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Antioxidative, inflammatory and immune responses in hydrogen peroxide-induced liver injury of tilapia (GIFT, *Oreochromis niloticus*)

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ABSTRACT

Oxidative stress has been implicated in the pathogenesis of many liver diseases in fish, but the molecular mechanism is still obscure. Here, we used hydrogen peroxide (H_2O_2) as a reactive oxygen species (ROS) to induce liver injury and assess underlying molecular mechanism linking oxidative stress and liver injury in fish. Tilapia were injected with various concentrations of H_2O_2 (0, 40, 120, 200, 300 and 400 mM) for 72 h. The blood and liver were collected to assay biochemical parameters and genes expression after 24, 48 and 72 h of injection. The results showed that treatments with higher H_2O_2 levels (300 and/or 400 mM) significantly increased the levels of GPT, GOT, AKP and MDA, and apparently decreased the levels of TP, ALB, SOD, GSH, CAT, GST and T-AOC throughout of the 72 h. The gene expression data showed that treatments with 200, 300 and/or 400 H_2O_2 suppressed Nrf2/keap1 pathway and its downstream genes including *ho-1*, *nqo1* and *gsta*, activated inflammatory response via enhancing the mRNA levels of *nf-κb*, *tnf-α*, *il-1β* and *il-8*, and attenuating *il*-10 mRNA level, and caused immunotoxicity through downregulating the genes expression of *c3*, *hep*, *lzm* and *lgm* for 24, 48 and/or 72 h. Additionally, there was a mild or strong increase in levels of *nrf2* and its subsequent antioxidant genes or enzymes such as *ho-1*, *nqo1*, *gst*, CAT and SOD in treatments with lower concentrations of H_2O_2 (40 or 120 mM) for 24 and/or 48 h. Overall results suggested that H_2O_2 hepatotoxicity was mainly concerned with lipid peroxidation, impairment antioxidant defense systems, inflammatory response and immunotoxicity, and Nrf2/Keap1 and NF-κB signaling pathways played important roles in oxidative stress-induced liver injury in fish.

1. Introduction

Oxidative stress is an inescapable component for aerobic organisms. It results from an imbalance between the production of reactive oxygen species (ROS) and the protective ability of the biological system to cell damage induced by ROS [1,2]. Under physiological condition, ROS is generated during aerobic metabolism and plays a positive role in many biochemical processes as a signaling molecule [3]. But adverse stimulation can cause abnormal antioxidation status of cell and accumulation of ROS, inducing oxidization of lipid, protein and DNA, eventually leading to cell damage or death [4]. In aquaculture activity, oxidative stress is also inevitable for most fish owing to variations of culture environment, such as temperature, salinity, nitrite and stocking density [2,5–7]. A strong oxidative stress has been implicated in the pathogenesis of many liver diseases in fish [8,9]. Moreover, numerous xenobiotics in aquatic environment, like heavy metal, pesticides, ammonia, pathogenic microorganism, etc., have been reported to cause hepatic damage in fish, which was associated with ROS accumulation, lipid peroxidation and inactivation of antioxidant enzymes [2,10–13].

Hydrogen peroxide (H_2O_2), a major ROS, can oxidize many biological molecules to generate the hydroxyl radical (˙OH) that further oxidizes proteins, lipids, carbohydrates, DNA and RNA, and causes significant oxidative damage [1]. Thus, H_2O_2 is extensively used as a model chemical in the study of oxidative stress or oxidative damage *in vivo* and *in vitro* [14,15]. In mammals, hepatocyte or liver damage induced by H_2O_2 is the best-characterized system of oxidative stress-induced hepatotoxicity, which is widely used to screen hepatoprotective and antioxidative agents [16,17]. In aquaculture, H_2O_2, as a

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Abbreviations

ROS	reactive oxygen species
Nrf2	nuclear erythroid 2-related factor 2
Keap1	Kelch-like ECH-associated protein-1
HO-1	heme oxygenase 1
NQO1	NAD(P) H:quinone oxidoreductase 1
GST	glutathione S-transferase
NF-κB	nuclear factor κB
GPT	glutamate pyruvate transaminase
GOT	glutamate oxalate transaminase
AKP	alkaline phosphatase
TP	total protein
Alb	albumin
SOD	superoxide dismutase
CAT	catalase
T-AOC	total antioxidant capacity
GSH	glutathione
GST	glutathione S-transferase
MDA	malondialdehyde
IL-1β	Interleukin 1β
TNF-α	tumor necrosis factor α
HEP	hepcidin
IgM	immunoglobulin M
C3	complement 3
LZM	lysozyme
HSP70	heat shock protein 70
CYP1A	cytochrome P450 1A

disinfectant, is commonly used due to its relatively lower toxicity and environmental impact [18]. Temporary or low level exposure to H_2O_2 can effectively control external bacteria and parasites in fish [19,20]. Moreover, in aquatic environment, H_2O_2 is an ecological factor, which is produced from UV-driven photoactivation of dissolved organic material [21,22] and may accumulate to micromolar concentration [23]. Ambient H_2O_2 can easily pass through cell membranes into cells by diffusion and cause oxidative stress [24,25]. Different studies have focused on H_2O_2 toxic effects in fish. For example, H_2O_2 exposure changed activity of antioxidative enzymes, enhanced lipid peroxidation and caused immunosuppression in gills of *Paralichthys olivaeceus, Oncorhynchus mykiss* and *Rhabdosarga sarba* [26–28]. Likewise, *in vitro* study showed that H_2O_2 treatment induced oxidative damage in intestinal epithelial cells of *Cyprinus carpio* [29]. Additionally, other physiological stress responses, such as plasma cortisol, lactate, glucose and ion concentration, were observed in *Dicentrarchus labrax* and *Salmo salar* under H_2O_2 exposure [30,31]. However, there is very limited information on H_2O_2-induced liver injury in fish, and the underlying molecular mechanism remains unclear.

Following a number of studies, it was shown that nuclear erythroid 2-related factor 2 (Nrf2) is a crucial regulator of antioxidative defense mechanism, and is involved in organism protection against oxidative stress-induced diseases [32,33]. Normally, Nrf2 activity is depressed by binding to Kelch-like ECH-associated protein-1 (Keap1) in the cytoplasm [34]. But upon stimulation, the Nrf2-Keap1 complex is disaggregated and Nrf2 is translocated into the nucleus, where it binds to the antioxidant response element (ARE) to regulate production of downstream antioxidant enzymes, such as heme oxygenase 1 (HO-1), NAD(P) H:quinone oxidoreductase 1 (NQO-1), glutathione S-transferase (GST), etc. [34,35]. The role of Nrf2/Keap1 signaling pathway in oxidative stress-induced cell and tissue damage has been found in mammals [35,36]. Also, the relationship between H_2O_2 toxicity and Nrf2/Keap1 signaling pathway has been elucidated in human and rat liver or hepatocyte [37,38]. Nonetheless, little is known about the involvement of Nrf2/Keap1 pathway in H_2O_2-induced liver injury of fish.

Tilapia (*Oreochromis niloticus*) is one of the major commercially cultured fish and widely cultured in China. In the intensive aquatic environment, several stressors, such as high stocking density, unfavorable temperature, excessive ammonia, etc., can cause oxidative stress in tilapia, which led to liver cell membrane damage and liver dysfunction [39–43]. However, the molecular mechanism of oxidative stress-induced liver damage is not elucidated yet in fish. Therefore, the present study aimed to explore underlying molecular mechanism linking oxidative stress and liver injury in fish, and further investigate the roles of Nrf2 and nuclear factor κB (NF-κB) signaling pathways in fish liver injury.

2. Materials and methods

2.1. Fish and experimental design

Juvenile tilapias (initial weight 107 ± 3.2 g) were obtained from the farm in Freshwater Fish Research Center of Chinese Academy of Fishery Sciences (Wuxi, China) and hold in a recirculation system (temperature 30 ± 2 °C; dissolved oxygen > 6 mg/L; pH 7.4–8.1) for 2 weeks. During the acclimation period, the fish were fed with a commercial diet containing 28% crude protein, 5% crude lipid, 15% crude ash, 12% crude fiber, 0.6% P and 12.5% water (Tongwei Co., LTD., Chengdu, China) twice per day.

The fish were assigned at random to six groups and injected intraperitoneally (i.p.) with six H_2O_2 (Keygen Biotech Co., Ltd., Nanjing, China) concentrations of 0 (control), 40, 120, 200, 300 and 400 mM, respectively. Each treatment contained ninety fish and was tested in triplicate. After injection for 24, 48 and 72 h, fifteen fish were randomly collected from each treatment (per time point) and immediately anesthetized in tricaine methane sulfonate (100 mg/L, Sigma, MO, USA). The blood was collected to separate serum by centrifugation (5000 rpm, 4 °C and 10 min). And then, the liver of the sampled fish was rapidly taken. All samples were stored at −80 °C until use. The experiment was performed taking into consideration the welfare of animal, and the use of fish was approved by the Institutional Animal Care and Use Committee (IACUC) of Chinese Academy of Fishery Science.

2.2. Liver injury parameters analysis

To assess hepatotoxicity, the levels of glutamate pyruvate transaminase (GPT), glutamate oxalate transaminase (GOT), alkaline phosphatase(AKP), total protein (TP) and albumin (ALB) were measured in serum using commercially available kits (Jiancheng Institute of Biotechnology, Nanjing, China) [9,44].

2.3. Oxidative stress parameters analysis

Oxidative stress parameters including superoxide dismutase (SOD), catalase (CAT), total antioxidant capacity (T-AOC), glutathione (GSH), glutathione S-transferase (GST) and malondialdehyde (MDA) were measured in serum and liver. SOD activity was determined by the SOD assay kit (Jiancheng Institute of Biotechnology) according to the WST-1 method described by He et al. [45]. CAT activity was assayed by detecting the decomposition of H_2O_2 as described previously [46]. The level of T-AOC was tested by a commercial kit (Jiancheng Institute of Biotechnology) with the ABTS method developed by Re et al. [47]. GSH content was estimated by monitoring 2-Nitro-5-thiobenzoic acid (TNB) formation using enzymatic recycling method [48]. The GST activity was measured according to the method of Habig et al. [49], using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate (Jiancheng Institute of

Biotechnology). Formation MDA was detected using the thiobarbituric acid-reactive substances (Jiancheng Institute of Biotechnology) assay [50]. Protein level of the liver tissue was measured via the method reported by Bradford [51].

2.4. Quantitative real-time PCR analysis

Total RNA was isolated from tilapia liver with RNAiso Plus (TaKaRa Bio, Inc., Dalian, China) in accordance with the manufacture's protocol. The quality and concentration of the isolated RNA were estimated by measuring the absorbance at 260 and 280 nm on a spectrophotometer (Eppendorf, Inc., Hamburg, Germany) and calculating the A260/A280 ratio. Purified RNA (1 μg for each sample) was used to synthesize cDNA by PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa) depending on the manufacturer's instruction.

The relative expression of gene was quantified by quantitative real-time PCR (qPCR) using a qPCR kit (TB Green™ Premix Ex Taq™ II, TaKaRa), which was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules CA, USA). In brief, each qPCR reaction contained 2 μL cDNA, 12.5 μL TB Green Premix Ex Taq II, 1 μL forward and reverse specific primers, and 8.5 μL RNase-free water. The reaction condition was as follows: pre-denaturation at 95 °C for 30 s, then 40 cycles of 95 °C for 5 s and 57–61 °C for 1 min. β-actin was used as reference gene to normalize the Cq value and the relative mRNA level was calculated by the $2^{-\triangle\triangle Cq}$ method [52]. Specific tilapia primers in this study were shown in Table 1.

2.5. Statistical analysis

The data was expressed as mean ± standard deviation (SD) and all statistical analyses were performed using SPSS version 18.0 software. Isolated and interactive effects of H_2O_2 concentration and injection time were assessed by using two-way ANOVA. When significant effects were obtained for a factor, Tukey's multiple range tests were used to determine the differences between means. A significant level of $P < 0.05$ was used in all the analyses. All data from the experiment were subjected to principal component analysis (PCA) to detect any intercorrelation between experimental variables. The standardized scores of the first two components which explained the highest variation were used to make biplots.

3. Results

3.1. Changes of hepatic damage parameters

The hepatic damage was evaluated by determining the levels of GPT, GOT, AKP, TP and ALB in serum, and the results were shown in Fig. 1. The activities of GPT, GOT and AKP showed higher values in tilapia treated with 300 and 400 mM H_2O_2 for 24 h and thereafter compared with their control groups ($P < 0.05$), similar higher

Table 1
The primers sequences used in the present study.

Type	Gene	Primer sequence (5′-3′)	GenBank number	References
Oxidative stress	*nrf2*	F: CTGCCGTAAACGCAAGATGG R: ATCCGTTGACTGCTGAAGGG	XM_003447296.4	
	keap1	F: CTTCGCCATCATGAACGAGC R: CACCAACTCCATACCGCACT	XM_003447926.3	
	nqo1	F: TGGATTTCAGGTTCTGGCTCC R: TCCTGTGGAGATGCCGAGA	XM_019361560.1	
	gsta	F: TAATGGGAGAGGGAAGATGG R: CTCTGCGATGTAATTCAGGA	NM_001279635.1	[115]
	ho-1	F: CTTGCCCGTGTGGAATCACT R: AGATCACCGAGGTAGCGAGT	XM_013270165.2	
NF-κB	*nf-κb2*	F: GAACATCAGACCGACGACCA R: TCTCCGCCAGTTTCTTCCA	XM_003457469.4	
	rel-b	F: TCACTGCCTCCACCTTTGCT R: ATCCTCATAGTTCCTCTTCCGTTTT	XM_005459330.3	
	p65	F; CAGATGAATACAGGCTGAGTGAGAA R: AGGTGCTGTCTATCTTGTGGAGTG	XM_005463161.3	
	nf-κb1	F: GCAGAAGGAGGCAGTGGAAG R: GACCTGCTGTGTTGGTTTGGT	XM_019363515.1	
	rel	F: GGTCAACAGAAATAGCGGAAGTG R: CCCAGCCATCAGGAGAGAAG	XM_019366581.1	
Inflammation	*tnf-α*	F: AAGCCAAGGCAGCCATCCAT R: TTGACCATTCCTCCACTCCAGA	NM_001279533.1	[116]
	il-1β	F: TCAGTTCACCAGCAGGGATG R: GACAGATAGAGGTTTGTGCC	XM_019365842.1	[117]
	il-8	F: CTGTGAAGGCATGGGTGTGGAG R: TCGCAGTGGGAGTTGGGAAGAA	NM_001279704.1	[116]
	il-10	F: CAGCAGCAGGAGCATCAGCATT R: CACAGGAGGACGGTCTGAGAAGT	KP645180.1	[116]
Detoxification	*hsp70*	F: ATTTCAGACGGAGGGAAGCC R: CAGCGTTGGACACCTTTTGG	XM_019357557.1	
	cyp1a	F: CGTCGTCGTCTCTGTTGCC R: CATCGTCGTGGTGGTCATAGC	NM_001279489.1	[116]
Immune	*lzm*	F: AAGGGAAGCAGCAGCAGTTGTG R: CGTCCATGCCGTTAGCCTTGAG	XM_003460550.2	[118]
	hep	F: GACACAAGCGTGGCATCAAG R: GTTGAGGCAGTAACTGAGGACA	XM_019365122.1	[116]
	Igm	F: ACCGAATCGAAAAATGCGGC R: AACACAACCAGGACATTGGTTC	KJ676389.1	
	c3	F: GGTGTGGATGCACCTGAGAA R: GGGAAATCGGTACTTGGCCT	XM_013274267.2	
Internal reference	*β-actin*	F: CCTGAGCGTAAATACTCCGTCTG R: AAGCACTTGCGGTGGACGAT	KJ126772.1	[119]

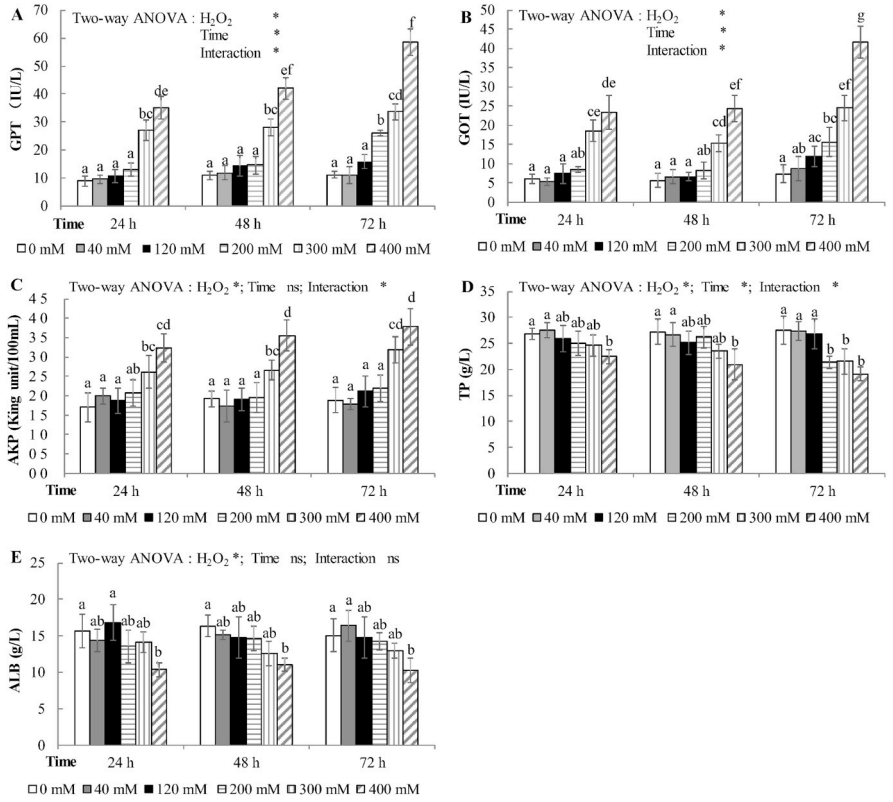

Fig. 1. Effects of different H_2O_2 concentrations on hepatic damage in tilapia. The values are means ± standard deviation (SD), n = 15. Data with different letters are significantly different ($P < 0.05$) among treatments. ns, not significant; *$P < 0.05$. The treatment with 0 mM H_2O_2 is control.

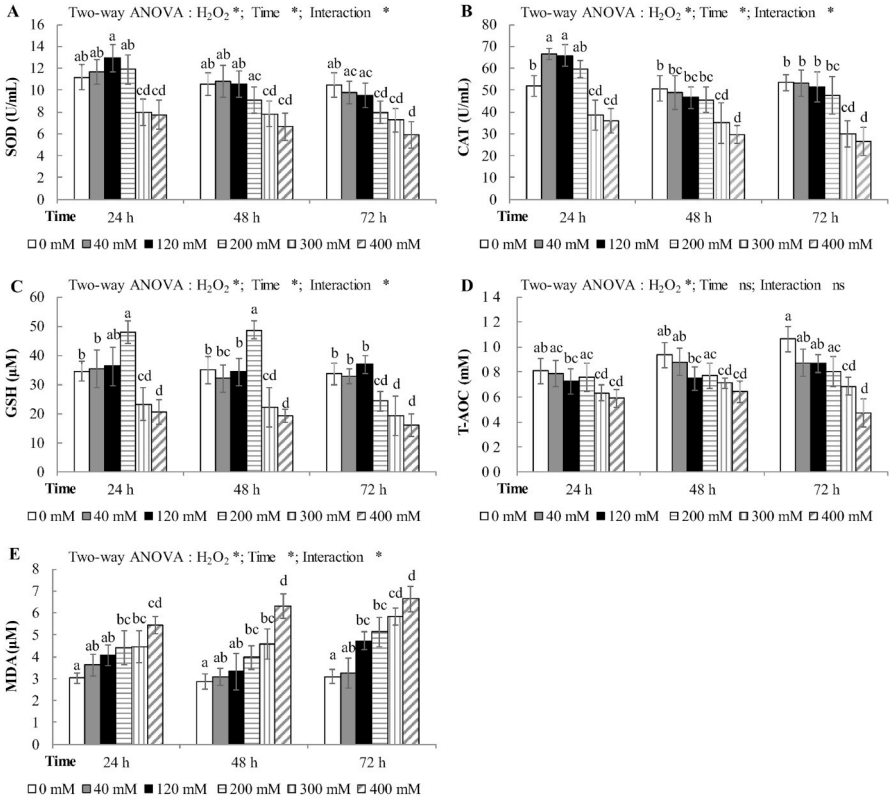

Fig. 2. Effects of different H_2O_2 concentrations on antioxidation status in the serum of tilapia. The values are means ± standard deviation (SD), n = 15. Data with different letters are significantly different ($P < 0.05$) among treatments. ns, not significant; *$P < 0.05$. The treatment with 0 mM H_2O_2 is control.

activities of GPT and GOT were also found in treatments with 200 mM H_2O_2 for 72 h. Inversely, the TP and ALB contents significantly decreased in fish treated with 400 mM H_2O_2 after 24 h injection ($P < 0.05$). Also, the decrease of TP content was visible in fish injected with 200 and 300 mM H_2O_2 for 72 h ($P < 0.05$).

3.2. Changes of antioxidation status in serum and liver

In serum, the antioxidant capacity and lipid peroxidation were clearly influenced by H_2O_2 concentrations and injection time ($P < 0.05$; Fig. 2). The levels of SOD, CAT, GSH and T-AOC showed a clear decline in fish treated with 300 and 400 mM H_2O_2 from 24 h onwards compared with their control groups ($P < 0.05$). Similar decreases of SOD and GSH levels were observed in treatments with 200 mM H_2O_2 for 72 h ($P < 0.05$). Moreover, there was a significant increase in CAT activity of treatments with 40 and 120 mM H_2O_2 for 24 h and in GSH content of treatments with 200 mM H_2O_2 for 24 and 48 h ($P < 0.05$). Also, an uptrend of SOD activity, but not significant, was seen in fish injected with 40 and 120 mM H_2O_2 for 24 h. The MDA level showed an uptrend with increasing H_2O_2 concentrations at 24, 48 and 72 h, and a marked enhancement was seen when the H_2O_2 concentration was from 200 to 400 mM ($P < 0.05$).

Likewise, the liver antioxidation status was differently changed by H_2O_2 treatment and injection time in individual as well as in interaction (Fig. 3). At and after 24 h of injection, treatments with 300 and 400 mM H_2O_2 apparently suppressed the antioxidative parameters including SOD, CAT, GSH, T-AOC and GST compared to their control groups ($P < 0.05$). When fish was injected with 200 mM H_2O_2 for 48 and/or 72 h, the levels of CAT, GSH, T-AOC and GST were also suppressed ($P < 0.05$). By contrast, a remarkable augmentation was noted in CAT activity of fish treated with 200 mM H_2O_2 for 24 and in GSH content of fish treated with 120 mM H_2O_2 for 24 and 40 mM H_2O_2 for 48 h ($P < 0.05$). The hepatic MDA level in tilapia subjected to 200, 300 and 400 mM H_2O_2 strongly increased at 24 h and thereafter ($P < 0.05$). In addition, administrations of H_2O_2 resulted in a non-significant evaluation in SOD activity from 0 mM to 200 mM at 24 h (Fig. 3A).

3.3. Expression of genes related to Nrf2/Keap1 signaling pathway

In order to further investigate the underlying mechanism of H_2O_2 on antioxidative effect, the mRNA levels of *nrf2, keap1, nqo1, ho-1* and *gsta* were measured by qPCR (Fig. 4). At 24 and 48 h of injection, the mRNA level of *nrf2* notably increased in treatments with 40 and 120 mM H_2O_2, and then sharply declined in treatments with 300 and 400 mM H_2O_2 ($P < 0.05$). At 72 h, the *nrf2* mRNA level displayed a non-liner fall with the H_2O_2 levels, and the lower values were recorded in fish challenged by 200–400 mM H_2O_2 compared with control fish ($P < 0.05$). The mRNA level of *keap1* visibly upregulated in fish treated with 300 and 400 mM H_2O_2 from 24 h to the end of the experiment ($P < 0.05$). Conversely, H_2O_2 treatments with 200–400 mM caused a drastic downregulation in *nqo1* gene expression throughout the injection period ($P < 0.05$), but a slight upregulation of the gene was observed in low level of H_2O_2 treatment (40 mM) at 24 and 72 h ($P > 0.05$). The *gsta* gene expression linearly increased relative to H_2O_2 levels (0–120 mM) after 24 and 48 h of injection, but such increase markedly decreased in fish subjected to higher levels of H_2O_2 (300 and 400 mM) ($P < 0.05$). The same decrease of *gsta* mRNA level was also detected in treatments with 200–400 mM H_2O_2 for 72 h ($P < 0.05$). Compared with the control, treatments with 120 mM H_2O_2 for 24 h or 40 mM H_2O_2 for 48 h caused marked upregulation of *ho-1* gene, but an opposite expression was found when fish were treated with 300 and 400 mM H_2O_2 for 24 and 48 h or 200–400 mM H_2O_2 for 72 h ($P < 0.05$).

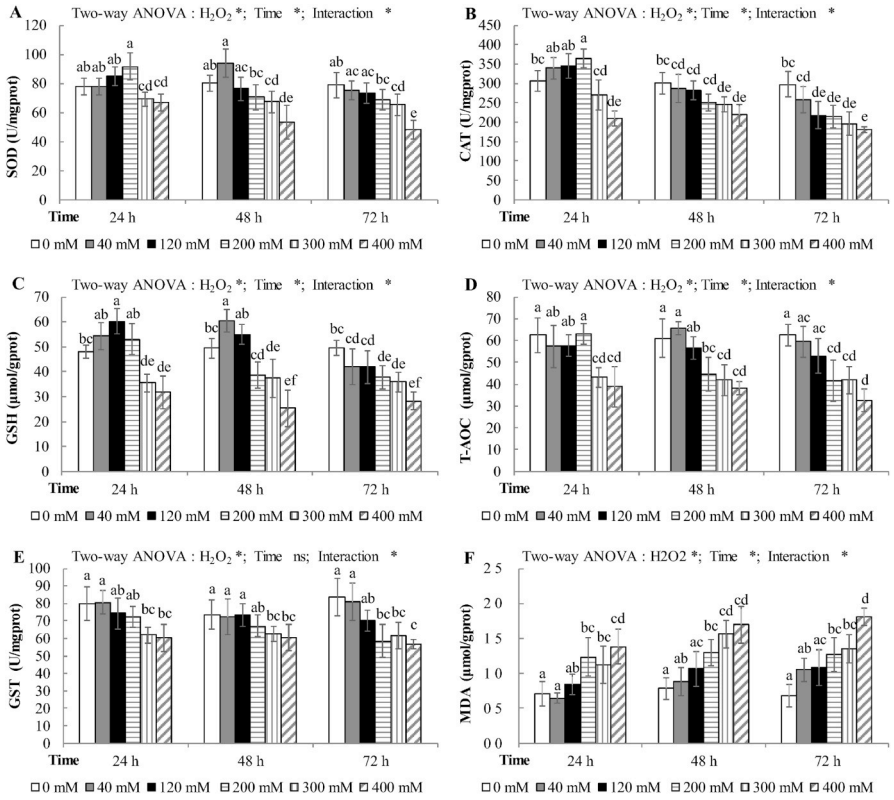

Fig. 3. Effects of different H_2O_2 concentrations on antioxidation status in the liver of tilapia. The values are means ± standard deviation (SD), n = 15. Data with different letters are significantly different ($P < 0.05$) among treatments. ns, not significant; *$P < 0.05$. The treatment with 0 mM H_2O_2 is control.

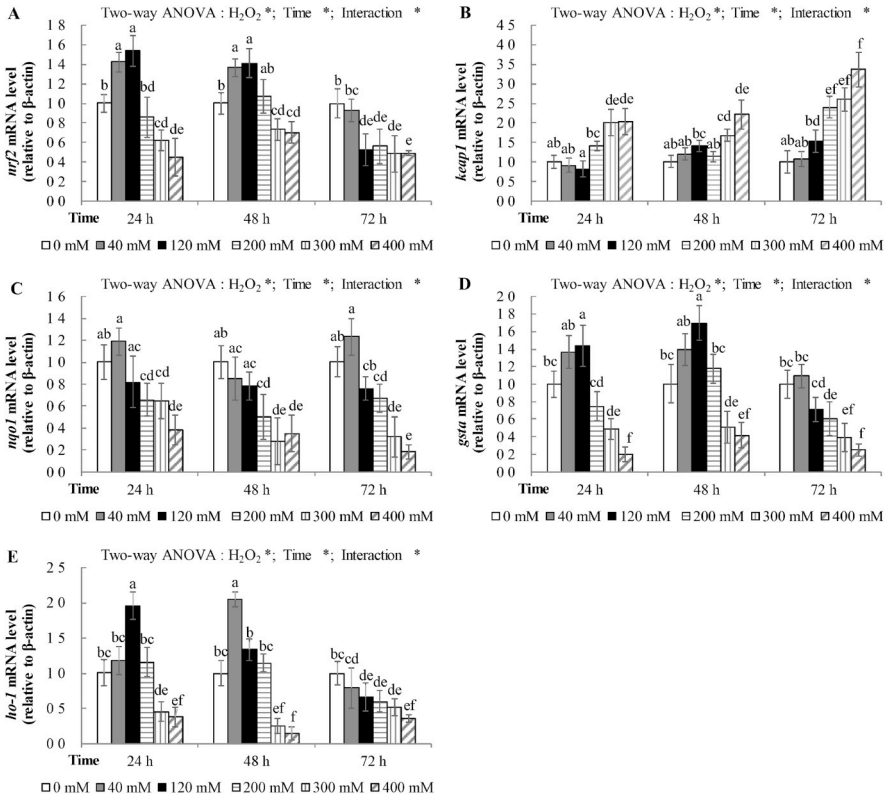

Fig. 4. Effects of different H_2O_2 concentrations on the antioxidant relevant pathway in the liver of tilapia. The values are normalized to control values and expressed as means ± standard deviation (SD), n = 15. Data with different letters are significantly different ($P < 0.05$) among treatments. *$P < 0.05$. The treatment with 0 mM H_2O_2 is control.

3.4. Expression of nf-κb genes

Nuclear factor κB (NF-κB) is a key transcription factor in inflammatory and immune response, and is made up of five proteins: Rel, P65, Rel-B, NF-κB1 and NF-κB2. QPCR analysis showed that the gene expression of the *nf-κb* subunits was differently upregulated by H_2O_2 challenge (Fig. 5). The transcript level of *rel* evidently increased in fish at concentration of 300 and 400 mM H_2O_2 for 24 and 72 h, or fish at concentrations of 400 mM H_2O_2 for 48 h ($P < 0.05$). The *p65* gene expression tended to be statistically higher in H_2O_2-treated fish (200–400 mM) throughout of the 72 h, except fish at 200 mM for 24 h, than corresponding controls ($P < 0.05$). The mRNA levels of *rel-b* and

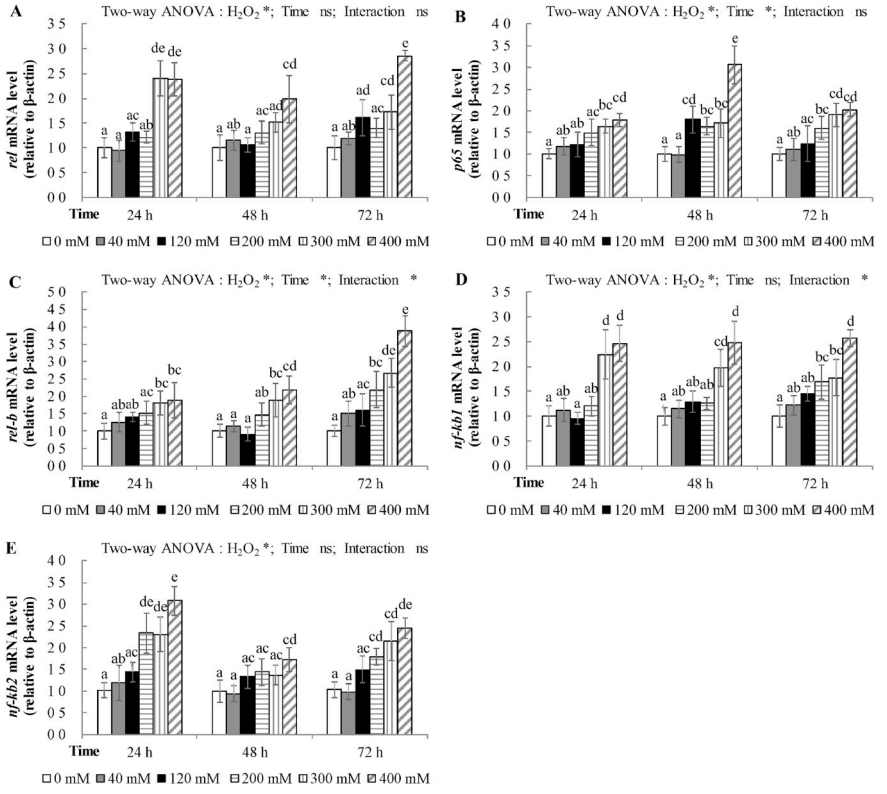

Fig. 5. Effects of different H_2O_2 concentrations on the *nf-κb* levels in the liver of tilapia. The values are normalized to control values and expressed as means ± standard deviation (SD), n = 15. Data with different letters are significantly different ($P < 0.05$) among treatments. ns, not significant; *$P < 0.05$. The treatment with 0 mM H_2O_2 is control.

nf-κb1 exhibited a semblable upregulation tendency in response to the increased levels of H_2O_2, and the values were significantly different from their controls when fish were treated with H_2O_2 at 200 mM or higher concentrations during the whole experimental period ($P < 0.05$). In like manner, a pronounced upregulation in *nf-κb2* gene expression was seen in treatments with 200–400 mM H_2O_2 for 24 and 72 h, or 400 mM H_2O_2 for 48 h ($P < 0.05$).

3.5. Expression of genes related to inflammation factors

Higher inflammation as a secondary response frequently occurred when cells undergo injury; therefore, some inflammatory cytokines were measured in liver (Fig. 6). Results revealed that treatments with higher levels of H_2O_2 (300 and 400 mM) resulted in considerable upregulation of *il-1β*, *tnf-α* and *il*-8 mRNA levels, and downregulation of *il*-10 mRNA level throughout the 72 h ($P < 0.05$). Also, treatment with 200 mM H_2O_2 evidently upregulated *il-1β* mRNA level at 24 h, and *tnf-α* and *il*-8 mRNA levels at 24, 48 and 72 h, but downregulated *il*-10 mRNA level at 48 and 72 h ($P < 0.05$).

3.6. Expression of genes related to immunity

The expression of genes involved in the immune responses in the liver of tilapia were shown in Fig. 7. The mRNA levels of *hepcidin* (*hep*) and *immunoglobulin M* (*Igm*) revealed obvious decrease in fish at concentrations of 200–400 mM H_2O_2 from 24 h onwards compared to fish at control ($P < 0.05$). Similar decreases of the two genes were evident in fish at concentration of 120 mM H_2O_2 for 72 h ($P < 0.05$). H_2O_2 treatments with 200 and higher levels apparently suppressed the *complement 3* (*c3*) and *lysozyme* (*lzm*) mRNA levels at 24 h and thereafter ($P < 0.05$). Meanwhile, the same changes in treatment with 120 mM H_2O_2 were observed in *lzm* mRNA level at 24 and 72 h, and in *c3* mRNA level at 24 h ($P < 0.05$).

3.7. Expression of hsp70 and cyp1a genes

H_2O_2 treatment resulted in an uptrend in *heat shock protein 70* (*hsp70*) gene expression at 24 h, and a downtrend at 48 and 72 h, excluding treatment with 40 mM H_2O_2 for 72 h, such increase or decrease was significantly different from its control when fish were subjected to 200–400 mM H_2O_2 ($P < 0.05$; Fig. 8A). The *cytochrome p450 1a* (*cyp1a*) mRNA level showed a linear decrease after H_2O_2 injection at 24, 48 and 72 h, respectively, but a mild increase in treatment with 40 mM H_2O_2 for 48 h. In the meanwhile, the decrease became prominent in treatments with 200–400 mM H_2O_2 from 24 h to the end of the experiment ($P < 0.05$; Fig. 8B).

3.8. Principal component analysis (PCA)

The PCA biplot depicted a clear separation of experimental groups representing overall data, and the first two components accounted for 81.44% of the variability of the data (Fig. 9). The first component (PC1, 73.4%) clustered most of the parameters in liver injury, oxidative stress, inflammation and immune, and the second component (PC2, 8.04%) was associated with the variables of *hsp70* and *cyp1a*. The results achieved showed the relationship among all biomarkers tested: (a) liver injury was positively correlated with inflammation and lipid peroxidation, and negatively correlated with antioxidation and immune indices; (b) the change of NF-κB pathway was negatively correlated with Nrf2 pathway and immune response.

4. Discussion

The liver is the main organ for material and energy metabolism in fish, and its detoxification and phagocytosis have a protective effect on the organisms. Liver damage or dysfunction of fish is one of the most serious problems in aquaculture and often leads to metabolic disorders, low disease resistance and even death [53]. Oxidative stress has been increasingly recognized as a contributing factor in the pathophysiology of acute liver injury [2]. Thus H_2O_2, as an oxidizing agent, was used to induce oxidative stress and reveal the molecular mechanism of fish liver injury in the present study.

H_2O_2 is readily metabolized to hydroxyl radicals by Fenton-like reaction [1], which causes lipid peroxidation and protein oxidation on the cell membrane, impairs cell membrane structure and function, results in the leakage of cytosolic enzymes (e.g., GPT, GOT and AKP) [16]. Indeed, in our study, the significant elevation of GPT, GOT and AKP activities in serum was seen in fish treated with higher H_2O_2 levels (300 and 400 mM), which demonstrated the occurrence of liver injury in tilapia. This phenomenon was supported by earlier findings in H_2O_2-treated hepatocyte and liver of mice or rat [16,54,55]. Another explanation for the phenomenon considered that the elevation of these enzymes might be ascribed to the corrosive damage of H_2O_2 on liver cell membrane leading to discharge of the intracellular enzymes into blood [55]. Moreover, protein synthesis, as a major function of liver, was seriously influenced when liver was damaged by oxidative stress or other xenobiotics [56,57]. The reduction of TP and ALB levels in tilapia at higher concentrations of H_2O_2 (300 and/or 400 mM) suggested that protein synthesis was inhibited in fish liver injury, which might be due to fragmentation of endoplasmic reticulum and disruption of ribosomes

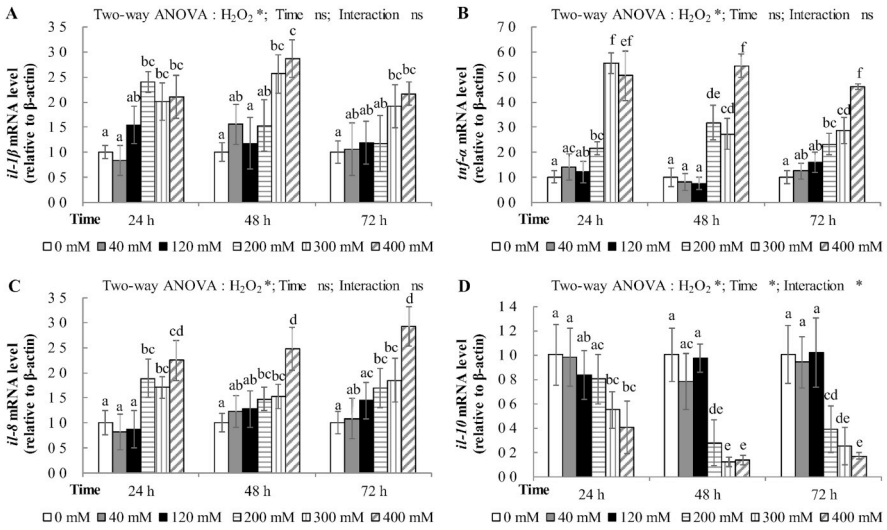

Fig. 6. Effects of different H_2O_2 concentrations on the inflammatory cytokines levels in the liver of tilapia. The values are normalized to control values and expressed as means ± standard deviation (SD), n = 15. Data with different letters are significantly different ($P < 0.05$) among treatments. ns, not significant; *$P < 0.05$. The treatment with 0 mM H_2O_2 is control.

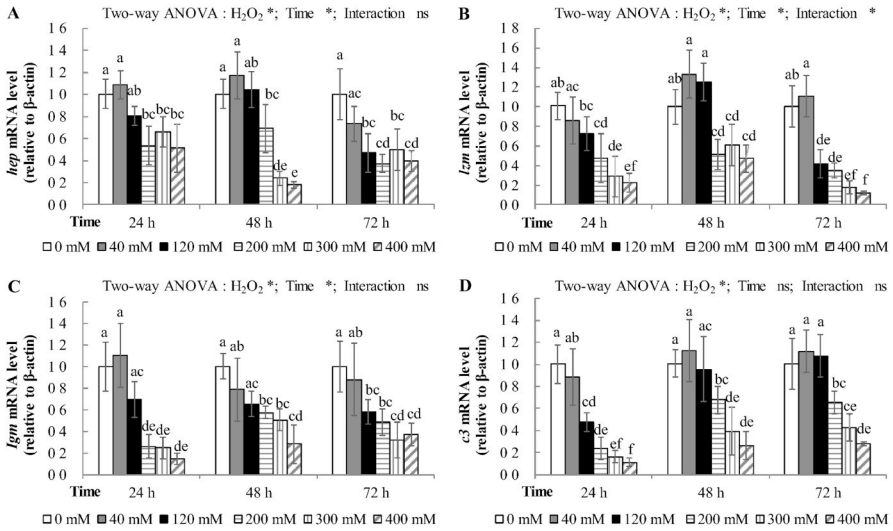

Fig. 7. Effects of different H_2O_2 concentrations on expression of immune-related genes in the liver of tilapia. The values are normalized to control values and expressed as means ± standard deviation (SD), n = 15. Data with different letters are significantly different ($P < 0.05$) among treatments. ns, not significant; *$P < 0.05$. The treatment with 0 mM H_2O_2 is control.

under oxidative stress [58,59].

Generally, antioxidative defense system dysfunction is a major reason in H_2O_2-induced liver injury. In biological systems, H_2O_2 and its metabolites can readily oxidize proteins, lipids, carbohydrates, DNA and RNA, which will lead to a disruption of redox signaling and significant oxidative damage, when there is an imbalance between oxidants and antioxidants [60]. It has been reported that overwhelming ROS including H_2O_2 and $^{\cdot}HO$ depleted the antioxidant enzymes (e.g., SOD and CAT) and non-enzymatic antioxidant components (e.g., GSH), which was likely to further result in cell death and tissue injury [8,24,61]. In the present study, treatments with 300 and 400 mM H_2O_2 remarkably depressed the levels of SOD, CAT, GSH, T-AOC and GST in serum and/or liver throughout of 72 h. This observation indicated that antioxidative defense systems of tilapia liver were impaired and the antioxidant capacity declined under H_2O_2-induced liver injury. In line with our results, H_2O_2 treatment resulted in a significant disruption in the oxidative balance by inhibiting SOD, GSH and CAT levels in rat liver and HepG2 [55,62]. Meanwhile, it was worthy to note that the antioxidant components such as SOD, CAT or GSH showed slight or obvious increase when fish were treated with lower H_2O_2 levels (40 or 120 mM) at 24 h or 48 h. Such increase might imply an adaptive response to scavenge the generated ROS [63].

Lipid peroxidation is also considered as a vital sign of H_2O_2 deleterious effect, which is often assessed by MDA formation [64]. Excess of un-eliminated H_2O_2 and $^{\cdot}HO$ can initiate lipid peroxidation via reacting with unsaturated fatty acids in cell membranes, which impairs membrane function and structural integrity [65]. This perspective was wildly accepted in liver cells of human and rats [16,62,66], and also conformed in our work where the MDA level clearly increased in serum and liver of tilapia subjected to 200–400 mM H_2O_2 at 24 h and thereafter.

Emerging evidence has revealed that the Nrf2/Keap1 signaling pathway regulated the antioxidative defense systems and several oxidant signaling proteins to impact oxidant homeostasis and a number of programmed cellular functions [67]. As follows, several antioxidants or hepatoprotective agents protected hepatocyte or liver from oxidative damage by augmenting Nrf2 signaling pathway [68,69], while silence or knockout of Nrf2 substantially increased the susceptibility to a broad range of chemical toxicity associated with oxidative pathology in mice [70,71]. Activation of Nrf2 and its cytoprotective response have been intensively investigated in a variety of settings under oxidative stress (Reviewed by Ma et al. [72]). Existed research has found evidence that H_2O_2 treatment resulted in upregulation of Nrf2 protein expression in intestinal epithelial cell, piglets jejunum, PC12 and astrocytes [73–76]. However, a conflicting viewpoint considered that Nrf2 was activated as a typical adaptive response only during mild to moderate oxidative stress, but it was suppressed under severe oxidative stress [77]. Such suppression of Nrf2 was observed in H_2O_2-treated rat liver, SAECs (small airway epithelial cells), L02, HepG2, zebrafish and SH-SY5Y cell [55,69,77,78], appearing to increase ROS production and DNA damage [61]. Interestingly, this viewpoint was supported by our data showing that the mRNA level of *nrf2* apparently upregulated in treatment with lower H_2O_2 levels (40 or 120 mM), but obviously downregulated in treatment with higher H_2O_2 levels (300 and 400 mM). The downregulation possibly imputed to dysfunction of Nrf2 system induced by severe and/or chronic oxidative stress [77,79]. In addition, some researchers discovered that Keap1, as a Nrf2 repressor, increased during H_2O_2-induced oxidative stress, thus leading to inactivation of Nrf2 via forming Keap1-Nrf2 complex [69,80], which was confirmed by upregulation of *keap1* mRNA level after H_2O_2 injection in our study.

It is well known that Nrf2/Keap1 pathway mediates phase II detoxifying/antioxidant enzymes such as HO-1, NQO1 and GST to prevent oxidative damage [81]. Corresponding to Nrf2 change, the mRNA levels of *ho-1*, *nqo1* and *gsta* showed mild or strong elevation in fish treated with lower doses of H_2O_2 (40 or 120 mM) at 24 h or 48 h, but prominent reduction in fish treated with higher doses of H_2O_2 (300 and 400 mM) throughout the experiment. These findings were also consistent with the SOD, CAT and GSH in serum and liver under same condition. The

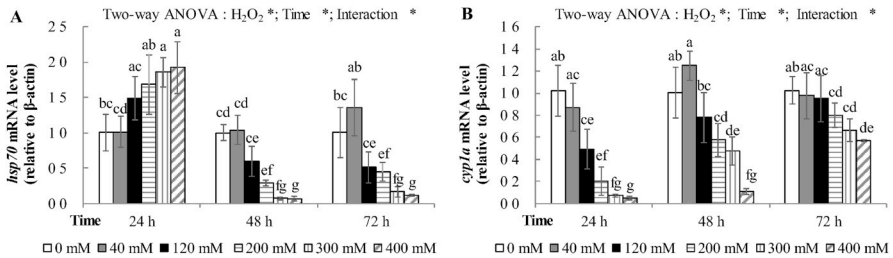

Fig. 8. Effects of different H_2O_2 concentrations on expression of *heat shock protein 70* (*hsp70*) and *cytochrome p450 1a* (*cyp1a*) in the liver of tilapia. The values are normalized to control values and expressed as means ± standard deviation (SD), n = 15. Data with different letters are significantly different ($P < 0.05$) among treatments. *$P < 0.05$. The treatment with 0 mM H_2O_2 is control.

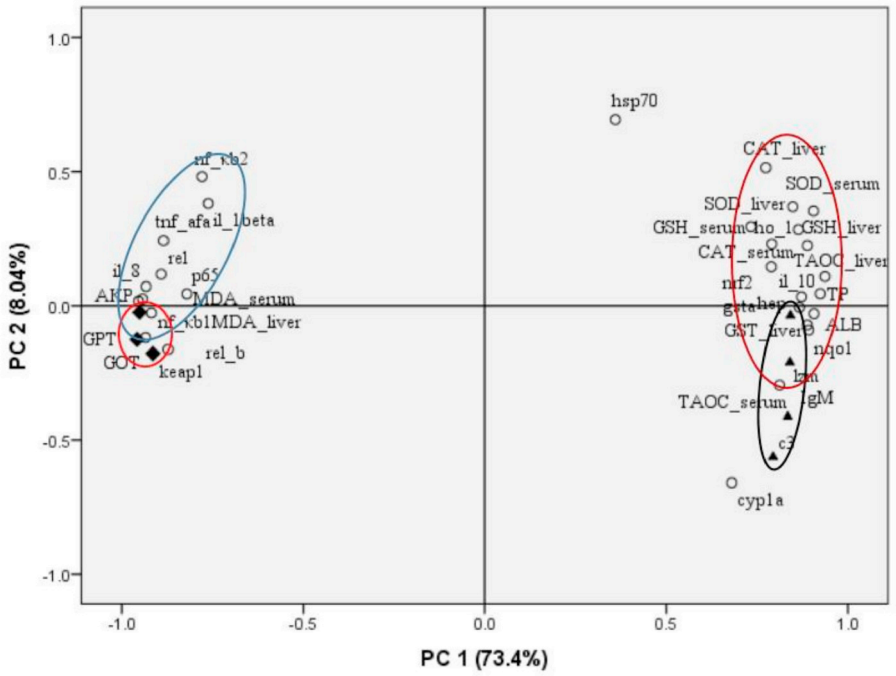

Fig. 9. Principal Component Analysis (PCA) representing the contribution of biochemical parameters and genes expression for H_2O_2-treated fish.

elevation of these enzymes was regard as an early antioxidation response to counteract the redundant ROS and maintain cellular redox state homeostasis under mild oxidative stress, while the reduction reflected hepatic injury and depression of antioxidant or detoxification ability in H_2O_2-intoxicated fish [32,81,82].

Apart from the oxidative damage, the inflammation was frequently activated as a secondary response, and played a pivotal role in H_2O_2-induced liver injury [64,83]. A wealth of data manifested that the NF-κB signaling pathway was implicated in the initiation, amplification and resolution of inflammation, and was activated by various different stimuli [84]. NF-κB activation and its subsequent inflammatory cytokines production in oxidative stress has been the focus of several studies in different models [85]. Early reports showed that H_2O_2 promoted NF-κB activity in human T cells, and might act as a modulator of the NF-κB pathway [86,87]. Meanwhile, we and many others have shown that NF-κB was activated under oxidative stress in liver injury induced by H_2O_2 and other toxicants [9,64]. Parallel with these findings, the results from this study revealed that the mRNA levels of *nf-κb* including *rel*, *p65*, *rel-b*, *nf-κb1* and *nf-κb2* evidently increased in fish at concentration of 300 and/or 400 mM H_2O_2 throughout the 72 h. Furthermore, worthy to note that the NF-κB signaling pathway was also regulated by Nrf2 and the molecular mechanisms showed a cell-type dependent manner [88]. Low Nrf2 can enhance NF-κB activity causing high cytokine production [89]. In this study, correlation analysis showed that *nf-κb* variation negatively correlated with *nrf2* variation, indirectly displaying abrogation of Nrf2 is conducive to NF-κB activation under oxidative stress.

It has been shown that the activated NF-κB would further trigger a series of cellular genes related to inflammatory and cytotoxic cytokines including IL-1β, IL-8 and TNF-α, resulting in dysfunction of liver [9,64,90]. IL-1β and TNF-α are prototypical proinflammatory cytokines, which are firstly produced at the beginning of inflammation, and promote the spread of the inflammatory response [91]. Higher IL-1β and TNF-α levels can induce NF-κB activation and thus regulate the transcription of target genes involved in immune, inflammation and cell survival [92]. IL-8 also is a proinflammatory cytokine which is rapidly synthesized by hepatocytes, Kupffer cells and macrophages to participate in the inflammatory disease response in liver injury [82,93]. Here, we found the mRNA levels of *il-1β*, *il-8* and *tnf-α* were highly induced after the treatment with 200, 300 and/or 400 mM H_2O_2. The upregulation of these cytokines was acute response to tissue damage and also revealed a direct correlation between cytokines and inflammatory response in liver injury [90]. On the other hand, IL-10 is an anti-inflammatory cytokine that suppresses expression of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-8, etc., and thus confers protection against several pathophysiological conditions [94]. In the present study, we observed that H_2O_2 treatments with 300 and 400 mM decreased the hepatic gene expression of *il-10*. The decrease might be attributed to the enhanced expression of NF-κB and pro-inflammatory cytokines, since anti-inflammatory cytokines also were regulated by NF-κB signaling pathway.

Overproduction of ROS is usually associated with the depression of immunity. Several immune parameters, such as LZM, HEP, C3 and IgM, have been successfully employed to evaluate the immune state of fish subjected to oxidative stress. LZM, HEP and C3 are representative of innate immune molecules, which play a key role in protecting fish from microbial invasion and other environmental stress, and are widely distributed in many tissues [95,96]. IgM is a most typical immunoglobulin in bony fish, playing a primary role in mediating humoral adaptive immune response, and participating in protection against potentially harmful conditions [97]. Previous reports have shown that H_2O_2 treatment led to reduction of LZM activity in *Salmo salar* plasma [98], inhibition of IgM secretion in murine B cell lymphoma, and downregulation of HEP mRNA in mice liver [99]. Moreover, following the exposure to hepatotoxicant, the generated oxidative stress suppressed these immune parameters levels in injured liver [100–102]. In line with previous studies, the results from the present experiment found dramatic decreases in *lzm*, *hep*, *c3* and *Igm* mRNA levels in liver of fish treated with H_2O_2 (200–400 mM) for 24–96 h. These results suggested that H_2O_2 caused immunotoxicity and inhibition of humoral immunity in tilapia. Interestingly, some evidences suggested that these immune parameters also were regulated by NF-κB pathway, despite the mechanism was remains unclear [103–106]. Our findings showed a negative correlation between immune parameters and NF-κB pathway, which reflected the immunosuppress might be related to the activation of NF-κB pathway.

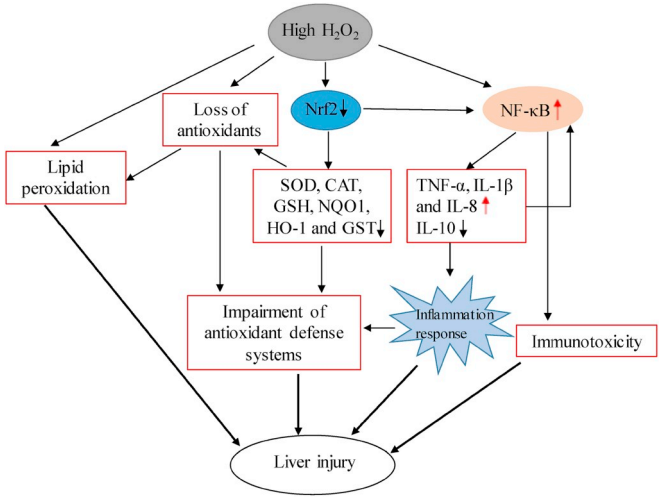

Fig. 10. Schematic model of H_2O_2-induced hepatotoxicity in tilapia. High H_2O_2 induced liver injury major through lipid peroxidation, impairment antioxidant defense systems, inflammation response and immunotoxicity. Further, in the liver injury, Nrf2 signaling pathway and NF-κB signaling pathway played pivotal role.

Additionally, oxidative stress is accompanied by change of various stress proteins including cytochromes P450 (CYP) and heat shock proteins (HSPs). CYP1A, a major CYP enzyme, plays central role in xenobiotic metabolism and biosynthesis of bioactive molecules [107]. It also is used to evaluate the presence of contaminants, physiological pressures and immune response in fish [44,82]. It has been known that H_2O_2 treatment repressed the expression of the *cyp1a* gene through heme degradation or oxidizing the heme thiolate ligand [107–109]. In liver or hepatocyte damage induced by H_2O_2, CCl_4 or other hepatotoxicants, the drastic decline in the activity of CYP1A was also observed [109–111]. Similarly, in our study, a linear decrease in hepatic *cyp1a* mRNA level was found in tilapia after H_2O_2 injection at 24, 48 and 72 h, which might repress the control of oxygen availability and the adaptation of cells to oxidative stress [107]. Furthermore, such downregulation detrimentally affected detoxification and other cellular functions [108].

HSP70, a main HSP in fish, is induced by various stressors including chemical stress, heat stress and crowding [44,112]. Its induction is a protective way through non-specific mechanism and often related to early cellular toxicity [113]. Results of the present study showed that *hsp70* mRNA level increased with increase of H_2O_2 concentration at 24 h. The results were consistent with previous studies showing higher *hsp70* gene expression in *Salmo salar* and neuroblastoma cells after H_2O_2 exposure [98,114]. Nevertheless, at 48 and 72 h, the gene expression of *hsp70* downregulated with H_2O_2 concentrations in tilapia, which might be due to failure of protein synthesis system in liver under sever oxidative stress.

5. Conclusion

The present study demonstrated that the mechanism of liver injury induced by H_2O_2 was mainly concerned with lipid peroxidation, impairment antioxidant defense systems, inflammation response and immunotoxicity (Fig. 10). Specifically, high H_2O_2, as a ROS, not only directly caused loss of antioxidant components and lipid peroxidation, but also suppressed Nrf2 signaling pathway to downregulate the gene expression of phase II detoxifying/antioxidant enzymes such as *ho-1*, *nqo1* and *gsta*, eventually leading to failure of antioxidant defense systems. Meanwhile, H_2O_2 treatment activated the NF-κB signaling pathway, resulting in cellular inflammation, immunotoxicity, and finally severe hepatotoxic damage to the fish. Additionally, high H_2O_2 suppressed the *hsp70* and *cyp1a* mRNA levels bringing about reduction of adaptive response of cells.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.084.

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