



Full length article

Dietary Radix Bupleuri extracts improves hepatic lipid accumulation and immune response of hybrid grouper (*Epinephelus lanceolatus* ♂ × *Epinephelus fuscoguttatus* ♀)

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ABSTRACT

In this study, two experiments were performed to explore the effect of Radix Bupleuri extracts (RBE) on growth, lipid deposition and metabolism and immune response of hybrid grouper (*Epinephelus lanceolatus* ♂ × *Epinephelus fuscoguttatus* ♀) using in vitro and in vivo models. In vitro, we used 2 ml/L 20% lipid emulsion (LE)-induced steatosis in hybrid grouper primary hepatocytes, then RBE (200, 400 and 800 µg/ml) was added to the hepatocytes after (post-treatment) the incubation with 20% LE (2 ml/L) in the culture medium. We found that RBE markedly increased cell viability, which were consistent with hepatocytes morphological structure examination and lipid metabolism and immune related genes study. The above result suggested that RBE has a protective effect on this model of hepatocytes damage. In vivo, five graded levels of RBE at 0, 200, 400, 800 and 1600 mg/kg diet were supplemented to a basal diet with 15% lipid levels (high lipid), and fed to a total of 300 hybrid grouper with an average initial weight of 25.58 ± 0.05 g for 8 weeks. Growth performance, liver histology, plasma biochemical parameters, and expression of genes involved in lipid metabolism and immune-related were measured. The study indicated that dietary RBE significantly improved growth performance and feed utilization and reduced hepatosomatic index. Dietary supplementation with 200–800 mg/kg RBE diets effectively decreased serum ALP, ALT, AST and LDH contents in fish. Furthermore, adipogenesis relative mRNA levels of DGAT2, G6PD, ME1 and DGKα in fish fed 200–400 mg/kg RBE diets were lower ($P < 0.05$) than in those fed RBE0 diets, while dietary supplementation with 200–800 mg/kg RBE diets up-regulated lipolysis-related genes (CPT1, LPL and PPARα) expression in the liver of hybrid grouper. Moreover, dietary RBE down-regulated the expression of apoptosis-related genes (caspase-9), up-regulated the expression of antioxidant genes (CAT) and immune-related genes (MHC2, IKKα and TGF-β1). Thus, our data suggest that RBE suppressed lipid accumulation and enhanced immune capability in hybrid grouper both in vitro and in vivo. These results offer new insight into RBE as a hepatoprotective in fish.

1. Introduction

Lipid is an important source of energy, and it plays a key role in fish nutrition to provide essential fatty acid (FA) and phospholipid [1]. High-lipid diets is the current trend for the protein-sparing effects in intensive aquaculture [2,3]. Protein is a relatively expensive source of energy, so high-lipid diets have increasingly been used for cost-effective farming in aquaculture in recent years [4,5]. Indeed, increasing dietary lipid within certain limits will support the higher growth rates of fish

[6]. However, high-lipid diets often lead to ectopic lipid accumulation in the tissues of farmed fish, including the liver and abdominal adipose tissue, causes metabolic disturbances, abnormal oxidative status and suppress the immune system, thus posing serious threats to the sustainable development of aquaculture [7,8]. Previous study showed that excessive lipid deposition also affect the quality of harvest in some fish farms [9]. In addition, some diseases related to fish lipid metabolism, such as fatty liver syndrome, increase yearly in cultured fish [10].

Groupers are the most important marine aquaculture fish species

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and their whole production reached approximately 100,000 tons in China in 2016, increase of 13.48% over the previous year [11]. The hybrid grouper, *Epinephelus fuscoguttatus*♂ × *Epinephelus lanceolatus*♀ is a popular cultured marine fish in the Southeast Asia and China. It has integrate the advantages of the selected parents, with a fast growth rate, highly disease resistant, highly nutritious and good meat quality [12]. Thus, it has been largely cultured due to its huge potential market value. Hybrid grouper is intensive rearing along the coasts often with diets utilizing increased lipid content to both spare protein and enhance growth. In fish, the liver plays an important role in lipid metabolism, including both the synthesis and degradation of fatty acids [13]. Based on our previous studies, 7–13% fat has been determined to be optimal for the growth and effective protein utilization of hybrid grouper, and fat accumulation in the liver occurs when the diet reaches 15% fat [14,15]. High-lipid diets are known to resulting in a high susceptibility of fatty liver in this fish species [16]. Furthermore, fatty liver may induces anomalies of metabolism and physical properties and often closely positively correlates with a high rate of mortality or poor growth, which further affects fish health [17,18]. Previous researchers have shown that structural lesions of the liver can alter metabolic and nutritional status in fish [19]. Therefore, studies about the mechanism of hepatic lipid deposition are important, it is necessary to clarify the nature underlying the disorder and seek ecofriendly disease-preventative measures to ensure the sustainability of aquaculture.

Recently, growing interest has arisen for medicinal plants that offer an alternative because of their immunomodulatory effects, to instead of the drugs, chemicals and antibiotics currently used in aquaculture to control diseases [20]. For example, some medicinal plants have the ability to reduce lipid deposition and increase resistance to disease by enhancing nonspecific and specific immunity in fish [21–23]. Medicinal plants are used in aquaculture as feed additives, because they contains a wide variety of nutrients [24,25]. So they came as a promising and substitute method for the control of fish disease. Radix Bupleuri is one of the most important plant medicines in China used for over a thousand years. The earliest record about Radix Bupleuri in China appeared in Shen Nong Ben Cao Jing. Contemporary pharmacological research indicated that Radix Bupleuri possesses many pharmacological functions, such as clearing heat, improving liver and circulatory system function, regulating the liver-qi, and lifting yang-qi, hepatoprotection, balancing different organs and energies within the body [26,27]. Previous studies reported that Radix Bupleuri and saikosaponins have marked immunostimulating effects on immune cells in human and mammal [28,29]. Yang et al. also reported that Compounds Radix Bupleuri could reduce lipid accumulation in rats [30]. In aquaculture, study showed that *Bupleurum chinense* were efficient in elevating the immune function of *Sciaenops ocellatus* [31]. In addition, many studies in animal models or on cell lines have demonstrated that Radix Bupleuri extracts (RBE) has cytoprotective effects in experimental liver injuries [32]. Our previous study reported that RBE can protect hepatocyte injury induced by D-GalN/LPS in hybrid grouper [33]. However, to our knowledge, little is known about the effects of RBE as a promising feed additive on the improvement of growth, lipid metabolism, and health status in fish fed the diets with elevated lipid levels.

Therefore, the aim of this study was to evaluate the regulatory role of RBE in lipid accumulation and immune function in hybrid grouper by both in vivo and in vitro methods. First, this study revealed the effect of RBE on hepatocytes in vitro by analyzing hepatocytes morphological structure, triglyceride accumulation, mRNA expression of lipid metabolism related genes and immune-related genes induced by 2 ml/L 20% LE. Furthermore, in support of the in vitro study, we investigated the effect of RBE on inhibition of lipid accumulation in liver tissue induced by high-lipid diets with an in vivo study. The findings of this study will provide a theoretical basis for the development of new feed additive for fish, and enhancing its innovation and practical significance.

2. Materials and methods

2.1. In vitro study

Primary hepatocytes from hybrid grouper were isolated by following the protocol of Xu et al. [34]. The cell pellet was resuspended in MEM culture medium (Gibco, Thermo Fisher, Suzhou) (containing 100 IU mL⁻¹ penicillin and 100 IU mL⁻¹ streptomycin) supplemented with 10% fetal bovine serum (FBS) and counted using a hemocytometer. In case of the viability of cells was > 92% as assessed with Trypan Blue exclusion, the cells were used for subsequent experiments. Then, the hepatocytes were cultured at 25 °C in an incubator with 5% (v/v) CO₂, the medium was replaced every 2–3 days. Upon reaching 80–90% confluency (about 10–12d), cells were harvested in 0.25% (w/v) trypsin-EDTA, and suspensions were seeded onto 6-well. After 48 h of the culture, the following treatments were done: control (hepatocytes neither treated with 2 ml/L 20% lipid emulsion (LE) nor RBE); model group (hepatocytes treated with 2 ml/L 20% LE alone for 72h); recovery group (hepatocytes treated with 2 ml/L 20% LE for 48h, then incubated with normal medium for 24h); post-treatment with RBE groups (hepatocytes were incubated with 2 ml/L 20% LE for 48h, then post-incubated with 200, 400 and 800 µg/mL of RBE for 24 h). 20% LE was purchased from Panyu Armed Police Hospital. RBE (20:1) was purchased from NANJING DASF BIO-TECHNOLOGY CO., LTD. Sampling occurred after 72 h of treatment.

2.2. In vivo study with dietary RBE addition

2.2.1. Diet preparation

The composition of the basal diet is given in Table 2. The tested Radix Bupleuri extracts (RBE) (20:1) was supplied by NANJING DASF BIO-TECHNOLOGY Co., Ltd. Six experimental diets were prepared with RBE supplement levels at 0 (RBE0), 200 (RBE200), 400 (RBE400), 800 (RBE800) and 1600 (RBE1600) mg/kg. Then, all diets were air-dried at room temperature (25–30 °C) and stored at –20 °C until used.

2.2.2. Experimental procedures

Hybrid grouper were obtained from Marine Fisheries Development Center of Guangdong Province (Huizhou, China). All fish were acclimatized for two weeks before the feeding trial in outdoor cement ponds (10 m × 3 m × 1 m) with running water and continuous aeration. In this experiment, 300 individuals (25.58 ± 0.05 g) were randomly distributed into five groups (15 floating cages: L100 cm × W100 cm × H70 cm) and were coded as RBE0, RBE200, RBE400, RBE800 and RBE1600. Each group was assembled in triplicate. The fish were fed two times each day at 8:30 and 16:30. The feeding trial lasted for 56 days.

At the termination of the feeding study, fish were fasted for 24 h before sampling, then were euthanized with MS222 at a concentration of 100 mg L⁻¹ prior to samplings. The body weight, body length, liver and viscera weight and weight gain (WG) were determined. Then, the condition factor (CF), hepatosomatic index (HSI), and viscerosomatic index (VSI) were calculated, respectively. Blood samples were collected from the caudal vein of fish in each group. Hepatic histology (hematoxylin and eosin staining) and histochemical (Oil Red O staining) observations were undertaken. The six fish livers from each floating cage was excised, frozen in liquid nitrogen, and stored at –80 °C to analyse lipid metabolism and immune related gene expression.

2.3. Sample analysis

2.3.1. Measurement of cell viability and hepatocytes function test

Hybrid grouper primary hepatocytes were seeded into 96-well plates, and 20% LE or RBE (appropriate concentrations) was added 72 h later. Cell viability was determined by Cell Counting Kit-8 (CCK-8, ABP Biosciences, Virginia, USA) assay by adding 10 µL CCK-8 solution into

each well for 2–4 h at 25 °C. Then, a microplate reader (Thermo, MA, USA) was used to determine the OD450. Proliferation inhibition rate (%) = experiment well A450/control well A450 × 100%.

Hepatocytes were incubated for an appropriate period with 20% LE or RBE (appropriate concentrations) and then subjected to low-temperature sonication, followed by centrifugation at 12,000 rpm for 5 min. The supernatant was collected, and the cellular levels of TG and CHOL were determined according to the instructions of an TG assay kit (Zhicheng Biological Technology, Shanghai, China) and CHOL assay kit (Beckman Coulter, Suzhou, China) respectively.

2.3.2. The hepatocytes morphological structure

Primary hepatocytes of hybrid grouper were done using cells grown in 6-well plates cover glass. Hepatocytes hematoxylin and eosin (H&E) staining were performed according to our previously described methods [33]. Cells were washed three times in 0.1M PBS for 2 min each time and then fixed in 4% paraformaldehyde for 20 min, washed in 0.1M PBS four times for 2 min each time. Finally, the morphological structure changes of hepatocytes were observed with an optical microscope (200 ×, MshOt MS60).

2.3.3. Growth performance and morphometric parameters

The fish were weighed at the beginning and the end of the experiment. The parameters of weight gain (WG), feed efficiency (FE), condition factor (CF), hepatosomatic index (HSI) and viscerosomatic index (VSI) were calculated as per following formulae:

Weight gain (WG, %) = $100 \times (\text{final body weight} - \text{initial body weight}) / \text{initial body weight}$;

Feed efficiency (FE) = wet weight gain (g)/dry feed intake (g);

Condition factor (CF, g/cm³) = $100 \times (\text{body weight, g}) / (\text{body length, cm})^3$;

Viscerosomatic index (VSI, %) = $100 \times (\text{viscera weight, g}) / (\text{whole body weight, g})$;

Hepatosomatic index (HSI, %) = $100 \times (\text{liver weight, g}) / (\text{whole body weight, g})$.

2.3.4. Chemical analysis

Whole body and muscle moisture were analyzed by drying the samples to a constant weight at 105 °C. Crude protein (N × 6.25) was measured by the Kjeldahl method after acid digestion using Kjeldahl (FOSS 8400, Hoganos, Sweden). Crude lipid was measured through ether extraction using Soxtec™ 2055 (FOSS, Hoganos, Sweden). Moisture was determined by drying in an oven at 105 °C for 24 h. Oven-dried feed, whole body, Muscle were ashed at 550 °C for 24 h in a muffle furnace (FO610C, Yamato Scientific Co., Ltd., Tokyo, Japan).

2.3.5. Serum biochemical analysis

The fish blood was first stored at 4 °C for 8 h, then centrifuged at 3000 rpm for 15 min at 4 °C to obtain serum. Alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), cholesterol (CHOL), lactate dehydrogenase (LDH) and triglycerides (TG) levels were determined by standard spectrophotometric procedures in Guanzhou First People's Hospital.

2.3.6. Liver histological and histochemical analyses

Individual liver samples were fixed in 4% neutral-buffered formalin. For histological observation, samples were dehydrated in graded ethanol concentrations and embedded in paraffin. Then, they were cut into 5- to 6-μm thick slices. The pieces were stained with hematoxylin and eosin (H&E) for optical examination. For histochemical observation, frozen liver sections were sectioned (9 μm) on a cryostat microtome. Sections were fixed in 4% buffered formaldehyde for 10 min, and rinsed in distilled water, and then immersed briefly in 60% isopropanol. After being stained with oil red O and then prepared for light microscopy. The relative area of lipid droplets in oil red O staining was

Table 1a

Primer design for lipid metabolism related genes in this study.

Primers	qPCR primers, forward/reverse (5'to3')
DGAT2	F: CATCTTCTGCTTTGGTGCTTTC R: GCATTTCCTGTCCTCCGTTA
FAS	F: CGGGTGTCTACATTGGGGTG R: GAATAGCGTGGAAAGCGTTT
G6PD	F: GCTTCACATCCTTGTATCTGCTC R: GCGTTCCTTTTATTCTCCG
ME1	F: GAAGTTGTTCTACCGCTTGCTG R: AGAGTCTCTGTTGCTCTCTGA
DGKα	F: TCCACGGCAGGTAACAACACC R: TATCTCTCTCCCATCGCA
ATGL	F: ATTGAGCACCTTCCACCCA R: CCGAATCCATCCCACATCTT
CPT1	F: TCCTTACCGTTGGTCCCTCT R: CTTTCCATCTGCTGCTATCTC
LPL	F: TTCAACAGCACCTCCAAAACC R: GTGAGCCAGTCCACCAGAT
ACO1	F: CGGCATGGACTTCTGTATG R: CCTGGTGTGCGTGTGTGTT
PPARα	F: CATCGACAATGACGCCCTC R: GCCGCTATCCCGTAAACAAC
β-Actin	F: TACGAGCTGCCTGACGGACA R: GGCTGTGATCTCTTCTCG

DGAT2, acyl CoA diacylglycerol acyltransferase 2; FAS, fatty acid synthase; G6PD, glucose 6-phosphate dehydrogenase; ME1, malic enzyme 1; DGKα, diacylglycerol kinase alpha; ATGL, adipose triglyceride lipase; CPT1, carnitine palmitoyltransferase 1; LPL, lipoprotein lipase; ACO1, acyl-CoA oxidase 1; PPARα, peroxisome proliferator-activated receptor alpha.

analyzed by Image-Pro Plus 6.0.

2.3.7. RNA isolation and gene expression analysis

Liver samples from six fish in each floating cage (or hepatocytes samples) were used for total RNA isolation by Trizol reagent (Invitrogen, Carlsbad, CA). The quantity of isolated RNA was assessed by measuring spectrophotometric (A260: 280 nm ratio) analysis (NanoDrop Technologies, USA). The quality of total RNA was detected using agarose gel (1.2%) electrophoresis. Subsequently, the reverse transcription from total RNA was utilized to synthesize the cDNA by utilizing EasyScript First-Strand cDNA Synthesis SuperMix (Transgen Biotech, Beijing, China) according to the instructions of the manufacturer. The cDNA was stored at –80 °C until further use.

Relative mRNA levels were assayed by quantitative real-time PCR (qPCR) method. The appropriate primers of each gene are listed in Table 1a (unpublished data) and Table 1b [23]. The real-time PCR reactions were carried out in a final volume of 20 μl, using 1 × Power SYBR Green PCR MasterMix buffer (ABI, USA) on a Step-one PCR amplifier (ABI, USA). Cycling parameters were as follows: 1 min at 94 °C, followed by 40 cycles of 10 s at 94 °C; 40 cycles of 20 s at 60 °C; and 40 cycles of 30 s at 72 °C. All assays were carried out in triplicate. After finishing the program, the comparative Ct values were obtained from each sample. The expression results were analyzed using the 2^{-ΔΔCt} method [35].

2.4. Statistical analyse

The results were expressed as the mean ± SD. All data were performed using One-way ANOVA and SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). If a significant difference was identified, differences among means were compared by Duncan's multiple range tests at P < 0.05.

Table 1b
Primer design for immune related genes in this study.

Primers	qPCR primers, forward/reverse (5'to3')
CAT	F: GCGTTTGGTTACTTTGAGGTGA R: GAGAAGCGGACAGCAATAGGT
MnSOD	F: TACGAGAAGGAGAGCGGAAGA R: ATACCGAGGAGGGGGATGA
Keap1	F: CCAGAAGGAATGTGTGGCTAAA R: TGGTTGGTCATCGGGTTGTA
TGF-β1	F: AACATCCGCTACCTCGCTT R: TCCGCTCATCTCATTCCT
IKKα	F: ACACCGACACAACGGCTCAT R: CCAGACGGCACAGTTTCACAG
MHC-2	F: CCACCCGAACAAACAGACC R: TGATGCCCTCCAACT
TLR3	F: TCTCCATTCCGTCACCTCC R: TCATCCAGCCGTTACTATCC
Caspase-3	F: CGCAAAGAGTAGCGACGGA R: CGATGCTGGGAAATTCAGAC
Caspase-9	F: TTTTCTGGTTATGTTTCGTGG R: TTGCTTGTAGAGCCCTTTGC
P53	F: GGCACCAACAAACAAAAAAC R: GTCAAGCAACTCCAGACCATCA
β-Actin	F: TACGAGCTGCCTGACGGACA R: GGCTGTGATCTCTCTCTGC

CAT, catalase; MnSOD, manganese superoxide dismutase; Keap1, Kelch-like-ECH-associated protein 1; TGF-β1, transforming growth factorβ1; IKKα, IκB kinase α; MHC-2, major histocompatibility complex 2; TLR3, toll-like receptor 3.

3. Results

3.1. In vitro

3.1.1. Cell viability and intracellular lipid content test

To confirm that RBE could mitigate the LE-induced decrease in cell viability, we pre-incubated hepatocytes for 48 h with 2 ml/L 20% LE and then incubated hepatocytes for 24 h using a range of RBE concentrations; next, the cell viability was analyzed (Fig. 1A). The results showed that hepatocytes viability of model group was $84.67 \pm 0.71\%$, and significantly decreased compared to control group ($P < 0.05$). Compared with the model group, the cell viability was higher in the recovery group, but it significantly decreased compared to RBE400 group ($P < 0.05$).

To quantify the intracellular lipid content, TG and CHOL contents in the cell lysis solution were detected (Fig. 1B). TG and CHOL contents demonstrated an obvious increase of the model group compared with the control group. Compared with the model group, TG and CHOL

contents were lower in the recovery group, whereas TG and CHOL were lowest contents in the RBE400 group ($P < 0.05$).

3.1.2. Hepatocytes structure examination

The hybrid grouper primary hepatocytes structure were examined after H.E. Staining (Fig. 2). In the control group, hepatic cells were arranged neatly with clear contour, and the cells nuclear were normal. In the model group, cell structure was destroyed, parts of the nucleoli of hepatocytes disintegrated, and vacuole formation was observed. In the recovery group, cell structure injury and nuclear hypertrophy had recovered a little, but the cell state is not very good. When the hepatocytes were treated with 3 different concentrations of RBE, different changes in cell structure were observed. In the RBE200 and RBE400 groups, cell structure injury was markedly decreased. However, in the RBE800 group, the cell showed disturbed architecture and swollen.

3.1.3. The expression of lipid metabolism and immune related genes

To determine the effect of RBE on the molecular metabolism of lipid accumulation, expressions of adipogenesis and lipolysis related genes are shown in Fig. 3. The mRNA levels of G6PD and ME1 were significantly up-regulated in model group compared to control group ($P < 0.05$). They were lower in recovery group compared to model group ($P < 0.05$). However, treatment with RBE 400 μg/ml significantly down-regulations the expression of G6PD, ME1 and FAS compared to recovery group ($P < 0.05$). Compared with the control, the mRNA expression levels of the lipolysis genes encoding LPL and PPARα were significantly decreased in the model group ($P < 0.05$). Gene expression of ATGL and PPARα were significantly increased in the recovery group compared to the model group. Compared with the recovery group, the mRNA expression of ATGL, LPL and PPARα were significantly up-regulated in the RBE200 and RBE400 groups ($P < 0.05$).

Immune-related genes mRNA levels in hybrid grouper primary hepatocytes were presented in Fig. 4. The model group exhibited significantly higher mRNA expression of caspase-3, caspase-9 and P53 compared with the control group, while the expression of caspase-3, caspase-9 and P53 were significantly down-regulated in the recovery group compared to the model group. Compared with the recovery group, the expression of caspase-3, caspase-9 and P53 in hepatocytes were significantly down-regulated in the RBE400 group ($P < 0.05$). Compared with the control group, model group down-regulated the mRNA levels of CAT and MnSOD, but up-regulated the mRNA levels of Keap1 ($P < 0.05$). There were no significant differences in CAT and MnSOD mRNA levels between model and recovery groups ($P > 0.05$). The decreased mRNA levels of CAT and MnSOD and increased mRNA

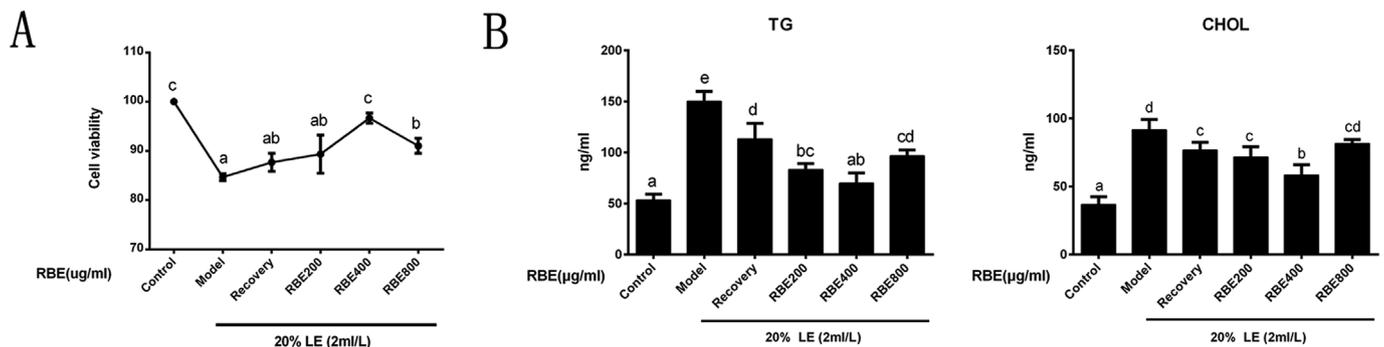


Fig. 1. Effects of Radix Bupleuri extracts (RBE) on the cell viability, TG and CHOL contents of the primary hepatocytes from hybrid grouper. (A) Cell viability assayed using Cell Counting Kit-8; the 20% lipid emulsion (LE) (2 ml/L) was used to pre-incubate hepatocytes for 48 h, and then, RBE (200, 400 and 800 μg/ml) were used to incubate the cells for 24 h. (B) The cell lysis solution was collected to determine the levels of TG and CHOL. Control: hepatocytes neither treated with 20% LE (2 ml/L) nor RBE; Model: hepatocytes treated with 20% LE (2 ml/L) alone for 72h; Recovery group: hepatocytes treated with 20% LE (2 ml/L) for 48h, then incubated with normal medium for 24 h; RBE(200, 400 and 800): hepatocytes were incubated with 20% LE (2 ml/L) for 48h, then post-incubated with 200, 400 and 800 μg/mL of RBE for 24 h. Cell viability was determined using the CCK-8 assay according to the absorbance value. Each experiment was repeated five times. Means in the same raw with different superscripts are significantly different ($P < 0.05$).

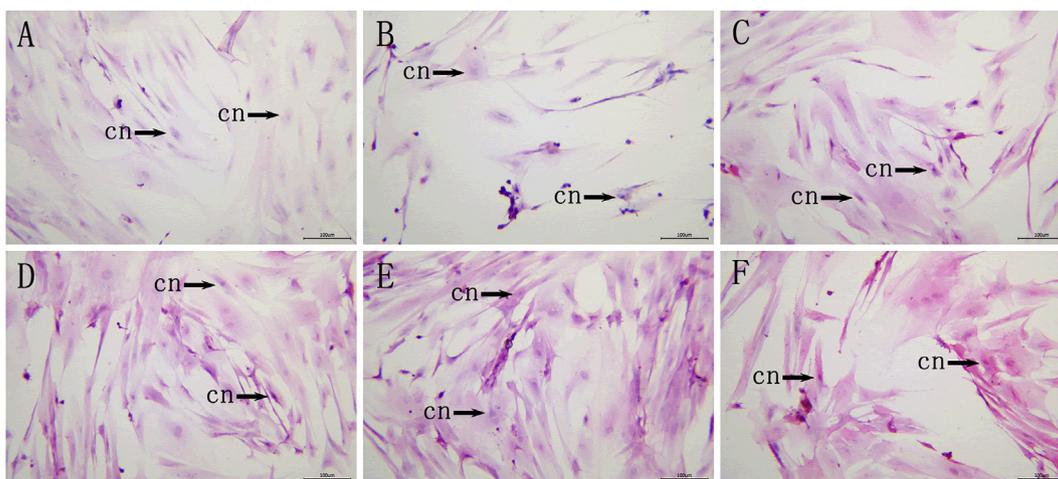


Fig. 2. Effect of Radix Buplei extracts on morphological changes of 20% LE-treated hepatocytes. Hematoxylin and eosin (H&E) staining was performed, and the cell morphology was observed under an inverted microscope (original magnification 200×). (A) Control: hepatocytes neither treated with 20% lipid emulsion (LE) (2 ml/L) nor RBE; (B) Model: hepatocytes treated with 20% LE (2 ml/L) alone for 72h; (C) Recovery group: hepatocytes treated with 20% LE (2 ml/L) for 48h, then incubated with normal medium for 24 h; (D) RBE (200 µg/ml) + 20% LE (2 ml/L); (E) RBE (400 µg/ml) + 20% LE (2 ml/L); (F) RBE (800 µg/ml) + 20% LE (2 ml/L); Each experiment was repeated three times. cn: cell nucleus; bar = 100 µm.

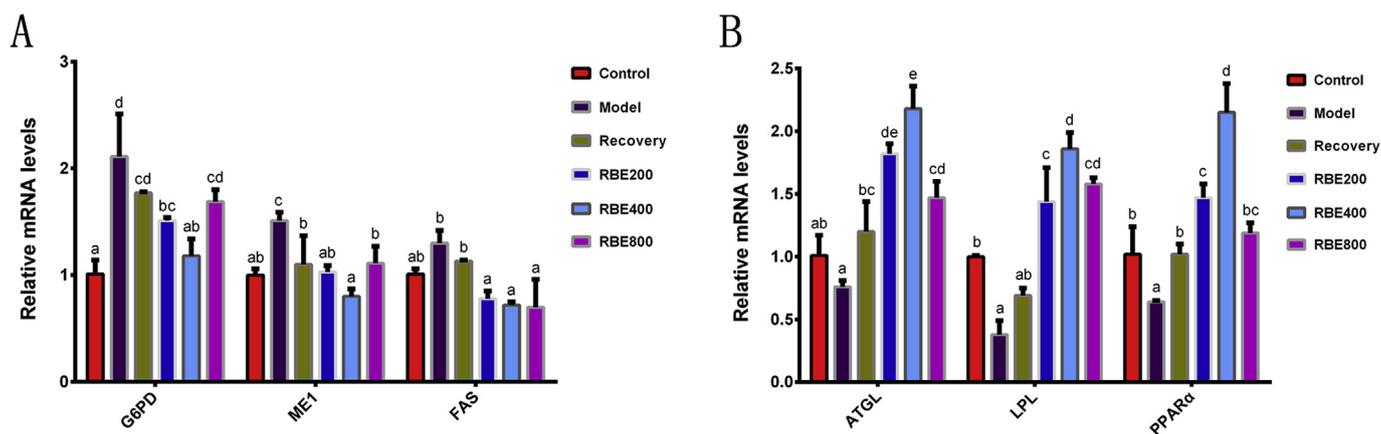


Fig. 3. Effect of Radix Buplei extracts on 20% LE-induced variation on the mRNA levels of genes involved in lipid metabolism in the primary hepatocytes of hybrid grouper. (A) adipogenesis genes (G6PD, ME1 and FAS), (B) lipolysis genes (ATGL, LPL and PPARα). After preincubation with the 20% lipid emulsion (LE) for 48 h, the cells were incubated with RBE for 24 h in MEM medium. Means in the same row with different superscripts are significantly different ($P < 0.05$). G6PD: glucose 6-phosphate dehydrogenase; ME1: malic enzyme 1; FAS: fatty acid synthase; ATGL: adipose triglyceride lipase; LPL: lipoprotein lipase; PPARα: peroxisome proliferator-activated receptor alpha.

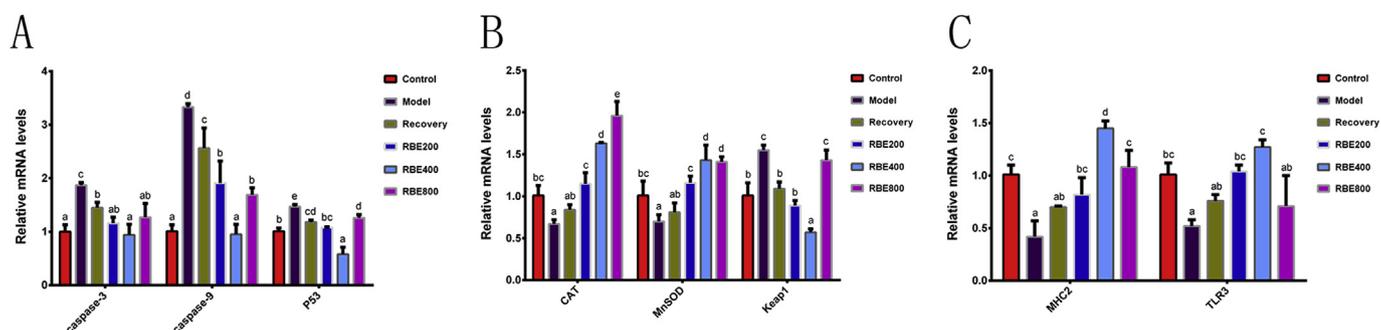


Fig. 4. Effect of Radix Buplei extracts post-treatment on hepatocytes mRNA expression of (A) apoptosis genes (caspase-3, caspase-9 and P53), (B) antioxidant enzyme genes (CAT, MnSOD and Keap1) and (C) immune genes (MHC2 and TLR3) against β-actin. Means in the same row with different superscripts are significantly different ($P < 0.05$). CAT, catalase; MnSOD, manganese superoxide dismutase; Keap1, Kelch-like- ECH-associated protein 1; MHC2, major histocompatibility complex 2; TLR3, toll-like receptor 3.

Table 2
Composition and nutrient levels of experimental diets (g/kg).

Ingredients	RBE0	RBE200	RBE400	RBE800	RBE1600
Fish meal	450.00	450.00	450.00	450.00	450.00
Soybean meal	180.00	180.00	180.00	180.00	180.00
Flour	200.00	199.80	199.60	199.20	198.40
Soybean oil	50.00	50.00	50.00	50.00	50.00
Fish oil	50.00	50.00	50.00	50.00	50.00
Beer yeast powder	20.00	20.00	20.00	20.00	20.00
Monocalcium phosphate	10.00	10.00	10.00	10.00	10.00
Lecithin	10.00	10.00	10.00	10.00	10.00
Choline chloride (50%)	5.00	5.00	5.00	5.00	5.00
Vitamin C	5.00	5.00	5.00	5.00	5.00
Vitamin and mineral premix	20.00	20.00	20.00	20.00	20.00
Radix Bupleuri extracts	0.00	0.20	0.40	0.80	1.60
Nutrient levels (%)					
Moisture	5.65	5.86	5.57	5.26	5.88
Crude protein	47.44	48.04	47.12	47.35	47.40
Crude lipid	14.95	14.90	14.94	14.91	14.88
Ash	12.49	12.13	12.08	12.11	12.14

Vitamin and mineral premix provided by Guangzhou Chengyi Aquatic Co., Ltd., China.

levels of Keap1 in the recovery group were reversed in the RBE groups ($P < 0.05$). Similar results were observed in the mRNA expression of MHC2 and TLR3, they were significantly down-regulated in the model group compared to the control group. There were no significant differences in MHC2 and TLR3 mRNA levels between model and recovery groups ($P > 0.05$). Compared with the recovery group, MHC2 and TLR3 mRNA levels were remarkably increased with treated RBE levels up to 400 $\mu\text{g}/\text{ml}$ ($P < 0.05$), and then decreased with levels higher than 400 $\mu\text{g}/\text{ml}$.

3.2. In vivo

3.2.1. Growth performance, feed utilization and morphological parameters

Effects of graded concentrations of dietary RBE on hybrid grouper growth performance, feed utilization and morphological parameters are presented in Table 3 and Table 4, respectively. The final body weight (FBW) and weight gain (WG) of fish in the RBE400 and RBE800 groups were significantly higher than that in the RBE0 group ($P < 0.05$). The feed efficiency (FE) in the RBE0 group was significantly lower than that of fish in the other experimental groups ($P < 0.05$), except for RBE1600 group ($P > 0.05$). The hepatosomatic index (HSI) and viscerosomatic index (VSI) of the RBE800 and RBE1600 groups were significantly lower than in the RBE0 group ($P < 0.05$).

3.2.2. Hematological parameters

The contents of alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), cholesterol (CHOL), lactate dehydrogenase (LDH) and triglycerides (TG) in serum were shown in Table 5. The highest AST, CHOL and LDH in serum were observed in RBE0 group, and it was significantly higher than those of the other groups ($P < 0.05$). ALT of RBE800 group was significantly lower than that observed in RBE0 group ($P < 0.05$).

Table 3
Effects of dietary Radix Bupleuri extracts (RBE) on growth performance and feed utilization in hybrid grouper.

Items	RBE0	RBE200	RBE400	RBE800	RBE1600
IBW(g)	25.60 \pm 0.10	25.63 \pm 0.25	25.57 \pm 0.25	25.49 \pm 0.08	25.59 \pm 0.34
FBW(g)	83.00 \pm 1.00 ab	87.33 \pm 2.08bc	88.62 \pm 4.66c	94.13 \pm 2.50d	80.46 \pm 2.05a
WG (%)	216.89 \pm 9.84a	235.68 \pm 9.34 ab	246.76 \pm 21.40b	269.32 \pm 9.33c	214.42 \pm 3.88a
FE	0.88 \pm 0.01a	0.98 \pm 0.05b	1.01 \pm 0.08b	1.02 \pm 0.05b	0.94 \pm 0.02 ab

Values are means \pm SD (n = 6) of three replications. Means in the same raw with different superscripts are significantly different ($P < 0.05$). IBW: initial body weight; FBW: final body weight; WG: weight gain rate; FE: feed efficiency.

3.2.3. Whole body and muscle composition

Whole body and muscle proximate compositions in the different experimental groups were shown in Table 6. There were no significant differences in moisture and protein contents of whole body among all groups ($P > 0.05$). Crude lipid contents of whole body and muscle significantly decreased as dietary RBE levels increased ($P < 0.05$). No significant differences were observed in muscle moisture and ash contents among all experimental treatments ($P > 0.05$).

3.2.4. Histology and histochemistry of the liver

Then liver histopathological characters were examined to further evaluate the effects of RBE on hybrid grouper (Fig. 5). Administration of RBE0 caused apparent histological changes in liver tissue, including the hepatocyte swelling and extensive vacuolization with the disappearance of nuclei. Liver histology was significantly improved by RBE supplementation, especially at the doses of 400–800 mg/kg. The RBE400 and RBE800 groups showed well-preserved hepatocytes and tissue architecture with less necrosis and inflammatory cell infiltration, the cell size recovered and became more and more uniform. However, the liver sections in RBE1600 group presented the fuzzy cell outline, inflammatory infiltration and the loss of hepatic architecture, which served as evidence of severe necrosis in liver.

By Oil red O staining (Fig. 6), the liver from the RBE0 group showed a higher occurrence rates lipid accumulation. However, RBE400 and RBE800 groups significantly reduced while RBE1600 tended to increased lipid accumulation in the liver.

3.2.5. Liver lipid metabolism and immune related gene

Liver tissues mRNA levels of lipid metabolism related genes are shown in Fig. 7. Acyl CoA diacylglycerol acyltransferase 2 (DGAT2), malic enzyme 1 (ME1) and diacylglycerol kinase alpha (DGK α) mRNA levels in fish were remarkably decreased with dietary RBE levels up to 400 mg/kg diet, and then sharply increased with RBE levels further increasing ($P < 0.05$). Compared with the RBE0 group, the mRNA levels of glucose 6-phosphate dehydrogenase (G6PD) were significantly down-regulated in fish fed dietary supplementation with RBE ($P < 0.05$). The mRNA levels of carnitine palmitoyltransferase 1 (CPT1) and lipoprotein lipase (LPL) were significantly up-regulated in the RBE200, RBE400 and RBE800 groups, compared to that of RBE0 group. Acyl-CoA oxidase 1 (ACO1) mRNA levels in fish were remarkably increased with dietary RBE levels up to 800 mg/kg diet. Compared with the RBE0 group, the mRNA levels of peroxisome proliferator-activated receptor alpha (PPAR α) were significantly up-regulated in fish fed dietary supplementation with RBE ($P < 0.05$), and no significant differences were found among 200–1600 mg/kg groups ($P > 0.05$).

Immune-related genes mRNA levels in the liver of fish were presented in Fig. 8. Compared with the RBE0 group, the RBE200 and RBE400 groups down-regulated the mRNA levels of caspase-9. The mRNA levels of CAT was found no significant differences when dietary supplementation with 0–800 mg/kg RBE diets ($P > 0.05$), and then increased with levels further increasing ($P < 0.05$). Dietary supplementation with RBE significantly increased MHC2 and IKK α mRNA levels in fish ($P < 0.05$), compared to those of the RBE0 group.

Table 4
Effects of dietary Radix Bupleuri extracts (RBE) on morphometric parameters in hybrid grouper.

Items	RBE0	RBE200	RBE400	RBE800	RBE1600
HSI (%)	3.52 ± 0.22b	3.11 ± 0.15 ab	2.82 ± 0.19a	2.77 ± 0.28a	3.01 ± 0.32a
VSI (%)	10.16 ± 0.15b	9.58 ± 0.27 ab	9.58 ± 0.08 ab	9.13 ± 0.47a	9.55 ± 0.05a
CF (g/cm3)	2.77 ± 0.34 ab	2.84 ± 0.10b	2.78 ± 0.12 ab	2.65 ± 0.05a	2.82 ± 0.08 ab

Values are means ± SD (n = 6) of three replications. Means in the same row with different superscripts are significantly different ($P < 0.05$). HSI: hepatosomatic index; VSI: viscerosomatic index; CF: condition factor.

4. Discussion

Because of plants extract have beneficial effects on farmed animals, such as pigs, poultry and fish, they have gained considerable attention in the feed industry as feed additives [36,37]. Many plants extract have been reported to favor various activities like growth promotion, antistress, appetite stimulation, immunostimulation and anti pathogen properties in aquaculture [38]. Several studies have demonstrated the powerful effect of medicinal plants to stimulate the immune system in fish against diseases [39]. Moreover, the advantages of plants extract being safer, eco-friendly and more cost effective than chemicals and synthetic drugs gave more encouragement for application in large scale aquaculture [40]. Radix Bupleuri extracts (RBE), a widely used traditional Chinese medicinal herb, possesses various pharmacological properties and comprehensive immunomodulatory action. Toxicity studies in mice and other animal models, as well as controlled human studies, have shown that appropriate concentrations RBE is safe and that there are no deleterious side effects [32]. In this study, we revealed the important role of RBE on growth, lipid deposition and metabolism and immune response in hybrid grouper using in vitro and in vivo models.

Previous reports showed that oleic acid, mixtures of oleic acid and linoleic acid, D-galactosamine/lipopolysaccharide and carbon tetrachloride could all be successfully established cell model [33,41,42]. Zhou et al. imitated a high-fat diet and successfully established a model of fatty degeneration in human L-02 hepatocytes induced by high concentrations of serum [43]. Similar results were found in hepatocytes of the grass carp after treatment with media containing lipid emulsion (LE) [44]. In our study, hybrid grouper hepatocytes were treated with 2 ml/L 20% LE to induce fatty degeneration. After 48 h, the results showed that a large number of lipid droplets accumulated in cells by inverted microscope observation and Oil Red O staining. On the other hand, lipid deposition occurs when there is an imbalance between adipogenesis and lipolysis [45]. Triglyceride (TG), a water-insoluble energy-rich lipid, acts as a short-term indicator of nutritional status, is an important lipid disposal pathway [46]. In the present study, TG content detection showed that the TG content markedly increased after exposed to 2 ml/L 20% LE in model group and that the hepatocytes morphological changes were synchronous with the changes in TG content. However, treatments of the hepatocytes with RBE (400 µg/mL) markedly decreased TG contents induced by LE. These results indicated that hybrid grouper hepatocytes steatosis and the characteristics TG increase can be successfully induced by 2 ml/L 20% LE and RBE could

inhibit lipid accumulation in hepatocytes.

In this study, hepatocytes morphological examination showed that LE caused cell morphological changes, including the disappearance of nuclei, cell membrane rupture and extensive vacuolization, which were all common apoptotic characteristics. To further clarify the hepatoprotective mechanism of RBE, we explored the effects of RBE on the hepatocytes mRNA expression of apoptosis, antioxidant and immune genes induced by LE. Some studies have demonstrated that hepatocyte apoptosis is an important element of liver damage and is related to the initiation and progression of the general immune inflammatory response [47]. Caspase activity is a useful marker for detecting stress-induced apoptosis of fish. There are two general pathways of apoptosis: the extrinsic death receptor pathway and intrinsic mitochondrial pathway [48]. The extrinsic death receptor pathway is directly activates the initiator caspase-8 by recognition of extracellular ligands with transmembrane receptors [49]. The intrinsic mitochondrial pathway is released of cytochrome c, and then active downstream effector caspase-9 and caspase-3. Caspases have been categorized as pro-apoptotic factors, in which caspase-3 activation in hepatocytes plays a central role in the initiation of apoptosis, resulting in fibrogenesis and eventually fibrosis in mammals and fish [50,51]. Previous studies reported some plants extract significantly decreased the activity of caspase-3 and attenuated mitochondrion-initiated apoptosis in the cell [23]. Similarly, our research have also demonstrated that RBE mitigates liver injury by inhibiting apoptosis in the D-GalN/LPS-induced liver injury of hybrid grouper [33]. In this study, serious hepatocytes steatosis were observed in the model group, combined with high caspase-3, caspase-9 and P53 genes expression in hepatocytes. After 24 h of RBE post-treated, caspase-3, caspase-9 and P53 mRNA levels in RBE (400 µg/mL) were significantly down-regulated, and we also observed that most of the hepatocytes in RBE (400 µg/mL) had normally morphological structure. These results suggest that RBE may act as a therapeutic agent for caspase-dependent apoptotic pathway which may be involved in LE induced apoptosis of hybrid grouper primary hepatocytes.

To our knowledge, antioxidant enzyme activities partly rely on antioxidant enzyme mRNA expression in mammal [52]. In rat, a high-fat diet can decrease antioxidant enzyme activities (such as MnSOD, CAT and GR), which may be partly ascribed to their down-regulated mRNA levels in the immune organs. The antioxidant system is crucial for fish to combat oxidative damage. Ni et al. reported that high levels of lipids could induce apoptosis and impair antioxidant system and decrease the immune function of fish [53]. In addition, previous studies in our lab indicated that dietary high levels of lipids could induce

Table 5
Effects of dietary Radix Bupleuri extracts (RBE) on hematological parameters in hybrid grouper.

Items	RBE0	RBE200	RBE400	RBE800	RBE1600
ALP (U/L)	73.50 ± 6.36	57.50 ± 6.36	59.00 ± 5.66	53.50 ± 3.54	65.50 ± 16.26
ALT (U/L)	2592.50 ± 126.57b	2206.50 ± 177.48 ab	2138.00 ± 149.91 ab	2027.00 ± 197.99a	2398.50 ± 265.17 ab
AST (U/L)	43.00 ± 2.83b	29.67 ± 5.51a	23.50 ± 0.71a	25.00 ± 1.41a	26.67 ± 5.51a
CHOL (U/L)	9.21 ± 0.58b	7.39 ± 0.42a	7.56 ± 0.52a	7.71 ± 0.15a	8.13 ± 0.21a
LDH (U/L)	325.00 ± 21.21b	209.50 ± 19.09a	215.50 ± 19.09a	250.00 ± 24.04a	252.50 ± 2.12a
TG (U/L)	1.72 ± 0.09 ab	1.35 ± 0.35 ab	1.26 ± 0.25a	1.56 ± 0.10 ab	1.94 ± 0.36b

Values are means ± SD (n = 6) of three replications. Means in the same row with different superscripts are significantly different ($P < 0.05$). ALP: alkaline phosphatase; ALT: alanine transaminase; AST: aspartate transaminase; CHOL: cholesterol; LDH: lactate dehydrogenase; TG: triglycerides.

Table 6

Effects of dietary Radix Bupleuri extracts (RBE) on whole body and muscle proximate composition in hybrid grouper.

Items	RBE0	RBE200	RBE400	RBE800	RBE1600
Whole body(%)					
Moisture	66.75 ± 2.41	68.40 ± 0.94	68.50 ± 2.30	67.23 ± 1.03	70.35 ± 0.85
Crude protein	17.70 ± 0.23	17.42 ± 0.45	17.23 ± 0.88	17.80 ± 0.32	16.86 ± 0.32
Crude lipid	8.87 ± 0.18b	7.47 ± 0.47a	7.05 ± 0.80a	7.30 ± 0.35a	7.01 ± 0.17a
Ash	4.83 ± 0.02c	4.65 ± 0.08 ab	4.59 ± 0.03a	4.70 ± 0.02b	4.59 ± 0.05 ab
Muscle(%)					
Moisture	76.67 ± 0.43	76.74 ± 0.36	76.76 ± 0.17	76.96 ± 0.62	77.73 ± 0.04
Crude protein	20.30 ± 0.27 ab	20.30 ± 0.13 ab	20.23 ± 0.17 ab	20.53 ± 0.43b	19.85 ± 0.15a
Crude lipid	2.91 ± 0.08c	2.57 ± 0.08bc	2.39 ± 0.26b	1.72 ± 0.26a	1.63 ± 0.19a
Ash	1.13 ± 0.04	1.16 ± 0.06	1.16 ± 0.14	1.21 ± 0.05	1.11 ± 0.03

Values are means ± SD (n = 6) of three replications. Means in the same row with different superscripts are significantly different (P < 0.05).

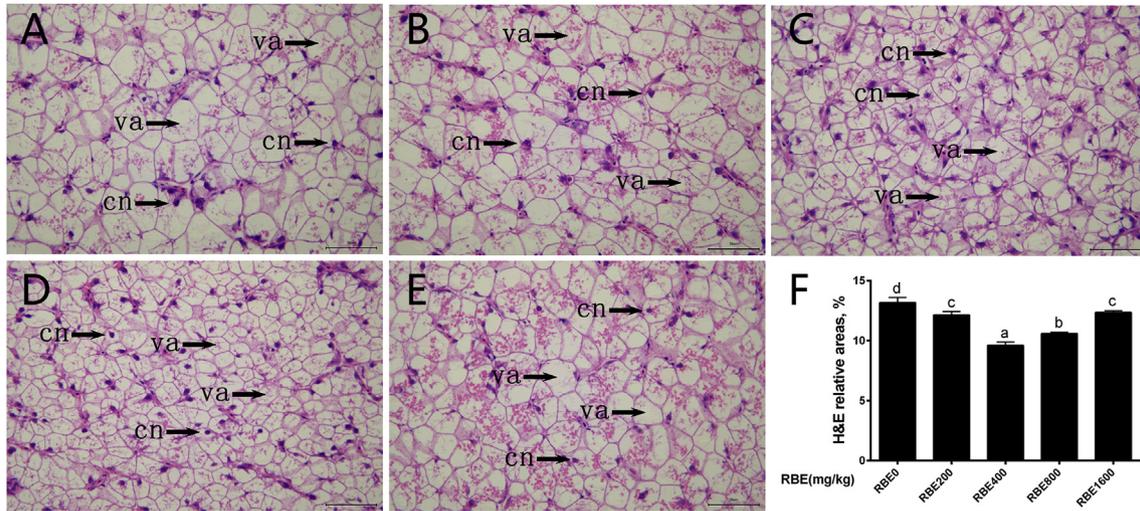


Fig. 5. Effects of Radix Bupleuri extracts diet on the liver histology (hematoxylin and eosin staining, original magnification 400 ×) of hybrid grouper after 8 weeks. (A) Liver from fish fed 0 mg/kg RBE (RBE0); (B) Liver from fish fed 200 mg/kg RBE (RBE200); (C) Liver from fish fed 400 mg/kg RBE (RBE400); (D) Liver from fish fed 800 mg/kg RBE (RBE800); (E) Liver from fish fed 1600 mg/kg RBE (RBE1600); (F) Relative areas for hepatic vacuoles in H&E staining. Means in the same row with different superscripts are significantly different (P < 0.05). cn: cell nucleus; va: vacuolation. bar = 50 μm. All slides are 400 × magnification.

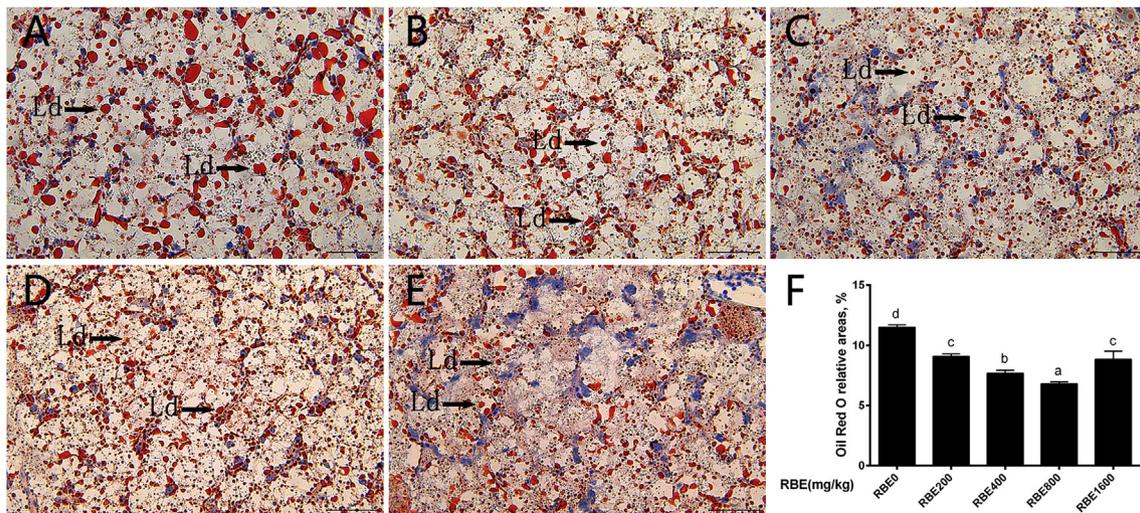


Fig. 6. Effects of Radix Bupleuri extracts diet on the liver histochemistry (Oil Red O staining, original magnification 400 ×) of hybrid grouper after 8 weeks. (A) Liver from fish fed 0 mg/kg RBE (RBE0); (B) Liver from fish fed 200 mg/kg RBE (RBE200); (C) Liver from fish fed 400 mg/kg RBE (RBE400); (D) Liver from fish fed 800 mg/kg RBE (RBE800); (E) Liver from fish fed 1600 mg/kg RBE (RBE1600); (F) Relative areas for lipid droplets in Oil Red O staining. Lipids appear red, and nuclei appear blue after staining with Oil Red O. Means in the same row with different superscripts are significantly different (P < 0.05). Ld: lipid droplet. bar = 50 μm. All slides are 400 × magnification.

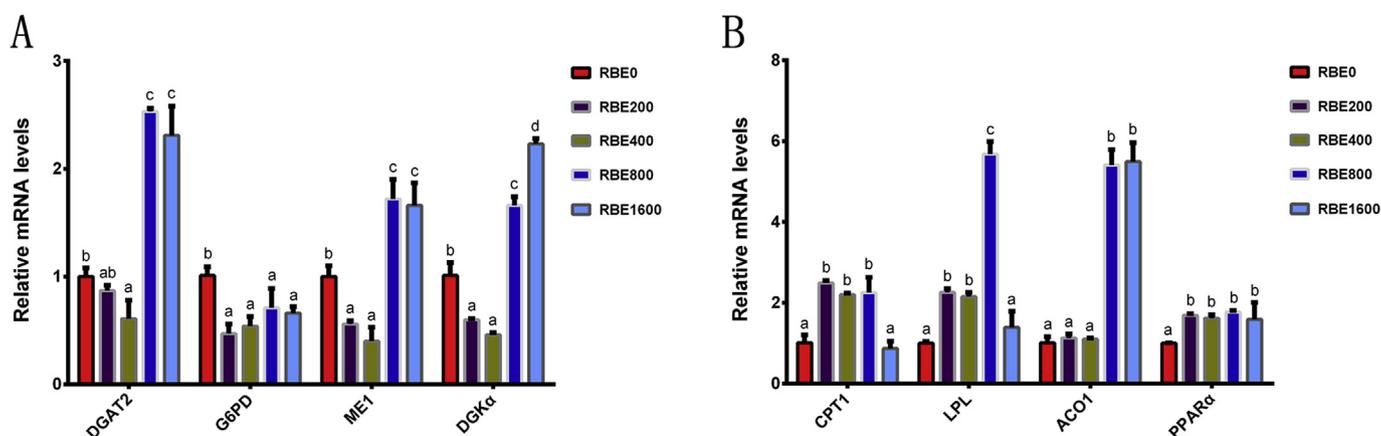


Fig. 7. The expression of genes (A and B) involved in lipid metabolism in the liver of hybrid grouper fed diets varying in *Radix Bupleuri* extracts concentration for 8 weeks. The results are presented as the mean ± SD of three replications. Means in the same row with different superscripts are significantly different ($P < 0.05$). DGAT2: acyl CoA diacylglycerol acyltransferase 2; G6PD: glucose 6-phosphate dehydrogenase; ME1: malic enzyme 1; DGKα: diacylglycerol kinase alpha; CPT1: carnitine palmitoyltransferase 1; LPL: lipoprotein lipase; ACO1: acyl-CoA oxidase 1; PPARα: peroxisome proliferator-activated receptor alpha.

oxidative damage in the liver of golden pompano [54]. Hence, we further examined the effects of RBE on antioxidant system in the hepatocytes of hybrid grouper. In this study, compared with control group, model group reduced CAT and MnSOD contents and increased the Keap1 activities in hepatocytes. However, RBE increased the attenuated levels of CAT and MnSOD mRNA expression, implicating that the protective effect of RBE might be associated with its enhancing antioxidant ability in fish.

High-fat diet could trigger inflammatory responses and decrease the immune function and normal structure of fish immune organs, which finally suppressing resistance to disease in fish [54]. MHC2 molecules can stimulate B cells by microbial antigens and may play a role in transducing signals to B cells by affecting T cell function [55,56]. TLR3 play an important role in fish immune system, and it can recognize and activate innate immunity when pathogens invading. Tan et al. reported that dietary supplementation with ginkgo biloba leaf extract significantly increased MHC2 and TLR3 mRNA levels in hybrid grouper [23]. Similarly, in our present study, MHC2 and TLR3 mRNA levels were significantly increased in hepatocytes treatment with RBE.

These results implied that RBE could suppressed lipid accumulation and enhanced immune response in primary hepatocytes of hybrid grouper induced by LE. However, the underlying mechanism by which RBE influences in fish is still unknown and needs further study.

Additionally, in support of the in vitro study, we assessed the effect of RBE on lipid storage and immune capacities in liver tissue induced by high-lipid diets with an in vivo study. Growth enhancement is a trait of particular interest in aquaculture, as it is inherently linked to the productivity and profitability of enterprises. Several plants extract have

been tested for their growth promoting activity in aquatic animals. Tan et al. observed that dietary supplementation of *Panax notoginseng* extract increased the growth rate in hybrid grouper [57]. Giri et al. showed that guava leaves could promote growth of *Labeo rohita* [58]. Dietary dandelion extract and hawthorn extract were reported to have positive effects on growth rate in hybrid grouper [54,59]. Similar results were observed using emodin can increased both food intake and final weight of *Labeo rohita* [60]. The present study showed that diets supplemented with a relatively low dose (400 and 800 mg/kg, respectively) of RBE resulted in increased growth performance compared to RBE0 group, but higher level (1200 mg/kg) resulted in decreased growth performance in hybrid grouper. So, overdose of RBE will produce negative effect on the growth performance of animals. Liu et al. reported that *Radix Bupleuri* exhibits liver toxicity by taking a large dose for a long period [61]. Similarly, previous studies reported that saikosaponins induce the hepatotoxicity through causing liver cell damage and oxidative damage mechanism [62,63]. Moreover, the diets supplemented with RBE is useful for improving the feed efficiency of hybrid grouper and the groups RBE200, RBE400 and RBE800 had better effects. The improved fish growth performance and feed utilization were likely attributable to RBE possessing many bioactive components, which have effects of growth promotion and appetite stimulation. At present, few reports exist on the effect of RBE treatment on culture experiment of fish, which makes comparison rather difficult. Due to the complexity of components in the plants extract, the reasons of growth-promoting were not clear until now and should be further researched in the future.

Fed high lipid diets may leads to excessive fat deposition in the

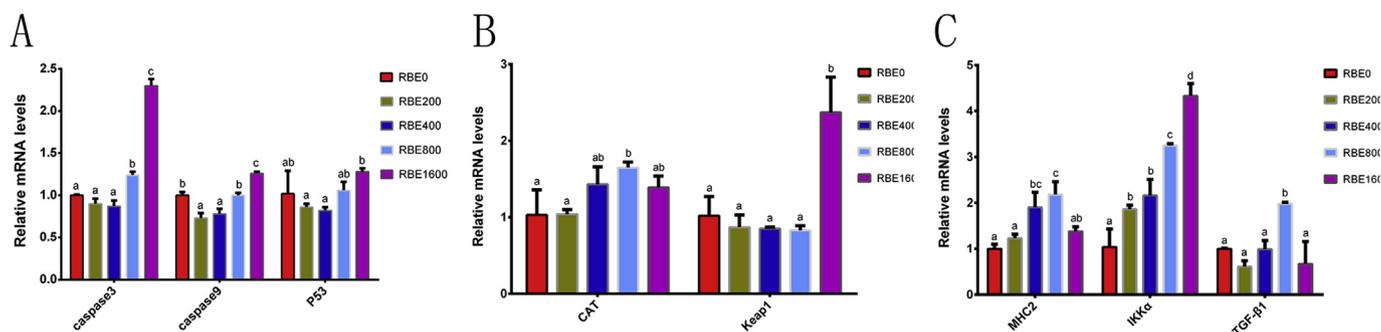


Fig. 8. The relative mRNA expression of the (A) apoptosis genes (caspase-3, caspase-9 and P53), (B) antioxidant enzyme genes (CAT and Keap1), (D) immune inflammation genes (MHC2, IKKα and TGF-β1) in the liver of hybrid grouper, determined by RT-qPCR. The results are presented as the mean ± SD of three replications. Means in the same row with different superscripts are significantly different ($P < 0.05$). CAT, catalase; Keap1, Kelch-like- ECH-associated protein 1; MHC2, major histocompatibility complex 2; IKKα, IκB kinase α; TGF-β1: transforming growth factor β1.

visceral cavity and tissues [6]. Wang et al. reported that large yellow croaker was significantly increased fat accumulation in the liver when fed high lipid diets [64]. Similarly, in this study, the whole body and muscle lipid deposition were significantly increased by high-dietary lipid level. However, dietary supplementation with 400–800 mg/kg RBE diets significantly decreased whole body and muscle lipid contents in fish. In addition, dietary RBE showed have marked influences on morphological parameters (such as HSI and VSI). This indicated that dietary adding RBE can improved lipid deposition in fish.

Serum biochemical parameters reflects physical and chemical changes occurring in organisms, which is widely used in clinical diagnosis of fish physiology to determine the general status of health [65]. In our study, the serum alanine transaminase (ALT) and aspartate transaminase (AST) activities were higher in fish fed the RBE0 diet than dietary supplementation with 800 mg/kg RBE diet. Blood serum enzymes ALT and AST primarily exist in liver cells under normal conditions [66]. Once liver cells are damaged, both ALT and AST are transferred through the cell membrane into the serum, thus resulting in a significant increase in the serum levels and indicating organ dysfunction in aquatic organisms during stress condition [67]. Often their values are used in estimating the health and condition of fish [68]. Cholesterol (CHOL), lactate dehydrogenase (LDH) and triglycerides (TG) activities in the serum of fish are considered important diagnostic characters [69]. According to Mensinger et al., CHOL levels can indicate disorders of lipid and lipoprotein metabolism and especially liver dysfunction [70]. In many previous studies, CHOL has also been used as a commonly used diagnostic tool for the health status of farmed fish [71]. Increasing blood TG levels in fish is always considered to be signs of declining health status of teleosts [72]. As in human studies, TG are mainly synthesized in the liver and stored in fatty tissues, thus they are often used in fish studies as a indicator of liver disease. Previous study showed that fed high lipid diets significantly increased serum TG contents in hybrid grouper [23]. Similarly, results of the present study showed that RBE had hypolipidemic effect in hybrid grouper, thus suggesting its clinical potential.

Liver is the key organ for metabolic processes, playing a critical role in nutrients digestion, metabolism and storage and normal hepatic function can be affected by various factors [73]. Hepatic lipid accumulation is considered wasteful, as it represents a diversion of energy from growth and results in the reduction of the edible yield, low feed efficiency, and poor growth [74]. This pathological condition is deleterious to the health of farmed fish [75]. Tan et al. found that there were hepatocyte swelling, hepatocyte vacuolization, and nuclei shifting to the cellular periphery cytoplasmic vacuolization, in hybrid grouper fed the high-fat diets [23]. The magnitude of pathological lesion was reversed in dietary supplementation with ginkgo biloba leaf extract (GBE) treated animals [23]. In another study, anomalies such as the hepatocyte vacuolization, and nuclei shifting to the cellular periphery cytoplasmic vacuolization were observed in fish fed oxidized fish oil [76]. Our results were supported by histopathological examination that fish fed RBE200, RBE400 and RBE800 showed regular hepatocyte morphology and polygonal cells possessed round nucleus with prominent nucleolus. However, a higher occurrence rates of the amount of cytoplasmic vacuolation, nuclear hypertrophy, parts of the nucleoli of hepatocytes disintegrated, and the loss of hepatic architecture were observed in the hepatocytes of fish fed RBE0 and RBE1600 in the H&E samples. These observations were further confirmed by the Oil Red O stained for lipid droplets in the liver samples. The amount of lipid droplets in the liver was reduced with increasing dietary RBE concentrations (up to 800 mg/kg) compared to RBE0 group, but higher inclusion level (up to 1600 mg/kg) resulted in increased lipid droplets in the liver of hybrid grouper. The dietary RBE addition reduced the amount of hepatic lipid droplets. He et al. reported that saikosaponin a could significantly decrease low-density lipoprotein (ox-LDL)-induced Lipid uptake, cholesterol efflux, immune-inflammatory response in THP-1 cells [77]. However, to our knowledge, no study to date has

investigated the effects of dietary RBE reduces hepatic lipid accumulation in fish, which makes comparison rather difficult. Based on these results, we consider the alterations in the nucleus and hepatocyte vacuolization observed in our fish to be a hepatic lesion due to fat overload in RBE0 group.

Lipids play a key role in growth and development in fish. However, excessive body fat deposition causes many adverse effects, thus posing serious threats to the sustainable development of aquaculture [78]. Dietary RBE supplementation reduced the lipid content in the liver of hybrid grouper. To investigate the mechanism for the variation of lipid accumulation as a response to RBE addition, the mRNA expression of genes related to adipogenesis and lipogenic were analyzed in fish. DGAT2 has been proven to catalyses the final and only committed step in the biosynthesis of TAG [79]. G6PD and ME1 play a predominant role in generating NAD(P)H, which is indispensable for lipogenesis [80]. Previous studies showed that a high-fat diet significantly up-regulated lipogenesis-related genes mRNA expression in the liver of yellow catfish [81]. The results from our study showed that DGAT2, G6PD, ME1 and DGK α mRNA expression significantly down-regulated with the supplement of RBE (RBE200 group and RBE400 group) compared with the RBE0 group, indicated that treated RBE could inhibit the lipogenic rate, which in turn reduced lipid deposition in the liver of hybrid grouper.

On the other hand, in this study the mRNA levels of lipolytic genes such as CPT1, LPL, ACO1 and PPAR α were determined to further confirm the relationship between RBE and lipid metabolism. In vertebrates, CPT I is located on the inner side of the outer mitochondrial membrane and it is the main regulatory enzyme in fatty acid oxidation because it catalyses the conversion of fatty acid-CoAs to fatty acyl-carnitines [82]. Then, fatty acyl-carnitine entry into the mitochondrial matrix and converted back to fatty acyl-CoA by the enzyme CPT II, thus, CPT I is thought to be a major regulating step in mitochondrial fat oxidation [83]. The high-lipid diets can significantly down-regulated the gene expression of CPT1 mRNA in blunt snout bream and large yellow croaker, indicated that supplemented with high-lipid diets may suppressed lipolysis [84,85]. The results from our study showed that dietary RBE significantly up-regulated the mRNA levels of CPT I in the liver of fish, compared with the RBE0 group. Similarly, dietary supplementation with silymarin increased the mRNA levels of CPT I [86]. The up regulation of LPL expression corresponded well with the enhancement of the mRNA abundances of the lipolytic gene CPT1. Lipoprotein lipase (LPL) hydrolyzes triacylglycerols and supplies free fatty acids for storage in adipose tissue, or for oxidation in other tissues, and plays a pivotal role in regulating lipid catabolic metabolism and lipogenesis [87]. LPL determined how dietary lipids were partitioned toward storage or utilization, and it was considered as a key rate-limiting enzyme in the provision of tissue fatty acids [88]. Our study also indicated that treated RBE up-regulated PPAR α mRNA levels. The increased lipolytic genes mRNA expression in the present study might indicate an increase in import of lipids from liver to nearby tissues for energy mobilization, which, in turn, would reduce hepatic lipid deposition. Thus, these data suggest that RBE has positive effects on improving lipolysis and fatty acid oxidation of the liver in fish fed with a high-fat diet. Additionally, we used primary hepatocytes of hybrid grouper to explore the signaling pathways by which RBE influences lipid metabolism. The down regulation of lipogenesis and the up regulation of lipolysis following RBE treatment were observed in the present in vitro study, in agreement with our in vivo study.

In conclusion, the present study provides novel evidence that RBE can suppress lipid accumulation by down-regulating lipogenesis and up-regulating lipolysis genes transcriptional expression of hybrid grouper both in vitro and in vivo. In addition, RBE improves health status via hepatoprotective effects and enhance immune response of fish induced by high lipid intake. These results might help researchers better understand the complex regulatory mechanism in lipid metabolism and provide useful information for dietary interventions to inhibit

excessive lipid accumulation and improve the health of fish.

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