Fish and Shellfish Immunology 91 (2019) 293–305

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Xiaochaihu Decoction reduces hepatic steatosis and improves D-GalN/LPS-induced liver injury in hybrid grouper (*Epinephelus lanceolatus*♂ × *Epinephelus fuscoguttatus*♀)

Cuiyun Zou, Minglei Xu, Leling Chen, Qingying Liu, Yuanyuan Zhou, Zhenzhu Sun, Huaqun Ye, Ningning Su, Chaoxia Ye*, Anli Wang**

Institute of Modern Aquaculture Science and Engineering, Guangzhou Key Laboratory of Subtropical Biodiversity and Biomonitoring, Guangdong Provincial Key Laboratory for Healthy and Safe Aquaculture, School of Life Science, South China Normal University, Guangzhou, 510631, China

ARTICLE INFO

Keywords:
Fatty liver diseases
Xiaochaihu decoction
Hybrid grouper
D-GalN/LPS

ABSTRACT

Excessive lipid accumulation and chemical abuse can induce fatty liver diseases in fish, but the underlying mechanism and therapies are unknown. The present study aims to evaluate the effects of Xiaochaihu Decoction (XCHD) on the growth performance, lipid metabolism and antioxidant function of hybrid grouper *in vitro* and *in vivo*, and provide evidence as to whether it can be potentially used as a medicine for liver diseases in aquaculture. *In vitro*, steatosis model of hybrid grouper primary hepatocytes were incubated for 48 h in control or lipid emulsion (LE)-containing medium with or without 24 h post-treatment with XCHD. XCHD treatment reversed the LE-induced intracellular lipid accumulation, cell viability and hepatocytes morphological structure. *In vivo*, a total of 300 hybrid grouper with an average initial weight of 25.43 ± 0.18 g were fed diets containing five graded levels of XCHD at 150–1200 mg/kg diet for 8 weeks. After that, a challenge trial was conducted by injection of D-GalN/LPS to induce liver injury. As a result, dietary supplementation with 150–300 mg/kg XCHD diets can significant improve growth performance and feed utilization ($P < 0.05$). Dietary XCHD down-regulated the expression of lipogenic-related genes (G6PD, DGAT2 and ME1) and up-regulated lipolysis-related genes (ATGL, PPARα and LPL) expression in the liver of hybrid grouper. Livers challenged with D-GalN/LPS exhibited extensive areas of vacuolization with the disappearance of nuclei and the loss of hepatic architecture. These pathological alterations were ameliorated by XCHD treatment. XCHD significantly down-regulated the D-GalN/LPS induced apoptosis-related genes caspase-3, caspase-9 and p53 mRNA expression and up-regulated the antioxidant-related genes CAT and MnSOD mRNA expression in dose dependent manner, respectively. XCHD potently reduced hepatic lipid accumulation and enhanced antioxidant capability in hybrid grouper and may be a potential fish-feed additive to prevent fatty liver diseases onset and progression.

1. Introduction

In most fish species, lipids are essential nutrient, both as the source of energy and essential fatty acids for normal growth and development [1]. In recent years, high-lipid diets have increasingly been used for cost-effective farming in aquaculture [2]. High dietary lipids have growth-promoting effect in short time, however, it often leads to excessive lipid accumulation in the livers (or other tissues), inducing a condition referred to as fatty liver diseases [3,4]. The liver is a major lipid storage organ, it plays important role in the health of fish [5]. Fatty liver diseases is a pathological condition because it is deleterious to the health of farmed fish [6]. It often lead to low feed efficiency, poor growth and reduction of the edible yield [7]. Fatty liver diseases are a major problem in aquaculture causing heavy loss to fish farmers. In addition, in an attempt to control diseases, antibiotics and chemotherapeutics have been generally used in the fish culture [8]. That can result in a series of adverse effects for the cultured species, the environment and the final consumer, such as the development of a drug-resistant bacteria, environmental pollution, residues in fish [5]. According to Li et al., fatty liver diseases also occurs when fish are abused chemical drugs [9]. Therefore, it is necessary to seek safe and secure disease-preventative measures to ensure the sustainability of aquaculture. Although studies have been carried out for decades, the underlying mechanisms related to nutrition and drug/chemical-induced

* Corresponding author.
** Corresponding author.
E-mail addresses: 316903556@qq.com (C. Ye), wanganl@scnu.edu.cn (A. Wang).

https://doi.org/10.1016/j.fsi.2019.05.025
Received 31 January 2019; Received in revised form 16 April 2019; Accepted 13 May 2019
Available online 15 May 2019

liver injury are not fully understood. There are no effective therapeutic strategies or specific medicines for these liver diseases.

The application of herbals has a long history in our country and it is widely used in the treatment of diseases. Many Chinese medicinal herbs have been used for the treatment of liver diseases in China [10–12]. They have the advantages of having little side effects, absence of bioaccumulation being easily degradable, causing disease resistance, and being easily available [13]. In aquatic animals, growing interests have also been paid to the use of Chinese medicinal herbs for controlling the liver diseases in recent years [14]. Xiaochaihu Decoction (XCHD) is an important multiherbal medicine in traditional Chinese medicine. It was first described in the *Shang Han Lun*, a treatise on exogenous febrile diseases by the famous physician Zhang Zhongjing, who lived from 150 to 219 A.D. during the Chinese Eastern Han Dynasty [15]. XCHD is composed of seven herbs including *Radix bupleuri*, *Radix scutellariae*, *Radix glycyrrhizae*, *Radix ginseng*, *Rhizoma pinelliae*, *Rhizoma zingiberis recens*, and *Fructus jujubae* [16]. Radix Bupleuri accounted for the largest proportion in preparations of Xiaochaihu Decoction, which is nearly three times as heavy as the other single herbs. Therefore, it can be said that Radix Bupleuri is the most important component in XCHD. Previous study showed that the XCHD contains considerable active components such as saikosaponin, baicalin, polysaccharide, ginsenoside, glycyrrhizin, and so on [17]. XCHD is widely used to treat chronic liver diseases [18]. In addition, many studies in animal models or cell lines have demonstrated that XCHD has cytoprotective effects in experimental liver injuries [19,20]. It also has an anti-inflammatory effect on chronic hepatitis which may decrease hepatic fibrosis via the inhibition of hepatic stellate cells and prevent lipid peroxidation in mammalian hepatocytes [21]. However, the effects of XCHD against liver diseases in aquatic animals and its associated mechanisms particularly at the molecular level, are scarce in fish. This is the first time to use the *in vitro* and *in vivo* models to study the hepatoprotective effects of XCHD in fish.

The hybrid grouper, *Epinephelus fuscoguttatus* × *Epinephelus lanceolatus* is a commercially important cultivated marine teleost in the Southeast Asia [22]. It has several advantages than its original type as an aquaculture species especially in terms of faster growth, high disease resistance and high tolerance to low salinity water [23]. Although they are relatively expensive, but there is a high demand for it [24]. When a shift from natural to artificial diets occurs, lipid deposition is a very frequent finding. However, excessive hepatic lipid deposition in fish may affect disease resistance, the quality of harvest and induce liver disease [2]. Liver disease in fish had become one of the major limiting factor for the sustainable development of the species in the world [25]. Studies about the liver diseases are important, not only to clarify the mechanisms underlying this disorder, but also from a commercial point of view. In order to further elucidate possible hepatoprotective mechanisms of Chinese medicinal herbs in fish, steatosis and liver damage models induced by high-lipid diets and D-GalN/LPS in hepatocytes and hybrid grouper were established. We explored the molecular mechanisms by Chinese medicinal herbs influences lipid metabolism and antioxidant response *in vitro* and *in vivo*. Therefore, the objectives of the present study were to evaluate the efficacy and tolerance of dietary XCHD by its effects on growth performance, lipid metabolism, antioxidant response, apoptosis and hepatosis in hybrid grouper. We also aimed to explore XCHD's potential as a feed additive in aquaculture.

2. Materials and methods

2.1. In vitro study

Hepatocyte primary isolation and culture was carried out as previously described [26]. Hepatocytes were cultured in MEM medium (Gibco, Thermo Fisher, Suzhou) supplemented with 10% fetal bovine serum (FBS V/V, Sigma) and 100 IU mL^{-1} of each penicillin and streptomycin (Sigma Chemical Company, St. Louis, MO), then maintained at 25 °C in an incubator, 5% CO2 atmosphere. After 10–15 d, cells were counted using Trypan Blue (Gibco, Life Technologies, USA) to confirm that cell viability exceeded 92% for subsequent experimentation. To determine the effects of Xiaochaihu Decoction (XCHD) influencing lipid metabolism and hepatoprotective, *in vitro* experiment was performed. The hybrid grouper primary hepatocytes received 1 of 6 treatments: control, model, recovery, XCHD100, XCHD200 and XCHD400. Control group (hepatocytes neither treated with 2 ml/L 20% lipid emulsion (LE) nor XCHD), model group (hepatocytes treated with 2 ml/L 20% LE alone for 72 h), recovery group (hepatocytes treated with 2 ml/L 20% LE for 48 h, then incubated with normal medium for 24 h) and XCHD (100, 200 and 400) groups (hepatocytes were incubated with 2 ml/L 20% LE for 48 h, then post-incubated with 100, 200 or 400 μg/mL of XCHD for 24 h) were set for experiment. Sampling occurred after 72 h of treatment. 20% lipid emulsion (LE) was purchased from Panyu Armed Police Hospital. XCHD (40:1) was purchased from NANJING DASF BIO-TECHNOLOGY CO., LTD.

2.2. In vivo study with dietary XCHD addition

2.2.1. Diet preparation and experimental procedures

The composition of experimental diets are presented in Table 2. Five experimental diets were prepared with XCHD supplemental levels at 0, 150, 300, 600 and 1200 mg/kg. Then, all diets were air-dried at room temperature (25–30 °C) and stored at −20 °C until used. Hybrid grouper were obtained from Marine Fisheries Development Center of Guangdong Province (Huizhou, China) and transported to the outdoor cement ponds (10 m × 3 m × 1 m) with running water and continuous aeration. At the beginning of the feeding experiment, 300 fish (25.43 ± 0.18 g) were randomly distributed into 15 floating cages (three cages for each treatment). The floating cages were rectangular polyvinylchloride cages (L100 cm × W100 cm × H70 cm). The experimental feed consisted of five diets and were coded as XCHD0, XCHD150, XCHD300, XCHD600 and XCHD1200. The fish were reared under natural photoperiod for 56 days. The water temperature ranged from 24 to 28 °C over the course of the feeding experiment.

At the end of the growth trial, fish from each cage were fasted for 24 h, then were batch weighed. Eighteen fish for each treatment (6 fish from each cage) were randomly selected and anaesthetized with MS222

Table 1a
Primer sequences for real-time PCR.

Primers	qPCR primers, forward/reverse (5′to3′)
DGAT2	F: CATCTTCTGCTTTGGTGCTTTC
	R: GCATTTCCCGTCCCGTTA
FAS	F: CGGGTGTCTACATTGGGGTG
	R: GAATAGCGTGGAAGGCGTTT
G6PD	F: GCTTCACATCCTTGTATCTGCTC
	R: GCGTTCCTTTCATTCTCCG
ME1	F: GAAGTTGTTCTACCGCTTGCTG
	R: AGAGTCCTCGTGGTCTCCTGA
ATGL	F: ATTGAGCACCTTCCACCCA
	R: CCGAATCCATCCCACATCTT
LPL	F: TTCAACAGCACCTCCAAAACC
	R: GTGAGCCAGTCCACCACGAT
ACO1	F: CGGCATGGACTTCCTGTATG
	R: CCTGGTGTGCGTGTTGTGTT
PPARα	F: CATCGACAATGACGCCCTC
	R: GCCGCTATCCCGTAAACAAC
β-Actin	F: TACGAGCTGCCTGACGGACA
	R: GGCTGTGATCTCCTTCTGC

F: forward primer; R: reverse primer. DGAT2, acyl CoA diacylglycerol acyltransferase 2; FAS, fatty acid synthase; G6PD, glucose 6-phosphate dehydrogenase; ME1, malic enzyme 1; ATGL, adipose triglyceride lipase; LPL, lipoprotein lipase; ACO1, acyl-CoA oxidase 1; PPARα, peroxisome proliferator-activated receptor alpha.

Table 1b
Primer sequences for real-time PCR.

Primers	qPCR primers, forward/reverse (5′to3′)
CAT	F: GCGTTTGGTTACTTTGAGGTGA
	R: GAGAAGCGGACAGCAATAGGT
MnSOD	F: TACGAGAAGGAGAGCGGAAGA
	R: ATACCGAGGAGGGGGATGA
Keap1	F: CCAGAAGGAATGTGTGGCTAAA
	R: TGGTTGGTCATCGGGTTGTA
Caspase-3	F: CGCAAAGAGTAGCGACGGA
	R: CGATGCTGGGGAAATTCAGAC
Caspase-9	F: TTTTCCTGGTTATGTTTCGTGG
	R: TTGCTTGTAGAGCCCTTTTGC
P53	F: GGCACCAAACAAACCAAAAAAC
	R: GTCAAGCAACTCCAGACCATCA
β-Actin	F: TACGAGCTGCCTGACGGACA
	R: GGCTGTGATCTCCTTCTGC

F: forward primer; R: reverse primer. CAT, catalase; MnSOD, manganese superoxide dismutase; Keap1, Kelch-like-ECH-associated protein 1.

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

Ingredients	XCHD0	XCHD150	XCHD300	XCHD600	XCHD1200
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	199.85	199.70	199.40	198.80
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
Xiaochaihu Decoction	0.00	0.15	0.30	0.60	1.20
Nutrient levels (%)					
Moisture	5.67	5.38	6.07	6.08	5.31
Crude protein	47.71	47.69	46.94	47.83	47.37
Crude lipid	15.27	15.17	15.31	15.30	14.90
Ash	12.31	12.09	12.11	12.00	12.13

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

(100 mg L^{-1}). The body weight, body length, liver and viscera weight were recorded individually to calculate condition factor (CF), hepatosomatic index (HSI), and viscerosomatic index (VSI), respectively. Blood samples were drawn from the caudal part of the sedated fish in each group. Two liver samples in each cage were collected to hematoxylin and eosin staining and Oil Red O staining. The six fish livers from each floating cage was excised, fast frozen in liquid nitrogen for RNA isolation. All samples were stored at −80 °C until analysis.

2.2.2. Challenge trial

After the growth trial, ten fish were picked out randomly from each cage for the challenge test. Fish were arbitrarily divided into six groups. Then, fish were injected by intraperitoneal injection with D-GalN (500 mg/kg, Yuanye Biologigal, Shanghai, China) and LPS (20 μg/kg, Sigma, USA) in sterile phosphate buffered saline (pH 7.4). Control group (without feeding XCHD) were given the same volume injection of sterile saline only. Model group (without feeding XCHD) received D-GalN/LPS and served as toxic control. XCHD groups were prophylactically treated with XCHD at a dose of 150, 300, 600 or 1200 mg/kg each, respectively, and then received D-GalN/LPS. After 24 h of D-GalN/LPS induced hepatotoxicity, blood and liver samples were collected.

2.3. Sample analysis

2.3.1. Measurement of cell viability and hepatocytes function test

Hepatocytes were seeded into 96-well plates, and 2 ml/L 20% LE or XCHD (appropriate concentrations) was added 72 h later. Then, 10 μl of CCK-8 (ABP Biosciences, Virginia,USA) was added, and the cells were incubated for 2–4 h. The microplate reader (Thermo, MA, USA) was used to determine the OD450. Proliferation inhibition rate (%) = experiment well A450/control well A450 × 100%. Each experiment was repeated five times.

The cells under the different treatment conditions were collected, 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 triglycerides (TG) and cholesterol (CHOL) were determined according to the manufacturer's instructions (TG: Zhicheng Biological Technology, Shanghai, China; CHOL: Beckman Coulter, Suzhou, China).

2.3.2. The hepatocytes morphological structure

Hematoxylin and eosin (H&E) staining was carried out to detect the degree of hepatocyte steatosis and injury. Hepatocytes grown in 6-well plates were collected, washed with phosphate buffered saline (PBS), immobilized with 4% paraformaldhyde for 20 min, washed in 0.1 M PBS four times for 2 min each time. Then, dyed with hematoxylin and eosin for 3–5 min, immersed with isopropanol for 15s-1min, and then pictured with microscope (200 × , MshOt MS60). Tests were performed in triplicates.

2.3.3. Growth performance and morphometric parameters

The fish in each cage were weighed and counted at the beginning and the end of the experiment. Six fish per cage were randomly selected for proximate analysis. The fish were dissected to calculate the weight gain (WG), feed efficiency (FE), condition factor (CF), hepatosomatic index (HSI) and viscerosomatic index (VSI). The formulae were calculated as following:

Weight gain (WG, %) = 100 × (final body weight-initial body weight)/initial body weight;

Feed efficiency (FE) = wet weight gain (g)/dry feed intake (g);
Condition factor (CF, g/cm^3) = 100 × (body weight, g)/(body length, $cm)^3$;

Viscerosomatic index (VSI, %) = 100 × (viscera weight, g)/(whole body weight, g);

Hepatosomatic index (HSI, %) = 100 × (liver weight, g)/(whole body weight, g).

2.3.4. Chemical analysis

Whole body and muscle tissue were sampled for moisture, protein, lipid and ash contents analyse. The moisture was analyzed by drying the samples to a constant weight at 105 °C. Crude protein (N × 6.25) was determined using a Kjeldahl (FOSS 8400, Hoganos, Sweden) by the Kjeldahl method after acid digestion. Crude lipid was analyzed through ether extraction using Soxtec™ 2055 (FOSS, Hoganos, Sweden). Ash was analyzed in a muffle furnace (FO610C, Yamato Scientific Co., Ltd., Tokyo, Japan) at 550 °C for 24 h.

2.3.5. Serum biochemical analysis

Blood samples were centrifuged at 3000 rpm for 15 min at 4 °C to obtain serum. Hematological parameters, including serum alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), cholesterol (CHOL), lactate dehydrogenase (LDH), triglycerides (TG), Cholinesterase (CHE) and total biliary acid (TBA) levels were

determined by standard spectrophotometric procedures in Guanzhou First People's Hospital.

2.3.6. Liver histological and histochemical analyses

Liver specimens were dissected and fixed immediately in 4% neutral buffered formalin after the fish were sacrificed. After the fixation, the liver samples were dehydrated in ethanol, embedded in paraffin, sectioned via hematoxylin and eosin (HE) stained. Formalinfixed (not embedded in paraffin) and frozen fresh samples were cut in cryostat for intracellular lipid detection by staining with Oil Red O. The relative area of vacuolation and lipid droplets were analyzed by Image-Pro Plus 6.0.

2.3.7. RNA isolation and gene expression analysis

Total RNA was extracted from hepatocytes (or liver tissue) using Trizol reagent (Invitrogen, Carlsbad, CA), and complementary DNA (cDNA) was obtained using a RT-PCR kit (Transgen Biotech, Beijing, China) according to the manufacturer's instructions. Relative mRNA levels were assayed by quantitative real-time PCR (qPCR) method. The primers used in this study are listed in Table 1a [27] and Table 1b [28]. SYBR dyes were purchased from YEASEN (Yeasen Biotech Co., Ltd., Shanghai, China) for real-time RT-PCR detection. The program was set to run for one cycle at 94 °C for 1 min, 40 cycles at 94 °C for 10 s, 40 cycles at 60 °C for 20 s and at 72 °C for 30 s. The relative amounts of mRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method [29].

2.4. Statistical analyse

Statistical analyses were carried out using SPSS version 17.0 software. Results are expressed as mean ± SD. The differences between different groups were analyzed using one-way analysis of variance (ANOVA). P value < 0.05 was considered statistically significant.

3. Results

3.1. In vitro

3.1.1. Effect of XCHD on LE-induced hepatocytes steatosis

To determine the cytotoxic effect, we used CCK-8 to detect the viabilities of primary hepatocytes treated with different concentrations of XCHD (Fig. 1A). The results showed that with 50–400 μg/ml of XCHD were non-toxic to hepatocytes. However, XCHD at doses over 800 μg/ml reduced cell viabilities. Thus, we chose 100, 200 and 400 μg/ml XCHD as the low-, medium-, and high-dose groups for next experiments. To confirm that XCHD could mitigate the lipid emulsion (LE) induced decrease in hepatocytes viability, we pre-incubated hepatocytes for 48 h using 2 ml/L 20% LE and then with a range of XCHD concentrations incubated hepatocytes for 24 h; next, the cell viability was analyzed (Fig. 1B). The results showed that XCHD significantly reversed the LE-induced decrease in hepatocytes viability.

The cell lysis solution of both triglycerides (TG) and cholesterol (CHOL) increased in model group to 2.1-fold and 1.6-fold, respectively, compared with those in the control group (Fig. 1C). However, pretreatment with XCHD attenuated these increases.

As showed in Fig. 2, hepatocytes hematoxylin and eosin (H&E) staining was performed to judge the hepatocytes structure of fatty degeneration. There were remarkable differences between the normal and model groups. The normal hepatocytes had almost no cell structure injury and vacuole formation were observed in the model group. Hepatocyte swelling and hepatocyte vacuolization were slightly improve in recovery group compared to model group. The cell structure injury relieved significantly in XCHD treated groups compared with recovery group. The relief of XCHD200 group was more obvious than those of the other two groups.

3.1.2. Effect of XCHD on the expression of genes related to lipid metabolism

The mRNA levels of lipid metabolism related genes in hepatocytes were determined by quantitative real-time PCR (Fig. 3). Adipogenesis related genes (G6PD, ME1, FAS) mRNA levels in the model groups were 1.67, 1.97 and 1.30 times higher, respectively, than in the control group ($P < 0.05$). Compared with the model group, the ME1 and FAS mRNA levels were decreased in the recovery groups ($P < 0.05$), and the G6PD mRNA levels showed no significant changes in these two groups ($P > 0.05$). However, the mRNA expression of G6PD, ME1 and FAS were clearly suppressed in the XCHD200 group, compared to the recovery groups ($P < 0.05$). There were no significant differences in ATGL and PPARα mRNA levels among control, model and recovery groups ($P > 0.05$). But the mRNA levels of ATGL and PPARα in hepatocytes showed an increasing trend with XCHD levels up to 200 μg/ml. The mRNA levels of LPL were significantly down-regulated in model group, compared with the control group ($P < 0.05$). In the recovery group, the expression of LPL was significantly higher than in the model group ($P < 0.05$). Whereas the mRNA levels of LPL was significantly higher in the XCHD post-treatment groups than in the recovery group ($P < 0.05$).

3.2. In vivo

3.2.1. Growth performance, feed utilization and morphological parameters

Results of growth performance, feed utilization and morphometric parameters for hybrid grouper fed different experimental diets for 8 weeks were shown in Table 3 and Table 4, respectively. Weight gain (WG) of fish in the XCHD150 and the XCHD300 groups were significantly higher than that of fish in the XCHD0 group ($P < 0.05$). Fish in the XCHD0 group had the lower feed efficiency (FE) values than that of fish in the other experimental groups ($P < 0.05$), except XCHD1600 group ($P > 0.05$). There was no significant difference in hepatosomatic index (HSI) among all groups ($P > 0.05$).

3.2.2. Whole body and muscle composition as well as serum parameters

The effects of dietary XCHD on whole body and tissue composition were shown in Table 5. Fish in the XCHD600 group contributed to reduced whole-body and muscle lipid levels ($P < 0.05$). The crude protein contents of whole-body and muscle in the XCHD300 and XCHD600 groups were significantly higher than that of fish in the XCHD0 group ($P < 0.05$). No significant differences were obtained in moisture and ash contents in whole body and muscle ($P > 0.05$).

There were significant differences in alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), cholesterol (CHOL) and triglycerides (TG) concentrations among the treatment groups (Table 6). ALP and AST of fish in the XCHD0 group was significantly higher than that of fish in the XCHD300, XCHD600 and XCHD1200 groups ($P < 0.05$). ALT and CHOL of fish in the XCHD300 and XCHD600 groups were significantly lower than that of fish in the XCHD0 group ($P < 0.05$). The lowest TG contents were observed of fish in the XCHD300 group, they were significantly lower than those of the other groups ($P < 0.05$).

3.2.3. Histology and histochemistry of the liver

Findings of histopathological changes in liver of fish fed XCHD diets are shown in Fig. 4. The results showed the XCHD0 group displayed hepatocyte swelling, hepatocyte vacuolization and inflammatory cell collection. However, compared to that of the XCHD0 group, dietary supplementation with XCHD significantly improved vacuolation areas ($P < 0.05$), especially with the 300 and 600 mg/kg dose XCHD.

The dietary XCHD addition reduced the amount of hepatic lipid droplets (Fig. 5A–E). These observations were further confirmed by the areas quantified for lipid droplets in the Oil Red O-stained samples (Fig. 5F). Quantification of the lipid content in the liver of fish in the XCHD300 and XCHD600 groups were significantly lower than those in the XCHD0 group ($P < 0.05$).

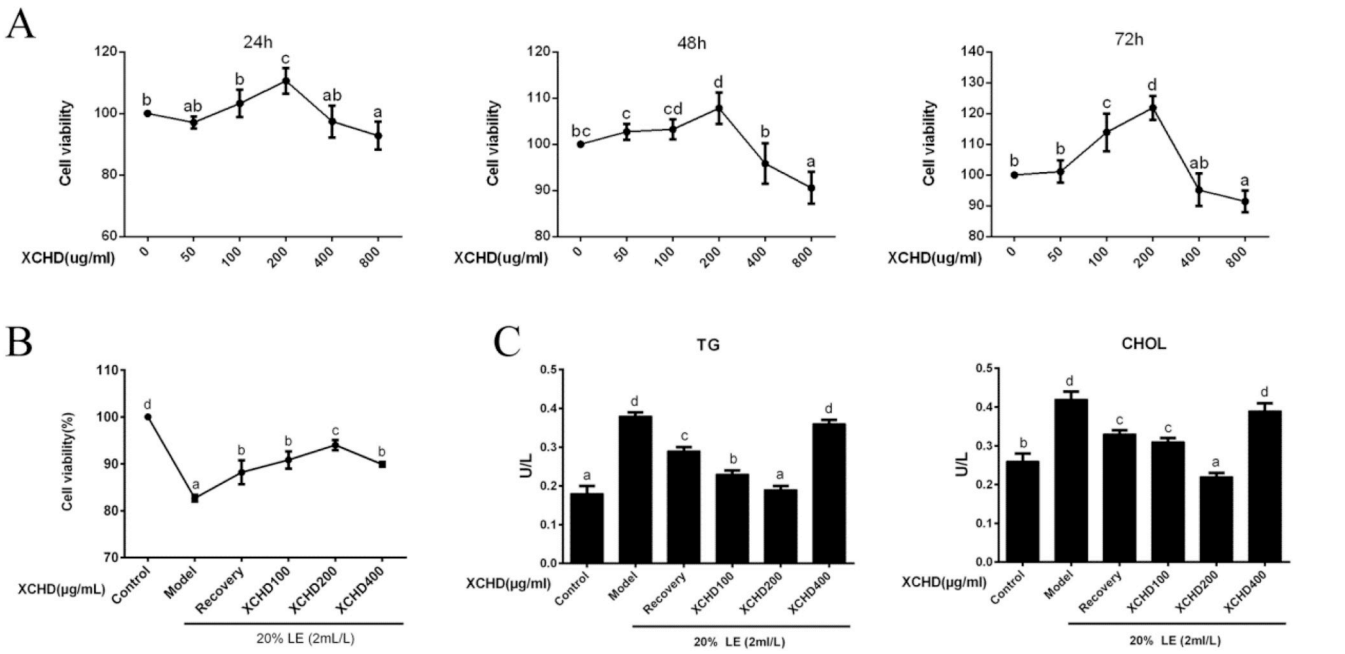

Fig. 1. Effects of XCHD on the cell viability and biochemical parameters of the primary hepatocytes from hybrid grouper. (A) Hepatocytes were incubated with XCHD (50, 100, 200, 400 and 800 μg/ml) for 24 h, 48 h and 72 h. (B) Lipid emulsion (LE) (2 ml/L) was used to pre-incubate hepatocytes for 48 h, and then, XCHD (100, 200 and 400 μg/ml) was used to incubate the cells for 24 h. (C) Effects of XCHD on the cell lysis solution TG and CHOL contents in 20% LE-induced hepatocytes. Control: hepatocytes neither treated with 20% LE (2 ml/L) nor XCHD; Model: hepatocytes treated with 20% LE (2 ml/L) alone for 72 h; Recovery: hepatocytes treated with 20% LE (2 ml/L) for 48 h, then incubated with normal medium for 24 h; XCHD (100, 200 and 400): hepatocytes were incubated with 20% LE (2 ml/L) for 48 h, then post-incubated with 100, 200 and 400 μg/mL of XCHD for 24 h. Cell viability was determined using the CCK-8 assay according to the absorbance value. Fig. 1A and B experiments were repeated five times. Fig. 1C experiments were repeated three times. Means in the same raw with different superscripts are significantly different ($P < 0.05$).

3.2.4. Liver lipid metabolism related gene

Considering the important relationship between liver and lipid metabolism, we further investigated the effect of XCHD on the transcriptions of adipogenesis and lipolysis related genes (Fig. 6). The results showed the mRNA expression of adipogenesis-related genes (G6PD, DGAT2 and ME1) were up-regulated in the XCHD0 group, while concomitant with the improvement of steatosis, their expression reduced in the XCHD300, XCHD600 and XCHD1200 groups. In contrast, lipolysis-related genes (ATGL, PPARα and LPL) transcription were notably up-regulated by XCHD treating.

3.2.5. Challenge trial

Table 7 summarized the effect of XCHD and subsequent treatment of D-GalN/LPS on the serum enzymatic in the experimental animals. The parameters were abnormally altered due to D-GalN/LPS exposure. The levels of alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH) and total biliary acid (TBA) in serum increased significantly ($P < 0.05$) in model group, compared with the control group. This trend was inhibited by the addition of XCHD, and from the results, XCHD at the concentration of 300 mg/kg reduced the levels of these serum indices more than the other concentrations.

The extent of liver damage caused by D-GalN/LPS toxicity was clearly evident through the hematoxylin-eosin staining in the histopathological studies (Fig. 7A and B). Extensive hepatocellular damage in the model group could be described through the presence of hepatocyte vacuolization and deformed cellular margins. The signs of hepatic damage were also visible in the XCHD treated groups, however the extent of damage was much lower than the model group. This demonstrated amelioration of D-GalN/LPS induced liver damage by the XCHD.

The mRNA levels of liver caspase-3, caspase-9, P53, catalase (CAT), manganese superoxide dismutase (MnSOD) and Kelch-like-ECH-associated protein 1 (Keap1) are presented in Fig. 7C. The hepatic levels of caspase-3, caspase-9 and P53 mRNA in control animals were low, but

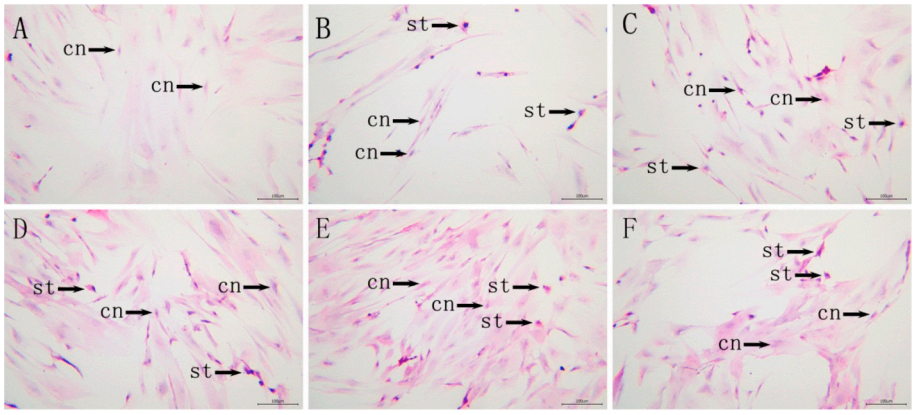

Fig. 2. Hematoxylin and eosin (H&E) staining to visualize the morphology of hepatocytes. (A) Control: hepatocytes neither treated with 20% LE (2 ml/L) nor XCHD; (B) Model: hepatocytes treated with 20% LE (2 ml/L) alone for 72 h; (C) Recovery: hepatocytes treated with 20% LE (2 ml/L) for 48 h, then incubated with normal medium for 24 h; (D) XCHD100: XCHD (100 μg/ml) + 20% LE (2 ml/L); (E) XCHD200: XCHD (200 μg/ml) + 20% LE (2 ml/L); (F) XCHD400: XCHD (400 μg/ml) + 20% LE (2 ml/L); Each experiment was repeated three times. cn: cell nucleus; st: structure injury; bar = 100 μm. All slides are 200× magnification.

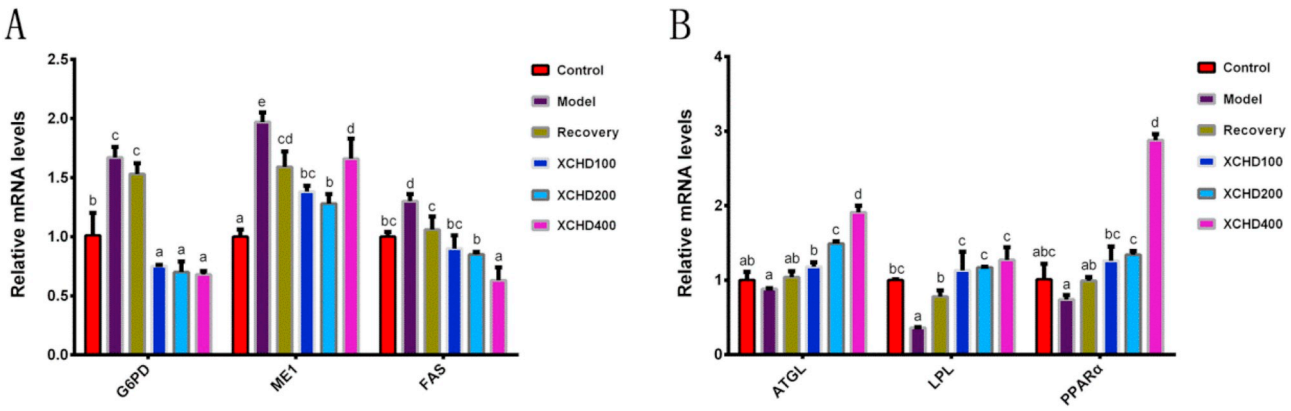

Fig. 3. XCHD suppressed lipogenesis while enhancing lipolysis in 20% LE-treated hepatocytes. (A) Adipogenesis genes (G6PD, ME1 and FAS) and (B) lipolysis genes (ATGL, LPL and PPARα) mRNA levels were measured by qRT-PCR. After pre-incubation with the 20% LE for 48 h, the cells were incubated with XCHD for 24 h in MEM medium. Each experiment was repeated three times. Means in the same raw 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.

24 h after D-GalN/LPS injection, the levels of caspase3, caspase9 and P53 mRNA expression increased to 1.51-, 3.58- and 1.3-fold, respectively, compared with those in the control group. These increases were attenuated by pretreatment with XCHD. CAT and MnSOD showed a significant reduce 24 h after D-GalN/LPS injection to 0.61 and 0.47 times, respectively, compared those of the control group. These reduces were increased by XCHD pretreatment.

4. Discussion

Liver plays a pivotal role in metabolism and detoxification of endogenous and exogenous hepatotoxicants [30,31]. Recently, fish fatty liver diseases have been frequently reported and caused dramatic economic loss in China [32,33]. It was proposed that high-lipid diets and chemicals drug abuse may be the important causes of the disease [34]. So far, no effective methods have been found for the treatment for fatty liver, and much attention has been focused on the use of medicinal herbs to prevent and control this disease [35,36]. Our previous study showed that Radix Bupleuri extracts (RBE) have significantly reduce lipid accumulation effect and hepatoprotective in fish (unpublished data). Importantly, Radix Bupleuri is usually used as monarch drug in Xiaochaihu Decoction (XCHD) prescriptions [11]. The XCHD has been widely used for several centuries as a folk medicine to ameliorate hepatitis in China [37], whereas there have been no studies on the hepatoprotective effect of this medicinal herbs in fish. Therefore, in the present investigation, the hepatoprotective effects of XCHD were studied using a lipid emulsion (LE)-induced steatosis model in primary cultured hybrid grouper hepatocytes and a D-GalN/LPS-induced hepatic damage model in hybrid grouper which were fed high lipid diets.

Lipid emulsion (LE) could be successfully used for cell model construction. Previous reports showed that fish hepatocytes treated with different concentration gradients of LE can induce fatty degeneration [38]. The present study represents the first attempt to use the *in vitro* model of LE-induced hepatocyte steatosis to study the hepatoprotective effects of XCHD in fish. The levels of TG and CHOL in the LE model group were significantly different from those in the control group, indicating that hepatocytes had accumulated a lot of lipid. In general, TG accumulation depends on the balance of hepatic lipid formation and usage [39]. Similarly, previous study showed that hepatic triglycerides had markedly accumulated in the treated fatty degenerated hepatocytes of the grass carp *Ctenopharyngodon idellus* [40]. The hepatocytes morphological changes were synchronous with the changes in TG and CHOL contents. This observation indicated that hybrid grouper hepatocytes fatty degeneration and the characteristics TG and CHOL increase can be successfully induced by 20% LE. As expected, our results confirmed that XCHD reduced LE-induced hepatocytes steatosis in a dose-dependent manner, as evidenced by its restoring the cell viability, reducing the lipid accumulated and improving the cell morphology. The hepatocytes morphological structure strongly support these results; LE caused various histological changes to the hepatocytes, including multiple cell structure injury and karyopyknosis. These alterations were attenuated by XCHD post-treatment with the affected hepatocytes showing only mild necrosis and mild cell structure injury. Our results suggest that XCHD may have potential applications for treating liver diseases in fish.

Many studies have shown that hepatic steatosis is tightly associated with lipid homeostasis [41]. Under normal physiological conditions, there is a balance between the lipogenic and lipolysis of lipid, however, the blocking lipid of the liver lipolysis is responsible for hepatic lipid accumulation [42]. In addition to test lipid contents increase, our study also provides evidence that treatment with XCHD could effectively regulate the expression of certain lipid metabolism-related genes in hepatocytes. At the molecular level, we found that XCHD significantly down-regulated the expression of G6PD, ME1 and FAS, the key genes involved in adipogenesis in hepatocytes. Reduced lipogenic enzymatic expressions indicated that treatment XCHD could inhibit the lipogenic rate, which in turn reduced lipid deposition in hepatocytes. Thus, the reduction in lipid content for XCHD groups were the consequences of

Table 3
Effects of dietary Xiaochaihu Decoction (XCHD) on growth performance and feed utilization in hybrid grouper.

Items	XCHD0	XCHD150	XCHD300	XCHD600	XCHD1200
IBW(g)	25.42 ± 0.26	25.44 ± 0.31	25.37 ± 0.09	25.39 ± 0.17	25.53 ± 0.14
FBW(g)	78.91 ± 0.74a	85.89 ± 2.45bc	89.50 ± 2.12c	82.09 ± 4.12 ab	81.93 ± 4.34 ab
WG (%)	210.50 ± 3.55a	237.56 ± 5.76bc	252.62 ± 6.59c	222.12 ± 14.93 ab	219.91 ± 16.41 ab
FE	0.90 ± 0.01a	0.95 ± 0.07b	0.99 ± 0.02c	1.01 ± 0.04c	0.93 ± 0.01 ab

Values are means ± 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.

Table 4
Effects of dietary Xiaochaihu Decoction (XCHD) on morphometric parameters in hybrid grouper.

Items	XCHD0	XCHD150	XCHD300	XCHD600	XCHD1200
HSI (%)	3.32 ± 0.19	2.99 ± 0.15	3.35 ± 0.08	3.21 ± 0.11	3.33 ± 0.31
VSI (%)	10.19 ± 0.04a	10.18 ± 0.11a	10.78 ± 0.04b	10.44 ± 0.19 ab	10.35 ± 0.50 ab
CF (g/cm3)	2.98 ± 0.11b	2.75 ± 0.09a	2.82 ± 0.08 ab	2.90 ± 0.07 ab	2.86 ± 0.05 ab

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

decrease in the mRNA expression of genes encoding lipogenic enzymes. Additionally, the mRNA levels of three pivotal genes involved in lipolysis, including ATGL, LPL and PPARα, were significantly up-regulated in hepatocytes. Previous evidences indicated that glycerol mainly originated from the hydrolysis of triglycerides [43]. ATGL is the rate-limiting enzyme that catalyzes the first step of TG hydrolysis to diacylglycerol and fatty acids [44]. The suppressed ATGL expressions may reflect a decrease in fatty acids from intracellular TG for oxidation and export [45]. In fish, it was reported that dietary lipid levels could affect LPL expression, lipid catabolic metabolism and lipogenesis [46,47]. LPL acts on the triacylglycerols to release fatty acids that are transported to the adipose tissue for storage or to other organs for oxidation [48]. PPARα is the key transcription factors that are involved in lipid metabolism, which plays key roles in the regulation of catabolism of fatty acids [49]. It activates lipid catabolism by regulating the expression of target genes encoding enzymes in the liver [50]. Kim et al. found that, PPARα mRNA is generally up-regulated by a high-fat diet in mammals, which is in contrast to the current study [51]. Our study showed that PPARα mRNA concentrations increased with increasing addition XCHD concentrations. The up regulation of PPARα expression corresponded well with the enhancement of the mRNA abundances of the lipolytic gene ATGL and LPL. Thus, it was possible that treatment XCHD might increase PPARα expression and up-regulate ATGL and LPL mRNA concentrations and stimulate lipolysis, which in turn reduces lipid deposition. Taken together, our results indicated that XCHD reduced the expression of lipid biosynthesis genes and enhanced the expression of key lipolysis-related genes, which affect lipid metabolism in the hepatocytes of hybrid grouper.

In fish, the *in vitro* model of lipid emulsion (LE)-induced hybrid grouper hepatocyte steatosis is established and applied in the present study for the evaluation of the hepatoprotective effects of XCHD. However, the effects of dietary XCHD on growth performance, serum biochemical parameters, liver histology, and lipid metabolism, apoptosis and antioxidant-related genes expression of hybrid grouper are unknown. So, a basal diet supplemented with XCHD at 0, 150, 300, 600 and 1200 mg/kg was fed to hybrid grouper for 8 weeks, and then a challenge trial was conducted by injection of D-GalN/LPS. Some studies have shown that suitable dietary lipid requirement of hybrid grouper was estimated to be 7–13% and that higher lipid levels in the diets induce negative effects on growth and body lipid deposition [52,53]. The lipid content was significantly increased in the whole body and liver when dietary lipids were increased to 15%, which was supported by the findings of Tan et al. in the same fish species [54]. Bou et al. and Guo et al. reported that high-lipid diet intake lead to an unwanted lipid accumulation in tissue [55,56]. Increases in lipid accumulated also increase the occurrence of fatty liver diseases [57]. Fatty liver diseases not only suppress growth but also affects the health of fish [2]. Like other fish, hybrid grouper fed high-lipid diets showed poor growth performance and feeding efficiency [58]. During the 8-week feeding trial, XCHD had significant effect on the growth rate of hybrid grouper. Hybrid grouper fed XCHD150 and XCHD300 diets possessed markedly higher weight gain (WG) than XCHD0 groups. Overdose of a XCHD will produce negative effect on the growth performance of fish. Our previous studies reported that dietary medicinal herbs supplementation resulted in higher WG but excess dose had adverse effects on growth and feed utilization [28]. Punitha et al. showed that fish fed with a diet supplemented with a mixture of medicinal herbs had higher weight gain than the control fish, indicating that feeding with the herbal mixture promotes weight gain [59]. Moreover, the feed efficiency (FE) increased with increasing dietary XCHD concentrations. Previous study showed that medicinal herbs significantly improved digestibility and availability of nutrients resulting in an increase in feed conversion and leading to a higher protein synthesis [60,61]. These results indicated that XCHD can promote weight gain when they are administered to cultured fish.

In fish, de novo lipogenesis mainly takes place in the liver and is negligible in muscle and adipose tissue [62]. Long-term lipid accumulation in hepatocytes induces liver dysfunction, which develops into pathological damage [63]. Previous study showed that excessive accumulation of fat in the cytoplasm of liver was generally accompanied by nuclear atrophy to a level such that the fish can be described as having a pathological liver [64]. Blanchard et al. reported that when the nucleus does not occupy the center of the cell is suggested pathological accumulation of lipid [63]. When animals ingest high-fat diets, large amounts of triglyceride are synthesized and deposited in vacuoles, leading to steatosis [65]. Tan et al. also reported that the administration of high lipid diets significantly increased occurrence rates of the hepatocyte swelling, hepatocyte vacuolization [28]. In the present study, histological examination of liver morphology with H&E staining indicated that XCHD treatment resulted in a reduction in indices of

Table 5
Effects of dietary Xiaochaihu Decoction (XCHD) on whole body and muscle proximate composition in hybrid grouper.

Items	XCHD0	XCHD150	XCHD300	XCHD600	XCHD1200
Whole body (%)					
Moisture	70.82 ± 1.20	68.61 ± 2.61	70.48 ± 1.12	69.18 ± 0.53	68.50 ± 1.09
Crude protein	16.30 ± 0.18a	17.09 ± 0.38 ab	17.90 ± 1.23b	17.89 ± 0.39b	17.61 ± 0.95 ab
Crude lipid	7.53 ± 0.44b	6.93 ± 0.28 ab	6.62 ± 0.35 ab	6.36 ± 0.45a	7.27 ± 0.21 ab
Ash	4.45 ± 0.05	4.64 ± 0.25	4.59 ± 0.31	4.61 ± 0.15	4.66 ± 0.11
Muscle (%)					
Moisture	77.91 ± 1.12	77.19 ± 0.34	76.60 ± 0.54	76.69 ± 0.88	77.34 ± 0.35
Crude protein	19.13 ± 0.33a	20.14 ± 0.12 ab	20.66 ± 0.64b	20.96 ± 0.87b	20.01 ± 0.08 ab
Crude lipid	2.63 ± 0.04c	2.50 ± 0.08bc	2.39 ± 0.08 ab	2.30 ± 0.06a	2.29 ± 0.07a
Ash	1.04 ± 0.08a	1.09 ± 0.03 ab	1.07 ± 0.01 ab	1.17 ± 0.07b	1.11 ± 0.03 ab

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

Table 6
Effects of dietary Xiaochaihu Decoction (XCHD) on hematological parameters in hybrid grouper.

Items	XCHD0	XCHD150	XCHD300	XCHD600	XCHD1200
ALP (U/L)	175.67 ± 3.21b	149.50 ± 12.02 ab	128.50 ± 0.71a	140.50 ± 26.16a	138.00 ± 18.38a
ALT (U/L)	2741.00 ± 36.77b	2237.50 ± 62.93a	2216.50 ± 195.87a	2348.00 ± 260.22 ab	2498.50 ± 242.54 ab
AST (U/L)	165.00 ± 8.89b	165.50 ± 10.61b	119.50 ± 24.75a	123.50 ± 4.95a	126.00 ± 11.31a
CHOL (U/L)	9.24 ± 0.52b	7.44 ± 0.62a	7.31 ± 0.58a	8.11 ± 0.49 ab	8.46 ± 0.39 ab
TG (U/L)	2.40 ± 0.25b	1.71 ± 0.43 ab	1.15 ± 0.09a	1.70 ± 0.24 ab	1.80 ± 0.26 ab

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

hepatocellular damage such as swelling of hepatocytes, reduced hepatocyte vacuolization and marked decrease nuclei shifting to the cellular periphery, which suggested that XCHD is important for the prevention of lipid accumulation in hepatocyte store after fed high lipid diets. These findings indicate that XCHD treatment may normalize liver size. Tian et al. and Jiang et al. also reported that degree of liver fibrosis of the treatment XCHD were markedly lessened on the experimental hepatic injury induced by carbon tetrachloride in rats [66,67]. XCHD could significantly decrease liver distortion in architecture, ameliorate cirrhotic nodule formation and preserve liver function in rats induced by dimethylnitrosamine (DMN) [68]. Previous study also showed that dietary supplementation with the optimum levels of medicinal herbs could significantly improve apoptosis occurred in hepatocytes in GIFT tilapia, *Oreochromis niloticus* [69]. In general, increases in dietary lipid levels are associated with increases in lipid deposits in the tissue [70,71]. In the present study, we used Oil Red O staining to examine the effect of XCHD on lipogenesis in fish. Previous studies have indicated that Xiaochaihu decoction low and high dose groups (0.6 and 1.2 g/mL) were notably mitigated the degree of hepatocyte steatosis and induced the content of lipid drops in rats [72]. XCHD also could induce liver lipid deposition in diet-induced rat nonalcoholic hepatitis model [73]. To our knowledge, no study to date has investigated the effects of dietary XCHD on liver histochemistry in fish, which makes comparison rather difficult. However, lipid droplet content were remarkably reduced by the administration of XCHD, in good agreement with the results of biochemical tests and *in vitro* research. The XCHD supplementation significantly decreased the liver lipid content in the current study, which further confirms that XCHD has lipid-lowering effects. Dietary XCHD supplementation reduced the lipid content in the liver and hepatocytes. To investigate the mechanism for the variation of lipid accumulation as a response to dietary XCHD addition, the mRNA expression of genes related to lipid metabolism were analyzed. In the present study, dietary XCHD supplementation was found to down-regulate adipogenesis genes mRNA levels but to up-regulate lipolysis genes mRNA levels in the liver, which again correlated well with the reported results *in vitro*. The present findings demonstrated the hepatoprotective effects of XCHD against lipid deposition induced by high lipid diets in fish.

It is known that liver disease can also be induced by chemical [74]. However, the pathogenesis of chemical induced hepatic injury in fish is still unclear. D-GalN/LPS-induced hepatitis is a well-established model of liver injury in human and other mammals [75]. This model has been widely used to investigate the molecular mechanisms underlying the pathophysiology of liver injury and evaluate the biological activities of hepatoprotective agents [76]. Our previous study showed that the *in vitro* and *in vivo* models of D-GalN/LPS induced hybrid grouper liver injury was established and applied in study for the evaluation of the hepatoprotective of medicinal herbs [25]. At 24 h after the D-GalN/LPS injection, extensive apoptosis and necrosis injury occurred as a result of D-GalN/LPS-induced fulminant hepatic injury. Our results are in agreement with previous observations, where administration of D-GalN/LPS resulted in an increase in serum AST, ALT, LDH and TBA

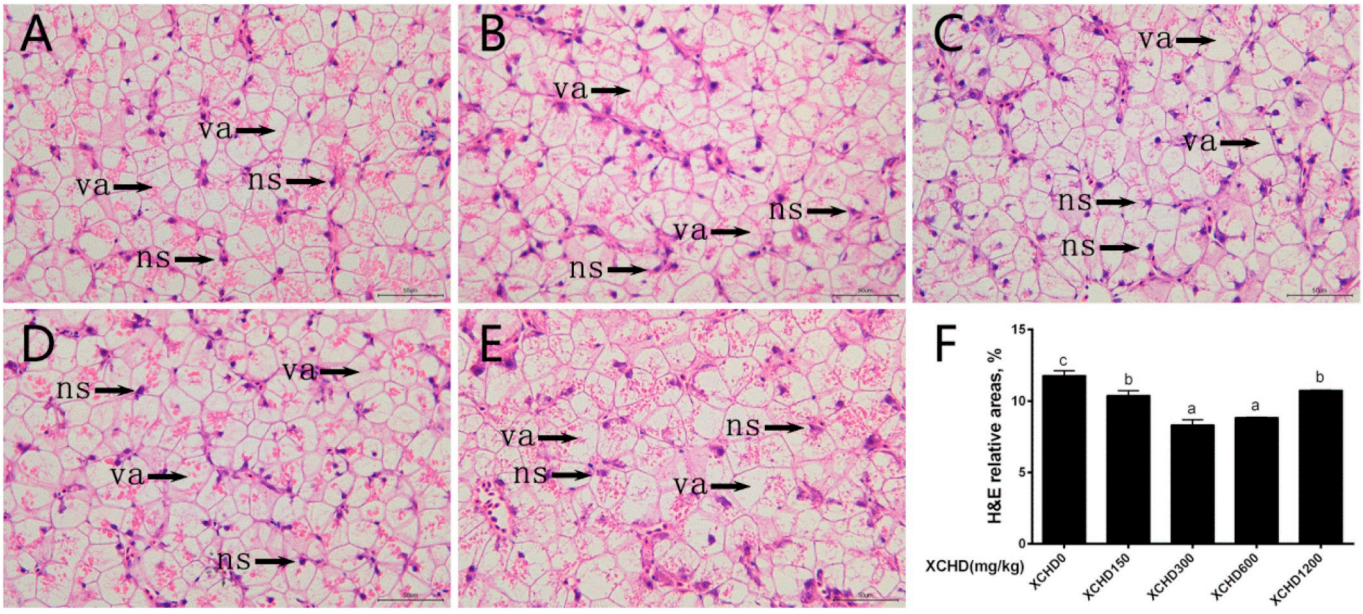

Fig. 4. Photomicrographs (400 ×) of the histopathological examinations in the liver samples of different groups. (A) Liver from fish fed 0 mg/kg XCHD (XCHD0); (B) Liver from fish fed 150 mg/kg XCHD (XCHD150); (C) Liver from fish fed 300 mg/kg XCHD (XCHD300); (D) Liver from fish fed 600 mg/kg XCHD (XCHD600); (E) Liver from fish fed 1200 mg/kg XCHD (XCHD1200); (F) Relative areas for hepatic vacuoles in H&E staining. Each experiment was repeated three times. Means in the same column with different superscripts are significantly different ($P < 0.05$). ns: nuclei shifted to the periphery of the hepatocytes; va: vacuolation. bar = 50 μm. All slides are 400× magnification.

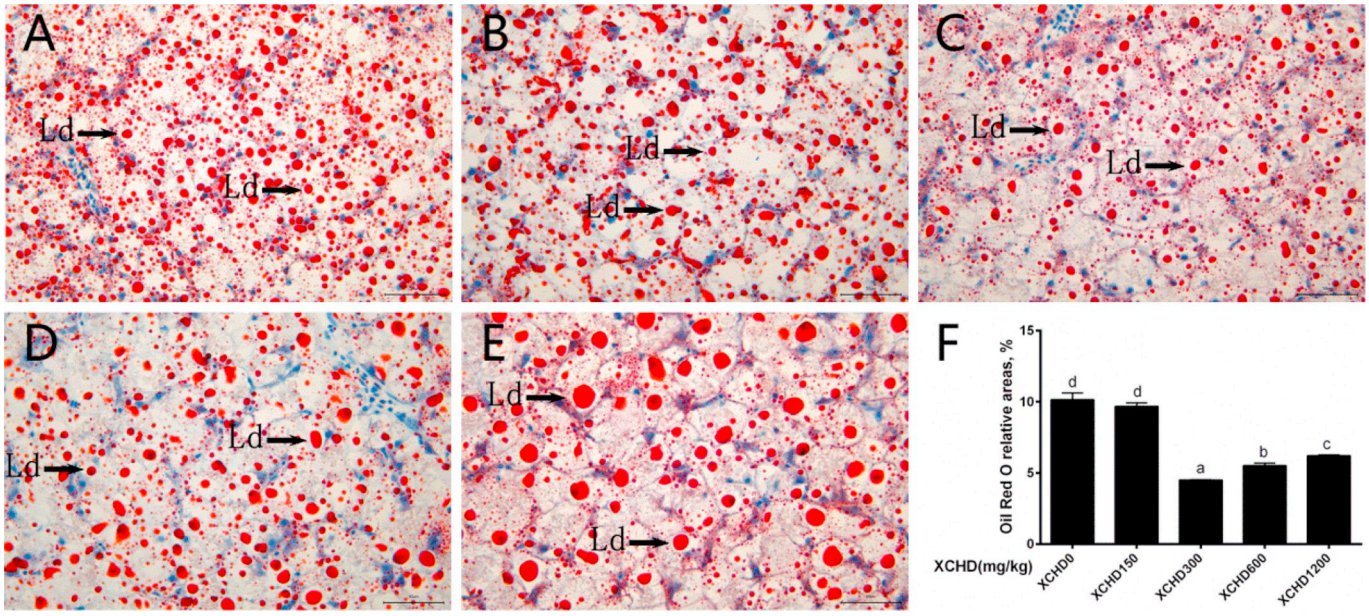

Fig. 5. Lipid visualization and quantification by Oil Red O staining show reduced lipid storage in the liver of hybrid grouper. (A) Liver from fish fed 0 mg/kg XCHD (XCHD0); (B) Liver from fish fed 150 mg/kg XCHD (XCHD150); (C) Liver from fish fed 300 mg/kg XCHD (XCHD300); (D) Liver from fish fed 600 mg/kg XCHD (XCHD600); (E) Liver from fish fed 1200 mg/kg XCHD (XCHD1200); (F) Relative areas for lipid droplets in Oil Red O staining. Lipids appear red, and nuclei appear blue after staining with Oil Red O. Each experiment was repeated three times. Means in the same raw with different superscripts are significantly different ($P < 0.05$). Ld: lipid droplet. bar = 50 μm. All slides are 400× magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

levels, suggestive of liver injury. In the whole animal model, the increased levels of AST, ALT, LDH and TBA are classical indicators of liver damage [7]. The hepatoprotective effect of XCHD was evidenced by its ability to decrease the AST, ALT, LDH and TBA levels after marked release of AST, ALT, LDH and TBA into the circulation during D-GalN/LPS intoxication. Simultaneously, according to histopathological examinations, livers challenged with D-GalN/LPS exhibited extensive areas of vacuolization with the disappearance of nuclei and the loss of hepatic architecture. However, severe hepatic lesions induced by D-GalN/LPS were remarkably reduced by the administration of XCHD, in good agreement with the results of biochemical tests. Apart from mild hepatic damage, the liver had a nearly normal appearance in D-GalN/LPS plus XCHD-treated hybrid grouper. These results were in accordance with our previous results as well [25]. XCHD pretreatment showed a potent protective effect against D-GalN/LPS-caused acute liver injury in fish, evidenced by a significant reduction of serum enzyme levels and the improvement of liver histopathology.

Oxidative stress is a recognized phenomenon in D-GalN/LPS-induced liver damage. Previous study indicated that D-GalN/LPS-treatment decreased the content of reduced the enzyme activities and the mRNA expression of genes related to antioxidative in hybrid grouper [25]. It has been documented that challenge with D-GalN/LPS resulted in overproduction of ROS which plays a critical role in the development of liver injury [77]. If there is an imbalance between the antioxidant defense systems and production of ROS, oxidative stress will occur and cause damage to cells through direct or indirect pathways [78]. Cells have many mechanisms to protect themselves from the toxic effects of free radicals, such as free radical scavengers and chain reaction terminators [79]. In this study, the D-GalN/LPS challenge caused a significant oxidative stress in the liver characterized by the formation of lipidperoxides. However, we observed that mRNA levels of CAT and MnSOD in the liver increased with increasing dietary XCHD levels up to a certain level, whereas down-regulated the mRNA levels of Keap1 in the liver of fish, compared to the model group. Therefore,

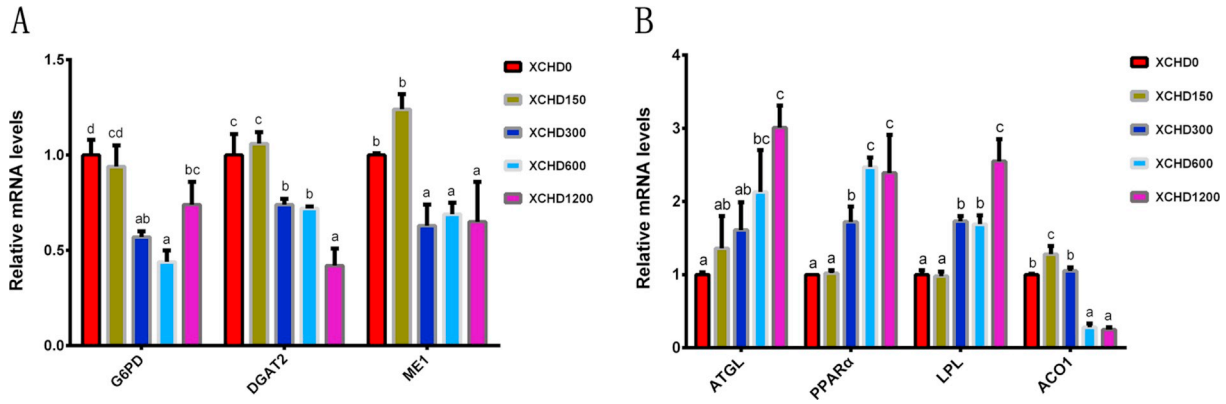

Fig. 6. XCHD improved the hepatic mRNA expression of lipid metabolism related genes in hybrid grouper.(A) Lipogenic-related genes; (B) Lipolysis-related genes. The results are presented as the mean ± SD of three replications. Means in the same column with different superscripts are significantly different ($P < 0.05$). G6PD: glucose 6-phosphate dehydrogenase; DGAT2: acyl CoA diacylglycerol acyltransferase 2; ME1: malic enzyme 1; ATGL: adipose triglyceride lipase; PPARα: peroxisome proliferator-activated receptor alpha; LPL: lipoprotein lipase; ACO1: acyl-CoA oxidase 1 .

Table 7
Effects of dietary Xiaochaihu Decoction (XCHD) on serum biochemical indices in hybrid grouper.

Items	Xiaochaihu Decoction (XCHD) concentration					
	Control (XCHD0)	Model (XCHD0 + D-GalN/LPS)	XCHD150 + D-GalN/LPS	XCHD300 + D-GalN/LPS	XCHD600 + D-GalN/LPS	XCHD1200 + D-GalN/LPS
ALP	143.00 ± 12.73a	185.00 ± 5.67b	152.00 ± 11.31 ab	139.50 ± 9.19a	166.50 ± 12.02 ab	172.50 ± 27.58 ab
ALT	2673.50 ± 74.25a	3497.67 ± 384.99b	2347.00 ± 208.02a	2542.50 ± 78.49a	2586.00 ± 216.37a	2797.00 ± 65.05a
AST	162.00 ± 5.66b	208.00 ± 9.90c	177.00 ± 2.83b	124.50 ± 2.12a	177.00 ± 8.49b	174.00 ± 5.66b
CHE	175.50 ± 3.54 ab	186.00 ± 14.14b	164.00 ± 8.49 ab	158.33 ± 6.51a	174.50 ± 9.19 ab	164.00 ± 19.80 ab
TBA	3.67 ± 0.23a	5.20 ± 0.14c	4.35 ± 0.49b	4.23 ± 0.15b	4.30 ± 0.14b	4.15 ± 0.07 ab
LDH	198.50 ± 3.54a	418.00 ± 89.10b	244.00 ± 16.97a	212.50 ± 65.76a	238.00 ± 16.97a	240.50 ± 24.75a

Note: Values are means ± SD (n = 6) of three replications. Means in the same raw with different superscripts are significantly different ($P < 0.05$). Control: injection of sterile saline only and without feeding XCHD; Model: injection of D-GalN (500 mg/kg) and LPS (20 μg/kg) only and without feeding XCHD; ALP: alkaline phosphatase; ALT: alanine transaminase; AST: aspartate transaminase; CHE: Cholinesterase; TBA: total biliary acid; LDH: lactate dehydrogenase.

hepatoprotection of XCHD may be achieved by scavenging free radicals and enhancing the activity of original natural hepatic-antioxidant enzymes to ameliorate oxidate stress from chemical-induced injury. It suggests that the beneficial effect of XCHD is partially associated with its anti-oxidative capacities.

In addition, we further investigated the mechanisms underlying XCHD-mediated hepatoprotection, which may be related to its anti-apoptotic properties. The anti-apoptotic property of XCHD was first examined in fish. Indeed, previous studies have revealed that XCHD can reduce apoptotic cell death in numerous injury models [79]. In D-GalN/

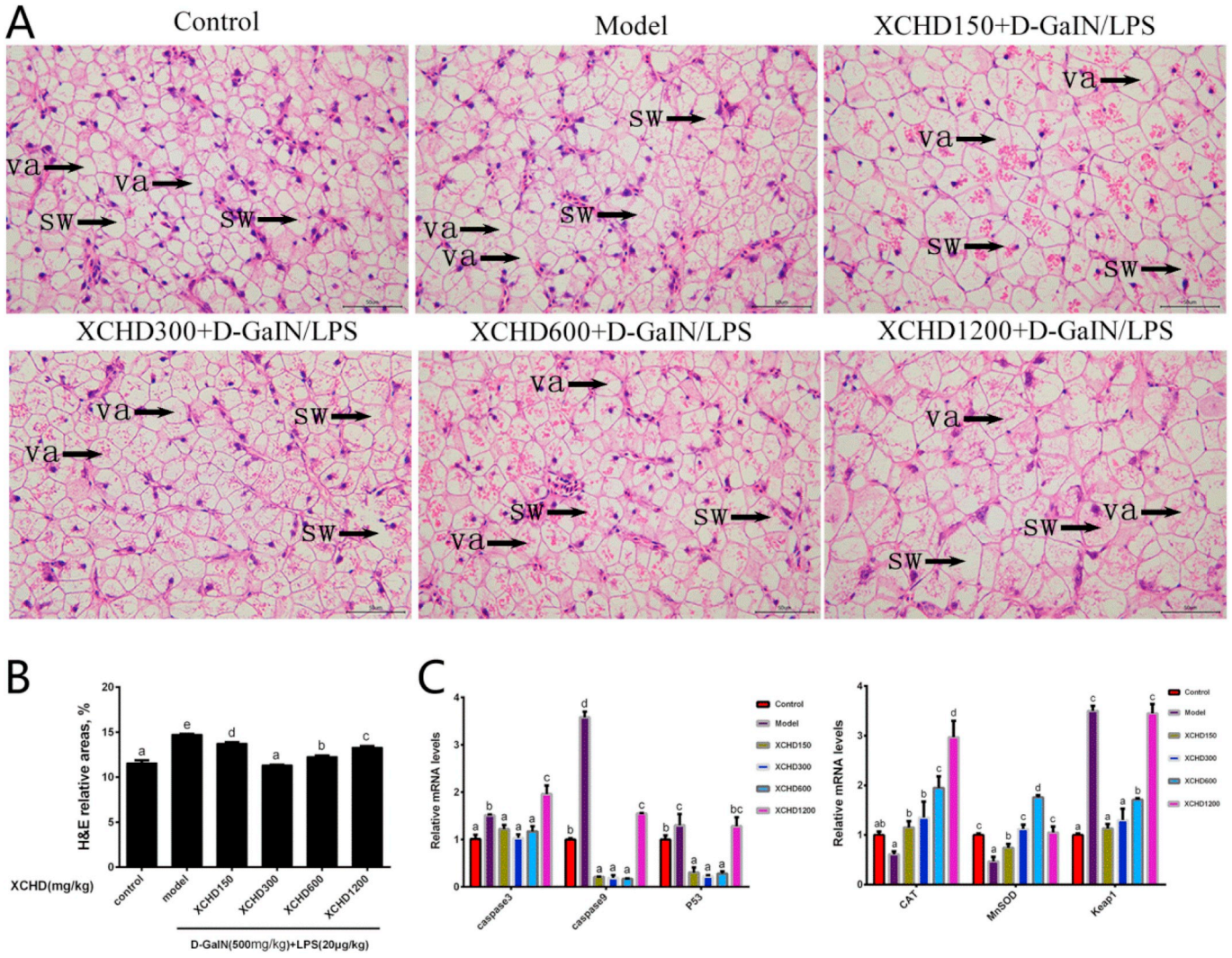

Fig. 7. Effects of XCHD on D-GalN/LPS-induced liver injury. (A) Photomicrographs (400 ×) of the histopathological examinations of the liver samples of different groups. (B) Relative areas for hepatic vacuoles in H&E staining. (C) The mRNA expression of apoptosis genes (caspase-3, caspase-9 and P53) and antioxidant enzyme genes (CAT, MnSOD and Keap1) were detected by qRT-PCR. Control: injection of sterile saline only and without feeding XCHD; (B) Model: injection of D-GalN/LPS only and without feeding XCHD. The results are presented as the mean ± SD of three replications. Means in the same raw with different superscripts are significantly different ($P < 0.05$). Typical images were chosen from each experimental group (original magnification 400 ×). sw: swelling cells; va: vacuolation; bar = 50 μm. All slides are 400 × magnification. CAT: catalase; MnSOD: manganese superoxide dismutase; Keap: Kelch-like- ECH-associated protein 1.

LPS model, hepatocyte apoptosis is also assumed to be a key feature involving liver damage and is associated with the initiation and progression of multiple inflammatory responses [80]. We also examined the expression of the caspase-3, caspase-9 and p53 genes. As the most important apoptosis-inducing genes in organisms, they play a central role in inducing cell apoptosis [81]. In mammals, there are at least three distinct but interactive apoptotic pathways: mitochondrial-mediated, endoplasmic reticulum stress-mediated and death receptor-initiated pathways [82]. Previous studies demonstrated that LPS increases the permeability of cells and induces structural mitochondrial damage with caspase-mediated apoptosis [82]. Caspases are a family of genes important for maintaining homeostasis by regulating apoptosis and inflammation [83]. Hepatocyte apoptosis has been recognized as a major contributing mechanism for liver injury [84]. Caspases have been categorized as either pro-apoptotic or pro-inflammatory factors, depending on their participation in these cellular responses [85]. Caspase-3 is closely associated with both death receptor- and mitochondrion-dependent apoptosis [86]. It is the primary terminal cleavage and will lead to hepatocyte apoptosis and eventually induce liver damage [87]. In our present study, caspase-3, caspase-9 and P53 levels markedly increased in hybrid grouper challenged by D-GalN/LPS. These alterations were attenuated by XCHD pretreatment, which suggests that XCHD simultaneously down-regulates caspase-3, caspase-9 and P53 expression. P53 can stimulate a wide network of signals that act through at least two apoptotic pathways, the intrinsic, mitochondrial pathway and the extrinsic, death receptor pathway [88]. Similarly, previous studies reported that P53 mRNA levels in the HepG2 and Hep3B cells treated with saikosaponin D (one of radix bupleuri major pharmacologically-active components) significantly decreased [89]. Apoptotic cells were also revealed in the high dose of XCHD groups, which may be attributed to XCHD absorption through guts, resulting in liver impairment. Since caspase-3, caspase-9 and P53 expression were significantly up-regulated in fish fed high doses of XCHD, the present study also demonstrated that addition of high doses of XCHD may induce apoptosis and impaired the health of liver tissues.

The results presented herein demonstrate the hepatoprotective effects of XCHD in primary hybrid grouper hepatocytes against hepatotoxicant and in hybrid grouper with hepatic damage. The detailed mechanism of hepatoprotective action and the interactions among the different components responsible for the hepatoprotective effect of XCHD need to be investigated further. Additional, more detailed studies are currently in progress.

In conclusion, the present study demonstrates, for the first time in fish, XCHD reduces hepatic lipid accumulation and liver injury in hybrid grouper both *in vitro* and *in vivo*. It is showed that dietary administration of XCHD at concentrations of 150–300 mg/kg for 56 days has the potential to modulate growth parameters and expression of lipid metabolism genes in hybrid grouper. In addition, XCHD pretreatment also ameliorated the degree of structural damage and down-regulate the apoptotic genes in D-GalN/LPS inflicted liver injury. These results also provide useful information for dietary interventions to inhibit lipid accumulation and improve the health of fish. XCHD can be used as a hepatoprotective agent for treatment for fatty liver diseases.

Acknowledgement

This research was supported by National Key R&D Program of China (2018YFD0900400), Guangdong Provincial Oceanic Fisheries Science and Technology Project (A201601C04), Guangdong Provincial Natural Science Foundation (2018A030313222) and (2016A030313447).

References

[1] Q. Chen, Z. Luo, K. Wu, C. Huang, M. Zhuo, Y. Song, W. Hu, Differential effects of dietary copper deficiency and excess on lipid metabolism in yellow catfish *Pelteobagrus fulvidraco*, Comp. Biochem. Physiol., B 184 (2015) 19–28.

[2] J. Yan, K. Liao, T. Wang, K. Mai, W. Xu, Q. Ai, Dietary lipid levels influence lipid deposition in the liver of large yellow croaker (*Larimichthys crocea*) by regulating lipoprotein receptors, fatty acid uptake and triacylglycerol synthesis and catabolism at the transcriptional level, PLoS One 10 (2015) e129937.

[3] Z. Du, P. Clouet, W. Zheng, P. Degrace, L. Tian, Y. Liu, Biochemical hepatic alterations and body lipid composition in the herbivorous grass carp (*Ctenopharyngodon idella*) fed high-fat diets, Br. J. Nutr. 95 (2006) 905–915.

[4] T. Gaylord, D. Gatlin, Dietary lipid level but not L-carnitine affects growth performance of hybrid striped bass (Morone chrysops female × M. saxatilis male), Aquaculture 190 (2000) 237–246.

[5] E. Spisni, M. Tugnoli, A. Ponticelli, T. Mordenti, V. Tomasi, Hepatic steatosis in artificially fed marine teleosts, J. Fish Dis. 21 (1998) 177–184.

[6] Z. Du, Y. Liu, L. Tian, J. Wang, Y. Wang, G. Liang, Effect of dietary lipid level on growth, feed utilization and body composition by juvenile grass carp (*Ctenopharyngodon idella*), Aquacult. Nutr. 11 (2005) 139–146.

[7] K. Lu, W. Xu, J. Li, X. Li, G. Huang, W. Liu, Alterations of liver histology and blood biochemistry in blunt snout bream Megalobrama amblycephala fed high-fat diets, Fish. Sci. 79 (2013) 661–671.

[8] Q. Zheng, C. Han, Y. Zhong, R. Wen, M. Zhong, Effects of dietary supplementation with green tea waste on growth, digestive enzyme and lipid metabolism of juvenile hybrid tilapia, *Oreochromis niloticus* × *O. aureus*, Fish Physiol. Biochem. 43 (2017) 361–371.

[9] D. Li, L. Liu, Introduction to diagnosis and treatment of fish fatty liver diseas, Prog. Veter. Med. 37 (2016) 114–117.

[10] P. Xiao, H. Ji, Y. Ye, B. Zhang, Y. Chen, J. Tian, P. Liu, L. Chen, Z. Du, Dietary silymarin supplementation promotes growth performance and improves lipid metabolism and health status in grass carp (*Ctenopharyngodon idellus*) fed diets with elevated lipid levels, Fish Physiol. Biochem. 43 (2017) 245–263.

[11] B. Yuan, R. Yang, Y. Ma, S. Zhou, X. Zhang, Y. Liu, A systematic review of the active saikosaponins and extracts isolated from Radix Bupleuri and their applications, Pharmaceut. Biol. 55 (2016) 620–635.

[12] Y. Chen, J. Wang, L. Yuan, L. Zhou, X. Jia, X. Tan, Interaction of the main components from the traditional Chinese drug pair Chaihu-Shaoyao based on rat intestinal absorption, Molecules 16 (2011) (2011) 9600–9610.

[13] J. Du, L. Cao, R. Jia, G. Yin, Hepatoprotective and antioxidant effects of dietary Glycyrrhiza polysaccharide against TCDD-induced hepatic injury and RT-PCR quantification of AHR2, ARNT2, CYP1A mRNA in Jian Carp (Cyprinus carpio var. Jian), J. Environ. Sci. 51 (2017) 181–190.

[14] R. Jia, L. Cao, P. Xu, G. Jeney, G. Yin, In vitro and in vivo hepatoprotective and antioxidant effects of Astragalus polysaccharides against carbon tetrachloride-induced hepatocyte damage in common carp (*Cyprinus carpio*), Fish Physiol. Biochem. 38 (2012) 871–881.

[15] Z. Zhu, L. Zhao, X. Liu, J. Chen, H. Zhang, G. Zhang, Y. Chai, Comparative pharmacokinetics of baicalin and wogonoside by liquid chromatography-mass spectrometry after oral administration of Xiaochaihu Tang and Radix scutellariae extract to rats, J. Chromatogr. B 878 (2010) 2184–2190.

[16] Z. Taira, K. Yabe, Y. Hamaguchi, Effects of Sho-saiko-to extract and its components, Baicalin, baicalein, glycyrrhizin and glycyrrhetic acid, on pharmacokinetic behavior of salicylamide in carbon tetrachloride intoxicated rats, Food Chem. Toxicol. 42 (2004) 803–807.

[17] M. Sumino, Y. Saito, F. Ikegami, Y. Hirasaki, T. Namiki, Extraction Efficiency of Shosaikoto (Xiaochaihu Tang) and Investigation of the Major Constituents in the Residual Crude Drugs, Evidence-Based Complementary and Alternative Medicine, (2012).

[18] H. Tajiri, K. Kozaiwa, Y. Ozaki, K. Miki, K. Shimizu, S. Okada, Effect of Sho-saiko-to (Xiao-chai-hu-tang) on HBeAg clearance in children with chronic hepatitis B virus infection and with sustained liver disease, Am. J. Chin. Med. 19 (1991) 121–129.

[19] K. Woo, J. Lim, S. Suh, Y. Kwon, S. Shin, S. Kim, Differential inhibitory effects of baicalein and baicalin on LPS-induced cyclooxygenase-2 expression through inhibition of C/EBPbeta DNA binding activity, Immunobiology 211 (2006) 359–368.

[20] J. Chao, W. Su, H. Liu, Baicalein induces cancer cell death and proliferation retardation by the inhibition of CDC2 kinase and surviving associated with opposite role of p38 mitogen-activated protein kinase and AKT, Mol. Cancer Ther. 6 (2007) 3039–3048.

[21] W. Chang, Z. Shao, J. Yin, S. Mehendale, C. Wang, Y. Qin, Comparative effects of flavonoids on oxidant scavenging and ischemia-reperfusion injury in cardiomyocytes, Eur. J. Pharmacol. 566 (2007) 58–66.

[22] A. Othman, G. Kawamura, S. Senoo, F. Ching, Effects of different salinities on growth, feeding performance and plasma cortisol level in hybrid TGGG (Tiger grouper, *Epinephelus fuscoguttatus* × Giant grouper, *Epinephelus lanceolatus*) juveniles, Int. Res. J. Biol. Sci. 4 (2015) 15–20.

[23] Y. Sun, C. Guo, D. Wang, X.F. Li, L. Xiao, X. Zhang, X. You, Q. Shi, G. Hu, C. Fang, H. Lin, Y. Zhang, Transcriptome analysis reveals the molecular mechanisms underlying growth superiority in a novel grouper hybrid (*Epinephelus fuscogutatus*♀ × *E. lanceolatus*♂), BMC Genet. 17 (2016).

[24] J. Triastuti, K. Pursetyo, A. Monica, L. Lutfiyah, D. Budi, Abnormalities of hybrid grouper (Epinephelus fuscoguttatus × Epinephelus lanceolatus) in Situbondo, Asean-Fen Int. Fish. Symp. 137 (2018).

[25] C. Zou, X. Tan, H. Ye, Z. Sun, S. Chen, Q. Liu, M. Xu, C. Ye, A. Wang, The hepatoprotective effects of Radix Bupleuri extracts against Dgalactosamine/lipopolysaccharide induced liver injury in hybrid grouper (*Epinephelus lanceolatus*♂ × *Epinephelus fuscoguttatus*♀), Fish Shellfish Immunol. 83 (2018) 8–17.

[26] W. Xu, W. Liu, Z. Liu, Trichlorfon-induced apoptosis in hepatocyte primary cultures of Carassius auratus gibelio, Chemosphere 77 (2009) 895–901.

[27] X. Tan, Z. Sun, C. Ye, Dietary *Lycium barbarum* extract administration improved growth, meat quality and lipid metabolism in hybrid grouper (*Epinephelus*

lanceolatus♂ × *E. fuscoguttatus* ♀) fed high lipid diets, Aquaculture 504 (2019) 190–198.
[28] X. Tan, Z. Sun, Q. Liu, H. Ye, C. Zou, C. Ye, A. Wang, Effects of dietary ginkgo biloba leaf extract on growth performance, plasma biochemical parameters, fish composition, immune responses, liver histology, and immune and apoptosis-related genes expression of hybrid grouper (*Epinephelus lanceolatus*♂ × *Epinephelus fuscoguttatus*♀) fed high lipid diets, Fish Shellfish Immunol. 72 (2018) 399–409.
[29] K. Livak, T. Schmittgen, Analysis of relative gene expression data using realtime quantitative PCR and the $2^{-\triangle\triangle Ct}$ method, Methods 25 (2001) 402–408.
[30] Q. Chen, Z. Luo, X. Liu, Effects of waterborne chronic copper exposure on hepatic lipid metabolism and metal-element composition in Synechogobius hasta, Arch. Environ. Contam. Toxicol. 64 (2013) 301–315.
[31] Y. Liu, J. Du, L. Cao, R. Jia, Y. Shen, C. Zhao, P. Xu, G. Yin, Anti-inflammatory and hepatoprotective effects of Ganoderma lucidum polysaccharides on carbon tetrachloride-induced hepatocyte damage in common carp (Cyprinus carpio L.), Int. Immunopharmacol. 25 (2015) 112–120.
[32] H. Arnold, H. Pluta, T. Braunbeck, Simultaneous exposure of fish to endosulfan and disulfoton in vivo: ultrastructural, stereological and biochemical reactions in hepatocytes of male rainbow trout (*Oncorhynchus mykiss*), Aquat. Toxicol. 33 (1995) 17–43.
[33] H. Arnold, H. Pluta, T. Braunbeck, Sublethal effects of prolonged exposure to disulfoton in rainbow trout (*Oncorhynchus mykiss*): cytological alterations in the liver by a potent acetylcholine esterase inhibitor, Ecotoxicol. Environ. Saf. 34 (1996) 43–55.
[34] X. Meng, Q. Ding, The causes of fish “liver and gall syndrome” and methods for prevention and control, Res. Fish. (Seattle) 24 (2004) 65–67.
[35] G. Yin, L. Cao, P. Xu, G. Jeney, M. Nakao, Hepatoprotective and antioxidant effects of Hibiscus sabdariffa extract against carbon tetrachloride-induced hepatocyte damage in Cyprinus carpio, in Vitro Cell, Dev. Biol. Anim. 47 (2011) 10–15.
[36] G. Yin, L. Cao, P. Xu, G. Jeney, M. Nakao, C. Lu, Hepatoprotective and antioxidant effects of Glycyrrhiza glabra extract against carbon tetrachloride (CCl4)-induced hepatocyte damage in common carp (*Cyprinus carpio*), Fish Physiol. Biochem. 37 (2011) 209–216.
[37] X. Liu, X. Hu, H. Xu, X. Zeng, Xiaochaihu Decoction attenuates the vicious circle between the oxidative stress and the ALP inactivation through LPS-catecholamines interactions in gut, liver and brain during CCl4 + ethanol-induced mouse HCC, BMC Complement Altern. Med. 13 (2013) 375.
[38] R. Lu, X. Liang, M. Wang, Y. Zhou, X. Bai, Y. He, The role of leptin in lipid metabolism in fatty degenerated hepatocytes of the grass carp Ctenopharyngodon idellus, Fish Physiol. Biochem. 38 (2012) 1759–1774.
[39] M. Roden, Mechanisms of disease: hepatic steatosis in type 2 diabetes-pathogenesis and clinical relevance, Nat. Clin. Pract. Endocrinol. Metabol. 2 (2006) 335–348.
[40] J. Park, Y. Kim, J. Im, H. Lee, Oligonol suppresses lipid accumulation and improves insulin resistance in a palmitate-induced in HepG2 hepatocytes as a cellular steatosis model, BMC Complement Altern. Med. 15 (2015) 185.
[41] K. Lu, W. Xu, X. Li, W. Liu, L. Wang, C. Zhang, Hepatic triacylglycerol secretion, lipid transport and tissue lipid uptake in blunt snout bream (Megalobrama amblycephala) fed high-fat diet, Aquaculture 408–409 (2013) 160–168.
[42] E. Karavia, D. Papachristou, I. Kotsikogianni, I. Triantafyllidou, K. Kypreos, Lecithin/cholesterol acyltransferase modulates diet induced hepatic deposition of triglycerides in mice, J. Nutr. Biochem. 24 (2013) 567–577.
[43] M. Wang, Y. Lee, U. Roger, Novel form of lipolysis induced by leptin, J. Biol. Chem. 274 (1999) 17541–17544.
[44] D. Kratky, S. Obrowsky, D. Kolb, B. Radovic, Pleiotropic regulation of mitochondrial function by adipose triglyceride lipase-mediated lipolysis, Biochimie 96 (2014) 106–112.
[45] Q. Chen, Z. Luo, K. Wu, C. Huang, M. Zhuo, Y. Song, W. Hu, Differential effects of dietary copper deficiency and excess on lipid metabolism in yellow catfish Pelteobagrus fulvidraco, Comp. Biochem. Physiol., B 184 (2015) 19–28.
[46] X. Wang, Y. Wang, Y. Li, Adipose triglyceride lipase (ATGL) clone, expression pattern, and regulation by different lipid sources and lipid levels in large yellow croaker (*Pseudosciaena crocea* R.), Mar. Biotechnol. 15 (2013) 197–205.
[47] C. Han, X. Wen, Q. Zheng, H. Li, Effects of dietary lipid levels on lipid deposition and activities of lipid metabolic enzymes in hybrid tilapia (*Oreochromis niloticus* × *O. aureus*), J. Anim. Physiol. Anim. Nutr. 95 (2011) 609–615.
[48] A. Albalat, A. Saera-Vila, E. Capilla, J. Gutierrez, J. Perez-Sanchez, I. Navarro, Insulin regulation of lipoprotein lipase (LPL) activity and expression in gilthead sea bream (*Sparus aurata*), Comp. Biochem. Physiol. 148 (2007) 151–159.
[49] J. Reddy, T. Hasimoto, Peroxisomal β-oxidation and peroxisome proliferator-activated receptor α: an adaptive metabolic system, Annu. Rev. Nutr. 21 (2001) 193–230.
[50] M. Yoon, The role of PPARα in lipid metabolism and obesity: focusing on the effects of estrogen on PPARα actions, Pharmacol. Res. 60 (2009) 151–159.
[51] J. Kim, D. Hahm, D. Yang, H. Lee, Effect of crude saponin of Korean red ginseng on high fat diet-induced obesity in the rat, J. Pharmacol. Sci. 97 (2005) 124–131.
[52] S. Rahimnejad, I. Bang, J. Park, A. Sade, J. Choi, S. Lee, Effects of dietary protein and lipid levels on growth performance, feed utilization and body composition of juvenile hybrid grouper, Epinephelus fuscoguttatus × E. lanceolatus, Aquaculture 446 (2015) 283–289.
[53] S. Jiang, X. Wu, Y. Luo, M. Wu, S. Lu, Z. Jin, W. Yao, Optimal dietary protein level and protein to energy ratio for hybrid grouper (*Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂) juveniles, Aquaculture 465 (2016) 28–36.
[54] Z. Sun, X. Tan, H. Ye, C. Zou, C. Ye, A. Wang, Effects of dietary Panax notoginseng extract on growth performance, fish composition, immune responses, intestinal histology and immune related genes expression of hybrid grouper (*Epinephelus lanceolatus* ♂ × *Epinephelus fuscoguttatus* ♀) fed high lipid diets, Fish Shellfish Immunol. 73 (2018) 234–244.
[55] M. Bou, M. Todorčević, R. Fontanillas, E. Capilla, J. Gutiérrez, I. Navarro, Adipose tissue and liver metabolic responses to different levels of dietary carbohydrates in gilthead sea bream (*Sparus aurata*), Comp. Biochem. Physiol. A 175 (2014) 72–81.
[56] X. Guo, X. Liang, L. Fang, X. Yuan, Y. Zhou, J. Zhang, B. Li, Effects of dietary non-protein energy source levels on growth performance, body composition and lipid metabolism in herbivorous grass carp (*Ctenopharyngodon idella* Val.), Aquacult. Res. 46 (2015) 1197–1208.
[57] A. Rolo, J. Teodoro, C. Palmeira, Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis, Free Radic. Biol. Med. 52 (2012) 59–69.
[58] P. Xiao, Z. Yang, J. Sun, J. Tian, Z. Chang, X. Li, B. Zhang, Y. Ye, H. Ji, E. Yu, J. Xie, Silymarin inhibits adipogenesis in the adipocytes in grass carp Ctenopharyngodon idellus in vitro and in vivo, Fish Physiol. Biochem. 43 (2017) 1487–1500.
[59] S. Punitha, M. Babu, V. Sivaram, V. Shankar, S. Dhas, T. Mahesh, G. Immanuel, T. Citarasu, Immunostimulating influence of herbal biomedicines on nonspecific immunity in Grouper Epinephelus tauvina juvenile against Vibrio harveyi infection, Aquacult. Int. 16 (2008) 511–523.
[60] A. Talpur, M. Ikhwanuddin, A. Ambok Bolong, Nutritional effects of ginger (Zingiber officinale Roscoe) on immune response of Asian sea bass, Lates calcarifer (Bloch) and disease resistance against Vibrio harveyi, Aquaculture 400–401 (2013) 46–52.
[61] M. Reverter, N. Bontemps, D. Lecchini, B. Banaigs, P. Sasal, Use of plant extracts in fish aquaculture as an alternative to chemotherapy: current status and future perspectives, Aquaculture 433 (2014) 50–61.
[62] T. Wang, Y. Liu, L. Tian, K. Mai, Z. Du, Y. Wang, H. Yang, Effect of dietary lipid level on growth performance, lipid deposition, hepatic lipogenesis in juvenile cobia (*Rachycentron canadum*), Aquaculture 249 (2005) 439–447.
[63] K. Lu, W. Xu, L. Wang, D. Zhang, C. Zhang, W. Liu, Hepatic β-oxidation and regulation of carnitine palmitoyltransferase (CPT) I in blunt snout bream Megalobrama amblycephala fed a high fat diet, PLoS One 9 (2014) e93135.
[64] G. Blanchard, J. Gardeur, N. Mathis, J. Brun-Bellut, P. Kestemont, Ultrastructural features of hepatocytes in cultured Eurasian perch (*Perca fluviatilis* L.) as affected by nutritional and husbandry conditions, Br. J. Nutr. 100 (2008) 317–331.
[65] Z. Du, P. Clouet, W. Zheng, P. Degrace, L. Tian, Biochemical hepatic alterations and body lipid composition in the herbivorous grass carp (*Ctenopharyngodon idella*) fed high-fat diets, Br. J. Nutr. 95 (2006) 905–915.
[66] Z. Tian, F. Shuai, G. Sun, M. Zhang, Effects of shosaikoto on Timp-1 Mrna expression in rats with liver fibrosis:an experimental study, Acta Academiae Medic Inae Qingdao Unive Rs Itatis 40 (2004) 224–226.
[67] S. Jiang, F. Li, Effects of Xiaochaihu Decoction on hepatic fibrosis in rats, Pharmacol. Clin. Chin. Mater. Med. 29 (2013) 17–19.
[68] C. Liu, M. Sun, L. Wang, G. Wang, G. Chen, C. Liu, P. Liu, Effects of Yinchenhao Tang and related decoctions on DMN-induced cirrhosis/fibrosis in rats, Chin. Med. 3 (2008) 1–9.
[69] Y. Zheng, Z. Zhao, L. Fan, S. Meng, C. Song, L. Qiu, P. Xu, J. Chen, Dietary supplementation with rutin has pro-/anti-inflammatory effects in the liver of juvenile GIFT tilapia, Oreochromis niloticus, Fish Shellfish Immunol. 64 (2017) 49–55.
[70] R. Martino, J. Cyrino, L. Portz, L. Trugo, Effect of dietary lipid level on nutritional performance of the surubim, Pseudoplatystoma coruscans, Aquaculture 209 (2002) 209–218.
[71] P. Xiao, H. Ji, Y. Ye, B. Zhang, Y. Chen, J. Tian, P. Liu, L. Chen, Z. Du, Dietary silymarin supplementation promotes growth performance and improves lipid metabolism and health status in grass carp (Ctenopharyngodon idellus) fed diets with elevated lipid levels, Fish Physiol. Biochem. 43 (2017) 245–263.
[72] L. Lao, X. Xue, Y. Li, Z. Wang, Y. Zhang, Influence of Xiaochaihu decoction on fatty degeneration of liver cells and GRP78 expression in rats, Chin. J. Info. TCM 19 (2012) 37–40.
[73] G. Qiu, F. Ye, Y. Liu, Y. Cai, R. Wang, Y. Sun, Q. He, The experimental study on the effects of Xiaochaihu Soup on nonmacoholic rat fatty liver, J. Xi an Jiaotong Univ. (Med. Sci.) 34 (2013) 400–402.
[74] T. Braunbeck, G. Gorge, V. Storch, R. Nagel, Hepatic steatosis in zebra fish (*Brachydanio rerio*) induced by long-term exposure to gamma-hexachlorocyclohexane, Ecotoxicol. Environ. Saf. 19 (1990) 355–374.
[75] L. Liu, J. Zhang, J. Luo, H. Guo, H. Deng, J. Chen, S. Sun, A role of cell apoptosis in lipopolysaccharide (LPS)-induced nonlethal liver injury in D-galactosamine (D-GalN)-sensitized rats, Dig. Dis. Sci. 53 (2008) 1316–1324.
[76] H. Cho, J. Hong, J. Choi, H. Choi, J. Kwak, D. Lee, S. Lee, S. Lee, β-Caryophyllene alleviates D-galactosamine and lipopolysaccharide-induced hepatic injury through suppression of the TLR4 and RAGE signaling pathways, Eur. J. Pharmacol. 764 (2015) 613–621.
[77] Y. Wang, Y. Li, J. Xie, Protective effects of probiotic Lactobacillus casei Zhang against endotoxin and d-galactosamine-induced liver injury in rats via anti-oxidative and anti-inflammatory capacities, Int. Immunopharmacol. 15 (2013) 30–37.
[78] J. Quan, M. Jin, H. Xu, D. Qiu, X. Yin, BRP, a polysaccharide fraction isolated from Boschniakia rossica, protects against galactosamine and lipopolysaccharide induced hepatic failure in mice, J. Clin. Biochem. Nutr. 54 (2014) 181–189.
[79] C. Liu, M. Sun, L. Wang, G. Wang, G. Chen, C. Liu, P. Liu, Effects of Yinchenhao Tang and related decoctions on DMN-induced cirrhosis/fibrosis in rats, Chin. Med. 3 (2008).
[80] X. Xia, C. Su, J. Fu, P. Zhang, X. Jiang, D. Xu, L. Hu, E. Song, Y. Song, Role of α-lipoic acid in LPS/D-GalN induced fulminant hepatic failure in mice: studies on oxidative stress, inflammation and apoptosis, Int. Immunopharmacol. 22 (2014) 293–302.
[81] Q. Jin, S. Jiang, Y. Wu, T. Bai, Y. Yang, X. Jin, L. Lian, J. Nan, Hepatoprotective effect of cryptotanshinone from Salvia miltiorrhiza in Dgalactosamine/

lipopolysaccharide-induced fulminant hepatic failure, Phytomedicine 21 (2014) 141–147.
[82] K. Ferri, G. Kroemer, Organelle-specific initiation of cell death pathways, Nat. Cell Biol. 3 (2001) E225–E263.
[83] X. Lin, S. Zhang, R. Huang, S. Tan, S. Liang, X. Wu, L. Zhuo, Q. Huang, Protective effect of tormentic acid from Potentilla chinensis against lipopolysaccharide/d-galactosamine induced fulminant hepatic failure in mice, Int. Immunopharmacol. 19 (2014) 365–372.
[84] A. Canbay, H. Higuchi, S. Bronk, M. Taniai, T. Sebo, G. Gj, Fas enhances fibrogenesis in the bile duct ligated mouse: a link between apoptosis and fibrosis, Gastroenterology 123 (2002) 1323–1330.
[85] S. Thapaliya, A. Wree, D. Povero, M. Inzaugarat, M. Berk, L. Dixon, Caspase 3 inactivation protects against hepatic cell death and ameliorates fibrogenesis in a diet-induced NASH model, Dig. Dis. Sci. 59 (2014) 1197–1206.
[86] H. Jaeschke, G. Gores, A. Cederbaum, J. Hinson, D. Pessayre, J. Lemasters, Mechanisms of hepatotoxicity, Toxicol. Sci. 65 (2002) 166–176.
[87] X. Lin, S. Wu, Q. Wang, Y. Shi, G. Liu, J. Zhi, F. Wang, Saikosaponin-d reduces H2O2-induced PC12 cell apoptosis by removing ROS and blocking MAPK-dependent oxidative damage, Cell. Mol. Neurobiol. 36 (2016) 1365–1375.
[88] S. Haupt, M. Berger, Z. Goldberg, Apoptosis - the p53 network, J. Cell Sci. 116 (2003) 4077–4085.
[89] Y. Hsu, P. Kuo, L. Chiang, C. Lin, Involvement of p53, nuclear factor kappaB and Fas/Fas ligand in induction of apoptosis and cell cycle arrest by saikosaponin d in human hepatoma cell lines, Cancer Lett. 213 (2004) 213–221.