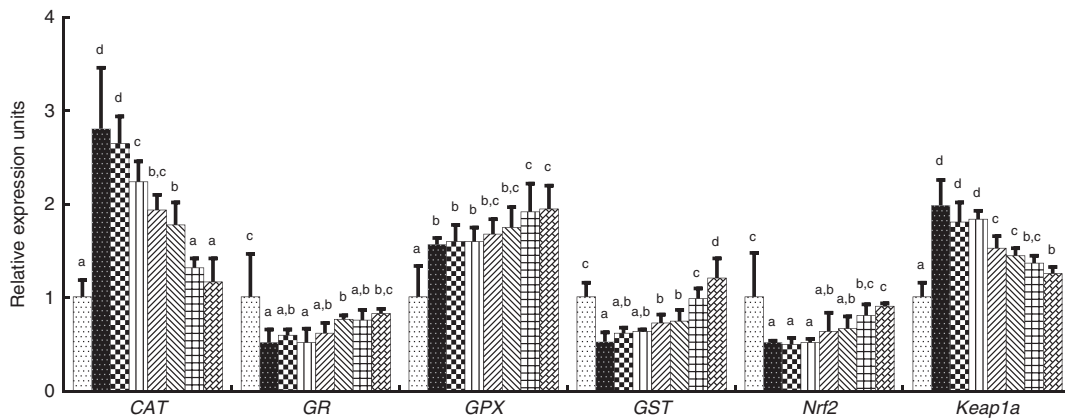


Fig. 4. Relative expression (shows to be relative to Ctrl/Ctrl, which was set at 1.0, in arbitrary units) of catalase (*CAT*), glutathione reductase (*GR*), glutathione peroxidase (*GPx*), glutathione S-transferase (*GST*), NF-E2-related nuclear factor 2 (*Nrf2*) and Kelch-like ECH-associated protein 1 (*Keap1*) mRNA in enterocytes cultured with medium containing graded levels of Glu for 72 h, followed by exposure to 6 mg/l Cu for 24 h. Values are means of six replicates, with their standard errors. ^{a,b,c,d} Mean values with unlike letters were significantly different ($P < 0.05$). □, Control; ■, 0 mmol/l; ▨, 2 mmol/l; ▩, 4 mmol/l; ▪, 6 mmol/l; ▫, 8 mmol/l; ▬, 10 mmol/l; ▮, 12 mmol/l.

in cells exposed to Cu. The result demonstrated that Cu-induced enterocyte oxidative damage may be partly related to disturbance of the antioxidant system. Glu pre-treatment prevented a Cu-induced decrease of these antioxidant enzyme activities. This may suggest that the antioxidative effect of Glu may be attributed to its ability to maintain the activity of radical scavenging enzymes. A variety of citrate cycle intermediates such as fumarate, oxaloacetate, malate and succinate were shown to modulate lipid peroxide production by interacting with endogenous iron ions, thus adjusting the iron redox cycle and, subsequently, free-radical generation⁽⁵⁸⁾. Glu can form α -ketoglutarate by deamination and enter into the citrate cycle to promote the production of citrate cycle intermediates⁽³⁾. Hence, the reduction in lipid peroxides with an increase in GSH on Glu pre-treatment could be because of the antioxidant effect of citrate cycle intermediates formed from Glu. Further, GSH was produced from exogenous Glu⁽²⁾. However, Cu exposure significantly elevated CAT and GPx activities. Glu pre-treatment of enterocytes further increased GPx activities. The reason might be attributed to an adaptive mechanism against stress. The enterocytes were directly exposed to Cu; increased CAT and GPx activities were required to inhibit the Cu-induced oxidative stress. The ASA and AHR activity are two indexes used to evaluate the total capacity of scavenging superoxide and hydroxyl radical, respectively⁽²²⁾. To investigate how Glu inhibited the Cu-induced oxidative damage in fish, we determined the ASA and AHR activities. Our results showed that Cu exposure significantly decreased the ASA and AHR activities in enterocytes. Glu pre-treatment prevented the Cu-induced decrease of ASA and AHR activities in a dose-dependent manner. These results indicated that the protective effects of Glu on Cu-induced oxidative damage may, at least in part, be because of the increased ASA and AHR activities.

The activities of antioxidant enzymes can be affected by the mRNA levels in fish⁽²⁷⁾. To further elucidate whether Glu regulated antioxidant enzyme activities at the gene level in fish, the mRNA levels of antioxidant enzyme of enterocytes after being challenged against Cu were investigated. The results of the current study demonstrated that Cu exposure significantly increased mRNA levels of *CAT* and *GPx* in enterocytes. Glu pre-treatment blocked the increase in *CAT* mRNA expression induced by Cu. However, Glu pre-treatment further elevated mRNA expression of *GPx* gene induced by Cu. The antioxidant enzyme gene expression exhibited a same pattern with their respective enzyme changes. The regulation of antioxidant gene mRNA levels may result from activating the antioxidant-related signalling molecules. Nrf2 is a master regulator of the antioxidant response through regulating the transcription of antioxidant gene in fish, including *SOD*, *CAT*, *GPx*, *GR* and *GST*⁽²⁶⁾. The present study showed that Cu exposure down-regulated the *Nrf2* gene expression in enterocytes. The result is very well in agreement with our previous report, which showed that Cu exposure markedly decreased the binding of nuclear Nrf2 to ARE⁽⁵⁹⁾. In addition, the negative effects of Cu-induced antioxidant gene mRNA expression may be partly ascribed to a decrease 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⁽²⁶⁾. Our study also showed that Cu induces the up-regulation of *Keap1a* mRNA level in enterocytes⁽⁶⁾. Glu pre-treatment elevated *Nrf2* mRNA expression and blocked the increase in *Keap1a* mRNA expression⁽⁶⁾. These results suggested that the antioxidant effect of Glu is mediated at least in part by Nrf2 signalling pathways in fish. To date, no information is available about the effect of Glu on *Nrf2* gene mRNA expression in fish enterocytes. The underlying mechanism needs further investigation.

On the basis of the beneficial effects of Glu against Cu-induced oxidative damage in the enterocytes, it was reasonable to hypothesise that Glu can protect fish against Cu-induced intestinal oxidative damage *in vivo*. The present study showed that Cu exposure could induce intestinal oxidative stress in grass carp. Similar results were observed that Cu exposure could induce oxidative stress in juvenile *Epinephelus coioides* intestine⁽⁶⁰⁾ and pacu *Piaractus mesopotamicus* liver⁽⁶¹⁾. The antioxidant enzyme activities of intestine were elevated in response to Glu supplementation. The *GR*, *GPx* and *GST* mRNA abundance in intestine was enhanced by Glu pre-treatment. The positive effects of Glu on antioxidant enzyme mRNA expression may be partly ascribed to promote Nrf2 nuclear translocation by down-regulating *Keap1a* mRNA expression. In the present study, a significant increase in mRNA levels of *Nrf2* was observed in the grass carp intestine with Glu supplementation. In contrast, Glu pre-treatment significantly decreased *Keap1a* mRNA expression. These results were in agreement with the present study statements *in vitro*. Studies from rats also indicated that Glu supplementation alleviates oxidative damage induced by isoproterenol⁽⁷⁾. However, it is of interest that the *in vivo* GPx activity in intestine was decreased by Cu exposure, which was the reverse of the pattern observed in enterocytes. The reason for these results is unclear. The differences may be because, in this study, the enterocytes were directly exposed to Cu, whereas the intestine is indirectly exposed to Cu. In other words, because the enterocytes were directly exposed to Cu, increased GPx activity was required to inhibit the Cu-induced oxidative stress. The previous study in grass carp showed that dietary Cu content (5.25 mg/kg), beyond requirement (4.78 mg/kg), induced intestinal oxidative stress, but increased GPx activity⁽⁹⁾. In addition, higher doses of Cu (6.70–8.33 mg/kg) induced intestinal oxidative stress and decreased the GPx activity at the same time⁽⁹⁾. Therefore, the enterocytes directly exposed to a certain amount of Cu by culture *in vitro* and oral administration *in vivo* can both induce oxidative stress, as well as increase GPx activity in fish intestine.

In conclusion, Cu exposure could induce oxidative damage, resulting in lipid peroxidation, protein oxidation and antioxidant enzymes activity alterations in intestine and the enterocytes of grass carp. Cu exposure down-regulated the mRNA abundance of *GR*, *GPx* and *GST* in the intestine of grass carp. Dietary and medium pre-supplementation with Glu could alleviate Cu-induced oxidative damage in fish intestine and the enterocytes, respectively. The protective effects of Glu on Cu-induced oxidative damage are associated with up-regulating the expression of antioxidant enzymes gene by regulating mRNA abundance of signalling molecule *Nrf2*. It provides a

theoretical basis for reducing oxidative stress of Cu in cultured fish by means of nutrition.

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J. J. and X.-Y. W. conducted the trial, performed the RT-PCR experiments and wrote the manuscript. Y. Z. and X.-Q. Z. contributed to the design of the study. L. F. and W.-D. J. assisted in the manuscript preparation. Y. L. assisted with all data analysis. P. W. assisted with the trial.

The authors declare that there are no conflicts of interest.

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