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Anti-oxidative, anti-inflammatory and hepatoprotective effects of Radix Bupleuri extract against oxidative damage in tilapia (*Oreochromis niloticus*) via Nrf2 and TLRs signaling pathway

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ABSTRACT

Radix Bupleuri extract (RBE) is one of the most popular oriental herbal medicines, which has anti-oxidative and anti-inflammatory properties. However, its protective effects and underlying molecular mechanisms on oxidative damage in tilapia are still unclear. The aims of the study were to explore the anti-oxidative, anti-inflammatory and hepatoprotective effects of RBE against oxidative damage, and to elucidate underlying molecular mechanisms in fish. Tilapia received diet containing three doses of RBE (0, 1 and 3 g/kg diet) for 60 days, and then were given an intraperitoneal injection of H_2O_2 or saline. Before injection, RBE treatments improved growth performance and partial anti-oxidative capacity in tilapia. After oxidative damage, RBE pretreatments were able to signally reduce the higher serum aminotransferases, alkaline phosphatase (AKP) and liver necrosis. In serum and liver, the abnormal lipid peroxidation level and antioxidant status induced by H_2O_2 injection were restored by RBE treatments. Furthermore, RBE treatments activated erythroid 2-related factor 2 (Nrf2) signaling pathway, which promoted the gene expression of heme oxygenase 1 (HO-1), NAD(P) H:quinone oxidoreductase 1 (NQO-1), glutathione-S-transferase (GST) and catalase (CAT). Meanwhile, RBE treatments reduced inflammatory response by inhibiting TLRs-MyD88-NF-κB signaling pathway, accompanied by the lower interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and IL-8 mRNA levels. In addition, RBE treatments upregulated complement (C3) gene expression and downregulated heat shock protein (HSP70) gene expression. In conclusion, the current study suggested that RBE pretreatments protected against H_2O_2-induced oxidative damage in tilapia. The beneficial activity of RBE may be due to the modulation of Nrf2/ARE and TLRs-Myd88-NF-κB signaling pathway.

1. Introduction

Liver of fish is a major organ involved in multiple metabolic functions and physiological processes, such as detoxification, metabolism of nutrients, biosynthesis and immunologic defense. It is more susceptible to injury due to the fact that liver is exposed to numerous xenobiotics including drugs and toxins [1]. Liver damage or dysfunction can result in metabolic disorder, growth inhibition, immunosuppression, and even death. In intensive aquaculture, fish liver damage is one of the most serious problems [2,3], and which is caused by many factors, such as toxic chemicals, pathogenic microorganisms, nutritional imbalance and uncomfortable farming condition [4–7]. Despite primary etiology of these liver damage is different, there are similar histological characteristics, including oxidative stress, lipid peroxidation, DNA damage, inflammation and hepatocellular death [8]. Among them, oxidative stress is considered to be a key process in the liver injury mechanism [9]. It is resulted from the imbalance between generation and scavenging in reactive oxygen species (ROS). Under oxidative stress, excessive

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ROS can lead to mitochondrial dysfunction, cell injury, lipid peroxidation and inflammation [10]. Thus, inhibiting oxidative stress may be a reasonable presumption for prevention of liver injury. Furthermore, because of involvement of oxidative stress in virtually all mechanisms of liver injury, lots of natural antioxidants are screened and used to prevent the deleterious effects of toxicants or other stressors via scavenging free radical [11,12].

Accumulating evidence reveals that nuclear erythroid factor 2-related factor 2 (Nrf2) is a pivotal regulator in oxidative stress-induced liver injury [13]. In brief, Nrf2 is anchored by binding to kelch like ECH-associated protein 1 (Keap1) in the cytoplasm under normal condition. Whereas, Nrf2 is translocated into the nucleus where it mediates the expression of downstream antioxdative genes and enzymes, such as heme oxygenase 1 (HO-1), NAD(P) H:quinone oxidoreductase 1 (NQO1), glutathione S-transferase (GST), etc. to inhibit oxidative stress [14]. Furthermore, long-term or severe oxidative stress can suppress Nrf2 expression leading to depression of antioxidant or detoxification ability [15,16].

The innate immune system has emerged as crucial component in the development of liver injury. Toll-like receptors (TLRs), a family of proteins regulating development and immunity, participate in early host defense against invading pathogens [17–21]. Seventeen TLRs have been identified in fish and grouped into six subfamilies (Reviewed by Fan et al. [22]). In liver, TLRs are expressed in various cells, such as hepatocytes, sinusoidal endothelial cells, Kupffer cells and hepatic stellate cells [23]. Multiple studies have shown the *in vivo* requirement of TLRs signaling in mediating oxidative stress-induced liver injury (Reviewed by Gill et al. [24]). Stimulation of TLRs, except TLR3, interacts with MyD88 (myeloid differentiation primary-response protein 88), triggers intracellular signaling cascades including activation of IL-1 receptor-associated kinases (IRAKs) and TNF receptorassociated factor 6 (TRAF6), finally leads to activation of nuclear factor κB (NF-κB) [19]. NF-κB is a vital transcription factor and its family is made up of five proteins: Rel, Rela, Relb, NF-κB1 and NF-κB2 [25]. It is activated in virtually every acute and chronic liver injury, including chemical, alcoholic, nonalcoholic and viral liver injury [26,27]. Activated NF-κB can regulate the expression of several pro-inflammatory and cytotoxic cytokines to involve in inflammation and cells damage [28]. In oxidative stress-induced liver injury, the activation of NF-κB signaling pathway is a possible mechanism [29–31].

Radix Bupleuri (Chaihu in Chinese), as an oriental folk medicine, has been used widely for the treatments of many diseases in China, Japan, Korea, and other Asian countries [32]. The root of Radix Bupleuri is usually the medicinal part, and which has been used in many traditional Chinese prescriptions, such as Xiao Chai Hu Tang, to treat liver diseases [33,34]. The pharmacological activities of extracts from Radix Bupleuri are frequently reported, and several valuable activities have been demonstrated, such as anti-oxidation, anti-inflammation, antipyretic and immunomodulatory [34,35]. All of these potent effects are due to its bioactive compounds including saponins, essential oils, flavonoids and sterols [36]. Among them, saikosaponins, the main class of secondary metabolites, are believed to be responsible for the most pharmacological activates of Radix Bupleuri Extract (RBE) [37]. It has been reported that RBE has protective effects against chemical-induced liver injury or liver cirrhosis in rats [38,39]. In fish, RBE can prevent dgalactosamine/lipopolysaccharide-induced liver injury [40], treat *Dactylogyrus intermedius* infection [41], and improve immunity [42,43]. However, no studies have reported the effects and molecular mechanisms of RBE against oxidative damage in fish. Therefore, it is of interest to examine the protective effects of RBE by using hydrogen peroxide (H_2O_2)-induced oxidative damage model in tilapia (*Oreochromis niloticus*), with focus on Nrf2 and TLRs singling pathways.

2. Materials and methods

2.1. Materials and reagents

RBE (6.7% saikosaponins and 4.9% flavonoids) was obtained from Y &L Biotech Co., Ltd. (Xi'an, China). H_2O_2 were purchased from Keygen Biotech Co., Ltd. (Nanjing, China). Tricaine methane sulfonate (MS-222) and saline were ordered from Sigma-Aldrich Corporation, St. Louis (MO, USA). Real-time quantitative PCR kits were obtained from Takara (Dalian, China). Commercial kits for glutamate pyruvate transaminase (GPT), glutamate oxalate transaminase (GOT), alkaline phosphatase (AKP), total protein (TP), albumin (Alb), superoxide dismutase (SOD), total antioxidant capacity (T-AOC) and glutathione (GSH) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Malondialdehyde (MDA) and catalase (CAT) detecting kits were bought from Beyotime Institute of Biotechnology (Nantong, China). All other reagents used in the experiment were of analytical grade.

2.2. Fish and treatments

The healthy tilapia (six-month-old, 96.7 ± 3.81 g) were obtained from the Freshwater Fish Research Center of Chinese Academy of Fishery Sciences (Wuxi, China) and acclimated to experiment condition in a circulating water aquaculture system (water temperature, 30 ± 2 °C; pH, 6.8–7.6; dissolved oxygen, > 6 mg/L) for two weeks. The fish were fed with commercial-pellet diet (31.6% crude protein, 4.6% crude lipid, 6.4% crude ash) two times per day prior to the experiment.

Tilapia were randomly assigned into 4 groups: normal control group, H_2O_2 treatment group, 1 g/kg RBE treatment group and 3 g/kg RBE treatment group. Each group consisted of 36 fish which were kept in three tanks for 60 days. Normal control group and H_2O_2 treatment group were fed with the basal diet, and two RBE-treated groups were fed with the basal diet supplemented with RBE at doses of 1 and 3 g/kg diet, respectively. After 60 days feeding, all fish were weighed to evaluate the growth performance. And then, the blood, gills, liver and muscle tissues were sampled from normal control group and RBE treatments (12 fish/group) to evaluate the anti-oxidative effects of RBE on tilapia.

Oxidative damage test: According to our previous experiment [44], the remaining fish in H_2O_2 treatment group and RBE-treated groups were injected intraperitoneally (i.p.) with H_2O_2 (300 mM), while the remaining fish in normal control group were administrated with saline. At 24 h after injection, the blood and liver tissues were collected from each treatment (12 fish/group) to investigate the protective effects of RBE against oxidative damage. All sampled fish were anesthetized in MS-222 (100 mg/L) and all samples were stored at −80 °C for further 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.

2.3. Growth performance

The growth parameters were evaluated by weight, specific growth rate (SGR) and feed conversion ratio (FCR). The calculation formulas are as follows: SGR = 100 × (ln final weight - ln initial weight)/test days; FCR = food consumed/biomass increment.

2.4. Biochemical analysis

Serum GPT and GOT activities were measured with commercial assay kits according to the Reitman–Frankel method [45]. Serum AKP activity was measured at 520 nm based on disodium phenyl phosphate method [46]. The levels Alb and TP in serum measured following bromocresol green method and bicinchoninic acid method, respectively

[47,48]. The levels of SOD, CAT, T-AOC, GSH and MDA in serum, liver, gills and muscle were determined with commercial kits according to the manufacturer's instructions by spectrophotometry. SOD activity was detected at 450 nm by the reduction of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) to water-insoluble formazan [21]. CAT activity was assayed by detecting the decomposition of H_2O_2 and production of a red product (N4-antipyryl-3-7 chloro-5-sulfonate-p-benzoquinonemonoimine) [49]. T-AOC level was tested by FRAP method thorough the reduction in the Fe^{3+}-TPTZ (2,4,6-tripyridyl-s-triazine) complex to the ferrous form Fe^{2+}-TPTZ [50]. GSH content was estimated at 412 nm by monitoring 2-Nitro-5-thiobenzoic acid (TNB) formation using enzymatic recycling method [51]. The amount of MDA was measured at 532 nm through the thiobarbituric acid-reactive substances (TBARS) method [52]. The levels of SOD, CAT, GSH, T-AOC and MDA in liver, gills and muscle were normalized to the total protein levels.

2.5. Real-time quantitative PCR

Total liver RNA for each fish was isolated using RNAiso Plus reagent (TaKaRa, Dalian, China). The amount of RNA was measured at 260 nm with a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA), and its quality was evaluated by agarose gel electrophoresis and the A260/A280 ratio (1.8–2.1). Following genomic DNA elimination (42 °C, 2 min), the RNA (1 μg for each sample) was reversely transcribed to first-strand cDNA using PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa) according to the manufacturer's instruction.

Real-time quantitative PCR (qPCR) was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.,Hercules CA, USA) using TB Green™ Premix Ex Taq™ II kit (TaKaRa). Briefly, each reaction contained 2 μL of cDNA, 12.5 μL of TB Green Premix Ex Taq II, 1 μL of forward and reverse primers (10 μM) and 8.5 μL of sterile purified water. The PCR thermal cycling conditions were as follow: initial denaturation 95 °C/30s, then 40 cycles at 95 °C/5s and 57–61 °C/1 min, and formation melt curve at the end of the last cycle. The relative mRNA levels of target gene were calculated on the basis of quantification cycle (Cq) value using the $2^{-\triangle\triangle Cq}$ method and β-actin was used as reference gene to normalize the Cq value [53]. The specific primers used for qPCR are shown in Table 1.

2.6. Histological evaluation

The liver tissue of fish from each group were fixed in Bouin's solution for 24 h, dehydrated in graded alcohol series, embedded in paraffin, and sectioned at 5–6 μm thickness according to standard procedures. The sections were stained with hematoxylin and eosin (H&E, Jiancheng Institute of Biotechnology) and examined under a light microscope.

2.7. Statistical analysis

The data from the experiment was analyzed using SPSS version 20.0 software. Results were expressed as mean ± SEM (standard error of mean). One way analysis of variance (ANOVA) was performed followed by the Tukey-kramer test in case of equal variances and Tamhane's T2 tests in case of unequal variances. The acceptable level of significance was established at $P < 0.05$.

3. Results

3.1. Effect of RBE on growth performance of tilapia

Before injection, the growth performance of tilapia fed with different doses of RBE was shown in Table 2. There were no differences in final weight among different treatments after 60 days. However, significant increase of the SGR in treatments with 1 and 3 g/kg RBE and significant decrease of FCR in treatments with 3 g/kg RBE were observed compared with the control group (0 g/kg RBE).

3.2. Effect of RBE on anti-oxidative status in different tissues of tilapia

The anti-oxidative status in different tissues of tilapia was listed in Table 3. In serum, compared to control group (0 g/kg RBE), SOD activity in 3 g/kg RBE treatment and T-AOC level in 1 and 3 g/kg RBE treatments were clearly improved ($P < 0.05$). In liver, 3 g/kg RBE treatment markedly increased the levels of SOD, T-AOC and GSH when compared with control group ($P < 0.05$); the similar increase of T-AOC was seen in 1 g/kg RBE treatment ($P < 0.05$). Likely, 3 g/kg RBE treatment also improved the gills SOD activity and muscle T-AOC level ($P < 0.05$).

3.3. Effects of RBE on liver injury maker of tilapia under oxidative damage

The histopathological examination showed that liver tissue from H_2O_2 treatment was seriously damaged, characterized by ambiguous cellular outline, cytomembrane breakage, karyopyknosis/hypochromatosis, and hepatocytes degeneration/necarosis. However, in RBE-treated group, the damage was notably alleviated (Fig. 1). Meanwhile, the activities of serum GPT, GOT and AKP showed a significant increase in H_2O_2 treatment, but a significant decrease in RBE-treated groups ($P < 0.05$; Fig. 2 ABC). In addition, the RBE treatments inhibited the reduction of Alb induced by H_2O_2 injection ($P < 0.05$; Fig. 2 D).

3.4. Effect of RBE on antioxidant capacity in tilapia under oxidative damage

After oxidative damage, the anti-oxidative status of tilapia was assessed by measuring the levels of SOD, GSH, T-AOC, CAT and MDA in serum and liver (Fig. 3). In serum, treatments with 1 and 3 g/kg RBE significantly restrained the reduced SOD and T-AOC, and formation of MDA induced by H_2O_2 injury ($P < 0.05$). Moreover, 3 g/kg RBE treatment also restrained the reduced GSH ($P < 0.05$).

Similarly, in liver, the levels of SOD, GSH, T-AOC and CAT in 3 g/kg RBE treatment were apparently higher than that in H_2O_2 treatment ($P < 0.05$); higher SOD and GSH were also observed in treatment with 1 g/kg RBE ($P < 0.05$). Nevertheless, the MDA formation in 3 g/kg RBE treatment was strongly lowered compared with H_2O_2 treatment ($P < 0.05$).

3.5. Effect of RBE on the antioxidant relevant pathway in tilapia under oxidative damage

To further assess the antioxidant response influence of RBE against the oxidative stress, the mRNA levels of antioxidant relevant genes Nrf2, HO-1, NQO1, GSTa and CAT were carried out in a qPCR assay (Fig. 4). In H_2O_2 treatment, the five genes were obviously down-regulated as compared with normal control. However, treatments with 1 and 3 g/kg RBE effectively upregulated the Nrf2, GSTa and CAT genes expression; similarly, the treatment with 1 g/kg RBE improved the HO-1 and NQO1 genes expression ($P < 0.05$).

3.6. Effect of RBE on toll-like receptor-related genes expression in tilapia under oxidative damage

To evaluate whether TLRs signaling pathway was involved in oxidative damage, we analyzed the transcript levels of toll-like receptors and their downstream genes (Fig. 5). QPCR analysis showed that the mRNA levels of TLR1, TLR2, TLR13, MyD88, IRAK1, IRAK4 and TRAF6 were evidently elevated in fish treated with H_2O_2 alone, while the elevation was repressed in RBE treatment at doses of 3 g/kg ($P < 0.05$). Also, the elevated TLR1, TLR13, IRAK4 and MyD88 mRNA levels were

Table 1
The primers sequences used in the present study.

Type	Gene	Primer sequence (5′-3′)	GenBank number	Reference
Oxidative stress	*Nrf2*	F: CTGCCGTAAACGCAAGATGG R: ATCCGTTGACTGCTGAAGGG	XM_003447296.4	
	NQO1	F: TGGATTTCAGGTTCTGGCTCC R: TCCTGTGGAGATGCCGAGA	XM_019361560.1	
	GSTa	F: TAATGGGAGAGGGAAGATGG R: CTCTGCGATGTAATTCAGGA	NM_001279635.1	[84]
	HO-1	F: CTTGCCCGTGTGGAATCACT R: AGATCACCGAGGTAGCGAGT	XM_013270165.2	
	CAT	F: ACATGCCACCAGGAATCGAG R: ATCTGCAGGTAGTTTGCCCC	XM_003447521	
TLRs pathway	*TLR1*	F: CTACAACGCCATCCAAACGC R: ACTGTGGCTGAAATCTCCCG	XM_005460356.3	
	TLR2	F: AAAAGCATAGATGAGTTCCACATCC R: GTAAGACAAGGCATCACAAACACC	JQ809459.1	
	TLR3	F: TCACCAACAAGACCTTAGCGT R: TCTGCCTGTCAAAGTTTGCTT	XM_019360096	[85]
	TLR5	F: CATTCAGCGGTCTCCCTAACT R: CGACATGGATACTGTTCATGG	XM_019353524.1	[85]
	TLR13	F: TCAACACGCTCTCAAGGGAC R: CAGAAATGCGGTTGTTGCGA	NM_001311317.1	
	Myd88	F: CAGGTTCCTGAGGTCGACAG R: CATTTCGTGGACGAACGCAA	KJ130039.1	
	IRAK1	F: CCAGTGATCCAGGTCCTTGT R: CGGGCAGGTTGAAGTACAAT	XM_003457627.4	[86]
	IRAK4	F: CTCAATGACTGGGGGACCAC R: TGGACTCGGGTAGCAGAACA	XM_003443911.4	
	TRAF6	F: AAGAGCCACCTAGAAGAGCA R: CTGACACTTCACACTGGCAA	XM_005455728.3	
NF-κB	*NF-κB2*	F: GAACATCAGACCGACGACCA R: TCTCCGCCAGTTTCTTCCA	XM_003457469.4	
	Rela	F; CAGATGAATACAGGCTGAGTGAGAA R: AGGTGCTGTCTATCTTGTGGAGTG	XM_005463161.3	
	Rel	F: GGTCAACAGAAATAGCGGAAGTG R:CCCAGCCATCAGGAGAGAAG	XM_019366581.1	
Inflammation	*TNF-α*	F: AAGCCAAGGCAGCCATCCAT R: TTGACCATTCCTCCACTCCAGA	NM_001279533.1	[87]
	IL-1β	F: TCAGTTCACCAGCAGGGATG R: GACAGATAGAGGTTTGTGCC	XM_019365842.1	[86]
	IL-8	F: CTGTGAAGGCATGGGTGTGGAG R: TCGCAGTGGGAGTTGGGAAGAA	NM_001279704.1	[87]
	IL-10	F: CAGCAGCAGGAGCATCAGCATT R: CACAGGAGGACGGTCTGAGAAGT	KP645180.1	[87]
Detoxification	*HSP70*	F: ATTTCAGACGGAGGGAAGCC R: CAGCGTTGGACACCTTTTGG	XM_019357557.1	
	CYP1a	F: CGTCGTCGTCTCTGTTGCC R: CATCGTCGTGGTGGTCATAGC	NM_001279489.1	[87]
Immune	*LZM*	F: AAGGGAAGCAGCAGCAGTTGTG R: CGTCCATGCCGTTAGCCTTGAG	XM_003460550.2	[88]
	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	[89]

Table 2
Effects of RBE on specific growth rate and feed conversion efficiency of tilapia.

Parameters	0 g/kg RBE	1 g/kg RBE	3 g/kg RBE
Initial average weight (g)	104.1 ± 6.81	103.9 ± 5.27	105.8 ± 4.15
Final average weight (g)	171.4 ± 4.25	181.4 ± 4.81	184.2 ± 6.07
Specific growth rate (%)	0.83 ± 0.05[a]	0.93 ± 0.04[b]	0.92 ± 0.07[b]
Feed conversion ratio	3.06 ± 0.31[a]	2.64 ± 0.08[ab]	2.56 ± 0.09[b]

Values are expressed as mean ± SEM (n = 12).
Data with different letters are significantly different ($P < 0.05$) among different treatments.

restrained in tilapia treated with 1 g/kg RBE ($P < 0.05$). In addition, the TLR3 and TLR5 genes expression was not influenced by H_2O_2 or RBE.

3.7. Effect of RBE on the levels of inflammation-related genes in tilapia under oxidative damage

To explore the anti-inflammatory effects of RBE, we examined the mRNA levels of NF-κB (Rela, NF-κB2 and Rel), interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), IL-8 and IL-10 in liver of tilapia under oxidative damage (Fig. 6). In our injury model, inflammatory markers such as NF-κB, IL-1β, TNF-α and IL-8 were induced, and IL-10 was suppressed upon liver injury ($P < 0.05$). With the treatment of 3 g/kg RBE, the genes expression of NF-κB (Rela, NF-κB2 and Rel), IL-1β, TNF-α was downregulated compared to the expression levels in H_2O_2 treatment ($P < 0.05$). With the treatment of 1 g/kg RBE, the genes

Table 3
Effects of RBE on anti-oxidative status tilapia.

	Parameters	0 g/kg RBE	1 g/kg RBE	3 g/kg RBE
Serum	SOD (U/mL)	10.15 ± 0.68[a]	12.38 ± 0.97 [ab]	13.08 ± 0.51[b]
	GSH (μM)	44.38 ± 5.22	46.88 ± 1.30	47.27 ± 2.58
	T-AOC (mM)	0.85 ± 0.06[a]	1.36 ± 0.22 [b]	1.26 ± 0.14 [b]
	MDA (μM)	5.41 ± 1.60	5.47 ± 1.11	5.38 ± 0.92
Liver	SOD (U/mgprot)	71.80 ± 1.93[a]	85.76 ± 5.07 [ab]	89.81 ± 1.67 [b]
	GSH (μmol/gprot)	19.16 ± 0.94[a]	27.13 ± 1.96[b]	25.62 ± 0.59 [b]
	T-AOC (μmol/gprot)	61.65 ± 7.53[a]	78.92 ± 6.29 [ab]	84.79 ± 3.63 [b]
	MDA (μmol/gprot)	0.43 ± 0.13	0.29 ± 0.04	0.32 ± 0.03
Gills	SOD (U/mgprot)	21.01 ± 0.50[a]	21.52 ± 0.59[a]	27.37 ± 2.96 [b]
	GSH (μmol/gprot)	9.16 ± 0.58	11.01 ± 2.33	10.10 ± 1.67
	T-AOC (μmol/gprot)	53.44 ± 1.36	48.19 ± 4.15	46.62 ± 4.79
	MDA (μmol/gprot)	1.59 ± 0.196	1.90 ± 0.37	1.83 ± 0.42
Muscle	SOD (U/mgprot)	13.84 ± 0.66	11.76 ± 1.17	13.61 ± 0.58
	GSH (μmol/gprot)	13.01 ± 0.94	11.24 ± 0.32	11.54 ± 1.67
	T-AOC (μmol/gprot)	13.47 ± 1.58[a]	13.15 ± 1.04[a]	16.26 ± 0.95 [b]
	MDA (μmol/gprot)	0.30 ± 0.05	0.31 ± 0.08	0.22 ± 0.07

Values are expressed as mean ± SEM (n = 12).
Data with different letters are significantly different ($P < 0.05$) among different treatments.

expression of Rela and IL-8 was also downregulated ($P < 0.05$). Moreover, there were no significant differences in IL-10 gene expression between H_2O_2 treatment and RBE treatments ($P > 0.05$).

3.8. Effects of RBE on the levels of immune-related genes in tilapia under oxidative damage

The effects of RBE on immune response in tilapia under oxidative damage were assessed by measuring immune parameters like lysozyme (LZM), complement 3 (C3) and immune globulin M (IgM). As detailed in Fig. 7, the mRNA levels of C3, LZM and IgM were much lower in the H_2O_2 treatment than the normal control group ($P < 0.05$), but the lower genes expression was upregulated in RBE treatments, although there were no clearly differences in these values except C3 in 1 g/kg RBE treatment.

3.9. Effect of RBE on HSP70 and CYP1A gene expressions in tilapia under oxidative damage

Compared with the normal control group, H_2O_2 treatment augmented heat shock protein 70 (HSP70) gene expression, and downregulated cytochrome P450 1a (CYP1a) gene expression ($P < 0.05$; Fig. 8). But the augmentation of HSP70 was availably suppressed when fish were pre-treated with RBE at doses of 1 and 3 g/kg ($P < 0.05$). CYP1a gene expression was not changed in RBE treatments.

4. Discussion

This work set out to evaluate anti-oxidative and protective effects of RBE in attenuating oxidative damage in tilapia, followed by assessing the underlying mechanisms. In the present study, we used H_2O_2 injection to induce oxidative damage model, and further analyzed the anti-oxidative status, inflammation and immune response as well as relevant signaling pathways (Nrf2 and TLRs pathway) in RBE and/or treated-tilapia. Accordingly, our experiments have demonstrated that RBE exerted anti-oxidative, anti-inflammatory and hepatoprotective effects against oxidative damage in fish.

It has been reported that RBE was used in the treatment of liver diseases, including chronic or acute hepatic injury. *In vivo* studies showed that the oral administration of RBE exerted a protective effect on inacetaminophen or diethylnitrosamine (DEN)-induced liver injury in rats via decreasing serum GPT and GOT [39,54]. *In vitro* studies demonstrated that RBE modulated the cell membrane constituents to affect the permeability of cultured BRL cells [55]. RBE also enhanced 5-fluorouracil-induced cytotoxicity in HepG2 to protect normal lymphocytes [56]. In fish, RBE as a hepatoprotective agent prevented dgalactosamine/lipopolysaccharide induced liver injury, and effectively suppressed the adverse alteration of GPT, GOT, AKP and Alb in serum [40]. In line with earlier studies, our work observed a sharp increase in activities of GPT, GOT and APK, and decrease in levels of TP and Alb in tilapia treated with H_2O_2 alone, indicating a serious liver injury. Meanwhile the injury was further confirmed by the histological changes. However, the injury phenomenon was visibly reversed in RBE treatments, where these injury markers almost returned to the levels of normal control, suggesting that RBE was able to ameliorate liver injury induced by oxidative stress in fish.

The accepted mechanism of RBE is recognized for its antioxidant activity and its ability as a free radical scavenger [57]. RBE could prevent the formation of ROS and reduction of SOD activity in SH-SY5Y Cells [58]. RBE also inhibited oxidative redox imbalance and lipid peroxidation in DEN-injured rats [39]. Similarly, our results showed excellent anti-oxidative properties of RBE in both tilapia before and under oxidative damage; RBE was able to depress oxidative damage, preserve the normal values of SOD, GSH, CAT and T-AOC, and prevent

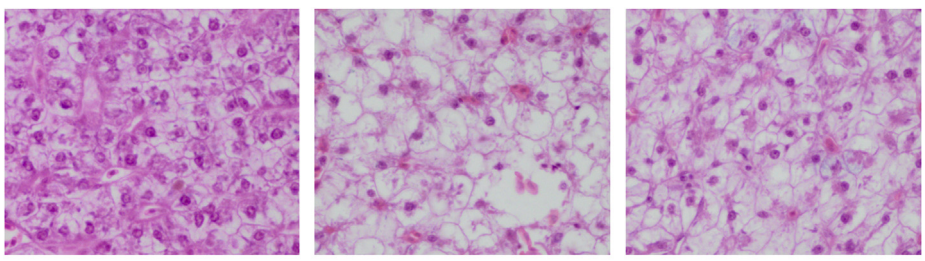

Fig. 1. Histopathological examination by H&E staining for liver injury of tilapia, × 400.

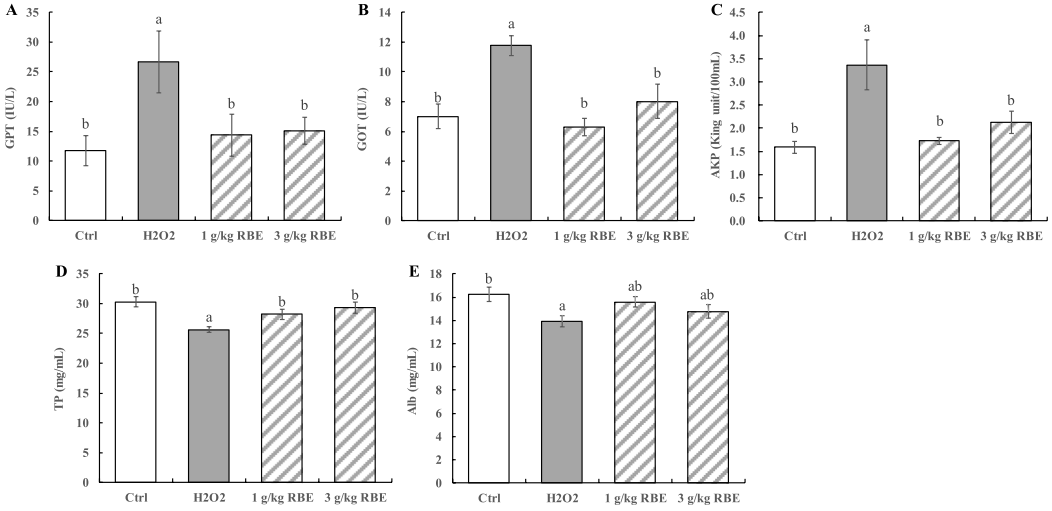

Fig. 2. Effects of RBE on blood biochemical parameters in tilapia under oxidative damage. The values are means ± SEM (n = 12). Different letters denote significant differences between different treatments ($P < 0.05$).

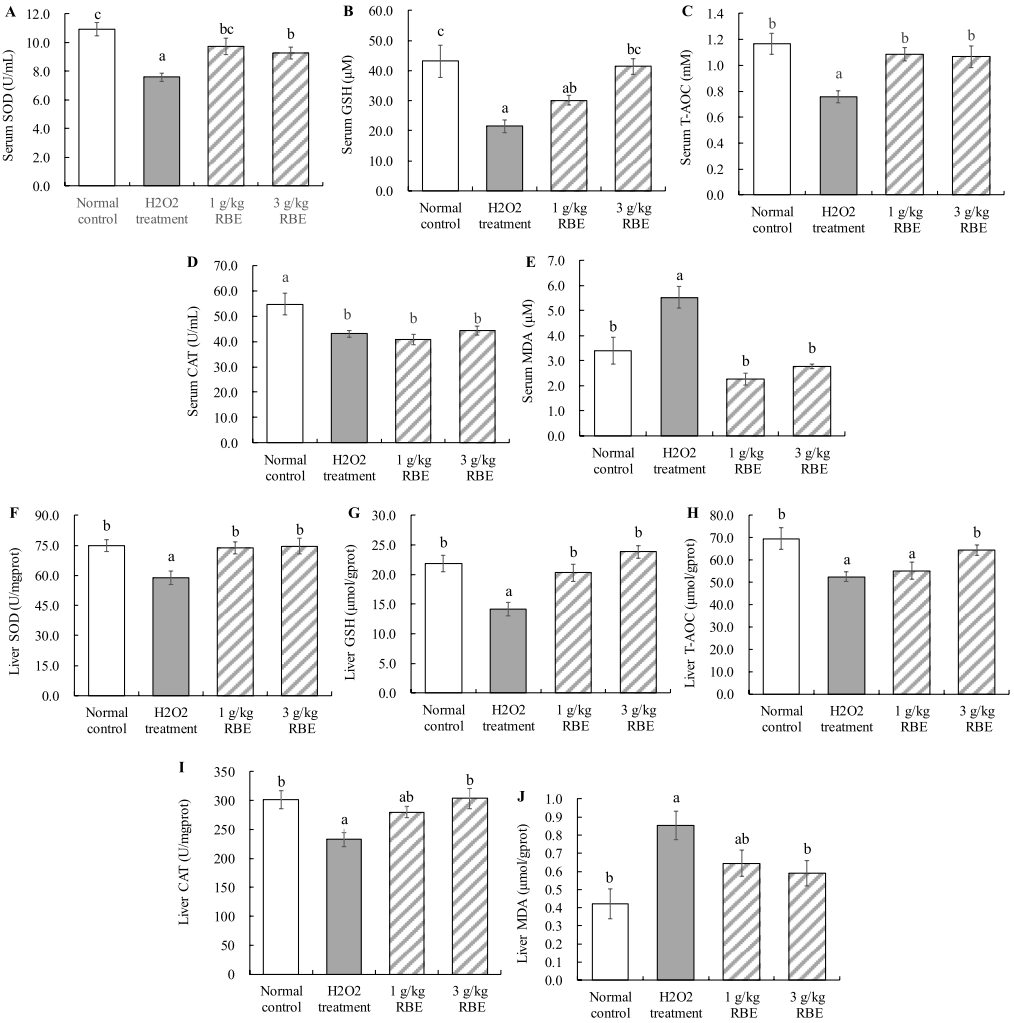

Fig. 3. Effects of RBE on antioxidative status in serum (A–E) and liver (F–J) of tilapia under oxidative damage. The values are means ± SEM (n = 12). Different letters denote significant differences between different treatments ($P < 0.05$).

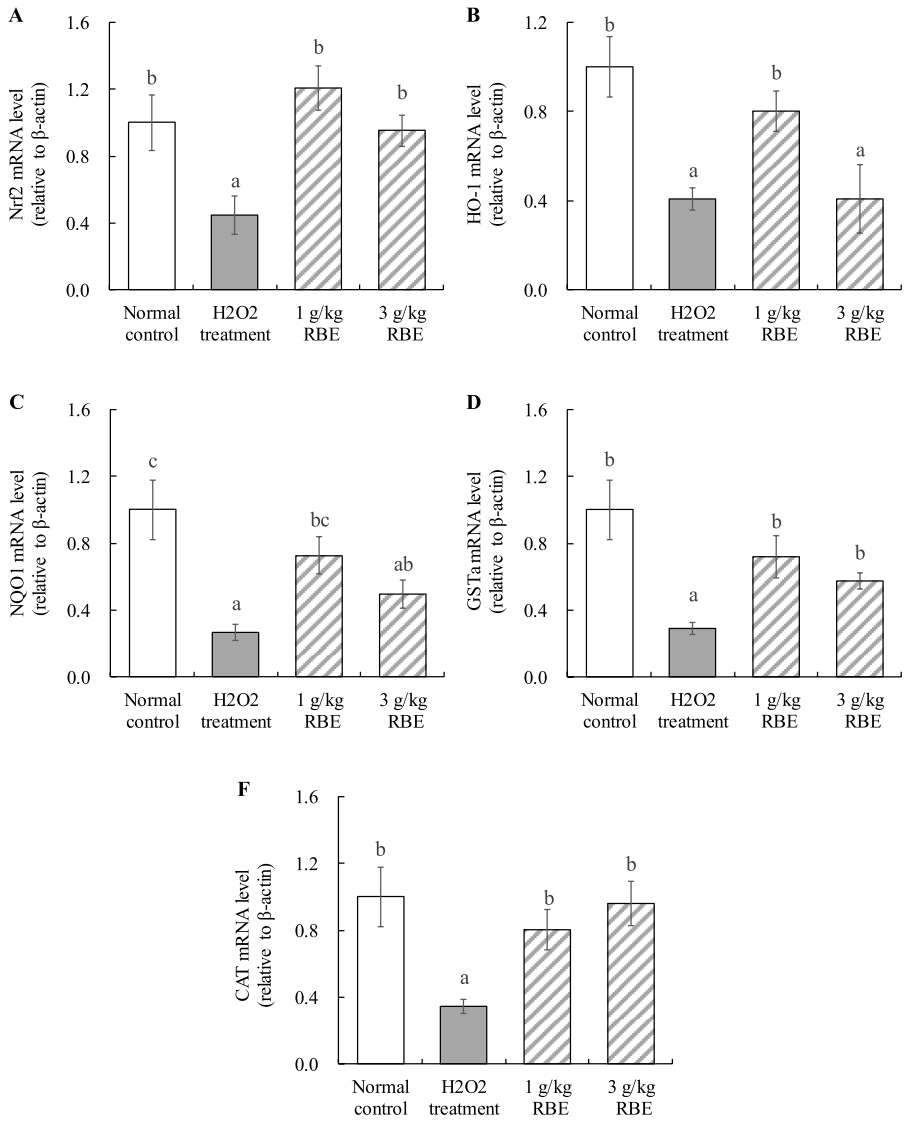

Fig. 4. Effects of RBE on the antioxidant relevant pathway in liver of tilapia under oxidative damage. The values are normalized to control values and expressed as means ± SEM (n = 12). Different letters denote significant differences between different treatments ($P < 0.05$).

MDA formation in liver and blood tissues. This is well in accordance with already published results where reduced antioxidant components (e.g. SOD, CAT and GSH) and elevated lipid peroxidation were signally attenuated by concomitant administration with RBE in DEN or acetaminophen-injured rats [54,59] and dgalactosamine-induced hybrid grouper [40]. In addition, the powerful anti-oxidative properties of RBE were further proven by its effectiveness to improve anti-oxidative capacities in blood, liver, gills and muscle of tilapia before oxidative damage.

Among these antioxidant enzymes, CAT plays a more significant role, as it can convert H_2O_2 into molecular oxygen and water. In oxidative stress, the CAT activity increases to keep the balance between H_2O_2 generation and elimination, which is an adaptive response. Nevertheless, the accumulation of H_2O_2 can accelerate the consumption of CAT, which may result in decrease of CAT activity [60]. In agreement with previous studies, our results showed a decreased CAT activity and mRNA level when tilapia were injected with H_2O_2 after 24 h. Interestingly, the activity and mRNA level of CAT were almost as well as normal level when tilapia were pretreated with RBE, which suggested that RBE could upregulate CAT gene expression and improve CAT activity. Moreover, the increase of CAT may be related to activation of Nrf2/ARE signaling pathway [16,61].

It has been suggested that Nrf2/ARE (antioxidant response element) pathway is a potential therapeutic target in various types of liver disease [62,63]. Numerous research have discovered that Nrf2 and its downstream genes were downregulated in oxidative damage [13,15,16]. Similarly, the results were also observed in the present study, where the levels of gene expression of Nrf2, HO-1, NQO1, GSTa and CAT were markedly downregulated in tilapia injured by H_2O_2. Inversely, RBE treatments enhanced these genes expression relative to H_2O_2 treatment. Consistent with our results, recent studies showed that saikosaponin A, a bioactive constituent of RBE, can prevent oxidative stress and inflammatory responses through activating Nrf2 signaling pathway in mice [64,65]. These results demonstrated that RBE may improve anti-oxidative capacity via activation of Nrf2/ARE signaling pathway in liver of tilapia.

On the other hand, inflammatory signaling pathway and innate immune response play pivotal role in development of liver injury [66]. Accumulating evidence manifested that pattern recognition receptors of the innate immune system, such as TLRs, might be involved in mediating inflammatory response in liver injury from oxidative stress [24]. Under oxidative stress, some TLRs are activated, and then utilize

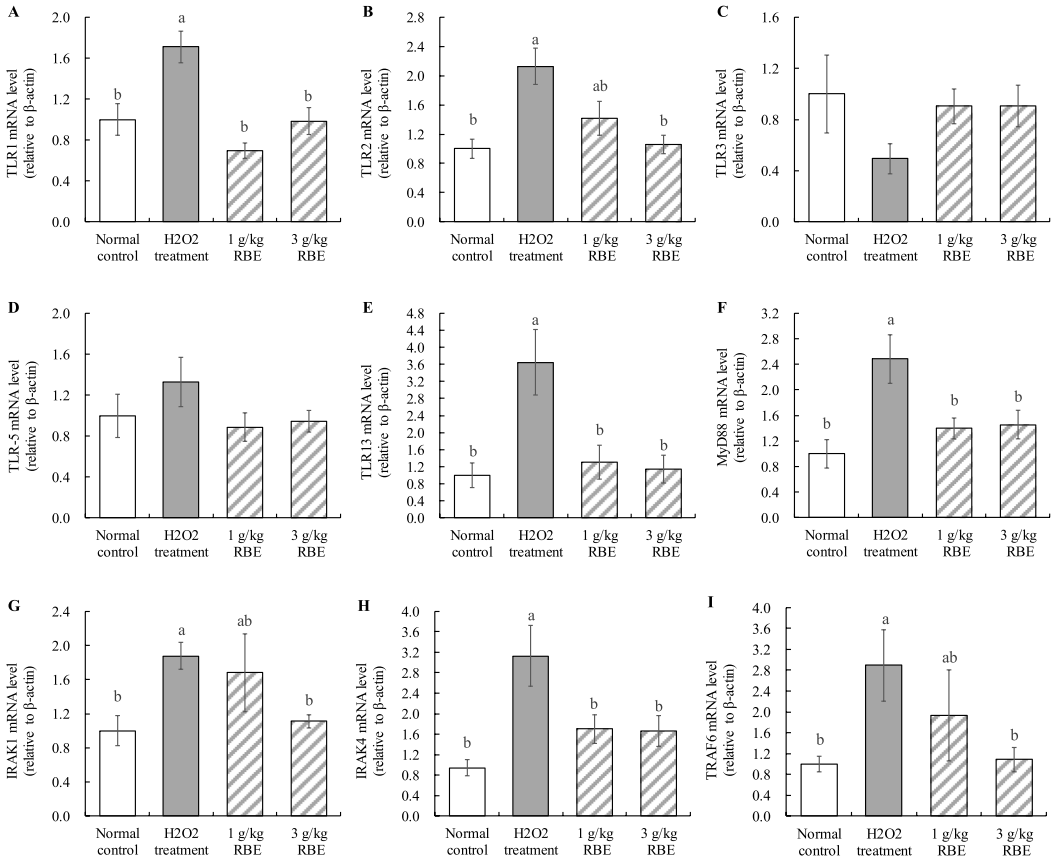

Fig. 5. Effects of RBE on the expression of toll like receptor-related genes in liver of tilapia under oxidative damage. The values are normalized to control values and expressed as means ± SEM (n = 12). Different letters denote significant differences between different treatments ($P < 0.05$).

MyD88 to initiate intracellular signaling cascade pathway including IRAK1, IRAK4, TRAF6, and finally NF-κB, which will lead to the production of inflammatory mediators [67]. In liver injury induced by various toxicants, TLRs-MyD88 signaling pathway was activated, and the TLRs (e.g.TLR2, TLR4 and TLR9) expression was upregulated [67–69]. Here, we also found the expression of TLR1, TLR2 and TLR13 was upregulated in liver injured by H_2O_2. Following the expression of TLRs, subsequent transcription of genes such as MyD88, IRAK1, IRAK4 and TRAF6 was also upregulated in H_2O_2 treatment. However, the expression of TLRs and their subsequent genes was evidently lowered by RBE pretreatments, suggesting that RBE attenuated inflammatory response and liver injury through inhibition of TLRs-MyD88 signaling pathway. We speculated that the property of RBE for inhibition of TLRs-MyD88 signaling pathway was possibly related to its bioactive constituent (saikosaponin A), as it can prevent TLR translocation [70].

Activation of TLRs-MyD88 signaling pathway further induces NF-κB to drive production of inflammatory cytokines such as IL-1β, IL-8 and TNF-α under oxidative stress [19]. Indeed, in H_2O_2 treated tilapia, the oxidative stress was capable of activating the NF-κB signaling pathway which in turn, upregulated the levels of pro-inflammatory cytokines like IL-1β, IL-8 and TNF-α. Interestingly, the upregulation of NF-κB and pro-inflammatory cytokines was depressed by RBE, further inducting the restoration of normal liver function under RBE treatment. Inhibition of pro-inflammatory cytokines is a common method in detecting liver recovery from injury. Under pathological condition, pro-inflammatory cytokines IL-1β and TNF-α contributes to the pathogenesis of liver inflammatory response, and high levels of IL-1β and TNF-α further promote the activation of NF-κB signaling pathway, which may worsen inflammation [25]. Studies have reported RBE administration relieved liver injury through downregulating pro-inflammatory cytokines (e.g. IL-1β, IL-6 and TNF-α) [40]. Also, Bui et al. reported that RBE blocked NF-κB signaling pathway to inhibit secretion of IL-1β, IL-4, IL-5, IL-6 and TNF-α, which ameliorated inflammatory response in a ovalbumin-induced allergic asthma mice model [71]. Moreover, some evidence suggested that anti-inflammatory effect of RBE seemed to be ascribed to its major pharmacologically-active components (saikosaponin A and D) that can attenuate inflammation and hepatotoxicity by suppressing activation of NF-κB signaling pathway [72,73]. Based on previous reports and our data, we hold the opinion that TLRs-MyD88-NF-κB signaling pathway played a vital role in anti-inflammatory and protective effects of RBE against oxidative damage in tilapia.

Beside anti-oxidative and anti-inflammatory effects, considerable evidence supported that RBE could enhance immune function in animal. RBE administration significantly promoted lymphocyte proliferation [74], increased Ig secretion [75], and improved non-specific immune responses [76] in mice or *Litopenaeus vannamei*. In the present study, RBE treatment only reversed the abnormal gene expression of C3, but not evidently influenced the expression of LZM and IgM genes in spite of the upward trend. Earlier investigations have demonstrated that the complement system participates in the pathogenesis of various liver injury, such as alcoholic liver injury, viral hepatitis, fibrosis, and liver ischemia/reperfusion injury [77,78]. C3 deficiency leads to severely defective liver regeneration, accompanied by transient or fatal liver failure [79]. Thus the restoration of C3 in RBE treatment might be beneficial to liver regeneration after liver oxidative damage induced by H_2O_2 in tilapia.

In addition, our study found that RBE effectively blocked the upregulation of HSP70 gene in liver injury induced oxidative stress, but not change the expression of CYP1a. The two genes were frequently used to evaluate cellular stress responses to various stimulus. CYP1a, a

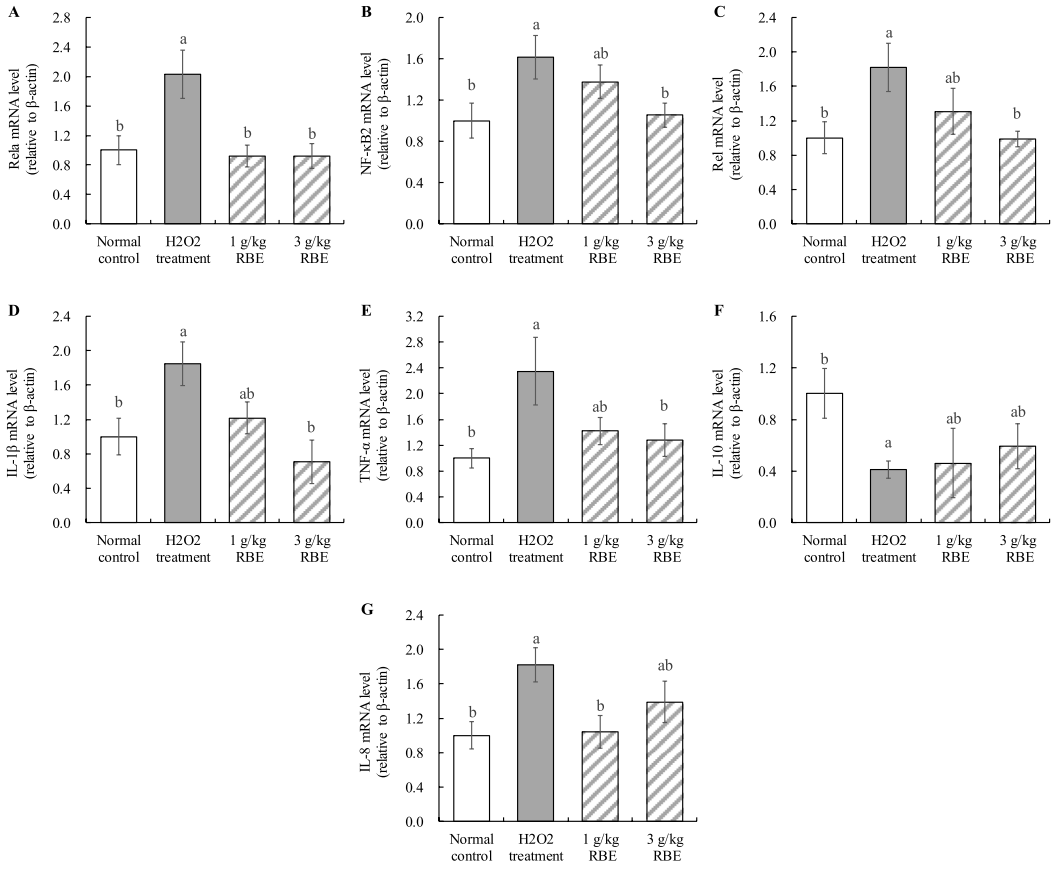

Fig. 6. Effects of RBE on the levels of inflammation-related genes in liver of tilapia under oxidative damage. The values are normalized to control values and expressed as means ± SEM (n = 12). Different letters denote significant differences between different treatments ($P < 0.05$).

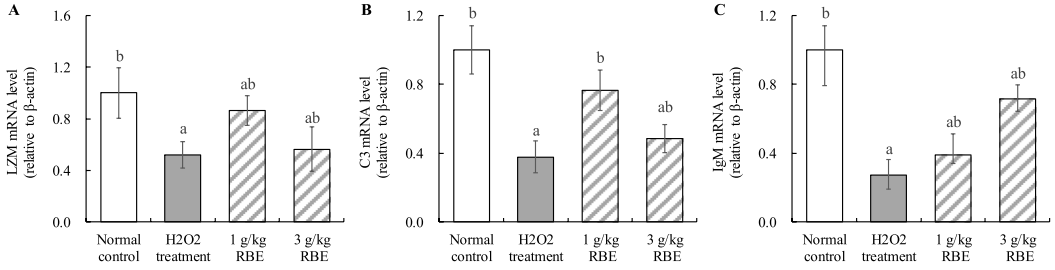

Fig. 7. Effects of RBE on the levels of immune-related genes in liver of tilapia under oxidative damage. The values are normalized to control values and expressed as means ± SEM (n = 12). Different letters denote significant differences between different treatments ($P < 0.05$).

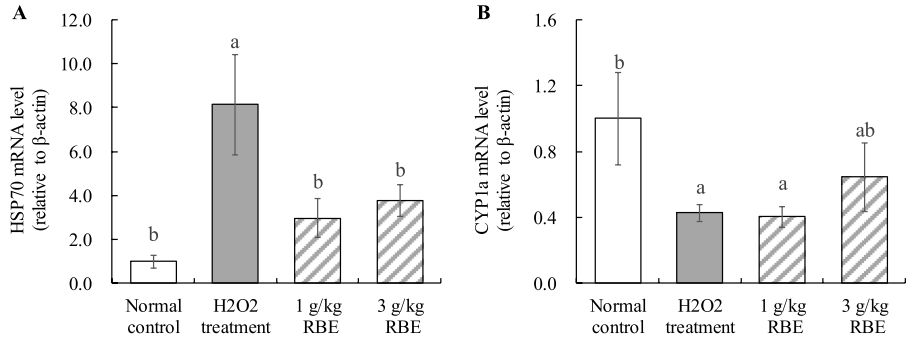

Fig. 8. Effects of RBE on HSP70 and CYP1a gene expressions in tilapia under oxidative damage. The values are normalized to control values and expressed as means ± SEM (n = 12). Different letters denote significant differences between different treatments ($P < 0.05$).

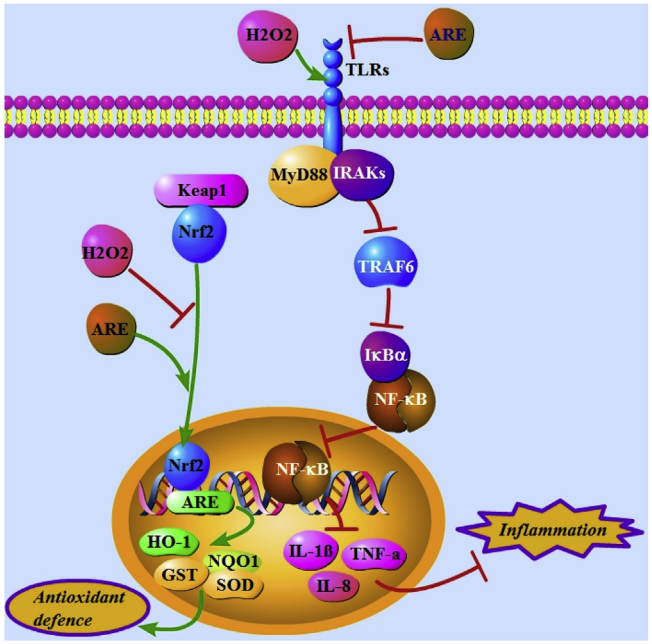

Fig. 9. Possible mechanisms of antioxidant and anti-inflammatory activities of RBE in the tilapia liver under oxidative damage. (→) indicates stimulatory modification, (T indicates stimulatory modification.

ubiquitous member of the P450 superfamily, is known to be reduced in oxidative stress-induced liver injury, implying the abnormal activity of drug-metabolizing enzymes [80,81]. HSP70, the most fundamental and prominent HSP in fish, is a pervasive adaptation mechanism of organisms to promote protein synthesis. Its induction under oxidative stress has been reported in previous our and other studies [44,82,83]. The downregulation of HSP70 in RBE treatments might be reflect the alleviation of oxidative damage in tilapia.

5. Conclusion

In summary, our results indicated that RBE exerted anti-oxidative, anti-inflammatory and hepatoprotective effects in tilapia under oxidative damage. The beneficial activity of RBE may be due to enhancing Nrf2/ARE signaling pathway and inhibiting TLRs-MyD88-NF-κB signaling pathway (Fig. 9). The enhancement of Nrf2/ARE pathway induced production of phase II detoxifying/antioxidant enzymes such as HO-1, SOD, NQO1 and GSTa, while the inhibition of TLRs-MyD88-NF-κB pathway led to reduction of pro-inflammatory cytokines like IL-1β, IL-8 and TNF-α.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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