Fish and Shellfish Immunology 93 (2019) 1100–1110

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

L-carnitine regulated Nrf2/Keap1 activation *in vitro* and *in vivo* and protected oxidized fish oil-induced inflammation response by inhibiting the NF-κB signaling pathway in *Rhynchocypris lagowski* Dybowski

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ARTICLE INFO

Keywords:
L-carnitine
Nrf2/Keap1
Oxidized fish oil
NF-κB
Rhynchocypris lagowski Dybowski

ABSTRACT

Nrf2/Keap1 pathway is associated with oxidative stress. L-carnitine is currently under preclinical evaluation as a antioxidant, but the use of L-carnitine in aquaculture has been poorly evaluated and so far no mechanism has been demonstrated. Here, we explored the effects of L-carnitine *in vitro* and *in vivo* and discussed the possible molecular mechanisms involved. Firstly, *Nrf2*-siRNA significantly knocked down the mRNA level of *Nrf2* in FHM cells. Thus, the activities of antioxidant enzymes (T-SOD, CAT, GSH-PX) and the level of antioxidant substance (GSH) and the level of MDA showed that *Nrf2*-siRNA pretreatment weakened the protective effect of L-carnitine. Moreover, the mRNA levels of *Keap1*, *Nrf2*, *Maf* and *HO-1* indicated that L-carnitine regulated Nrf2/Keap1 activation. Furthermore, oxidized fish oil remarkably suppressed growth in *Rhynchocypris lagowski* Dybowski, and the lower antioxidant capacity was also observed in liver. According to the results of immune related indexes (the levels of IL-1β, TNF-α, LZM, AKP) in serum and the mRNA levels of immune related genes (*NF-κB*, *IL-1β*, *TNF-α*, *IL-8*, *IL-10* and *TGF-β*) in liver, oxidized fish oil also induced inflammatory response in fish. Also, L-carnitine supplementation can relieve this bad condition. In conclusion, L-carnitine regulated Nrf2/Keap1 activation in *vitro* and in *vivo* and protected oxidized fish oil-induced inflammation response by inhibiting the NF-κB signaling pathway in *Rhynchocypris lagowski* Dybowski.

1. Introduction

With the improvement of science and technology, the global aquaculture industry is developing rapidly, the intensive degree of aquaculture is getting higher and higher. However, the inherent behavior, nutrition, psychology and other biological needs of fish are also undergoing severe challenges [1]. In recent years, more and more studies have been focused on various stresses (such as environmental factor, feed, feeding management) in aquaculture. So far, it has been proved that any strong stress is accompanied by oxidative stress [2]. Therefore, reactive oxygen free radical (ROS), as a cellular metabolite, has attracted many researchers' attention. ROS can break the redox state of cells by disrupting the reaction between molecules and macromolecules, leading to the pathogenesis of different diseases and aging [3–5]. In order to maintain the cell redox homeostasis, a series of antioxidant and detoxifying enzymes and genes of organism play a key role in the response to various environmental stresses, which are regulated by the nuclear transcription factor NF-E2-related factor 2 (Nrf2) [6]. Nrf2 belongs to the CNC family and has an alkaline leucine zipper structure, which is widely distributed in various tissues [7]. It is retained in the cytosol by binding to a cluster of proteins, including cytosolic inhibitor, Kelch like-ECH-associated protein 1(Keap1) [8]. Under oxidative stress, Nrf2 dissociates from Keap1 and moves to the nucleus to bind with the antioxidant-response element (ARE) to regulate the target genes(*HO-1*, *NF-κB*), suggesting that Nrf2 can regulate antioxidant and anti-inflammatory cellular responses, playing an important protective role on the development of oxidative stress [9,10].

The understanding of how nutrition may impact the prevention and/or treatment of various stresses in aquaculture has grown tremendously in recent years. The recommendation of a balanced and varied diet is based on the feed components that can bring health benefits to fish. In recent years increasing evidence has emerged suggesting that L-

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https://doi.org/10.1016/j.fsi.2019.08.041
Received 9 June 2019; Received in revised form 10 August 2019; Accepted 14 August 2019
Available online 15 August 2019

Table 1
Primer sequences of target gene for the real-time fluorescent quantitative PCR.

Gene	Primer sequence(5′-3′)		Amplification efficiency (%)	Annealing temperature (°C)	GenBank number
β-actin	Forward	CGGTATCCATGAGACCACCT	100.0	59.5	AAB97964.1
	Reverse	CTTCTGCATCCTGTCAGCAA			
Nrf2	Forward	GGAGAAGGAGCGATTGAAGA	100.1	59.5	MF150102.1
	Reverse	AGGCTTTCCCTTCTCGTCTC			
Keap1	Forward	CGCAGGAGGAGTTCTTCAAC	100.1	59.5	AAI52660.1
	Reverse	CGGTTCTCTGGCTCGTACTT			
Maf	Forward	TGGAGCAGCAGAAGAAGGAG	100.4	59.8	NM131844.2
	Reverse	GTGTTCAGACGGGGTGTTGT			
HO-1	Forward	TGTCAGGAGGACAAGTGCTG	99.7	59.7	JX257180.1
	Reverse	CAGCTGCTTGAATCTGTTGG			
NF-κBp65	Forward	GAAGAAGGATGTGGGAGATG	100.7	62.3	KJ526214
	Reverse	TGTTGTCGTAGATGGGCTGAG			
TNF-α	Forward	CCAGGCTTTCACTTCACG	100.6	56	FN543477
	Reverse	GCCATAGGAATCGGAGTAG			
IL-1β	Forward	CTGGAGCAATGCAATACAAA	100.3	56	AJ245635
	Reverse	AGGTAGAGGTTGCTGTTGGAA			
IL-8	Forward	ATGAGTCTTAGAGGTCTGGGTG	100.2	57.8	DQ453125
	Reverse	ACAGTGAGGGCTAGGAGGG			
IL-10	Forward	AATCCCTTTGATTTTGCC	100.3	57.9	AB110780
	Reverse	GTGCCTTATCCTACAGTATGTG			
TGF-β	Forward	TTGGGACTTGTGCTCTAT	99.5	55.9	EU099588
	Reverse	AGTTCTGCTGGGATGTTT			

Nrf2, nuclear factor erythroid 2-related factor 2; *Keap1*, Kelch-like ECH-associated protein 1; *Maf*, musculoaponeurotic fibrosarcoma oncogene; *HO-1*, haem oxygenases-1; *NF-κB p65*, nuclear factor kappa B p65; *TNF-α*, tumour necrosis factor α; *IL-1β*, interleukin 1β; *IL-8*, interleukin 8; *IL-10*, interleukin 10; *TGF-β*, transforming growth factor β.

carnitine can not only promote fatty acid β-oxidation, but also plays an important role in fish growth, antioxidation and immunity [11,12], such as common carp (*Cyprinus carpio*) [13,14], silver perch (*Bidyanus bidyanus*) [15], zebrafish (*Danio rerio*) [16], black sea bream (*Sparus macrocephalus*) [11], black seabream (*Acanthopagrus schlegelii*) [17], Nile tilapia (*Oreochromis niloticus*) [18]. Especially in terms of antioxidation, L-carnitine is a potent antioxidant (free radical scavenger) and thus may protect tissues from oxidative damage [19]. However, most deeply studies are concentrated on humans and terrestrial animals, the use of L-carnitine in aquaculture has been poorly evaluated and so far no mechanism has been demonstrated. Previous studies in our laboratory have shown that the regulation of L-carnitine on oxidative stress induced by H_2O_2 is related to Keap1-Nrf2-ARE signaling pathway [20,21]. Furthermore, many studies have shown that a series of oxidation products produced by lipid oxidation are seriously harmful to the health of fish and cause oxidative damage to fish, most of the reports focused on largemouth bass (*Micropterus salmoides*) [22], Japanese sea bass (*Lateolabrax japonicus*) [23], Atlantic halibut (*Hippoglossus hippoglossus*) [24], Siberian sturgeon (*Acipenser baeri*) [25], gilthead sea bream (*Sparus aurata* L.) [26], channel catfish (*Ictalurus punctatus*) [27]. What's more, fish oil is widely choosed as the target to explore the oxidative damage in aquaculture because it is rich in polyunsaturated fatty acids. Also, that lead to oxidative decay in fish oil, mediated by metal ions, light, molecular oxygen and heat [28].

On this basis, the H_2O_2 and oxidized fish oil were choosed as the inducing factor, the present study was conducted to evaluate the antioxidative and anti-inflammatory effect of L-carnitine in *Rhynchocypris lagowski* Dybowski, the mechanisms and signaling pathways underlying this property was also determined to provide basic data for revealing the molecular mechanism of L-carnitine antioxidant effect.

2. Materials and methods

2.1. Ex vivo study

2.1.1. Culture of FHM cells

FHM cells were preserved in Jilin Agricultural University (Changchun, China), and incubated in 25-cm^2 cell culture flasks that contained M199 media supplemented with 10% FCS. The cells were cultivated to 4–5 generations in an incubator at 27 °C with 2% CO_2 in 98% air. All the chemicals and reagents were same as the described by the Wang et al. [20].

2.1.2. Nrf2-siRNA transfection

FHM cells were transfected with siRNA (chemically synthesized by Shanghai GenePharma, Shanghai, China) using RNAi-Mate (Shanghai GenePharma, Shanghai, China) following the manufacturer's protocol. The sequences of the siRNA were GCA AGC GGA AGA UGG AUA ATT (sense, 5′-3′), UUA UCC AUC UUC CGC UUG CTT (antisense, 5′-3′). In the transfection experiments, FHM cells were transfected with *Nrf2*-siRNA using RNAi-Mate according to the manufacturer's instructions. The time when transfection commenced was considered as time 0. After incubation in medium containing transfection reagent for 6 h, the media were changed into normal growth medium. At 24 h after the transfection, cells were harvested.

2.1.3. Ex vivo effect of LC against H_2O_2-induced oxidative stress in FHM cells

The FHM cells in the logarithmic phase of growth were initially plated in 6-well cell culture plates at a density of 1.0×10^6 cells/well with M199 media supplemented with 10% FCS and allowed to adhere for 24 h in an incubator at 27°C with 2% CO_2 in 98% air. The cells were divided into 8 groups: group 1, 2, 3, 4, 5, 6, 7, 8. Among them, group 1–4 continued to be cultured with serum-free medium without any treatment, and the rest 4 groups (group 5–8) were treated with *Nrf2*-siRNA, all of them cultured for 24 h. After 24 h culture, the old medium was abandoned, group 3, 4, 7, 8 were pretreated with M199 medium containing L-carnitine (0.5 mM) for 6 h, and the other groups were cultured with serum-free M199 for 6 h. After 6 h, the old medium was abandoned, group 2, 4, 6, 8 were treated with H_2O_2 (1 mM) for 1 h, and the other groups were cultured with serum-free M199 for 1 h.

2.1.4. Antioxidant enzyme activity and antioxidant substance determination

Total levels of alonaldehyde (MDA) and glutathione (GSH), and total activities of superoxide dismutase (T-SOD), catalase (CAT), and glutathione peroxidase (GSH-PX) were analyzed by kit (Nanjing Jiancheng Bioengineering Institute).

2.1.5. Expression of genes related to Keap 1-Nrf2-ARE signaling pathway

Total RNA was isolated using RNAiso Reagent (Takara, Dalian, China) according to the manufacturer's instructions. The quantity of the RNA was evaluated by using NanoDrop2000 spectrophotometer (Nano Drop Technologies, USA). The RNA was reverse transcribed with ReverTra Ace RqPCR RT Kit from TOYOBO. The complementary DNA was stored at −80 °C for later analysis. Specific primers were shown in Table 1 or *β-actin* was used as a housekeeping gene. The RT PCR assays were performed on an ABI StepOnePlus Detection System (ABI 7500, USA) according to the following protocol: 10 μl of FastStart Universal SYBR Green Master (ROX) (2 ×), 0.6 μl forward and 0.6 μl reverse primer, 2 μl of cDNA template and 6.8 μl nuclease-free water. The cycling conditions were as follows: 95 °C for 15 s, 60 °C for 60 s. The mRNA levels of related genes were analyzed using the double standard curve method according to Schikorski et al. [29].

2.2. In vivo

2.2.1. Experimental diets

Seven semi-purified diets (I- VII) containing three oxidation levels of fish oil and two LC (L-carnitine) levels (FFO (fresh fish oil), OFO (oxidized fish oil)100, OFO400, OFO100 + LC500, OFO100 + LC1000, OFO400 + 500 and OFO400 + 1000) were formulated. Diets were isolipidic and isonitrogenous with 35% protein, 7% lipid and 10% ash respectively. The composition and proximate analysis of the experimental diets was shown in Table 2. The oxidized fish oil was prepared by heating fresh fish oil at 70 °C with vigorous aeration. The three oxidation levels of fish oil were monitored as 6.43 ± 0.92 (FFO), 106.89 ± 3.52 (OFO100) and 402.77 ± 11.76 (OFO400) meg/kg by the determination of the peroxide value (POV). All the materials passed the 60 mesh sieve, weighed and mixed with water according to the formulation. Then the mixture processed into 1.0 mm diameter particles, naturally air-dried and stored in −20 °C freezer for reserve.

2.2.2. Fish and feeding trial

The experiment was conducted at Aquaculture Department of Jilin Agricultural University. In the experiment, a total number of 630 juvenile *Rhynchocypris lagowski* Dybowski with an initial weight of (4.53 ± 0.11) g were purchased from Changshan Fishery in Jilin City. All the fishes were randomly assigned to seven groups with three replicates and 30 fishes per replicate. The fish were allowed to acclimatize with the experimental conditions for 15 d, followed by 56 d feeding with the aforementioned diets. Temperature and oxygen levels were monitored daily; both parameters varied in the range of 20–23 °C and 7–9 mg/L OD. During the period of temporary cultivation, the basal diet was fed, and the feeding was stopped for 1 day before the experiment began. Feeding rate was adjusted based on the water temperature and the fish weight.

2.2.3. Sample collection

At week four and eight of the feeding trial, six fish were randomly caught each replicate used for analysis. Fish were euthanized by an overdose of MS-222 (Ethyl 3-aminobenzoate) and weighted. Then, blood samples were drawn from the caudal vein, and serum was separated by centrifugation. Finally, the fish were dissected to isolate the liver samples. All samples were stored at −80 °C until analyzed.

2.2.4. Growth index assay

The replicate' biomass and feed intake (FI) were determined and growth factors such as body weight gain (BWG), specific growth rate (SGR), feed efficiency (FE) and protein efficiency (PE) were calculated as follows:

$$BWG\ (\%) = (100/IW) \times (FW–IW)$$

$$SGR\ (\%/d) = (100/\text{days of rearing}) \times (\ln FW - \ln IW)$$

$$FI\ (\%/d) = \{(100 \times FI)/[(IW + FW)/(2 \times \text{days of rearing})]\}$$

$$FE\ (\%) = 100 \times (FW–IW)/FI$$

$$PE\ (\%) = 100 \times (FW–IW)/(FI \times \text{feed protein content})$$

where IW is fish initial weight and FW is fish final weight.

2.2.5. Antioxidant related index assay

Total levels of antioxidant capacity (T-AOC), alonaldehyde (MDA)

Table 2
Composition and proximate analysis of experimental diets.

Ingredients	Experimental diets						
	I	II	III	IV	V	VI	VII
Fish oil	FFO	OFO100	OFO400	OFO100	OFO100	OFO400	OFO400
LC (mg/kg)	0.0	0.0	0.0	500	1000	500	1000
Fish meal (g/kg)	200.0						
Peeling soybean meal (g/kg)	330.0						
Corn gluten meal (g/kg)	150.0						
Wheat bran (g/kg)	60.0						
Fish oil (g/kg)	40.0						
Flour (g/kg)	100.0						
Dextrin (g/kg)	70.0						
Monocalcium phosphate (g/kg)	30.0						
Multi-nutrient premix[a] (g/kg)	10.0						
Lysine (g/kg)	2.0						
Methionine (g/kg)	5.0						
Choline chloride (g/kg)	3.0						
Proximate composition							
Crude lipid (g/kg)	71.9						
Crude protein (g/kg)	346.5						
Crude ash (g/kg)	95.8						
Gross energy (MJ/kg)	15.28						

[a] Multi-nutrient premix (vitamins and mineral included): vitamin A (IU/kg) 3600; vitamin D3 (IU/kg) 1200; vitamin E (mg/kg) 20, vitamin K3 (mg/kg) 5; vitamin B1 (mg/kg) 5; vitamin B2 (mg/kg) 7; vitamin B6 (mg/kg) 6; vitamin B12 (ug/kg) 20; calcium pantothenate (mg/kg) 20; nicotinic acid (mg/kg) 30; folic acid (mg/kg) 1.7; biotin (mg/kg) 0.05; vitamin C phosphate (mg/kg) 171.4; inositol (mg/kg) 90; Mg (mg/kg) 150; Fe (mg/kg) 120; Zn (mg/kg) 60; Mn (mg/kg) 30; Cu (mg/kg) 4; Co (mg/kg) 0.5; Se (mg/kg) 0.1; I (mg/kg) 1.

and glutathione (GSH), and total activities of superoxide dismutase (T-SOD), catalase (CAT), and glutathione peroxidase (GSH-PX) in liver were analyzed by kit (Nanjing Jiancheng Bioengineering Institute).

2.2.6. Immune related index assay

The levels of interleukin 1β (IL-1β) and tumour necrosis factor α (TNF-α) and the activities of lysozyme (LZM) and alkaline phosphatase (AKP) in serum were measured according to the kit instructions (Nanjing Jiancheng Bioengineering Institute).

2.2.7. Related genes mRNA expression assay

The mRNA levels of antioxidant related genes and the mRNA levels of immune related genes in liver were measured according to the description in 2.1.5.

2.3. Data analysis

All data were analyzed by one-way ANOVA (SPSS20.0) to assess significant difference at the 5% ($P < 0.05$) between oxidized fish oil -treated and control groups. All results were presented as means ± standard deviation (Means ± SD). Significance was analyzed by LSD and Duncan's and indicated by different letters.

3. Results

3.1. Ex vivo study

3.1.1. Nrf2-siRNA transfection

After *Nrf2*-siRNA transfection, *Nrf2*-siRNA significantly knocked down the *Nrf2* gene mRNA level in FHM cells when compared to the control group ($P < 0.05$) (Fig. 1).

3.1.2. Antioxidant enzyme activity and antioxidant substance determination

As shown in Fig. 2, it was clearly observed that L-carnitine pretreatment could significantly decreased the level of MDA in normol FHM cells ($P < 0.05$). At the same time, L-carnitine pretreatment could significantly decreased the increasing the level of MDA in FHM cells caused by H_2O_2 treatment ($P < 0.05$). However, *Nrf2*-siRNA pretreatment weakened the protective effect of L-carnitine: there was no significant difference between L-carnitine + *Nrf2*-siRNA group and *Nrf2* -siRNA group. And FHM cells treated with *Nrf2*-siRNA and H_2O_2 showed a significant increase in the level of MDA when compared to the H_2O_2 treatment group ($P < 0.05$), and the same result was observed between the L-carnitine + H_2O_2 + *Nrf2*-siRNA group and H_2O_2 + *Nrf2*-siRNA group.

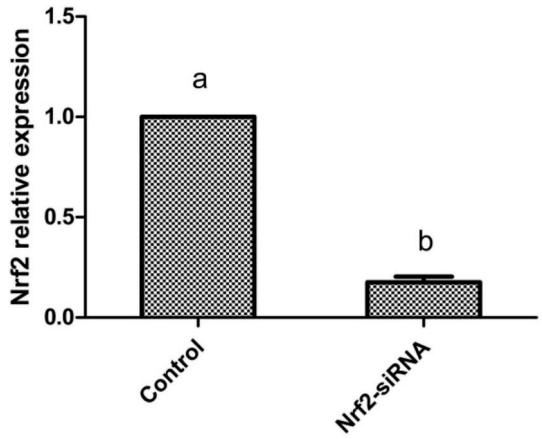

Fig. 1. *Nrf2*-siRNA knock down *Nrf2* gene expression in FHM cells. Note: Values are means ± SD (n = 3), values followed by different letters are significantly different ($P < 0.05$).

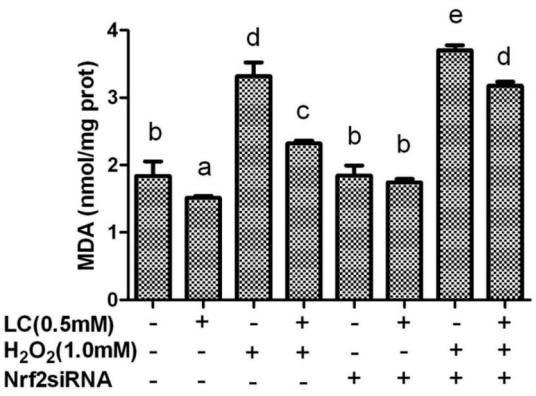

Fig. 2. Effects of LC on the level of MDA in FHM and *Nrf2*-siRNA-FHM cells after H_2O_2 exposure. Note: Values are means ± SD (n = 3), values followed by different letters are significantly different ($P < 0.05$).

As shown in Fig. 3, it was clearly observed that L-carnitine pretreatment could significantly increased the activities of CAT and GSH-PX in normol FHM cells ($P < 0.05$), and L-carnitine pretreatment could significantly increased the level of GSH, the activities of CAT and GSH-PX in H_2O_2 treated FHM cells ($P < 0.05$). At the same time, *Nrf2*-siRNA pretreatment weakened the protective effect of L-carnitine: there was no significant difference between L-carnitine + *Nrf2*-siRNA group and *Nrf2*-siRNA group. And FHM cells treated with *Nrf2*-siRNA and H_2O_2 showed a significant decrease in the level of GSH, the activities of CAT and T-SOD when compared to the H_2O_2 treatment group ($P < 0.05$), and the same result was observed in the level of GSH, CAT, the activities of T-SOD and GSH-PX between the L-carnitine + H_2O_2 + *Nrf2*-siRNA group and H_2O_2 + *Nrf2*-siRNA group.

3.1.3. Expression of genes related to Keap 1-Nrf2-ARE signaling pathway

The mRNA levels of related genes analysis showed that L-carnitine pretreatment could significantly decreased the mRNA level of *Keap1*and increased the mRNA levels of *Nrf2*, *Maf* and *HO-1* in normol FHM cells ($P < 0.05$), even in H_2O_2 treated FHM cells ($P < 0.05$). However, *Nrf2*-siRNA pretreatment not only decreased the mRNA levels of *Keap1*, *Nrf2*, *Maf* and *HO-1* in the four groups, and also weakened the protective effect of L-carnitine after H_2O_2 treatment: there were no significance in the mRNA levels of *Keap1*, *Nrf2*, *Maf* and *HO-1* between the L-carnitine + H_2O_2 + *Nrf2*-siRNA group and H_2O_2 + *Nrf2*-siRNA group (Fig. 4).

3.2. In vivo study

3.2.1. Effects of LC on growth performance

At the end of four weeks feeding experiment, BWG, SGR, FER and PER significantly decreased in group III, VI and VII ($P < 0.05$), and FI significantly increased in group III, VI and VII ($P < 0.05$). However, at the end of eight weeks feeding experiment, FI significantly increased in group II($P < 0.05$), then significantly decreased in group IV, V and VII ($P < 0.05$), and there was on significant difference between group Iand VI. At the same time, BWG, SGR, FER and PER significantly decreased in group III ($P < 0.05$), there was no significant difference in BWG and SGR among group I, IV, V, VI and VII, and no significant difference was found in FER and PER among group I, IV, VI and VII. But group V showed significantly higher FER and PER than the group I ($P < 0.05$) (Table 3).

3.2.2. Effects of LC on antioxidant related index

The antioxidant related index assay in liver was shown in Fig. 5. At the end of four weeks feeding experiment, the level of T-AOC in liver significantly increased in group II, group VI and group VII ($P < 0.05$), and group II showed significantly higher level of T-AOC than the group

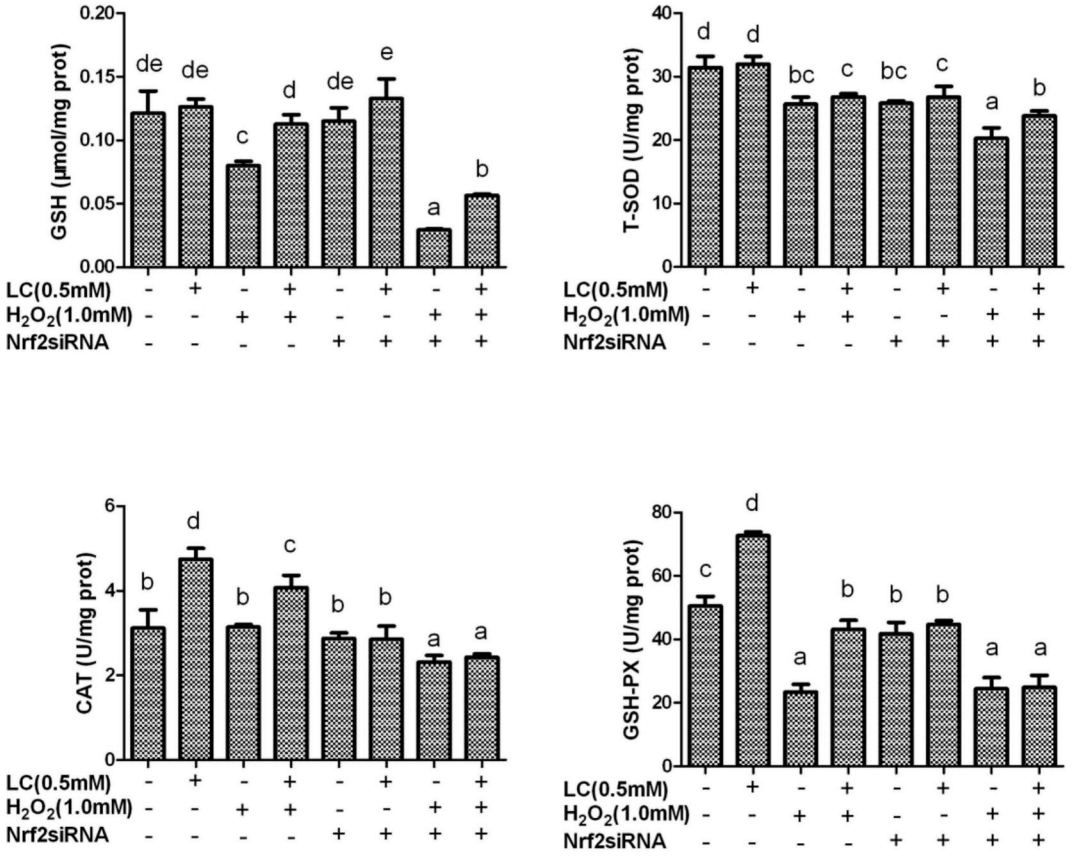

Fig. 3. Effect of LC pretreatment for 6 h on the antioxidant related indexes of FHM and *Nrf2*-siRNA-FHM cells following a H_2O_2 exposure. Note: Values are means ± SD (n = 3), values followed by different letters are significantly different ($P < 0.05$).

VI and group VII ($P < 0.05$). Only the activity of T-SOD significantly increased in group II ($P < 0.05$), and no significant difference were obsreved in the other groups. The activity of CAT in liver firstly significantly increased in group II ($P < 0.05$), then significantly decreased in group III ($P < 0.05$), and no significant difference were obsreved in the group I, III, IV, V and VII, but group VI still showed significantly lower activity of CAT than the group I ($P < 0.05$). Compared to the group I, the other groups showed no significant difference in the activity of GSH-PX. The level of GSH in the group III showed a significant lower than the groupI ($P < 0.05$), and no significant difference were obsreved in the group I, IV, Vand VII, but group VI still showed significantly lower activity of CAT than the group I ($P < 0.05$). However, the level od MDA in all other groups showed a significant higher than the group I ($P < 0.05$), especially the group III showed a highest value ($P < 0.05$).

At the end of eight weeks feeding experiment, the level of T-AOC, the activities of T-SOD, CAT, GSH-PX, and the level of GSH all significantly decreased in group II and III($P < 0.05$). No significant differences were found in the level of T-AOC and the activity of GSH-PX in the group I, IV, Vand VII, but group V showed significantly higher values than the group I ($P < 0.05$). The activity of T-SOD in group IV and Vshowed significantly higher values than the group I ($P < 0.05$), and there were no significant differences among group I, group VI and group VII. There was no significant difference in activity of CAT in group Iand IV, and the activity of CAT showed significantly increased in group III ($P < 0.05$), but the activity of CAT in group VI and group VII were still significantly lower than the group I ($P < 0.05$). There were no significant difference in the level of GSH in the group I, IV, V, VI and VII. However, the level of MDA in group VI, V, VI and VII all were significantly lower than the group I ($P < 0.05$).

3.2.3. Effects of LC on immune related index

Immune related index assay was shown in Table 4. After eight weeks feeding, the levels of IL-1β and TNF-α were significantly increased in group II and III ($P < 0.05$), the level of IL-1β in group IV, V, VI and VII and the level of TNF-α in group V all showed no significant difference with the group I, but the level of TNF-α in group IV, VI and VII were still significantly higher than the group I ($P < 0.05$). The activities of AKP and LZM were significantly decreased in group IIand III ($P < 0.05$), and there were no significant difference among group I, IVand V, but the activities of AKP and LZM in group VI and VII were still significantly lower than the group I ($P < 0.05$).

3.2.4. LC promoted the activation of Nrf2/Keap 1 signaling pathway

The mRNA levels of antioxidant related genes in liver were shown in Fig. 6. At the end of four weeks feeding experiment, group II, III, IV, V, VI and VII all showed a significant higher value in the mRNA levels of *Keap 1* and *HO-1* than the group I ($P < 0.05$). Meanwhile, group III, IV, V, VI and VII all showed a significant higher value in the mRNA levels of *Nrf2* and *Maf* than the group I ($P < 0.05$). At the end of eight weeks feeding experiment, the mRNA level of *Keap 1* in group II, III significantly increased ($P < 0.05$), then significantly decreased in group IV, V, VI and VII ($P < 0.05$), but the mRNA level of *Keap 1* in the four groups were still significant lower than the group I ($P < 0.05$). The mRNA level of *Nrf2* in group III significantly decreased ($P < 0.05$), then significantly increased in group IV, V, VI and VII ($P < 0.05$), and the mRNA level of *Nrf2* in group VI and VII were significant higher than the groupI ($P < 0.05$). The mRNA level of *Maf* in group II, III significantly decreased ($P < 0.05$), then significantly increased in group IV, V, VI and VII ($P < 0.05$), but the mRNA level of *Maf* in group IV, V, VI were still significant lower than the group I ($P < 0.05$), only group VII showed a significant higher value in the mRNA level of *Maf* than the

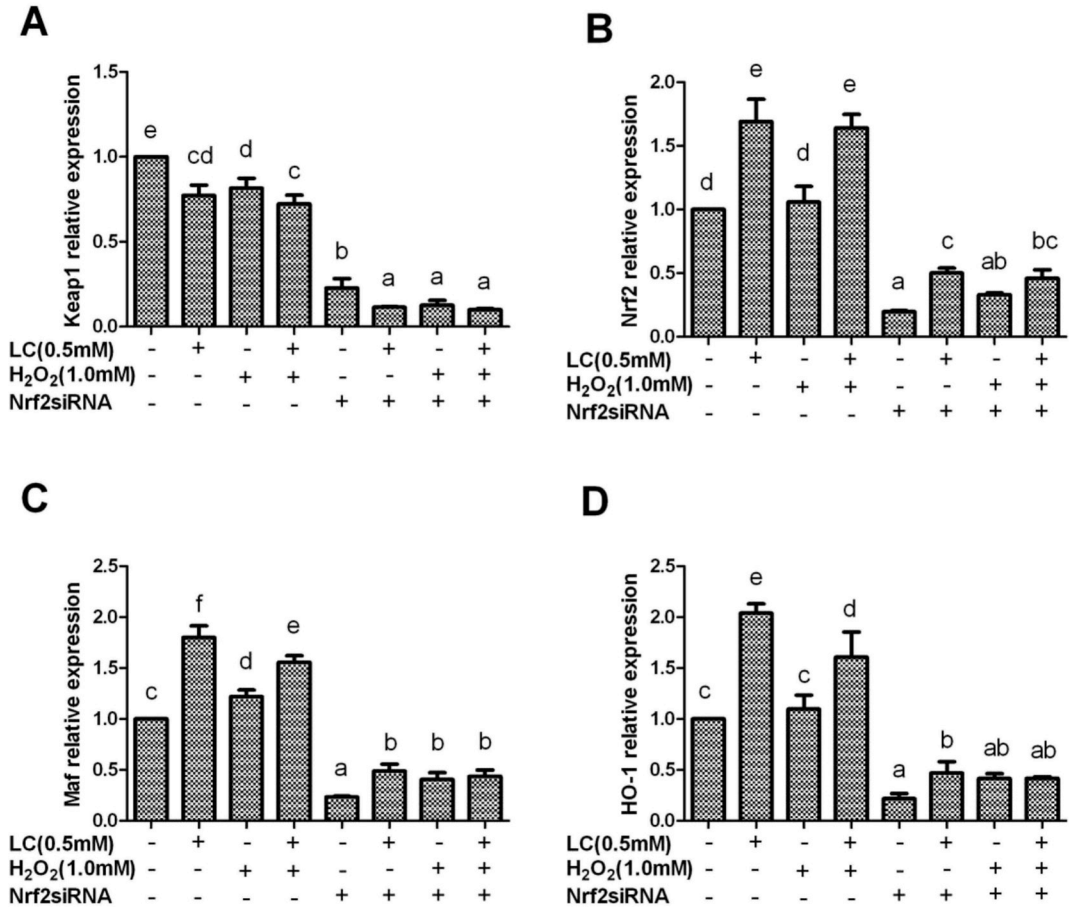

Fig. 4. Effect of LC pretreatment for 6 h on the mRNA levels of antioxidant related genes of FHM and *Nrf2*-siRNA-FHM cells following a H_2O_2 exposure. Note: Values are means ± SD (n = 3), values followed by different letters are significantly different ($P < 0.05$).

group I ($P < 0.05$). The mRNA level of *HO-1* in group II, III significantly decreased ($P < 0.05$), then significantly increased in group IV, V, VI and VII ($P < 0.05$), and group V, VI and VII showed a significant higher value in the mRNA level of *HO-1* than the group I ($P < 0.05$).

3.2.5. LC inhibited the activation of TNF-α/NF-κB signaling pathway

The mRNA levels of immune related genes in liver were shown in Fig. 7. After eight weeks feeding, the mRNA level of *NF-κBp65* significantly increased in group II, III ($P < 0.05$), then significantly decreased in group IV and V, and group Vshowed no significant difference with the group I, but group VI and VII showed a significant higher value in the mRNA level of *NF-κBp65* than the group I ($P < 0.05$). The mRNA level of *TNF-α* significantly increased in group II, III ($P < 0.05$), then significantly decreased in group IV, V, VI and VII, and group IV, Vshowed no significant difference with the group I, but group VI and VII still showed a significant higher value in the mRNA level of *TNF-α* than the group I ($P < 0.05$). The mRNA level of *IL-1β* significantly increased in group II, III ($P < 0.05$), then significantly decreased in group IV, V, VI and VII, but the four groups still

Table 3
Effects of oxidized fish oil and LC on growth performance of *Rhynchocypris lagowski* Dybowski.

Groups	I	II	III	IV	V	VI	VII
BWG/%							
4w	60.73 ± 13.22^{b}	58.79 ± 6.1^{b}	33.48 ± 9.31^{a}	45.68 ± 6.28^{ab}	48.14 ± 5.04^{ab}	40.34 ± 8.33^{a}	38.63 ± 4.78^{a}
8w	134.48 ± 10.09^{bc}	124.03 ± 9.01^{b}	102.61 ± 7.8^{a}	130.07 ± 8.97^{b}	150.58 ± 12.84^{c}	138.06 ± 13.57^{bc}	140.58 ± 10.9^{bc}
SGR/%							
4w	0.84 ± 0.15^{c}	0.83 ± 0.07^{c}	0.51 ± 0.12^{a}	0.67 ± 0.08^{abc}	0.70 ± 0.06^{bc}	0.60 ± 0.11^{ab}	0.58 ± 0.06^{ab}
8w	1.52 ± 0.08^{bc}	1.44 ± 0.07^{b}	1.26 ± 0.07^{a}	1.49 ± 0.07^{b}	1.64 ± 0.09^{c}	1.55 ± 0.1^{bc}	1.57 ± 0.08^{bc}
FI %/d							
4w	2.72 ± 0.12^{ab}	2.66 ± 0.09^{a}	2.79 ± 0.04^{bc}	2.70 ± 0.02^{ab}	2.76 ± 0.05^{ab}	2.9 ± 0.05^{c}	2.9 ± 0.04^{c}
8w	3.64 ± 0.04^{c}	3.72 ± 0.05^{d}	3.56 ± 0.03^{b}	3.55 ± 0.02^{b}	3.44 ± 0.01^{a}	3.61 ± 0.06^{bc}	3.54 ± 0.08^{b}
FER/%							
4w	61.23 ± 12.84^{c}	60.94 ± 5.94^{c}	36.88 ± 2.43^{a}	49.13 ± 5.58^{abc}	50.41 ± 5.31^{bc}	41.18 ± 6.83^{ab}	39.75 ± 3.73^{ab}
8w	39.42 ± 1.09^{bc}	36.43 ± 1.9^{ab}	34.69 ± 1.83^{a}	39.56 ± 1.64^{bc}	44.55 ± 2.04^{d}	40.37 ± 2.53^{c}	41.69 ± 0.77^{cd}
PER/%							
4w	1.77 ± 0.37^{c}	1.76 ± 0.17^{c}	1.07 ± 0.07^{a}	1.42 ± 0.16^{abc}	1.46 ± 0.15^{bc}	1.19 ± 0.2^{ab}	1.15 ± 0.1^{ab}
8w	2.28 ± 0.07^{bc}	2.1 ± 0.11^{ab}	2.00 ± 0.11^{a}	2.28 ± 0.09^{bc}	2.57 ± 0.12^{d}	2.33 ± 0.15^{c}	2.41 ± 0.05^{cd}

Note: values(means ± SD, n = 3) followed by different letters are significantly different ($P < 0.05$).

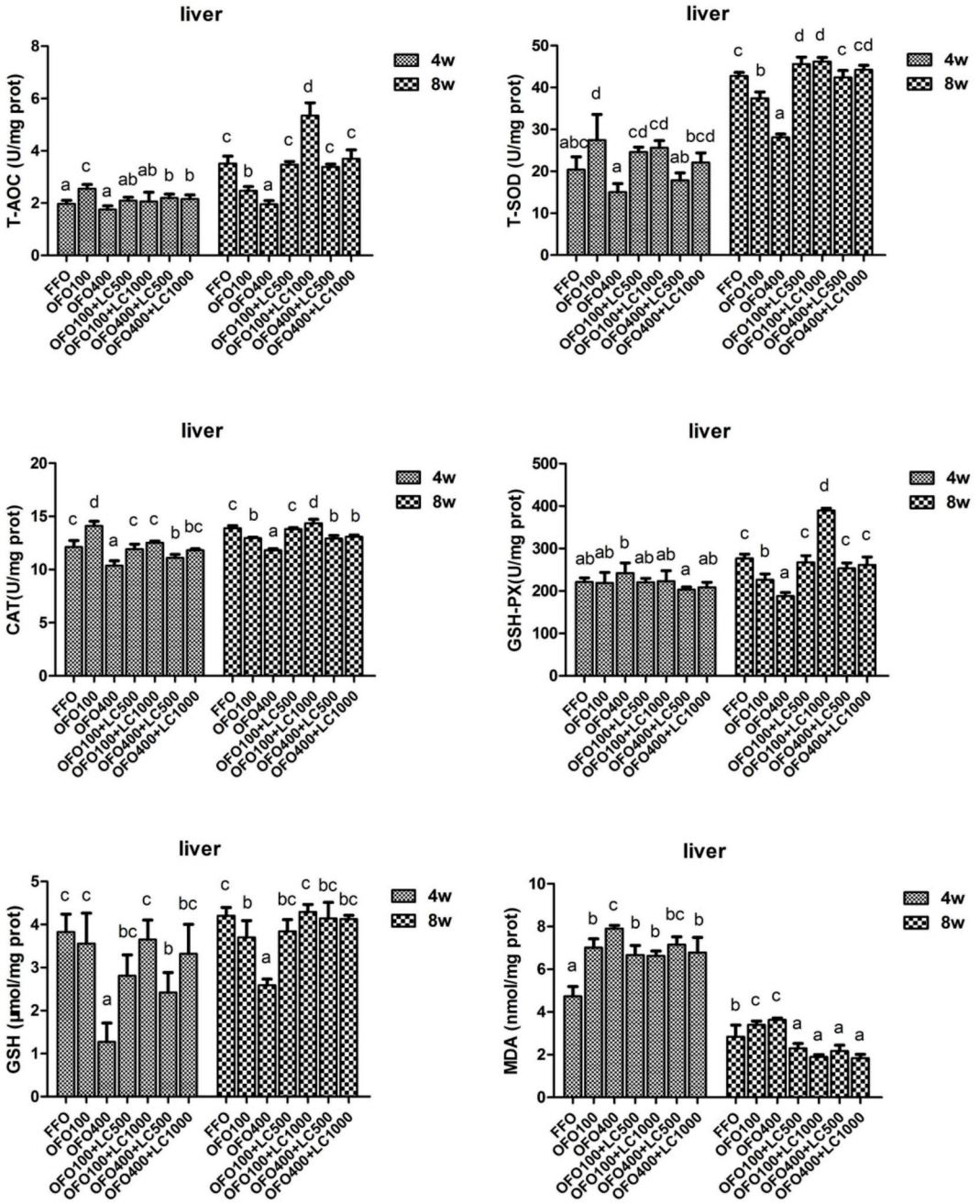

Fig. 5. Effects of oxidized fish oil and LC on the antioxidant related indexes in liver of *Rhynchocypris lagowski* Dybowski. Note: Values are means ± SD (n = 3), values followed by different letters are significantly different ($P < 0.05$).

Table 4
Effects of oxidized fish oil and LC on the immune related indexes in serum of *Rhynchocypris lagowski* Dybowski.

Groups	I	II	III	IV	V	VI	VII
IL-1β (pg/mL)	20.41 ± 1.84^{a}	27.83 ± 1.90^{bc}	32.41 ± 4.77^{c}	22.99 ± 3.03^{ab}	21.87 ± 2.06^{a}	25.74 ± 2.34^{ab}	23.59 ± 3.34^{ab}
TNF-α (pg/mL)	6.56 ± 0.20^{a}	7.35 ± 0.16^{cd}	7.55 ± 0.17^{d}	7.09 ± 0.17^{bc}	6.87 ± 0.16^{ab}	7.24 ± 0.13^{bc}	6.94 ± 0.35^{b}
AKP (U/mL)	1.21 ± 0.13^{d}	0.96 ± 0.10^{c}	0.37 ± 0.11^{a}	1.22 ± 0.08^{d}	1.25 ± 0.13^{d}	0.61 ± 0.06^{b}	0.83 ± 0.06^{c}
LZM (μg/mL)	2.31 ± 0.18^{c}	2.05 ± 0.08^{b}	1.47 ± 0.12^{a}	2.32 ± 0.17^{c}	2.35 ± 0.13^{c}	1.84 ± 0.11^{b}	1.96 ± 0.17^{b}

Note: values(means ± SD, n = 3) followed by different letters are significantly different ($P < 0.05$).

showed a significant higher value in the mRNA level of *IL-1β* than the group I ($P < 0.05$). The mRNA level of *IL-8* significantly increased in group III ($P < 0.05$), then significantly decreased in group IV, Vand VII, and group IV,V showed no significant difference with the group I, but group VI and VII still showed a significant higher value in the mRNA level of *IL-8* than the group I ($P < 0.05$). However, the mRNA levels of *IL-10* and *TGF-β* showed the opposite trend. The mRNA level of *IL-10* significantly decreased in group III ($P < 0.05$), then significantly increased in group IV, V and VII ($P < 0.05$), and the three groups showed no significant difference with the group I, but group VI still

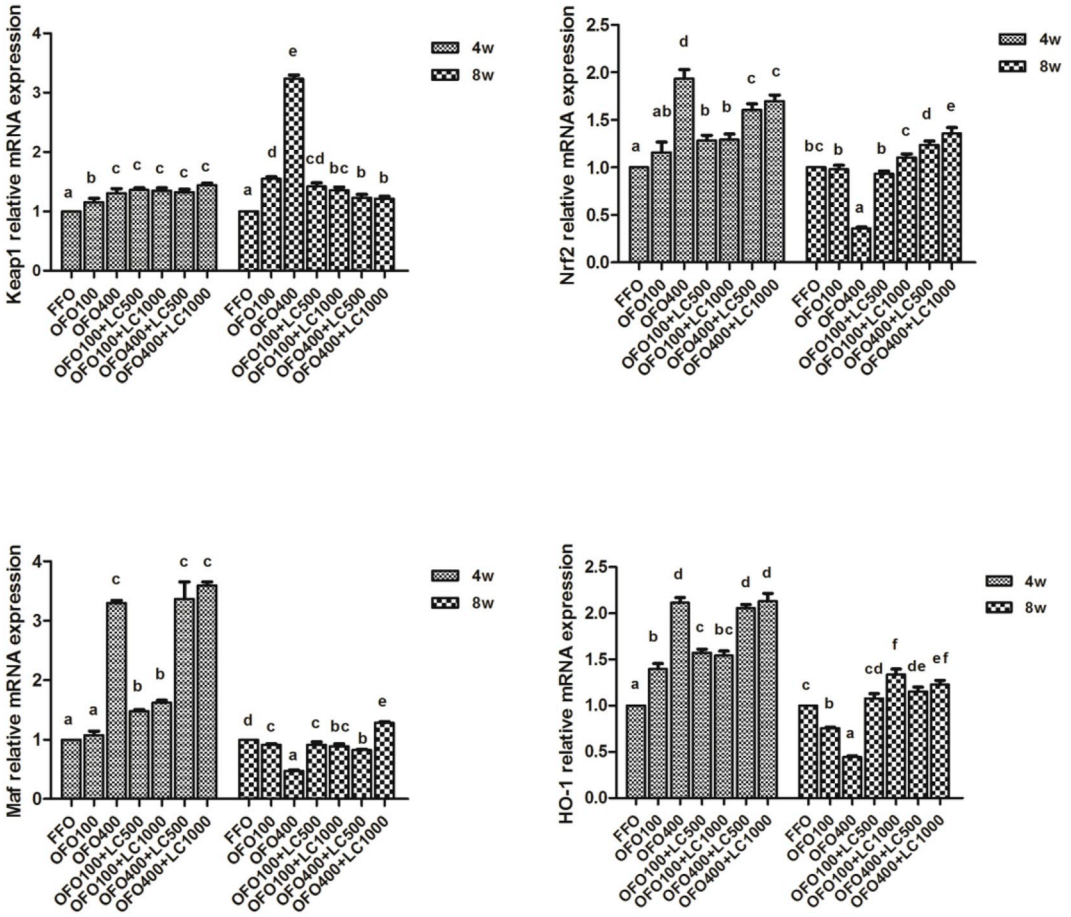

Fig. 6. Effects of oxidized fish oil and LC on the mRNA levels of antioxidant related genes in liver of *Rhynchocypris lagowski* Dybowski. Note: Values are means ± SD (n = 3), values followed by different letters are significantly different ($P < 0.05$).

showed a significant lower value in the mRNA level of *IL-10* than the group I ($P < 0.05$). The mRNA level of *TGF-β* significantly decreased in group IIand III ($P < 0.05$), then significantly increased in group IV, V, VI and VII, but group IV and VI still showed a significant lower value in the mRNA level of *TGF-β* than the group I ($P < 0.05$).

4. Discussion

It is well known that the classical physiological function of L-carnitine is to carry long-chain fatty acids into mitochondria for β-oxidation and ATP production in peripheral tissues [30,31]. However, it also plays an important role in antioxidant. Gülçin et al. [32] reported that L-carnitine could inhibit lipid peroxide by scavenging DPPH, superoxide anion radical, hydrogen peroxide. Furthermore, L-carnitine administration has been reported to protect the endogenous antioxidant defense system by promoting the activities of antioxidant enzymes (SOD, CAT and GSH-PX) and the levels of antioxidant (such as GSH), decreasing the level of MDA, especially in treatment of various kinds of disease [33–37]. In the present study, the same results were observed: L-carnitine supplementation exerts a cytoprotective effect against H_2O_2-induced oxidative stress in the fathead minnowmuscle cell line, which was also confirmed by Wang et al. [20]. This was also in line with the results in SH-SY5Y neuroblastoma cells [33], human hepatocyte HL7702 cell line [38] and human proximal tubule epithelial cell line [39].

So far, the anti-oxidation mechanism of L-carnitine is not clear. Considering the results of previous studies in our laboratory, L-carnitine seemed to be related to the Keap1-Nrf2-ARE signaling pathway [20,21]. Most researchers believe that the mechanism of Nrf2 activation is that after being stimulated by nucleophilic substances or ROS, Nrf2 is unbound to Keap1 and transported into the nucleus, then it binds to Maf protein and then to ARE, which mediates the transcription of II detoxification enzymes (such as HO-1) [40,41]. In the present study, L-carnitine pretreatment was found that it significantly decreased the mRNA level of *Keap1* and increased the mRNA levels of *Nrf2*, *Maf* and *HO-1* in normol FHM cells, even in H_2O_2 treated FHM cells. Similarly, Cao et al. [42] reported that LC treatment was associated with an increased the levels of *Nrf2*, *HO-1* and γ-*GCS* in high glucose stimulated RGCs. It is also supported by the published research reported by Hota et al. [43]. In order to evaluate the relationship between the L-carnitine and *Nrf2*, the *Nrf2*-siRNA was used to knock down the mRNA level of *Nrf2* gene in FHM cells. Qin et al. [44] also using *Nrf2*-siRNA to explore baicalein modulates Nrf2/Keap1 system. Similar results have been found in the investigation of Caffeic acid-induced protection against APAP-induced liver oxidative injury, and the involvement of the Keap1-Nrf2 signaling pathway [45]. In the present study, *Nrf2*-siRNA pretreatment not only decreased the mRNA levels of *Keap1*, *Nrf2*, *Maf* and *HO-1* in the four groups, and also weakened the protective effect of L-carnitine after H_2O_2 treatment: there were no significance in the mRNA levels of *Keap1*, *Nrf2*, *Maf* and *HO-1* between the L-carnitine + H_2O_2 + *Nrf2*-siRNA group and H_2O_2 + *Nrf2*-siRNA group. These results indicated that the antioxidant effect of L-carnitine treatment probably through Nrf2/Keap1 pathway.

To further explore the anti-oxidation role of L-carnitine, the fish oxidative stress model of *Rhynchocypris lagowski* Dybowski caused by oxidized fish oil was set up in the present study. Under the experimental conditions, oxidized fish oil significantly reduced the growth performance, feed efficiency and protein efficiency, indicating that oxidized

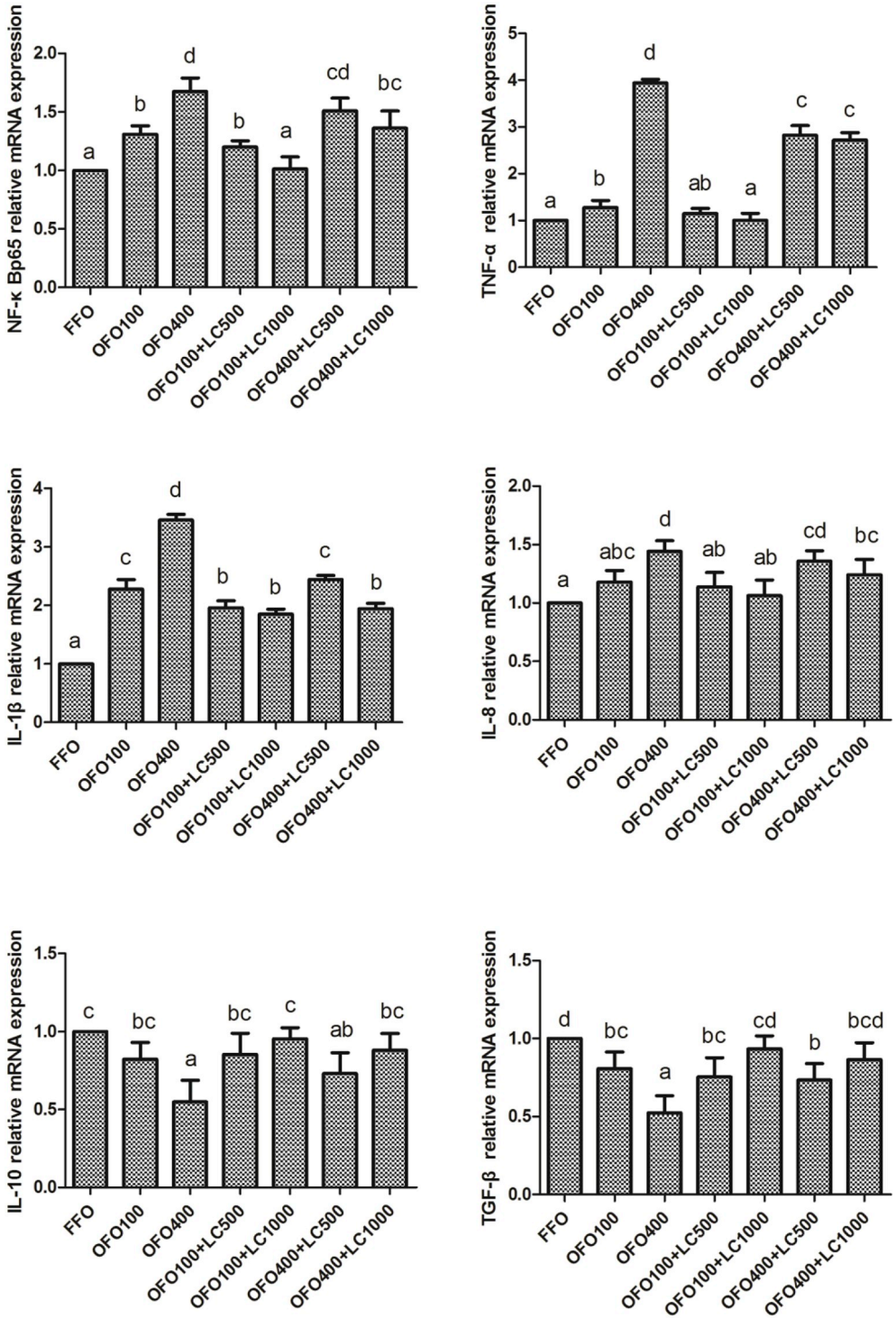

Fig. 7. Effects of oxidized fish oil and LC on the mRNA levels of immune related genes in liver of *Rhynchocypris lagowski* Dybowski. Note: Values are means ± SD (n = 3), values followed by different letters are significantly different ($P < 0.05$).

fish oil could significantly induce oxidative stress, which was clarified by Sutton et al. [46] and Gao et al. [23]. Of course, in line with the damage effects on growth caused by oxidized fish oil, the increasing of the level of MDA, the decreasing of activities of antioxidant enzymes (T-SOD, CAT and GSH-PX) and the level of GSH of *Rhynchocypris lagowski* Dybowskiwere also observed in the present study. This shows that the fish is in a state of oxidative stress, which is consistent with the results of low antioxidant enzyme activity and antioxidant level [22,23,26,47,48]. Moreover, the mRNA level of *Keap1* was significantly increased, while the mRNA levels of *Nrf2*, *Maf* and *HO-1* were significantly decreased after feeding on oxidized fish oil. In zebrafish, Nrf2 and MAPK signaling pathways were also activated by perfluorooctane sulfonic acid (PFOS), and the expression of *Nrf2* and *HO-1* increased significantly [2]. This suggests that oxidized fish oil activates the Keap1-Nrf2-ARE signaling pathway to regulate the body's antioxidant defense system.

Knowing that the fact of oxidative damage caused by oxidized fish oil in *Rhynchocypris lagowski* Dybowski, the nutritional feed additive is

very necessary. Previous studies have reported that L-carnitine could reduce mortality, increase feed efficiency, improve muscle quality in freshwater prawn (*Macrobrachium rosenbergii*) [49], juvenile silver perch (*Bidyanus bidyanus*) [50]. In this study, oxidized fish oil feed supplemented with 500 mg/kg and 1000 mg/kg L-carnitine significantly improved the growth, which reached a maximum weight gain rate and a specific growth rate in the OFO100 + L-carnitine 1000 group. This indicated that L-carnitine could promote growth, even if the subjects were in a state of oxidative stress. Furthermore, L-carnitine significantly increased the levels of antioxidant substances and the activities of antioxidant enzymes and decreased the level of MDA in *Rhynchocypris lagowski* Dybowski stimulated by oxidized fish oil, especially in OFO100 + L-carnitine 1000 group. Results of growth performance and antioxidant parameters suggested that L-carnitine could reduce the oxidative damage caused by oxidized fish oil. In consistence with these results, ALC could increase the endogenous antioxidant defense mechanism in rat and there by protect the animals from radiation-induced organs toxicity [51]. Moreover, Pignatelli et al. [52] demonstrated that L-carnitine could be helpful in modulating oxidative stress and platelet activation during major abdominal surgery-dependent oxidative damage. Although L-carnitine has been previously reported to enhance the antioxidant capacity in human and rat, we mainly focused on the antioxidant function of L-carnitine in fish. Guzmá n-Guillén et al. [18] found that L-carnitine is a chemoprotectantthat reduces hepatic and renal oxidative stress by regulating the activities of antioxidant enzymes (SOD, CAT and γ-GCS) and may be effective when used for the prophylaxis and treatment of CYN-related intoxication in tilapia. This result was also in agreement with the findings in juvenile narrow clawed crayfish (*Astacus leptodactylus leptodactylus* Eschscholtz, 182) [53], common carp [14]. However, further investigations are required to understand the exact mechanisms on how the L-carnitine supplementation cause the antioxidant effect. In accordance with these results *in vitro*, the mRNA levels of *Keap1*, *Nrf2*, *Maf* and *HO-1* in liver of fish were also determined, the mRNA level of *Keap1* was up-regulated and the mRNA levels of *Nrf2*, *Maf* and *HO-1* was significantly decreased by oxidized fish oil, and the mRNA level of *Keap1* was significantly down-regulated and the mRNA levels of *Nrf2*, *Maf* and *HO-1* was up-regulated with L-carnitine supplementation. Therefore, these results suggested that Nrf2/Keap1 played a role in the antioxidative mechanism of L-carnitine in *Rhynchocypris lagowski* Dybowski.

It seems that the immune system is particularly sensitive to the balance of oxidants and antioxidants, several studies have demonstrated that Nrf2 contributes to the anti-inflammatory process by orchestrating the recruitment of inflammatory cells and regulating gene expression through the ARE [54,55]. According to the results of a study conducted by Bellezza et al., increased expression of Nrf2-dependent down-stream HO-1 inhibited NF-κB activity when prostate cancer cells were briefly exposed to α-tochopheryl succinate [56]. Therefore, it is believed that the signaling pathways of Nrf2 and NF-κB interact to control the transcription or function of downstream target proteins. Evidence in literature have reported that the NF-κB signaling pathway is involved in the development of the classical pathway of inflammation, the activation of NF-κB signaling pathway lead to the increased the mRNA levels of *IL-1β*, *TNF-α* and *IL-8* and decreased the mRNA levels of *IL-10* and *TGF-β*, which play a central role in inflammation and wound-healing [57–60]. In this study, the mRNA levels of *NF-κB*, *IL-1β*, *TNF-α* and *IL-8* were increased with the increase of dietary oxidized fish oil levels, while the mRNA levels of *IL-10* and *TGF-β* were decreased. Moreover, the levels of *IL-1β* and *TNF-α* in serum significantly increased, and the activities of LZM and AKP in serum significantly decreased. However, L-carnitine supplementation reversed this trend, which suggested L-carnitine regulated Nrf2/Keap1 activation and protected oxidized fish oil-induced inflammation response by inhibiting the NF-κB signaling pathway in *Rhynchocypris lagowski* Dybowski. Similar results have been found in biochanin A and mangiferin [61,62]. However, more studies should be undertaken in order to gain a better understanding of the mechanism (s) involved in the protective effects of L-carnitine in oxidative stress in fish.

In conclusion, we found that L-carnitine exhibited anti-oxidative effects both *in vitro* and *in vivo*. Thus, L-carnitine may regulated Nrf2/Keap1 activation and protected oxidized fish oil-induced inflammation response by inhibiting the NF-κB signaling pathway in *Rhynchocypris lagowski* Dybowski. However, the underlying molecular mechanism (s) remain to be elucidated.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

The research was supported by National Natural Science Foundation of China (30671621), Industrial Technology Research and Development Project of Jilin provincial Development and Reform Commission (2011002-1), Foreign Trade Development Project of Northeast Old Industrial Base, Jilin Provincial Department of Science and Technology Key Project (20160204019NY), Scientific Research Foundation of Jilin Agricultural University (201704), Key Research & Development of Jilin Science and Technology Department (20180201017NY).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.08.041.

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