

# Effects of glutamate on growth, antioxidant capacity, and antioxidant-related signaling molecule expression in primary cultures of fish enterocytes

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**Abstract** The present study explored the effects of glutamate (Glu) on the growth, antioxidant capacity, and gene expression of NF-E2-related nuclear factor 2 (Nrf2) signaling molecule in enterocytes of Jian carp (*Cyprinus carpio* var. Jian). The enterocytes were incubated in media containing 0, 2, 4, 6, 8, and 10 mM/L Glu for 96 h. The results showed that Glu could promote fish enterocytes proliferation and differentiation. Additionally, activities of alkaline phosphatase, Na<sup>+</sup>, K<sup>+</sup>-ATPase,  $\gamma$ -glutamyl transpeptidase, and creatine kinase were significantly improved with the increase in Glu level up to 6 mM/L. Lactic acid dehydrogenase activity and malondialdehyde content in the medium and cellular protein

carbonyls were depressed by Glu. Moreover, optimum Glu significantly enhanced glutathione content and the activities and gene expression of catalase, glutathione reductase, and glutathione peroxidase in enterocytes. Finally, the expression level of Nrf2 in enterocytes was significantly elevated by appropriate Glu content in the medium. Furthermore, optimum Glu significantly decreased Kelch-like ECH-associated protein 1 mRNA level in enterocytes. In conclusion, Glu improved the proliferation, function, and antioxidant capacity and regulated antioxidant-related signaling molecule expression of fish enterocytes.

**Keywords** Growth · Antioxidant capacity · Signaling molecule · Enterocytes · Glutamate

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## Introduction

L-Glutamate (Glu) is a non-essential metabolically versatile amino acid (Brosnan 2000; Neu et al. 1996). In brain, Glu plays a major role in the development of central nervous system and serves as the major mediator of excitatory signals (Falkenberg et al. 2014). In intestine, Glu is a key transamination partner and is required for the synthesis of glutathione (GSH), an essential component in the defense against oxidative stress (Amores-Sánchez and Medina 1999; Johnson et al. 2003). Additionally, Glu is a vital substrate for the ammonia detoxification mechanism in nervous

tissues in Atlantic salmon *Salmo salar* (Kolarevic et al. 2012). Thus, Glu has been identified as a functional amino acid for humans and animals (Brosnan and Brosnan 2013; Wu 2010).

Piscine growth is correlated with digestion and absorption function (Ling et al. 2010). Recent reports show that Glu improves growth, digestive and absorptive capacities of grass carp *Ctenopharyngodon idella* (Zhao et al. 2014a), muscle cellularity, and textural properties of grass carp (Zhao et al. 2014b) and Atlantic salmon (Larsson et al. 2014). Oehme et al. (2010) also observed increased feeding rate, growth, and gut weight of Atlantic salmon fed a diet supplemented with a combination of arginine and Glu. However, the probable mechanism of Glu has not been elucidated. In piglet, dietary monosodium glutamate supplementation was shown to increase jejunal villus height (Rezaei et al. 2013). Our previous study has shown that Glu improves the intestinal weight, length, and enzyme activities in grass carp (Zhao et al. 2014a). These data suggested that the improvement in fish growth may at least be partly due to Glu-induced intestinal structural and functional integrity.

The intestinal epithelium is an important site for nutrient uptake and protecting the organism against harmful agents in the lumen. It consists mainly of absorptive columnar cells (enterocytes) (Al-Hussaini 1949). Nutrition plays a key role in the modulation of the development of the intestine and intestinal functions including nutrient absorption, cell growth, and differentiation (Buccigrossi et al. 2010). Glu was shown to elevate the growth and differentiation of human colon cancer cell (Viallard et al. 1986). However, there are few studies about the effect of Glu on proliferation, differentiation, and function of fish enterocytes. The structural and functional integrity of the intestine and enterocytes may be associated with antioxidant capacity. In general, fish antioxidant systems are composed of non-enzymatic compounds (GSH) and antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST), and glutathione reductase (GR) (Martinez-Alvarez et al. 2005). These antioxidant compound and enzymes play a key role in eliminating the reactive oxygen species (ROS) in fish enterocytes (Wu et al. 2014; Jiang et al. 2009, 2011; Chen et al. 2009). 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 (Kohen and Nyska 2002). Sivakumar et al. (2011) reported that Glu prevented isoproterenol-induced cardiac toxicity by alleviating oxidative stress in rats. Our previous study demonstrated that Glu improved SOD, CAT, GPx, and CAT activities in the intestine of grass carp (Zhao et al. 2014a), suggesting that Glu may promote piscine antioxidant ability of enterocytes. However, to our knowledge, there are few reports about the effect of Glu on piscine antioxidant ability of enterocytes.

NF-E2-related nuclear factor 2 (Nrf2) is an important transcription factor that can bind to the antioxidant-responsive element (ARE) and induce transcriptional of antioxidant enzyme genes, such as SOD, CAT, and GPx (Ramprasath et al. 2012). Kelch-like ECH-associated protein 1 (Keap1) was identified as an Nrf2-binding protein, which depresses Nrf2 translocation to the nucleus (Ma 2013). Our recent study found that Nrf2 and Keap1 exist in Jian carp *Cyprinus carpio* var. Jian and widely distribute in several organs including intestine (Jiang 2013). The regulation of antioxidant enzyme gene transcription by the Keap1/Nrf2–ARE signaling also exists in Jian carp (Jiang 2013). However, to date, no study has addressed the effect of Glu on piscine Nrf2 signaling pathway in enterocytes. Therefore, the present study was designed to investigate the effect of Glu on proliferation, differentiation, and antioxidant capacity and explore whether the antioxidant capacity is mediated through Nrf2 signaling pathways in primary cultures of fish enterocytes.

## Materials and methods

### Chemicals

Glu, insulin, collagenase, dispase, transferrin, benzyl penicillin, and streptomycin sulfate were purchased from Sigma (St. Louis, MO, USA). Hank's balanced salt solution (HBSS) and fetal bovine serum (FBS) were bought from Hyclone (Logan, UT, USA). DMEM medium was ordered from Beijing Tsing Skywing Bio. Tech. Co. Ltd (Beijing, China). 3-(4, 5-dimethylthiazol-

2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega Corporation (Madison, WI).

### Cell isolation and culture

Healthy Jian carp were obtained from a local hatchery and kept in 1000-L tanks supplied with running tap water (20–24 °C). The fishes were fed to satiety with a commercial carp feed four times daily under a natural photoperiod. Cell isolation and culture were carried out according to our previous method with minor modifications (Jiang et al. 2009). In brief, nine healthy carp (40–50 g) were maintained for approximately 24 h without feeding before the experiment and were killed by decapitation. The intestines were rapidly removed from the carcass, opened, and rinsed with Hanks containing 100 µg streptomycin and 100 U penicillin per mL. Enterocytes were isolated by enzymatic dissociation using collagenase and dispase followed by physical disaggregation. Cells were pooled and suspended in DMEM and washed five times with DMEM to remove enzymes and blood cells. Isolated enterocytes were seeded in 24-well culture plates (Falcon, Franklin Lake, NJ, USA) at the density of  $2 \times 10^3$  cells per well that had been previously coated with collagen (Sigma, St. Louis, MO, USA), as previously described by Jiang (2005). The cells were cultured in DMEM supplemented with 5 % FBS, 0.02 mg transferrin, 0.01 mg insulin, 100 µg streptomycin, and 100 U penicillin per mL at  $26 \pm 0.5$  °C in a biochemical incubator (Shanghai Boxun Industry & Commerce Co., Ltd, Medical Equipment Factory, Shanghai, China).

### Glu treatments

Glu stock solutions were prepared by dissolving Glu to a final concentration of 50 mM, and further dilutions were performed using DMEM medium at the desired concentrations (0, 2, 4, 6, 8, and 10 mM/L). All Glu solutions were stored in Eppendorf tubes at  $-80$  °C. To determine the effects of Glu on enterocytes, we ordered Glu-free DMEM medium with L-Glutamine 4.0 mM/L (Life Technologies). And the Glu-free medium group was set as the control. Cells were allowed to attach to plates for 72 h before trial. After inoculation for 72 h, the medium was replaced by a new medium containing different Glu concentrations. Then, the cells were cultured under the same

conditions for 96 h. Six replicate wells were prepared for each concentration of Glu.

### Cell viability assay

Cell growth was quantified by CellTiter 96<sup>®</sup> AQueous One Solution cell proliferation assay kit (Promega, Madison, WI). In brief, after treatment, 40 µL of MTS working solution was added and incubated for 2 h at 27 °C in a humidified atmosphere; the amount of formazan was estimated by optical density (OD) at 490 nm on a plate reader (Wellscan MK3, Labsystems, Finland).

### Cell function assays

After treatment, the cell lysates were prepared according to Jiang et al. (2009) and used to measure the following parameters. Protein concentrations of enterocytes (PCE) were measured using the method of Bradford with bovine serum albumin standards (Bradford 1976). Alkaline phosphatase (AKP) and  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activities were assayed according to Krogdahl et al. (2003). The  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT) and creatine kinase (CK) activities were determined with the  $\gamma$ -GT and a creatine kinase assay kits, respectively (Sigma, St. Louis, MO, USA). In the assay enzymes, one unit of activity was considered as the amount of enzyme required releasing 1 µmol of product  $\text{h}^{-1}$  at 37 °C, except  $\gamma$ -GT that was calculated as  $\text{min}^{-1}$ .

### GSH assay

Reduced GSH of the cell lysates were determined by using a method described by Chen et al. (2009) with a minor modification. The method is based on the formation of yellow color when dithionitro benzoic acid (DTNB) reacts with compounds containing sulfhydryl groups. The amount of GSH was expressed as nmoles of GSH per mg protein.

### Antioxidant enzyme activity assays

The activities of cellular superoxide dismutase (SOD) and catalase (CAT) were determined by our previous methods (Jiang et al. 2013). Glutathione peroxidase (GPx) activity was assessed according to the method by Rotruck et al. (1973). Briefly, a known quantity of enzyme preparation was allowed to react with hydrogen peroxide and GSH for a period of time, and GSH

content after the reaction was assayed using DTNB. Activity of glutathione-S-transferase (GST) was measured by monitoring the adduct formation between GSH and 1-chloro-2, 4-dinitrobenzene (CDNB). Glutathione reductase (GR) activity was measured following the method by Carlberg and Mannervik (1975). GR activity was assayed by measuring the GSH formed when the oxidized glutathione (GSSG) reduced by reduced nicotinamide adenine dinucleotide phosphate (NADPH), and the color intensity was read at 340 nm. The capacities of superoxide anion (SAS) and hydroxyl radical (HRS) scavenging abilities were measured by our previous methods (Jiang et al. 2009).

At the end of the experiment, the medium was collected and the release amount of lactate dehydrogenase (LDH) by the cells was measured following the method by Mulier et al. (1998). And malondialdehyde (MDA) content was analyzed as described by Zhang et al. (2008) using the thiobarbituric acid reaction. The content of protein carbonyl (PC) of enterocyte was determined according to the method described by Armenteros et al. (2009) using 2, 4-dinitrophenylhydrazine reagent.

#### Real-time quantitative PCR

Total RNA isolation was conducted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Two microliters total RNA was used to synthesize cDNA using the

PrimeScript® RT reagent Kit with gDNA Eraser [Takara Biotechnology (Dalian) Co., Ltd]. Real-time quantitative PCR analysis of CAT, GR, GPx1a, GPx1b, Nrf2, Keap1, and housekeeping gene (EF1a) was performed using CFX96 real-time PCR detection system (Bio-Rad, Laboratories, Inc.). The gene-specific primers used in this study are shown in Table 1. The PCR mixture consisted of 1 µL of the first-strand cDNA sample, 0.5 µL each of forward and reverse primers from 10 µM stocks, 3 µL RNase-free dH<sub>2</sub>O, and 5 µL 2 × Ssofast EvaGreen Supermix (Bio-Rad). Cycling conditions were 98 °C for 10 s, followed by forty cycles of 98 °C for 5 s, and annealing at a different temperature (Table 1) for each gene for 10 s and 72 °C for 15 s. A melting curve analysis was generated following each real-time quantitative PCR assay to check and verify the specificity and purity of all PCR products. Each sample was amplified in triplicate. Target gene mRNA concentration was normalized to the mRNA concentration of the reference gene EF1a. Threshold cycle (Ct) value was obtained from CFX96™ real-time PCR system software (Bio-Rad). The expression results were analyzed using the  $2^{-\Delta\Delta CT}$  method after verification that the primers amplified with an efficiency of approximately 100 % (Livak and Schmittgen 2001). The amplification efficiency of target and housekeeping gene was calculated according to the specific gene standard curves which were generated from the tenfold serial dilutions.

**Table 1** Primers and annealing temperature used for in real-time quantitative PCR

Name	Sequence (5'–3')	Annealing temperature (°C)	GenBank ID
CAT-QF	CTGGAAGTGGAATCCGTTTG	54	JF411604
CAT-QR	CGACCTCAGCGAAATAGTTG		
GR-QF	GAGAAGTACGACACCATCCA	56	JF411607
GR-QR	CACACCTATTGAACTGAGATTGAG		
GPx1a-QF	GTGACGACTCTGTGTCCTTG	60.4	JF411605
GPx1a-QR	AACCTTCTGCTGTATCTCTTGA		
GPx1b-QF	TATGTCCGTCCTGGCAATGG	60.4	JF411606
GPx1b-QR	ATCGCTCGGGAATGGAAGTT		
Nrf2-QF	TTCCCGCTGGTTTACCTTAC	60	JX462955
Nrf2-QR	CGTTTCTTCTGCTTGTCTTT		
Keap1-QF	GCTCTTCGGAAACCCCT	60	JX470752
Keap1-QR	GCCCCAAGCCCACTACA		
EF1a-QF	TCACCATTGACATTGCTCTC	56	AF485331
EF1a-QR	TGTTCTTGATGAAGTCTCTGT		

## Statistical analysis

The data were presented as mean  $\pm$  SEM. All data were subjected to one-way analysis of variance (ANOVA) using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences between groups, multiple comparisons were made with Duncan's multiple-range test at the level of  $P < 0.05$ .

## Results

The effects of Glu on cell growth and function are presented in Table 2. The results show that MTS OD values were the highest for enterocytes cultured in medium containing 6 mM/L Glu and the lowest for the control group ( $P < 0.05$ ). A similar pattern was observed for PCE. AKP activity was higher in enterocytes cultured in medium containing 8 mM/L Glu compared to other groups ( $P < 0.05$ ). Cell  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity was significantly enhanced with the increase in Glu level up to 6 mM/L ( $P < 0.05$ ) and decreased thereafter. The  $\gamma$ -GT activity was responsive to Glu content in medium by increasing with graded levels of Glu up to 6 mM/L ( $P < 0.05$ ), and there was no difference between 8 and 10 mM/L. Cellular CK activities were the highest for enterocytes cultured in medium containing 6 mM/L Glu and the lowest for the control group ( $P < 0.05$ ).

The effect of Glu on antioxidant parameters is presented in Table 3. Cellular T-SOD activity was significantly increased with the increased Glu content in medium up to 6 mM/L ( $P < 0.05$ ) and decreased thereafter. A similar pattern was observed for cellular CAT, GST, and GR activities. GPx activity was the highest for enterocytes cultured in medium containing 6 mM/L Glu ( $P < 0.05$ ), whereas no significant differences were found between the control group and 10 mM/L Glu group. In addition, ASA activity was markedly increased with the increase in Glu content in medium up to 6 mM/L ( $P < 0.05$ ), and there were no differences between the 8 and 10 mM/L Glu levels. A similar pattern was found in cellular HRS capacity. The effects of Glu on the oxidative status of enterocytes are shown in Table 4. Oxidative damage, indicated by LDH release and MDA content in the medium, was significantly lower in 4–8 mM/L Glu groups than in the control group ( $P < 0.05$ ). The PC was the highest in the Glu-unsupplemented group.

**Table 2** Effect of Glu (mM/L) on MTS OD, protein concentrations of enterocytes (PCE,  $\mu\text{g}$  protein per well), and the activities of alkaline phosphatase (AKP, U per g protein),  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase (U per g protein), gamma-glutamyl transpeptidase ( $\gamma$ -GT, U per g protein), and creatine kinase (CK, U per g protein) of carp enterocytes

Glu	0	2	4	6	8	10
MTS OD	0.1206 $\pm$ 0.0099 <sup>a</sup>	0.1268 $\pm$ 0.0037 <sup>ab</sup>	0.1297 $\pm$ 0.0041 <sup>ab,c</sup>	0.1380 $\pm$ 0.0097 <sup>c</sup>	0.1333 $\pm$ 0.0086 <sup>b,c</sup>	0.1282 $\pm$ 0.0073 <sup>ab</sup>
PCE	376.69 $\pm$ 8.23 <sup>a</sup>	387.96 $\pm$ 4.60 <sup>ab,c</sup>	401.71 $\pm$ 7.76 <sup>d</sup>	399.43 $\pm$ 10.27 <sup>c,d</sup>	389.83 $\pm$ 12.35 <sup>b,c</sup>	380.69 $\pm$ 11.32 <sup>ab</sup>
AKP	2.31 $\pm$ 0.07 <sup>a</sup>	2.35 $\pm$ 0.09 <sup>ab</sup>	2.39 $\pm$ 0.14 <sup>ab</sup>	2.54 $\pm$ 0.08 <sup>c,d</sup>	2.59 $\pm$ 0.10 <sup>d</sup>	2.46 $\pm$ 0.08 <sup>b,c</sup>
$\text{Na}^+$ , $\text{K}^+$ -ATPase	88.28 $\pm$ 2.16 <sup>a</sup>	125.65 $\pm$ 6.83 <sup>b</sup>	144.97 $\pm$ 4.27 <sup>c</sup>	152.41 $\pm$ 10.58 <sup>c</sup>	144.63 $\pm$ 6.36 <sup>c</sup>	124.02 $\pm$ 12.28 <sup>b</sup>
$\gamma$ -GT	3161.83 $\pm$ 201.25 <sup>a</sup>	3492.33 $\pm$ 211.83 <sup>b</sup>	3605.67 $\pm$ 138.08 <sup>b,c</sup>	3721.17 $\pm$ 108.10 <sup>c</sup>	3568.17 $\pm$ 147.15 <sup>b,c</sup>	3504.67 $\pm$ 172.91 <sup>b</sup>
CK	3.82 $\pm$ 0.10 <sup>a</sup>	5.26 $\pm$ 0.13 <sup>c</sup>	5.24 $\pm$ 0.10 <sup>c</sup>	6.37 $\pm$ 0.21 <sup>d</sup>	5.53 $\pm$ 0.49 <sup>c</sup>	4.81 $\pm$ 0.66 <sup>b</sup>

Values are mean  $\pm$  SEM,  $n = 6$ . Values within the same row with different superscripts are significantly different ( $P < 0.05$ )

**Table 3** Effect of Glu on total superoxide dismutase (T-SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST), glutathione reductase (GR) activities, superoxide anion scavenging ability (SAS), and hydroxyl radical scavenging ability (HRS) of carp erythrocytes

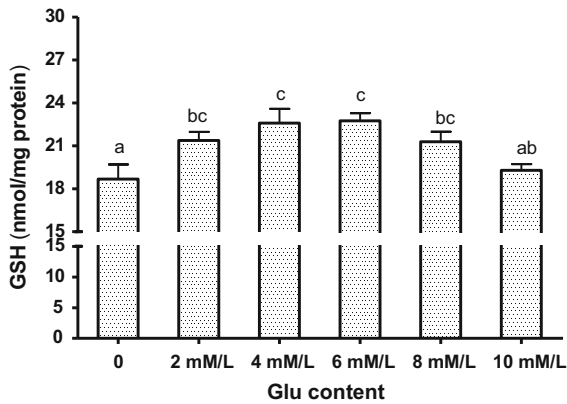
Glu (mM/L)	0	2	4	6	8	10
T-SOD (U per mg protein)	9.00 ± 0.17 <sup>a</sup>	9.43 ± 0.17 <sup>a</sup>	10.29 ± 0.15 <sup>b</sup>	10.39 ± 0.22 <sup>b</sup>	9.33 ± 0.43 <sup>a</sup>	9.09 ± 0.75 <sup>a</sup>
CAT (U per mg protein)	6.54 ± 0.17 <sup>b</sup>	6.82 ± 0.15 <sup>b</sup>	7.33 ± 0.13 <sup>c</sup>	7.43 ± 0.14 <sup>c</sup>	6.56 ± 0.28 <sup>b</sup>	5.50 ± 0.72 <sup>a</sup>
GPx (U per mg protein)	419.09 ± 11.99 <sup>a</sup>	467.84 ± 10.17 <sup>b</sup>	486.15 ± 6.13 <sup>b</sup>	500.90 ± 15.26 <sup>b</sup>	463.47 ± 16.18 <sup>b</sup>	424.33 ± 18.57 <sup>a</sup>
GST (U per mg protein)	214.17 ± 9.01 <sup>a</sup>	241.90 ± 4.37 <sup>b,c</sup>	260.70 ± 7.19 <sup>c,d</sup>	272.58 ± 7.62 <sup>d</sup>	236.47 ± 7.51 <sup>a,b</sup>	218.04 ± 7.85 <sup>a</sup>
GR (U per g protein)	18.28 ± 0.75 <sup>a</sup>	22.21 ± 0.85 <sup>b</sup>	24.18 ± 0.61 <sup>b,c</sup>	24.39 ± 0.58 <sup>c</sup>	22.51 ± 0.44 <sup>b,c</sup>	20.06 ± 0.73 <sup>a</sup>
SAS (U per g protein)	6.34 ± 0.11 <sup>a,b</sup>	6.50 ± 0.07 <sup>b</sup>	6.60 ± 0.08 <sup>b</sup>	6.98 ± 0.07 <sup>c</sup>	6.14 ± 0.03 <sup>a</sup>	6.15 ± 0.13 <sup>a</sup>
HRS (U per mg protein)	1277.58 ± 12.76 <sup>a</sup>	1279.96 ± 8.80 <sup>a</sup>	1290.12 ± 14.82 <sup>a,b</sup>	1322.49 ± 12.09 <sup>b</sup>	1283.46 ± 12.10 <sup>a</sup>	1268.88 ± 13.3 <sup>a</sup>

Values are mean ± SEM,  $n = 6$ . Values within the same row with different superscripts are significantly different ( $P < 0.05$ )

**Table 4** Effect of Glu on the lactate dehydrogenase (LDH) activity and malondialdehyde (MDA) in medium and protein carbonyl (PC) content in erythrocytes

Glu (mM/L)	0	2	4	6	8	10
LDH (U per g protein)	54.78 ± 4.09 <sup>c</sup>	53.38 ± 3.37 <sup>c</sup>	38.89 ± 3.42 <sup>a</sup>	36.51 ± 3.49 <sup>a</sup>	38.17 ± 3.19 <sup>a</sup>	45.43 ± 4.96 <sup>b</sup>
MDA (nmol per mL)	2.89 ± 0.10 <sup>d</sup>	2.85 ± 0.11 <sup>d</sup>	2.55 ± 0.16 <sup>b,c</sup>	2.15 ± 0.23 <sup>a</sup>	2.44 ± 0.22 <sup>b</sup>	2.72 ± 0.12 <sup>c,d</sup>
PC (nmol per mg protein)	0.2582 ± 0.0126 <sup>c</sup>	0.2406 ± 0.0318 <sup>b,c</sup>	0.2091 ± 0.0206 <sup>a</sup>	0.1951 ± 0.0172 <sup>a</sup>	0.2172 ± 0.0120 <sup>a,b</sup>	0.2340 ± 0.0144 <sup>b</sup>

Values are mean ± SEM,  $n = 6$ . Values within the same row with different superscripts are significantly different ( $P < 0.05$ )



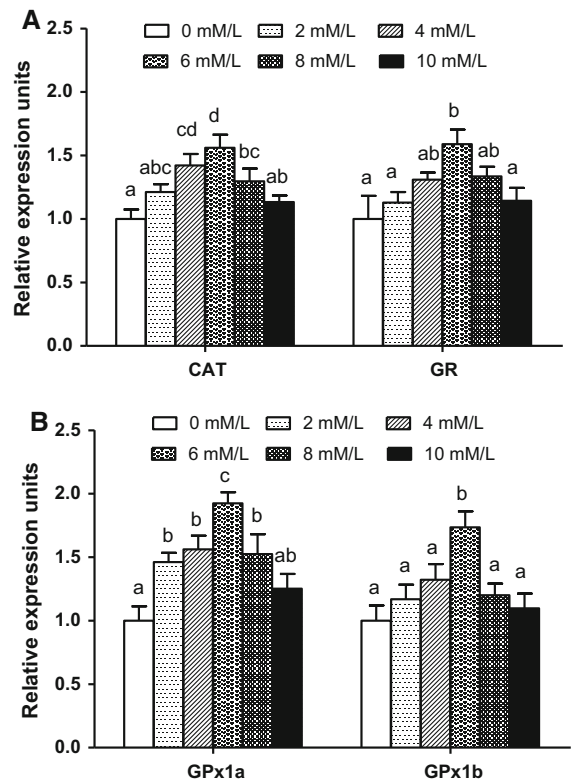
**Fig. 1** Effect of Glu on contents of reduced glutathione (GSH, nmole per mg protein) of carp enterocytes. Data represent mean  $\pm$  SEM, and values having different letters are significantly different ( $P < 0.05$ ,  $n = 6$ )

The lowest value was observed in the 6 mM/L Glu group ( $P < 0.05$ ).

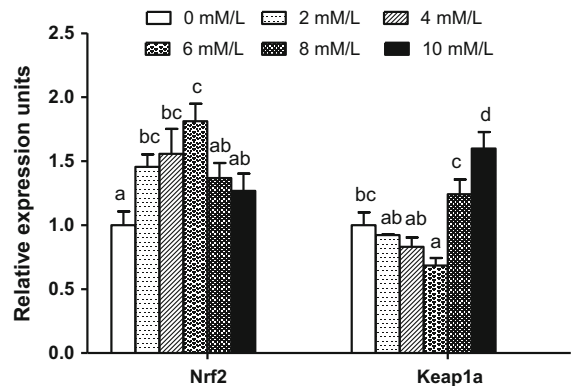
Figure 1 shows the effect of Glu on contents of GSH in carp enterocytes. GSH content was the highest for cell cultured in 6 mM/L Glu medium groups ( $P < 0.05$ ) and the lowest in the control group. Relative gene expressions of CAT, GR, GPx1a, and GPx1b of enterocytes in response to graded levels of Glu are presented in Fig. 2a, b. CAT and GR mRNA levels were increased in higher levels of Glu content in medium, and there were no differences between 8 and 10 mM/L Glu in medium. The GPx1a and GPx1b expression levels were significantly increased with increased Glu levels up to 6 mM/L in the medium and then decreased. The effect of Glu on relative expressions of Nrf2 and Keap1a gene in carp enterocytes is shown in Fig. 3. The relative expression level of Nrf2 in enterocytes was significantly up-regulated by the increasing Glu level up to 6 mM/L in the medium. Patterns of Keap1a mRNA were opposite compared with Nrf2 in enterocytes.

## Discussion

The life cycle of enterocytes includes proliferation, differentiation, and upward migration along the crypt-villus axis (Stehr et al. 2005). The measurement of cell proliferation and viability plays a fundamental role in all forms of cell culture (Stoddart 2011). An improved colorimetric assay (MTS) for assessing cell proliferation and viability is commonly used today (Buttke



**Fig. 2** Effect of Glu on CAT (a), GR (b), GPx1a (c), GPx1b, and (d) gene relative expression in carp enterocytes. Data represent mean  $\pm$  SEM, and values having different letters are significantly different ( $P < 0.05$ ,  $n = 6$ )



**Fig. 3** Effect of Glu on Nrf2 and Keap1a gene relative expressions in carp enterocytes. Data represent mean  $\pm$  SEM, and values having different letters are significantly different ( $P < 0.05$ ,  $n = 6$ )

et al. 1993). Using this assay, it was shown that Glu in an optimum level significantly improved the proliferation of fish enterocytes. To our knowledge, the

present study provides evidence that Glu could increase fish enterocytes proliferation for the first time. Glu is one of the glutamine precursors. Hence, it is probable that the supplemented Glu formed as a result of increased glutamine synthesis. Our previous study also showed that glutamine could promote fish enterocytes proliferation (Jiang et al. 2009). The proliferation of enterocytes may directly affect the formation of intestinal folds. Thus, the present result may partly explain our observation that Glu increases the intestinal fold height in grass carp, as showed in a previous study (Zhao et al. 2014a). The interesting phenomenon is that 10 mM/L Glu group showed lower proliferation of fish enterocytes than 6 mM/L Glu group in the present study. The reason remains yet unclear. Several research teams reported that Glu could induce apoptosis of astrocytes (Szydłowska et al. 2006; Mao et al. 2010). Excessive Glu can lead to neuronal and glial cell death in a variety of pathological conditions (Farber et al. 1998). Protein biosynthesis is the principal foundation of cell growth and proliferation. In good agreement with this argument, our results indicated that PCE was markedly increased by Glu. The value of PCE reflects protein synthesis and degradation. However, several reports in nerve cells showed that Glu inhibited protein synthesis (Bonova et al. 2013; Cheung 1989). The observed differences may attribute to a different effect in different types of cells. The intestine is the main organ of glutamine utilization and converts glutamine into other amino acids (Windmueller and Spaeth 1980). Increased glutamine synthesis requires a supply of Glu in intestine (Peh et al. 2010). Intestinal AKP is a marker enzyme of the brush border membrane (Vilanueva et al. 1997), which reflects the epithelium differentiation (He et al. 1993; Tengjaroenkul et al. 2000). The present study showed that Glu may induce the differentiation of enterocytes. The mechanism by which Glu increased the activity of AKP is unclear.  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase is the enzyme that provides the gradient for sodium movement and thus for a number of sodium-coupled transport events across the apical domain of the cell (Sweeney and Klip 1998).  $\gamma$ -GT is also an enzyme localized at the intestinal brush border and involved in amino acid transport via the  $\gamma$ -glutamyl cycle (Bell et al. 1987). CK plays a critical role in the energy metabolism of cells that have fluctuating energy requirements (Sun et al. 1998). The results of the  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase,  $\gamma$ -GT, and CK assays

showed that the absorption capacity was significantly improved by Glu supplementation, which was consistent with our results in vivo (Zhao et al. 2014a).

Several studies have indicated that the integrity and viability of cells are often accompanied by an increment in antioxidative capacity (Chen et al. 2009; Jiang et al. 2013, 2009). SOD, CAT, GPx, GST, GR, and GSH are important components of the antioxidant defense system (Martinez-Alvarez et al. 2005). The SOD is the first enzymes to respond against oxygen radicals which are important endogenous antioxidants for protection against oxidative stress (Winston and Di Giulio 1991). SOD degrades superoxide radicals by converting them into  $\text{O}_2$  and  $\text{H}_2\text{O}_2$ , which can be reduced to  $\text{H}_2\text{O}$  by CAT in the peroxisomes or by GPx in the cytosol (Olsvik et al. 2005). In the current study, activities of T-SOD and GPx were significantly increased up to a point and decreased thereafter as Glu levels increased. GST and GR are important glutathione-dependent enzymes and able to counteract the peroxidative damage (Nishinaka et al. 2007). GSH is a sulfhydryl-containing non-protein antioxidant, which detoxifies  $\text{H}_2\text{O}_2$  and lipid hydroxides. The present result showed that GSH contents and GST activity in enterocytes were significantly increased up to an optimum Glu levels. These results may contribute to the higher SAS and AHR activities in enterocytes treated with Glu. Our previous study also showed that Glu was beneficial for scavenging superoxide and hydroxyl radicals in the intestines in an in vivo experiment (Zhao et al. 2014a). In addition, Glu can form  $\alpha$ -ketoglutarate and could enter into the citrate cycle to promote production of citrate cycle intermediates. GSH can be formed from exogenous Glu. The properties of antioxidant enzymes are proteins, the activities of which can be affected by the mRNA levels in the rat (Tiedge et al. 1997). To further elucidate whether Glu regulated antioxidant enzymes activities at the gene level, the mRNA levels of the antioxidant enzyme of enterocytes in the absence or presence of Glu were investigated. The study showed that CAT, GST, GR, GPx1a, and GPx1b gene expression levels were up-regulated by Glu levels in the cell. These results matched a similar pattern to the respective enzyme activity changes. Thus, Glu might improve these antioxidant enzyme activities through up-regulation of gene transcription in enterocytes. Antioxidant genes transcription is regulated by Nrf2 signaling in terrestrial animals.



Thus, we next investigated the relationship between Glu and the major signaling molecules Nrf2 and Keap1 involved in the Nrf2 signaling pathway. Recently, our laboratory had been the first to clone the Nrf2 and Keap1a cDNA of carp and found that Nrf2 and Keap1a genes are expressed extensively in carp tissues, including the intestine. In human and mice, the Nrf2 activates the expression of many antioxidant genes which include CAT, SOD,  $\gamma$ -GT, GST, and GPx (Reisman et al. 2009; Ramprasath et al. 2012). These Nrf2 regulated genes are characterized by the presence of a cis-acting element called antioxidant-responsive element (ARE), which lies within the regulatory region. Data in this study displayed that the Nrf2 mRNA level was the highest for fish enterocytes cultured in 6 mM/L Glu medium and showed similar patterns as gene expressions of CAT, GPx, GST, and GR, suggesting that the benefits of Glu on antioxidant enzyme gene expressions may be partly due to up-regulating Nrf2 gene expression in enterocytes. To date, there is not any information about the relationship between Glu and Nrf2 gene expression in fish and terrestrial animals. In terrestrial animals, Keap1 could retain Nrf2 in the cytoplasm and prohibit the Nrf2 nuclear translocation (Haarmann-Stemmann et al. 2012). The down-regulation of Keap1 gene in mice could increase the Nrf2 nuclear translocation and result in transcriptional induction of antioxidant gene (Reisman et al. 2009). Our study firstly showed mRNA levels in enterocytes significantly decreased with Glu supplement to a certain level. These results suggest that the enhanced antioxidant enzyme gene expression in our study by Glu may be partly due to the promotion of Nrf2 nuclear translocation via depressing Keap1 gene expression. However, the detailed mechanism awaits further characterization.

Because there is a close relationship between cell structure and function, the integrity of the cell membrane contributes to the growth, development, and function of cells (Chen et al. 2009). In order to evaluate the integrity of cells, we measured LDH release in the medium (Koh and Choi 1987). In the present study, the cell damage in the absence of Glu has been examined in enterocytes, which gradually disappeared with increased Glu levels. It is well known that metabolic and other processes occurring in cells give rise to the formation of reactive oxygen species (ROS) that can destroy biological targets, such

as lipids and proteins (Kohen and Nyska 2002). MDA is a key to metabolite production and a useful marker of lipid peroxidation (Mourente et al. 2007). PC is one of the most extensively studied forms of protein oxidative modification (Wang and Powell 2010). We found that MDA and PC contents in carp enterocytes were increased by Glu deficiency, suggesting that Glu decreased lipid peroxidation and protein oxidation of fish enterocytes. This result was in good agreement with a report on the rat. A previous study reported that Glu could reduce oxidative stress in myocardial infarction-induced rats (Sivakumar et al. 2011). Hence, the reduction in lipid peroxides and protein oxidation by Glu treatment could be due to the antioxidant effect of citrate cycle intermediates formed from Glu. Further, GSH produced from exogenous Glu can also decrease the oxidative stress.

In conclusion, Glu could improve the proliferation, differentiation, function, and antioxidant capacity of fish enterocytes. Meanwhile, Glu increased the activities of antioxidant enzymes in enterocytes, which might be partly attributable to increased gene expressions of antioxidant enzymes by Glu. Additionally, the present study firstly showed that Glu regulated Nrf2 and Keap1a gene expression, which might mediate the signal transduction involving in increased gene expressions of antioxidant enzymes by Glu. The detailed mechanism by which Glu regulated these gene expressions in enterocytes needs to be further investigated.

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