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Effects of L-carnitine against H_2O_2-induced oxidative stress in grass carp ovary cells (*Ctenopharyngodon idellus*)

Qiuju Wang · Xue Ju · Yuke Chen · Xiaoqing Dong · Sha Luo · Hongjian Liu · Dongming Zhang

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Abstract This study was designed in vitro to investigate the effects of L-carnitine against H_2O_2-induced oxidative stress in a grass carp (*Ctenopharyngodon idellus*) ovary cell line (GCO). GCO cells were pre-treated with different concentrations of L-carnitine, followed by incubation with 2.5 mM H_2O_2 for 1 h to induce oxidative damage. The results indicated that adding L-carnitine at concentrations of 0.01–1 mM into the medium for 12 h significantly increased cell viability. Pre-treatment with L-carnitine at concentrations of 0.1–5 mM for 12 h significantly inhibited 2.5 mM H_2O_2-induced cell viability loss. The significant decreases in the level of reactive oxygen species and cell apoptosis were observed in 0.5 mM L-carnitine group compared to the H_2O_2 group. Malondialdehyde values of all of the L-carnitine groups were significantly lower than those of the H_2O_2 group, while total glutathione levels of all of the L-carnitine groups were significantly higher than of the H_2O_2 group. The activity of antioxidant enzymes, such as total superoxide dismutase (0.1 and 0.5 mM L-carnitine), catalase (0.5 mM L-carnitine) and γ-glutamyl cysteine synthetase (0.5 and 1 mM L-carnitine), was significantly increased. In addition, pre-treatment of L-carnitine in GCO cells exposed to 2.5 mM H_2O_2 significantly increased the mRNA expression of copper, zinc superoxide dismutase, catalase (0.5 mM L-carnitine), glutamate cysteine ligase catalytic subunit (0.1–1 mM) and glutathione peroxidase (0.1 mM L-carnitine). In conclusion, L-carnitine promotes GCO cell growth and improves antioxidant function, it plays a protective role against oxidative stress induced by H_2O_2 in GCO cells, and the appropriate supplemental amount of L-carnitine is 0.1–1 mM.

Keywords Fish · Antioxidant enzymes · Lipid peroxidation · Antioxidant compounds

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Introduction

Reactive oxygen species (ROS) are highly reactive and short-lived molecules containing oxygen (i.e., O_2^-, ·OH and H_2O_2) that are observed in all aerobic organisms, which can be continuously generated during normal cellular metabolism processing (Lushchak 2014). Oxidative stress is caused by an imbalance of the amount of ROS and antioxidant defense systems, which occurs in all aerobic biological system, including plants, animals and aerobic bacteria, when exposed to higher levels of ROS (Valavanidis et al.

2006). Oxidative stress can lead to damage at the molecular, cellular and tissue levels. This stress increases the susceptibility to lipid peroxidation, DNA damage and structural and functional changes in proteins, which leads to a series of consequences, such as membrane fluidity decrease, DNA fragmentation, enzymatic inactivation and cell death (Banerjee et al. 2012; Khalil et al. 2013). Recently, many studies noted that oxidative stress was associated with a variety of abnormal physiological states and diseases in human and livestock (Rajasekar et al. 2007; Ye et al. 2010; Lauritzen et al. 2003; Deaton et al. 2005).

In the modern mode of intensive aquaculture, fish are constantly exposed to various environmental stresses, such as water temperature, dissolved oxygen, salinity, heavy metal ions, pesticides and other toxic pollutants (Cui et al. 2014; Lushchak and Bagnyukova 2006; Martínez-Alvarez et al. 2002; Jiang et al. 2014; Monteiro et al. 2010; Guzmán-Guillén et al. 2013). However, it is known that in fact, any strong stress usually is accompanied by oxidative stress (Lushchak 2011). Meanwhile, fish cells are vulnerable to the challenge of ROS because they are rich in essential long-chain polyunsaturated fatty acids in the membrane, such as docosahexaenoic acid, eicosapentaenoic acid and arachidonic acid. Taken together, fish are more sensitive to oxidative stress compared to terrestrial mammals, and they therefore are a good animal model in this respect and may provide more experimental data for elucidating underlying negative effects of oxidative stress (Lackner 1998).

L-Carnitine (LC) (4-*N*-trimethylammonium-3-hydroxybutyric acid) is a small water-soluble organic solute that plays a major role in the transport of long-chain fatty acids across the inner mitochondrial membrane for the β-oxidation cycle and energy metabolism (Harpaz 2005). LC supplementation in fish feed had been advocated in aquaculture for several reasons. LC can be used to promote growth performance by providing a protein-sparing effect (Chatzifotis et al. 1995), improve nutrient utilization and body composition (Ozorio et al. 2001), protect cells from the toxic effects of ammonia and removal of organic acids and xenobiotics from the mitochondrion (Chapela et al. 2009), ameliorate stress related to water temperature extremes and facilitate better adaption for water temperature changes (Guderley et al. 1997), and enhance reproduction (Jayaprakas et al. 1996). Increasing evidence has emerged suggesting that LC supplementation may be beneficial in preventing diabetes (Broderick 2008), cardiovascular diseases (Miguel-Carrasco et al. 2010) and neurometabolic diseases (Eniko et al. 2010) derived from oxidative stress. Filipa et al. (2011) reported the effects of acetyl-LC on diepoxybutane-induced loss of viability in human lymphocytes. The data showed that viability of human lymphocytes simultaneously exposed to diepoxybutane (400 μg/ml) was significantly decreased. On the contrary, the administration of acetyl-LC (5 mM) for 4 and 5 h significantly improved lymphocyte viability. The activities of antioxidant enzymes of human proximal tubule epithelial cell line were clearly facilitated by LC (0.01, 0.05 and 0.1 mM for 12 h) administration when cells were exposed to 0.5 mM H_2O_2 for 0.5 h, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Ye et al. 2010). These findings may represent a new perspective for the use of LC as a potential antioxidant therapy for those diseases, but the mechanism has not been thoroughly elucidated.

To date, although the antioxidant effects of LC had been studied in the terrestrial animal, little work has been performed on the antioxidant capacity of LC in the aquatic organisms (Guzmán-Guillén et al. 2013). The data are still scarce concerning the effects of LC on the antioxidant system in fish cells, especially when considering its effects on the status of oxidative stress. In this study, H_2O_2 was used as the inducer and GCO cells were the model system chosen to set up a cell model of oxidative stress. The viability of GCO cells, the apoptosis and biological parameters, such as MDA and total glutathione (T-GSH), the activity and mRNA expression of antioxidant enzymes, including total superoxide dismutase (T-SOD), CAT, GPx and γ-GCS, were determined in GCO cells. Therefore, the primary purpose of the present study was to evaluate the effects of LC on growth and the antioxidant system in GCO cells and to provide evidence for elucidating the mechanism of how LC protects cells against oxidative damage in vitro.

Materials and methods

Cell culture

GCO cells were purchased from the Fisheries Research Institute of Tianjin (Tianjin, China). The

cells were seeded in 25 cm^2 cell culture flasks that contained M199 medium supplemented with 15 % fetal calf serum (FCS) and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin), which were cultivated in an incubator at 25 °C with 5 % CO_2 in 95 % air.

MTT assay

The MTT assay was used to test cell viability and proliferation, which is a sensitive and classical measurement of the normal metabolic status of cells. Briefly, GCO cells in the logarithmic phase of growth were initially plated in 96-well cell culture plates at a density of 2.5×10^4 cells/well and adhered for 48 h in an incubator at 25 °C with 5 % CO_2 in 95 % air. Next, the supernatant was discarded and cells were treated by M199 (without FCS) containing different concentrations of LC (0.001, 0.01, 0.1, 0.5, 1 and 5 mM; the concentrations of LC are obtained from the paper of Ye et al. 2010) or H_2O_2 (1, 1.5, 2, 2.5, 3 and 4 mM) for different hours at 100 μl/well. After the treatments, the cells were rinsed with fresh medium (without FCS) 1 time and then incubated with M199 containing 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) at 25 °C with 5 % CO_2 in 95 % air for 4 h at 100 μl/well. The formazan crystals generated by viable mitochondrial succinate dehydrogenase from MTT were dissolved using dimethyl sulfoxide. Finally, the absorbance was measured at a wavelength of 490 nm using a microplate reader (Bio-Rad imark, CA, USA). Each treatment group was set up in 6 wells, and all of the experiments were performed in triplicate. In this study, treatment of GCO cells with 2.5 mM of H_2O_2 for 1 h (viability was reduced by approximately 60 %) was chosen to establish oxidative stress.

Oxidative parameters and enzyme measurements

To analyze the oxidative parameters and the enzyme measurements, the cultured cells in the logarithmic phase of growth were harvested and seeded into 6-well cell culture plates at 1×10^6 cells/well and adhered for 48 h. The cells were pre-treated with or without different concentrations of LC (0.1, 0.5 and 1 mM for 12 h, the concentrations of LC was chosen based on the results of viability) and then stimulated with H_2O_2 (2.5 mM for 1 h). Each treatment group was set up in 3 wells of 2 ml/well. The cells were treated with the cell lysis buffer (Solarbio, Beijing, China) and centrifuged at $13000 \times g$ at 4 °C for 10 min. The supernatant was collected and stored at –80 °C.

TBARS was measured to indirectly estimate the content of MDA at 532 nm using the TBA method (Zhang et al. 2008). T-GSH was determined using the method as described by Atencio et al. (2008). Cellular T-SOD activity was measured at 505 nm using the xanthine oxidase–cytochrome c method (McCord and Fridovich 1969; Prieto et al. 2008). CAT activity was also calculated at 240 nm by measuring the initial rate of H_2O_2 (10 mM) decomposition (Prieto et al. 2008; Beers and Sizer 1952). GPx activity was assayed by the method described by Rotruck et al. (1973). The activity of γ-GCS was measured using the trace phosphorus method at 636 nm (Gutiérrez-Praena et al. 2011). The content of the total protein in the cells was quantified by the method of Bradford (1976) using bovine γ-globulin as the standard. The absorbance was measured with a spectrophotometer (Shanghaiyouke Co., LTD., Shanghai, China). The content of T-GSH, MDA and the enzyme activity were normalized to the total protein.

Analysis of the mRNA expression of the antioxidant enzymes

The mRNA expression of the antioxidant enzymes was also detected according to the experimental design of "Oxidative parameters and enzyme measurements" section. Total RNA was isolated from the cells using the TRIzol reagent (TaKaRa, Dalian, China). The purity of total RNA was analyzed by the A260/A280 ratio, and the integrity was verified on a 1 % agarose gel under a denatured status. According to the manufacturer's instructions, total RNA from the cells was reverse transcribed using a reverse transcription kit (Invitrogen Corp., New York, CA, USA). First-strand cDNA was stored at –80 °C for quantitative real-time PCR (qRT-PCR) analysis.

QRT-PCR was performed using a software program (Applied Biosystems Step One Plus, Life Technologies, New York, CA, USA). As shown in Table 1, the gene-specific primers of copper, zinc superoxide dismutase (CuZn-SOD), GPx, CAT, glutamate cysteine ligase catalytic subunit (GCLC) and β-actin were designed using the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto,

Table 1 Sequences of primers used for RT-qPCR amplification

Target gene	Product size (bp)	Accession no.	Primer sequences (from 5′ to 3′)	Reference
CuZn-SOD	157	Y12236	GGCCAACCGATAGTGTTAGA CCAGCGTTGCCAGTTTTTAG	Paul et al. (2007)
CAT	240	NM_130912.1	ACTACCAGTCAACTGCCCGTAC TTTAGCACCTGAGTGAAGAACG	This study
GSH-Px	136	AW232474	ACAGAGGGTGGGCGTTAT TTCGGGCACCAGAGGA	This study
GCLC	249	NM-199277	CGAATCGGACCACTTTGAG CGCTTCTGGGCTACCTTCA	This study
β-actin	111	AF057040.1	TGCGGAAACTGGCAAAGG GAGGGCAAAGTGGTAAACG	This study

CA, USA) for qRT-PCR. The housekeeping gene β-actin was used as an internal control for normalization. For each qRT-PCR, the 25 μl reaction mixture contained 12.5 μl SYBR Premix Ex Taq (Takara, Dalian, China), 2 μl of cDNA template, 2 μl of primers, 0.5 μl of SYBR Green Dye I and 8 μl of nuclease-free water. The amplification of qRT-PCR was conducted with the following program: 95 °C for 5 s, 58.3 °C for 45 s and 72 °C for 60 s followed by 40 cycles. The Ct values of all of the samples were collected and were used for calculating the mRNA relative expression values genes. The quantitative fold changes in mRNA expression were determined relative to the β-actin mRNA level in each corresponding group and calculated using the $2^{-\Delta\Delta Ct}$ method.

ROS and apoptosis measurements

ROS generation was monitored by flow cytometry using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Beyotime, Beijing, China). Experimental design was the same as "Oxidative parameters and enzyme measurements" section. In the apoptosis measurement trial, the concentration of LC was reduced (only 0.5 mM) based on the results of activity and mRNA relative expression of the antioxidant enzymes. After the treatments, single cells were collected by 0.1 % trypsin and washed in cold phosphate-buffered saline (PBS) twice. In addition, the cells were incubated with 2 ml 10 μM DCFH-DA or stained with 5 μl 100 mg/ml Annexin-V FITC (Biolegend, Hercules, CA, USA) and 50 μg/ml of PI (Biolegend, Hercules, CA, USA) for 20 min in the dark at room temperature. And then, the fluorescence intensity of 10000 cells was measured by means of flow cytometry (Becton–Dickinson, NJ, Franklin Lakes, USA) with a 488-nm argon laser and analyzed using CELL Quest software within 1 h. Finally, the cells were observed and photographed under the inverted fluorescence microscope (Olympus, Osaka, Japan). Apoptotic cells were recognized by the number of Annexin-V-positive cells (Annexin-V^+/PI^- and Annexin-V^+/PI^+), and the necrotic cells were quantified as the number of PI-positive cells (Annexin-V^-/PI^+) divided by the total number of cells.

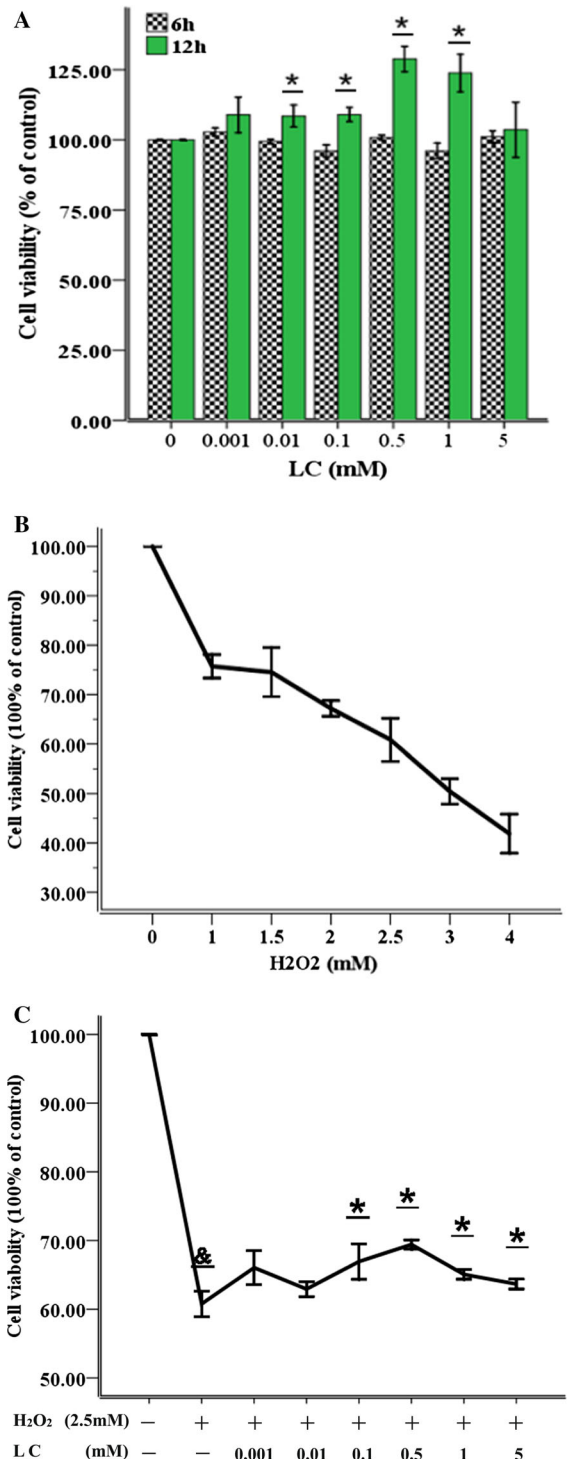
Statistical analyses

The data are presented as the means with standard errors (mean ± SEM). The viability data and the differences in the mean values between the groups were analyzed by a one-way analysis of variance (ANOVA) followed by a Student's t test ($P < 0.05$) using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The other results were analyzed by ANOVA, and Duncan's multiple range test was used to determine significant differences ($P < 0.05$) among the individual treatments.

Results

The effect of L-carnitine and H_2O_2 on the viability of GCO cells

The effect of different concentrations of LC treatment for 6 and 12 h on the viability of GCO cells was tested by the MTT method. As shown in Fig. 1a, there were no significant changes in cell viability in the 6-h group



◀**Fig. 1** Effect of LC treatment for 6 and 12 h on the viability of GCO cells (**a**). The effect of H_2O_2 treatment for 1 h on the viability of GCO cells (**b**). The effect of LC pre-treatment for 12 h on the viability of GCO cells following a 2.5 mM H_2O_2 challenge (**c**). The data are expressed as the mean ± SEM ($n = 6$) of the percentage of control cells, & ($P < 0.05$) means significantly different when compared to the control group, and *($P < 0.05$) means significantly different when compared to the H_2O_2 group

($P > 0.05$). However, treatment with 0.01–1 mM/L LC for 12 h significantly increased the cell viability in a dose-dependent manner ($P < 0.05$). These results showed that the best treatment time was 12 h; therefore, in the next experiment, we chose the concentrations of LC at 0.001–5 mM and a pre-treatment time of 12 h to detect the effects of LC following exposure to H_2O_2 in GCO cells.

The results of the cell viability revealed that H_2O_2 gradually damaged GCO cells with the increase in the concentrations of H_2O_2. In the current study, inhibition ratio of approximately 40 % was found when GCO cells were exposed to 2.5 mM of H_2O_2 for 1 h (Fig. 1b).

The effect of L-carnitine on the viability of GCO cells under H_2O_2-induced oxidative stress

Incubation of LC with the concentrations from 0.1 to 5 mM of LC for 12 h significantly increased the viability of GCO cells when compared to the 2.5 mM H_2O_2 group ($P < 0.05$). Although the viability of both groups of the 0.001 and 0.01 mM was not significantly increased ($P > 0.05$), the values were still higher than the 2.5 mM H_2O_2 group. The data are shown in Fig. 1c.

The effect of L-carnitine on the ROS levels of GCO cells under H_2O_2-induced oxidative stress

To further confirm the beneficial effects of LC on cells in a state of oxidative stress, we determined the ROS levels of GCO cells and observed the fluorescence intensities under the microscope. As shown in Fig. 2A, B (a), B (c), C (a), C (c), ROS levels of the cells in the H_2O_2 group were significantly increased when compared to the control group ($P < 0.05$), and the fluorescence signal was also strong. However, ROS generation was significantly inhibited by 0.5 and 1 mM treatment of LC ($P < 0.05$). As shown in Fig. 2B (d), C (d), the fluorescence intensity was also reduced in the 0.5 mM LC group when compared to the H_2O_2 group.

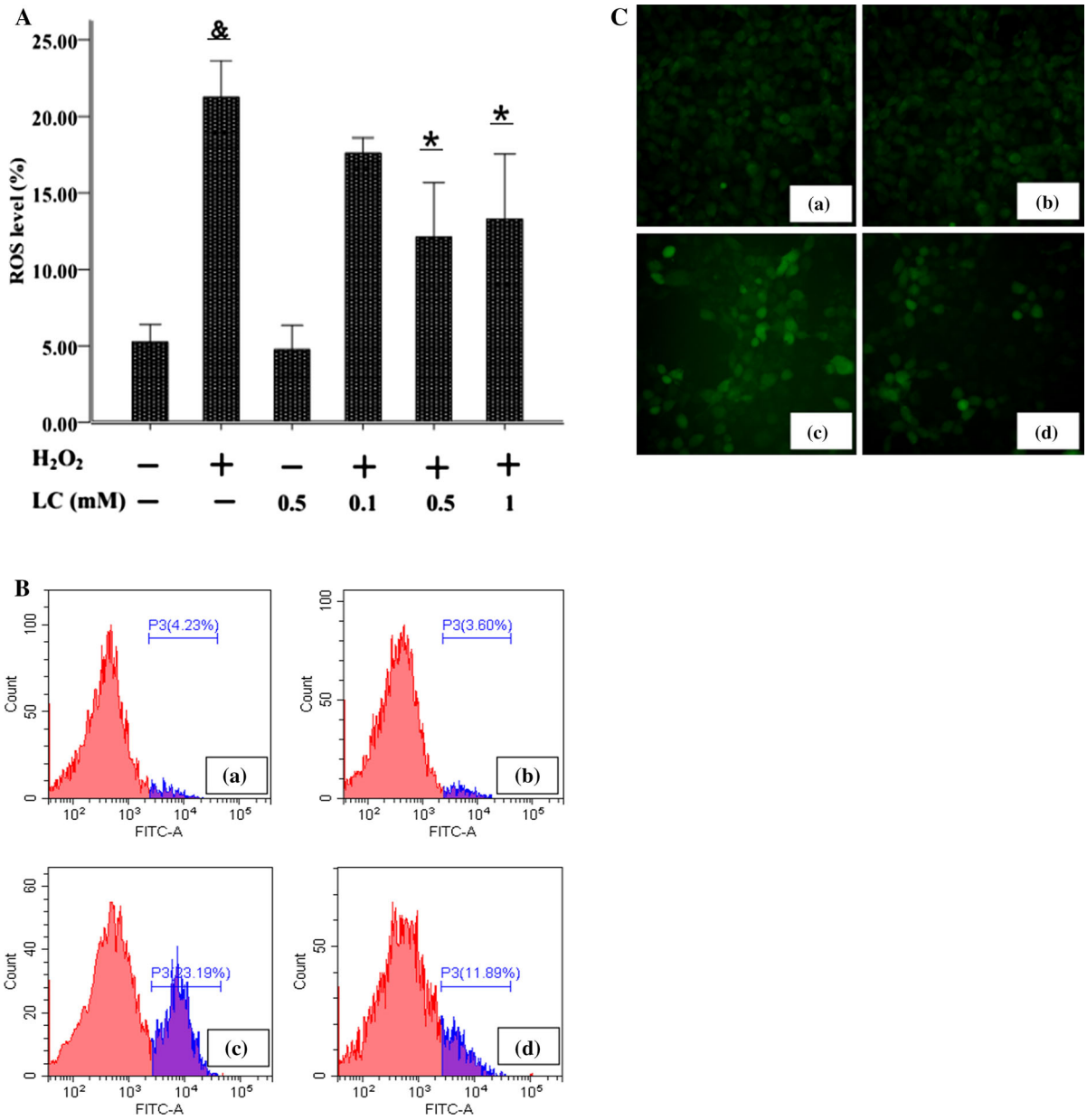


Fig. 2 Effect of LC pre-treatment for 12 h on the levels of ROS in GCO cells following a 2.5 mM H_2O_2 challenge. **A** Columns, **B** (**a**) control group, **B** (**b**) 0.5 mM LC group, **B** (**c**) 2.5 mM H_2O_2 group and **B** (**d**) 0.5 mM LC + 2.5 mM H_2O_2 group; **C** Image of the fluorescence signal, **C** (**a**) control group, **C** (**b**) 0.5 mM LC group, **C** (**c**) 2.5 mM H_2O_2 group and **C** (**d**) 0.5 mM LC + 2.5 mM H_2O_2 group. The data are expressed as the mean ± SEM ($n = 3$) of the percentage of control cells, & ($P < 0.05$) means significantly different when compared to the control group, and *($P < 0.05$) means significantly different when compared to the H_2O_2 group

The effect of L-carnitine on the oxidative parameters and the enzyme measurements of GCO cells under H_2O_2-induced oxidative stress

As markers of antioxidant status, the levels of MDA, T-GSH, and the activities of T-SOD, CAT, GPx, γ-GCS were measured for evaluating the presence of oxidative stress in GCO cells after exposure to H_2O_2 (Fig. 3). The MDA values increased significantly in the GCO cells exposed to 2.5 mM H_2O_2 compared with the control group, and significant decreases were observed in all of the LC groups compared to the H_2O_2 group ($P < 0.05$). Compared with the control group, a reduction in T-GSH content was found in the H_2O_2 group, and only 0.5 mM LC incubation significantly improved the T-GSH content ($P < 0.05$). Compared with 2.5 mM H_2O_2 group, pre-treatment with LC at concentrations of 0.1–1 mM for 12 h significantly increased the T-GSH content of the cells ($P < 0.05$). The data are shown in Fig. 3a, b.

H_2O_2 significantly affected the activities of T-SOD, CAT, GPx and γ-GCS of GCO cells when compared to the normal cells. Significant increases of CAT activity were found in the groups pre-treated with 0.5 mM LC for 12 h when compared to the 2.5 mM H_2O_2 cells ($P < 0.05$) (Fig. 3d). Similarly, the activities of γ-GCS in the groups pre-treated with 0.5 and 1 mM LC were significantly higher than in the 2.5 mM H_2O_2 group ($P < 0.05$) (Fig. 3f). The activity of T-SOD in the 2.5 mM H_2O_2 group was significantly reduced when compared to the normal cells (Fig. 3c), and the concentrations of LC (0.1 and 0.5 mM) significantly increased the level of T-SOD activity of the GCO cells subjected to 2.5 mM H_2O_2 ($P < 0.05$). Moreover, compared with the control group, 2.5 mM H_2O_2 did not change the GPx activity, and no significant difference was found in all of the LC groups ($P > 0.05$) (Fig. 3e).

The effect of L-carnitine on the antioxidant enzyme gene expression of the GCO cells under H_2O_2-induced oxidative stress

All of the gene expression data are expressed as fold change from the control. In the H_2O_2 group, significant changes in the gene expression of CuZn-SOD and GCLC were found when compared to the control group ($P < 0.05$). However, the gene expressions of GPx and CAT were not significantly affected ($P > 0.05$). Compared to the control group, only 0.5 mM LC treatment significantly up-regulated gene expression of CuZn-SOD, GPx and GCLC ($P < 0.05$). With increasing concentrations of LC (0.1–1 mM), the trend of gene expression CAT was decreased after an increase, and 0.5 mM LC pre-treatment group reached significant level ($P < 0.05$). The 0.1–1 mM LC pre-treatment groups significantly induced an increase in the mRNA levels of CuZn-SOD and GCLC, and the GPx mRNA levels were significantly up-regulated in the groups pre-treated with 0.1 mM LC when compared to the H_2O_2 group ($P < 0.05$). The data are shown in Fig. 4.

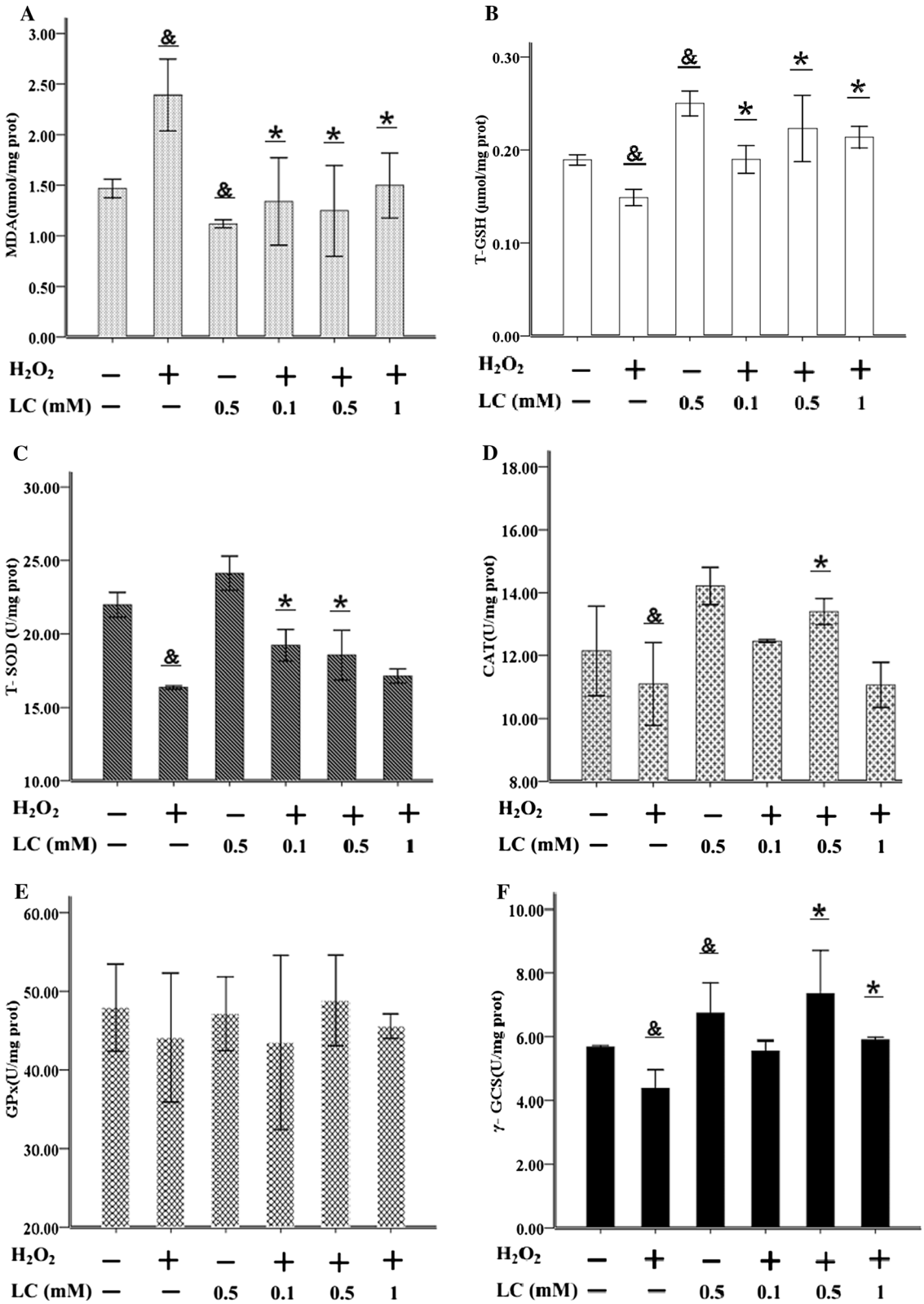
The effect of L-carnitine on apoptosis in GCO cells under H_2O_2-induced oxidative stress

At 1 h after 2.5 mM H_2O_2 treatment, the differences in apoptosis levels between the H_2O_2-treated and the control cells were significant ($P < 0.05$). The apoptosis level of the GCO cells in the H_2O_2 group was significantly increased ($P < 0.05$). However, 0.5 mM LC pre-treatment for 12 h significantly reduced the apoptosis level of the cells exposed to 2.5 mM H_2O_2 ($P < 0.05$). The data are shown in Fig. 5.

Discussion

In our current study, we investigated, for the first time, the effects of LC on the viability of a grass carp ovary cell line. An obvious promotion of viability was observed when LC was used at 0.01–1 mM for 12 h in the GCO cells. Similarly, after incubation with LC (0.001–1 mM) for 12 h, the viability of HK-2 cells was significantly increased (Ye et al. 2010). This conclusion was further confirmed by Ye et al. (2014).

He et al. (2011) reported that LC pre-treatment (1 mM) significantly reduced the loss of cell viability in nickel-treated Neuro-2a cells. A similar result was found in the present study. Our data indicated that the pre-treatment of cells with LC at concentrations from 0.1 to 5 mM for 12 h conferred a protective effect on the viability of GCO cells against H_2O_2-induced oxidative damage. In addition, the viability of GCO cells gradually reduced after an increase with increasing concentrations of LC. Incubation with LC at concentrations of 0.001–1 mM for 12 h significantly increased the viability of HK-2 cells exposed to


A
MDA(nmol/mg prot)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
B
T-GSH (μmol/mg prot)
0.30
0.20
0.10
0.00
C
T- SOD (U/mg prot)
30.00
25.00
20.00
15.00
10.00
D
CAT(U/mg prot)
18.00
16.00
14.00
12.00
10.00
8.00
E
GPx(U/mg prot)
60.00
50.00
40.00
30.00
20.00
F
γ- GCS(U/mg prot)
10.00
8.00
6.00
4.00
2.00
0.00
H2O2 – + – + + +
LC (mM) – – 0.5 0.1 0.5 1
&
*

◀**Fig. 3** Effects of LC pre-treatment for 12 h on the level of MDA (**a**) and T-GSH (**b**), activities of T-SOD (**c**), CAT (**d**), GPx (**e**) and γ-GCS (**f**) in GCO cells following a 2.5 mM H_2O_2 challenge. The data are expressed as the mean ± SEM ($n = 3$) of the percentage of control cells, & ($P < 0.05$) means significantly different when compared to the control group, and *($P < 0.05$) means significantly different when compared to the H_2O_2 group

0.6 mM H_2O_2, and the best concentrations were 0.05 and 0.1 mM (Ye et al. 2010). The results of Ye et al. showed the same tendency with our data. LC (0.03, 0.06, 0.09, 0.12 and 0.15 mM) had been shown to be effective for the protection of human peripheral leukocytes against DNA damage induced by propionic acidemia and methylmalonic acidemia (Graziela et al. 2010). Taken together, these results mean that LC exerts the protective role in viability against oxidative stress in a dose-dependent manner.

ROS plays an important role in the pathophysiological processes of many diseases in mammals and aquatic animals, which can mediate a number of chemical-, drug- and xenobiotic-induced toxicities.

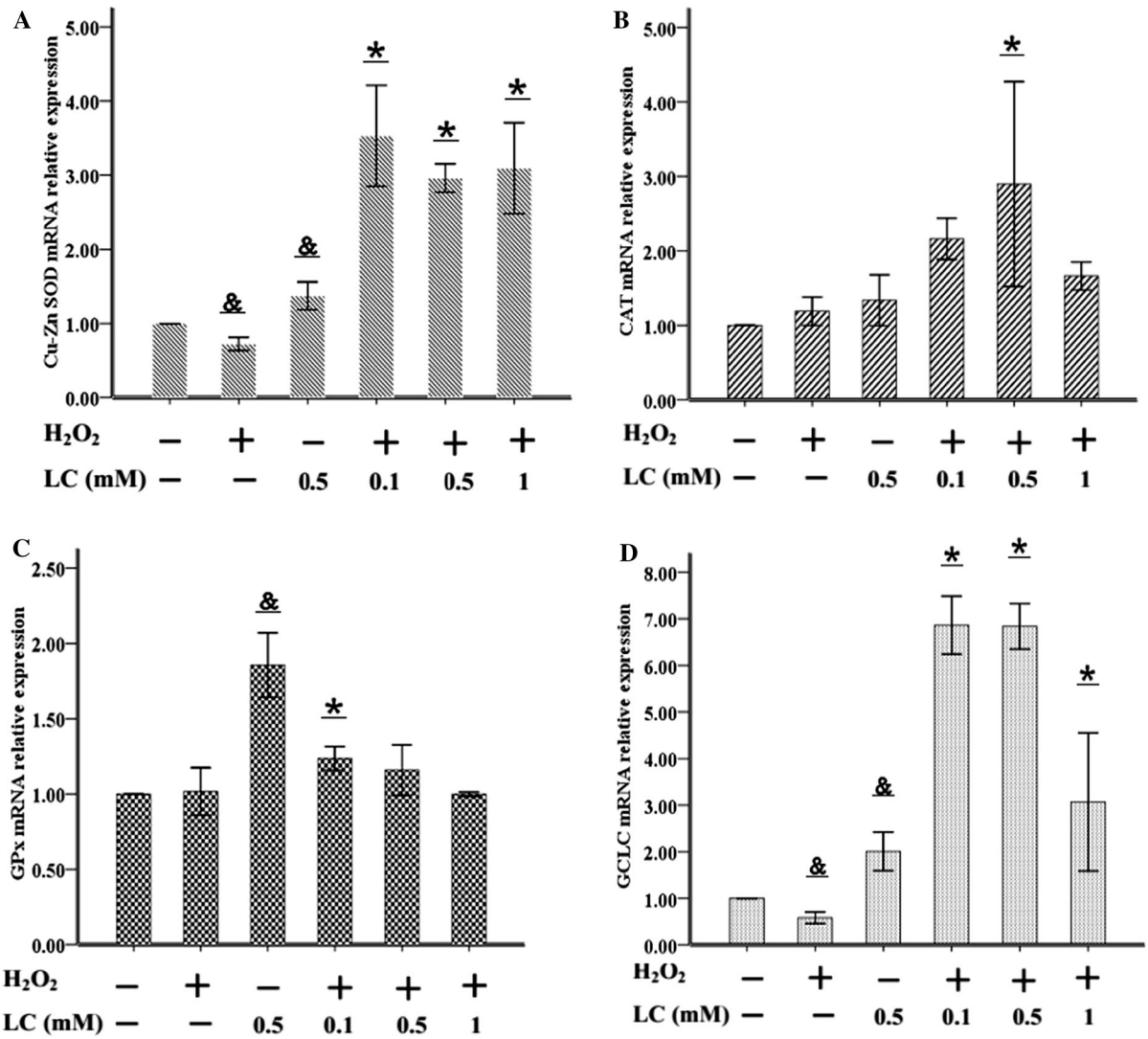


Fig. 4 Effect of LC pre-treatment for 12 h on the mRNA expressions of CuZn-SOD (**a**), CAT (**b**), GPx (**c**) and GCLC (**d**) in GCO cells following a 2.5 mM H_2O_2 challenge. The data are expressed as the mean ± SEM ($n = 3$) of the percentage of control cells, & ($P < 0.05$) means significantly different when compared to the control group, and *($P < 0.05$) means significantly different when compared to the H_2O_2 group

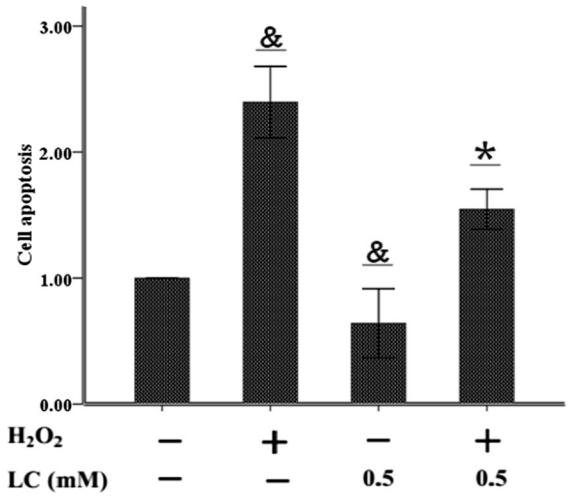


Fig. 5 Effect of LC pre-treatment for 12 h on the level of apoptosis in GCO cells following a 2.5 mM H_2O_2 challenge. The data are expressed as the mean ± SEM ($n = 3$) of the percentage of control cells, & ($P < 0.05$) means significantly different when compared to the control group, and *($P < 0.05$) means significantly different when compared to the H_2O_2 group

Although H_2O_2 is not a radical, it still belongs to the reactive species due to its higher oxidative activity. Therefore, H_2O_2 was used to induce oxidative damage and assess the resistance of GCO cells to oxidative stress after treatment with LC. Some studies in mammals have proved that LC exhibited strong antioxidant effects and prevented ROS generation in cells (Abdelrazik et al. 2009; You et al. 2012). Our results showed that treatment with LC significantly reduced ROS levels in GCO. Images of fluorescence intensities also supported this result. In addition, LC inhibited ROS accumulation in H_2O_2 or TM-treated SH-SY5Y cells by analysis of CM-H_2DCFCA staining, and the fluorescence intensity was also reduced in the 0.1 mM LC pre-treatment group (Ye et al. 2014). A previous study reported that LC had the stronger capabilities of 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) scavenging, superoxide anions scavenging, H_2O_2 scavenging, total reducing power and metal chelating on ferrous ion activities (Gülçin 2006).

In the present study, to further understand the extent of lipid peroxidation and the changes of the natural antioxidants in the cells, intracellular MDA values and T-GSH levels were measured. Our results revealed that pre-treatment with LC reduced the MDA value and maintained the GSH content in GCO cells. This was attributed to LC counteracting ROS, saving the number of intracellular GSH and thus sustaining the stores of GSH in the cells. γ-GCS is the rate-limiting enzyme of GSH synthesis, and GCLC is the catalytic subunit of γ-GCS, and then the expression of GCLC mRNA plays an important role in GSH synthesis in the cell. In a state of oxidative stress, the synthesis of natural antioxidants would be strengthened because of the increasing demand for natural antioxidants. LC treatment at 0.1–0.5 mM significantly up-regulated the expression of GCLC mRNA and improved the activity of γ-GCS in this study. These data revealed that exogenous supplementary LC strengthens GSH synthesis to maintain the intracellular GSH content via up-regulating GCLC mRNA expression and improving γ-GCS activity.

The responses of a cell against ROS usually involve increases in antioxidant enzyme activities, such as SOD, that convert the superoxide anions to H_2O_2, CAT or GPx that further change H_2O_2 to water. Several studies demonstrated that LC supplementation could improve the activities of several important enzymes in a state of oxidative stress induced by various factors, including SOD, CAT and GPx (Graziela et al. 2010; Agnieszka and Elzbieta 2009; Ye et al. 2010; Lamiaa et al. 2014). The results are consistent with our study. The data on the effects of LC on the expression of the antioxidant enzyme mRNA levels are still scant in fish. Then, we also detected the expression of CuZn-SOD, CAT and GPx mRNA in this study. Elanchezhian et al. (2010) reported the regulatory effect of acetyl-LC on the expression of CAT mRNA in rat lenses. LC up-regulated the mRNA expression of CuZn-SOD and GPx gene in the heart of spontaneously hypertensive rats (Miguel-Carrasco et al. 2010). Our data revealed that LC could increase the levels of CuZn-SOD and GCLC mRNA and did not significantly change the mRNA expression of CAT and GPx. The up-regulation of antioxidant enzyme mRNA expression induced by LC might involve the nuclear factor erythroid-2 related factor 2 (Nrf2)–antioxidant responsive element (Nrf2–ARE) signaling pathway. The Nrf2-mediated antioxidant response is a primary cellular defense mechanism against oxidative stress and serves to maintain intracellular redox homeostasis in mammals (Oyang et al. 2013). Under the state of oxidative stress, Nrf2 is activated and transfers into the nucleus to bind with the antioxidant responsive element (ARE) to regulate the gene expression of antioxidant enzymes,

suggesting that Nrf2–ARE signaling pathway plays an important role in protecting cells from oxidative stress (Quesada et al. 2011). It has been well documented that the increases in antioxidant genes were associated with up-regulation of Nrf2 mRNA expression (Hseu et al. 2015). Recently, a transcriptional regulation of Nrf2 on the antioxidant enzyme genes was found in fish (Jiang et al. 2014). Interestingly, the gene transcription of antioxidant enzymes and Nrf2 can be affected by dietary nutrients in aquatic animals (Deng et al. 2014; Jiang et al. 2014). Therefore, in our experiment we speculate that LC prevents oxidative stress in GCO cells via activating the Nrf2–ARE signaling pathway to up-regulate the mRNA expression of antioxidant enzymes and then improve the activity of the antioxidant enzymes. In accordance with our hypothesis, dose-dependent increases of LC (0.05, 0.1 and 0.2 mM) up-regulated the proteins of Nrf2 and γ-GCS (Cao et al. 2015). However, this molecular mechanism still needs to be proved by further study.

The data on cell apoptosis carried out using the AV/PI assays supported the results of the viability assay. Supplementation of LC inhibits cell apoptosis induced by 2.5 mM H_2O_2. Data from the literature showed the protective action of LC toward apoptosis involves cytochrome c release and immunoreactivity to caspase 3 (Pillich et al. 2005). Interestingly, another explanation was recently shown by Ye et al. (2014), stating that the protective effect of LC is mediated by CHOP/Bim or JNK/Bim-dependent ER stress signaling pathways. However, the antiapoptotic mechanisms of LC seem to remain unclear.

The results of this study indicated that LC with appropriate concentration promotes cells growth, clearly reduces levels of ROS and inhibits of lipid peroxidation and apoptosis in GCO cells. Meanwhile, LC with appropriate concentration improves the activity and gene expression of the antioxidant enzymes of GCO cells exposed to 2.5 mM H_2O_2. Our findings suggested that LC can ameliorate H_2O_2-induced oxidative stress in GCO cells mainly through its beneficial role in the antioxidant defense systems. Under these experimental conditions, the appropriate supplemental amount of LC is 0.1–1 mM. LC possesses antioxidation effects on fish cells and can serve as a potential and available food antioxidant that might help fish to overcome endogenous and exogenous oxidative stress. However, the regulatory mechanisms of LC on the antioxidant functions in fish cells are still not completely revealed, and further studies are warranted.

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