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Research Article

Choline Alleviates Disorders of Lipid Metabolism in Hybrid Grouper (♀ *Epinephelus fuscoguttatus* × ♂ *E. lanceolatus*) Caused by High-Lipid Diet

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This study was conducted to evaluate the effect of dietary choline levels on growth performance, liver histology, lipid metabolism and related gene expression of hybrid grouper (♀ *Epinephelus fuscoguttatus* × ♂ *E. lanceolatus*) fed high-lipid diets. The fish (initial body weight 6.86 ± 0.01 g) were fed diets containing different choline levels (0, 5, 10, 15, and 20 g/kg, named D1, D2, D3, D4, and D5, respectively) for 8 weeks. The results shows that (a) dietary choline levels had no significant effect on the final body weight (FBW), weight gain rate (WGR), and specific growth rate (SGR) compared with the control group ($P > 0.05$); (b) with the increase in choline levels, triglyceride (TG), total cholesterol (TC), lipoprotein lipase (LDL), and high-density lipoprotein (HDL) of serum, all showed a tendency to increase and then decrease, and the maximum values were obtained in the D4 group ($P < 0.05$); (c) TG and TC in the liver showed a tendency to decrease first and then increase with the dietary choline levels and reached the minimum value in D3 and D4 groups, respectively ($P < 0.05$); (d) with increased choline levels, hepatic lipase (HL), lipoprotein lipase (LPL), adipose triglyceride lipase (ATGL), and fatty acid synthetase (FAS) in the liver all showed a tendency to increase and then decrease, and the maximum values were obtained in the D4 group, except ATGL that the maximum value was obtained in the D3 group ($P < 0.05$); (e) oil red staining showed that dietary choline significantly reduced hepatic lipid deposition; (f) choline supplementation significantly upregulated the expression of lipolytic genes lipoprotein lipase (*lpl*), hormone-sensitive lipase (*hsl*), and peroxisome proliferator-activated receptor α (*pparα*) and inhibited the expression of lipid synthesis genes fatty acid synthetase (*fas*), acetyl-CoA carboxylase α (*accα*), and peroxisome proliferator-activated receptor γ (*pparγ*). In summary, choline can promote lipid metabolism of hybrid grouper by regulating lipid metabolism-related enzyme activity and gene expression and mitigating the negative effects of high-lipid diets.

1. Introduction

For farmed fish, protein is the most important and the most expensive component of the feed [1]. As the most important high-quality animal protein raw material for aquatic animals, fish meal is characterized by high protein content, balanced amino acids, rich unsaturated fatty acids, good palatability, no antinutritional factors, and easy digestion and absorption and is being used in large quantities of carnivorous or omnivorous aquatic animal feeds [2]. With the

improvement of the intensive farming level, the demand for fish meal in the aquaculture industry is developing [3]. The addition of considerable amounts of fish meal in the feed will increase the content of the nitrogen in aquaculture water; the imbalance of the nitrogen cycle in water will cause pollution of water environment, thus hindering the growth and development of farmed fish [4]; on the other hand, with the continuous increase in the human population and the increasing demand for aquatic products, the fish meal has become more and more scarce. This brings hidden dangers to the sustainable development of the aquaculture industry [5]. Therefore, when designing feed formulations, how to reduce the use of fish meals under the premise of maintaining healthy fish growth has become a new research hot spot.

Lipid, as one of the three major energy substances, has the following benefits when its content is increased in feed: improving protein utilization, improving feed efficiency, reducing feed costs, and reducing nitrogen load, and the utilization of lipid in marine fish is higher than that in freshwater fish; therefore, high-lipid feed is often used in marine fish culture to achieve the above purpose [6, 7]. However, a high-lipid diet can affect the health of fish [8]. Most studies have shown that excessive lipid and lipid oxidation products not only do not promote fish growth but also cause damage to fish organs, especially the liver, resulting in fish metabolic disorders, slow growth, reduced feed utilization, and much higher morbidity [9, 10]. For example, grass carp (*Ctenopharyngodon idella*) and tilapia (*Oreochromis niloticus*) fed with high-lipid diets (15% and 16%) exhibited reduced growth performance and suppressed immune capacity and altered lipid metabolism [11, 12]. Also, high-lipid intake (10% or 15%) leads to excessive lipid deposition in the liver of blunt snout bream (*Megalobrama amblycephala*) coupled with growth retard [10, 13]. In addition, common carp (*Cyprinus carpio*) Jin et al. [14], turbot (*Scophthalmus maximus*) [15], *Takifugu rubripes* [8], and black seabream (*Acanthopagrus schlegelii*) [16] also exhibit similar symptoms with the increase in dietary lipid. Therefore, research on effective methods to improve growth inhibition and lipid metabolism in fish is important for high-lipid diet.

As a basic nutrient of terrestrial and aquatic animals, choline has a variety of biological functions in the body: (a) as a component of lecithin, which is an important component of cell membranes and lipoproteins, involved in tissue lipid metabolism; (b) as a component of the neurotransmitter acetylcholine, involved in the transmission of information in the nervous system; (c) as a precursor of betaine synthesis which can provide unstable methyl groups for methylation reactions in the body [17–19]. Studies have shown that choline can not only promote fish growth and improve feed efficiency but also prevent lipid liver in fish [20, 21]. However, long-term consumption of choline-deficient feed can cause lipid deposits in the liver of fish, resulting in stunted growth, reduced feed utilization efficiency, and increased mortality, such as carp (*Cyprinus carpio* L) [22], grass carp (*Ctenopharyngodon idella*) [23], and freshwater eel (*Anguilla japonica*) [24].

Hybrid grouper (♀ *Epinephelus fuscoguttatus* × ♂ *Epinephelus lanceolatus*) is known as gentian or pearl spot. It is a marine carnivorous fish with good taste and high nutritional value ([25, 26]. Compared with the parental fish, hybrid grouper has the characteristics of better growth and resistance and therefore can obtain higher economic benefits [27]. However, in practice, to reduce the content of fishmeal in feed and costs, the feeds developed for this species in China usually contain high levels of lipid, which inevitably leads to severe body lipid deposition and the ongoing outbreak of lipid liver in recent years, which poses a major challenge to the healthy and sustainable development of Chinese farming industry. Therefore, it is urgent to promote the utilization of high-lipid diets by developing effective strategies to avoid an excessive deposition. The study was conducted to evaluate the effect of dietary choline levels on growth, liver structure, lipid metabolism, and related gene expression of hybrid grouper (♀ *Epinephelus fuscoguttatus* × ♂ *E. lanceolatus*) fed high-lipid diets. It is expected that the data obtained from this experiment will be useful for the actual aquaculture of this species.

2. Material and Method

2.1. Experimental Diets. The basal diet was prepared with casein and white fish meal served as the protein source and fish oil and corn oil as a lipid source. Five iso-nitrogenous and iso-lipid diets were formulated containing 0 (basal diet, control), 5, 10, 15, and 20 (mg/kg) choline designated as D1, D2, D3, D4, and D5, respectively. All ingredients were crushed and sieved through a sixty-mesh sieve, then accurately weighed, and thoroughly mixed using the progressive enlargement method, as described by Ayisi and Zhao (2017). After homogenization of feed, add lecithin and oil, rub them manually, put them into a V-type vertical mixer after sieving, and mix them evenly and then add distilled water (320–420 ml/kg) and choline to mix them evenly. The diets were processed into 2.5 mm diameter pellets by a twin-screw extruder (F–26, South China University of Technology, Guangdong, China), air-dried at room temperature, then ground and sieved to an appropriate size and stored in Ziploc bags at −20°C. Ingredients and approximate compositions of the trial diets are shown in Table 1.

2.2. Fish and Feeding Trial. We followed the "Guide for the Care and Use of Laboratory Animals" set by the National institutes of Health strictly. This study was approved by the Animal Ethics Committee of Guangdong Ocean University (Zhanjiang, China).

The experiment was conducted in an indoor mariculture system at the Donghai Island Breeding Base of Guangdong Ocean University. Hybrid groupers (♀ *Epinephelus fuscoguttatus* × ♂ *E. lanceolatus*) were purchased from a local hatchery at Donghai Island (Zhanjiang, China) and acclimatized in aerated cement pools (5 m × 3.5 m × 1.8 m) to the experimental conditions for one week during which they were fed with a commercial feed (Zhanjiang, China). After fasting for 24 h, 375 fish (6.86 ± 0.01 g) were randomly distributed into 15 tanks (1000 l; 0.8 m in water depth). Each kind of experimental feed was fed to triplicate groups of fish twice daily (08:00 and 16:00) until apparent satiation. The amount of feed ingestion was recorded for 8 weeks. To maintain the water quality, about

Table 1: Composition and nutrient content of test diets (dry matter g/kg).

Ingredient	D1	D2	D3	D4	D5
Fish meal	420	420	420	420	420
Wheat gluten	120	120	120	120	120
Casein	150	150	150	150	150
Wheat flour	122	122	122	122	122
Soybean lecithin	15	15	15	15	15
Fish oil	50	50	50	50	50
Corn oil	70	70	70	70	70
Gelatinized starch	25	20	15	10	5
Premix[a]	10	10	10	10	10
Vitamin C	0.5	0.5	0.5	0.5	0.5
$Ca(H_2PO_4)_2$	15	15	15	15	15
Antioxidant	1	1	1	1	1
Attractant	1.5	1.5	1.5	1.5	1.5
Choline chloride	0	5	10	15	20
Total	1000	1000	1000	1000	1000
Proximate composition[b]	0	0	0	0	0
Moisture	102.7	109.5	92.4	95.2	94.7
Crude protein	488.5	492.3	501.3	501.6	504.7
Crude lipid	150	148	152.3	146.8	147.8
Ash	121.7	123	119.9	128.6	120.2

[a]Premix was obtained from Qingdao Master Biotechnology Co, Ltd. (Qingdao, China). [b]Measured value.

60% of water was exchanged every day. Each tank was provided with one piece of polyvinylchloride (PVC) pipe of 20.0 cm (diameter) × 30.0 cm (length) as a shelter for fish [28]. With natural illumination, the temperature of water ranged from 28 to 31°C, salinity was 28, and dissolved oxygen was >7 mg/l (continuous inflation was provided during the experiment), while ammonia and nitrates remained <0.05 mg/l, which was detected by PTF-001B multiparameter water quality detector (WBD Biotechnology Co., Ltd., China).

2.3. Sample Collection. At the end of 8-week feeding trial, all fish were fasted for 24 hours before collecting samples. The number and weight of fish in each tank were recorded. After weighing, four fish from each tank were randomly selected for blood collection with 1 ml sterile syringes. Put the blood into 1.5 ml microcentrifuge tubes and place them at 4°C for 12 hours. After separation at 3500 r/min for 15 minutes, the serum was collected and stored in a refrigerator at -80°C for the determination of biochemical indices. The liver of another three fish were collected randomly to analyze biochemical indices and enzyme activities; then, the liver of another four fish from each tank were quickly removed and loaded in 2 ml enzyme-free centrifuge tubes containing RNAlater and then stored at -80°C for subsequent analysis of related gene expression. Besides, the liver of another two fish was immediately removed, after being washed with PBS, and fixed in 4% formaldehyde over 4 hours for histology analysis.

2.4. Methods of Analysis. The formulas for calculating growth performance were weight gain rate (WGR, %) = 100 × [final body weight (FBW) − initial body weight(IBW)]/IBW and $specific\ growth\ rate\ (SGR, \%/d) = 100 \times [(\ln\ (FBW) - \ln\ (IBW)]/days$ of *experiment*.

The contents of triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), lipoprotein lipase (LPL), hepatic lipase (HL), fatty acid synthase (FAS), adipose triglyceride lipase (ATGL), and very low-density lipoprotein (VLDL) in serum and liver were analyzed by using commercial ELISA kits (Shanghai Enzyme-Linked Biotechnology Co., Ltd., Shanghai, China). The test steps were operated according to the instructions of kits.

2.5. Oil Red Staining. Oil red staining was carried out to determine the amount of neutral lipid deposition in the liver. Sectioned liver tissues were fixed with 4% paraformaldehyde and then frozen with liquid nitrogen. The sectioned tissue (approximately 6–10 μm thickness) slides were stained with oil red solution (Servicebio, China) for 8–10 min at room temperature, and the nuclei were counterstained with hematoxylin solution (Servicebio, China) for 3–5 min. After staining, the slides were washed under running tap water. Finally, a cover glass was fixed to the slide with glycerol jelly mounting medium (Servicebio, China), and the lipid droplets were observed using a light microscope (Nikon Eclipse NieU, Japan) at 400x magnification. The deep red areas were scaled and tagged to calculate the proportions with ImageJ software (National Institutes of Health, USA) [29].

2.6. RNA Extraction and cDNA Synthesis. 1 ml TransZol UP (TransGen Biotech, Beijing, China) was added to the samples, and the total RNA was extracted at the instance of the manufacturer's specification. The quality and quantity of isolated RNA were detected at 260 and 280 nm with a NanoDrop 2000 spectrophotometer (Gene Company Limited, Guangzhou, China) by electrophoresis of 1% agarose gel, severally. The first-strand cDNA was synthesized by using PrimeScript™ RT Reagent Kits with cDNA Eraser (Takara, Japan). Then, the cDNA was reserved at −80°C for real-time quantitative polymerase chain reaction (RT-qPCR).

2.7. Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed in a 384-well plate with a 10 μl reaction volume containing 5 μl of SYBR® Green Real-Time PCR Master Mix, 0.8 μl of each primer, 1 μl of cDNA sample, and 3.2 μl of RNase Free dH_2O. The PCR conditions were set using a thermal programmer at 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 34 s. Each sample was tested in triplicate. Primers of the reference gene (β-actin) and target gene were designed according to published sequences of groupers (Table 2). Threshold cycle (Ct) values were collected from each sample after finishing the process. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [30].

2.8. Statistical Analysis. Results are presented as means ± standard error (SE). Before performing one-way analysis of variance (ANOVA), all data were tested for normality distribution

Table 2: Real-time quantitative PCR primers.

Primer names	Forward and reverse primer sequence (5′ to 3′)	Product size	TM (°C)	GenBank accession no.
β-Actin -F/R	ACTGCTGCCTCCTCTTCATC/ ACCGCAAGACTCCATACCAA	135	84.5	KU746361.1
hsl-F/R	TGCTCCGAGATTTCCGACAG/ AGGATGCCTCCGAAATGC	144	86.6	KF049203.1
lpl-F/R	CCACCTGTTCATCGACTCCC/ TCGGACGGACCTTGTTGAT	152	86	EU683732.1
pparα-F/R	AAAAGCACGGCTCATACTCAC/ GCTCTGGCAGCAGTGGAA	226	85.7	FJ196235.1
fas-F/R	TCGTTGACCAGCCTATTCC/ GCCTTCACTGCGTCCTCT	181	84.9	FJ196231.1
acc-F/R	CTCAGCAAGACGACCAACGC/ GGCAGCCATCCTGACAACCT	243	89.6	KX066238.1
pparγ-F/R	ACCGCAGCACGAAGAACAAC/ *TGGACGCCATAGTGAAACCC*	225	82	XM-022748373.1
*apo*B-100-F/R	ACCACATCCTCATTCCCTTCT/ CATCTTTATCCTGGACAACACTCT	180	80	KM593126.1

Notes: *hsl*: hormone sensitive lipase; *lpl*: lipoprotein lipase; *pparα*: peroxisome proliferator-activated receptor *α*; *fas*: lipidity acid synthetase; *accα*: acetyl-CoA carboxylase *α*; ppar*γ*: peroxisome proliferator-activated receptor *γ*; *apo*B-100: *apolipoprotein* B-100.

(Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test) and then were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. P value < 0.05 was considered significant. All statistical analyses were performed using SPSS version 20.0 (SPSS Inc., USA) for Windows.

3. Results

3.1. Growth Performance. The IBW, FBW, WGR, and SGR are shown in Table 3; as we can see, there was no significant difference of growth performance among choline-treated groups ($P > 0.05$).

3.2. Serum Biochemical Indices. As shown in Table 4, with the choline levels increased, TG, TC, LDL, and HDL all showed a trend of first increasing and then decreasing, achieving a maximum in the D4 group, and were significantly higher than those of D1 ($P < 0.05$); in addition, LDL of D4 and D5 groups was significantly higher than that in the control group ($P < 0.05$).

3.3. Liver Biochemical Indices and Lipid Metabolizing Enzyme Activities. Biochemical indices and liver enzyme activities related to lipid metabolism are shown in Table 5. With the increase in choline concentration, TG, TC, and VLDL in the liver showed a trend of first decreasing and then increasing and TG content was the lowest in the D3 group, while the lowest value of VLDL appeared in the D4 group and both of them were significantly lower than those of the control group ($P < 0.05$). TC content in all groups was significantly lower than that in the D1 group except the D2 group and reached the lowest value in the D4 group ($P < 0.05$). LPL, HL, FAS, and ATGL all showed a trend of increasing and then decreasing with the increase in choline concentration: LPL and HL achieved the maximum values in D3 and D4 groups, respectively, and were significantly higher than those in the control group. Except for the D5 group, FAS activity in choline-treated groups was significantly higher than that in the D1 group ($P < 0.05$) and was highest in the D3 group. ATGL activity in all groups except D2 was significantly higher than that of the control group ($P < 0.05$) and achieved a peak in the D4 group.

3.4. Oil Red Staining. The results of oil red-stained liver sections are shown in Figure 1. In oil red staining, nuclei and lipid are dyed blue and red, respectively. With the increase in choline levels, the number and area of lipid droplets in the liver decreased significantly ($P < 0.05$). In addition, the red areas in the control group were larger than those in choline-treated groups.

3.5. Lipid Metabolism-Related Gene Expression. As shown in Figure 2, the expression of hormone-sensitive lipase (*hsl*), lipoprotein lipase (*lpl*), and peroxisome proliferator-activated receptor *α* (*pparα*) was significantly upregulated with the dietary choline concentration, and all of them reached the maximum in the D4 group, while the expression of fatty acid synthetase (*fas*), acetyl-CoA carboxylase *α* (*accα*), and peroxisome proliferator-activated receptor *γ* (*pparγ*) was suppressed ($P < 0.05$). In addition, the expression of *apolipoprotein*B-100 (*apo*B-100) showed a trend of increasing and then decreasing, and the expression of *apo*B -100 in the D2 group was significantly higher than that in the control group ($P < 0.05$).

Table 3: Effect of choline levels in high-lipid diets on growth performance of hybrid grouper (g).

Items	D1	D2	D3	D4	D5
IBW (g)	6.85 ± 0.00	6.84 ± 0.00	6.85 ± 0.01	6.85 ± 0.01	6.85 ± 0.00
FBW (g)	31.48 ± 0.18	32.80 ± 1.09	32.89 ± 2.26	32.27 ± 2.09	31.90 ± 4.56
WGR (%)	359.20 ± 2.78	379.25 ± 15.79	380.64 ± 32.96	371.17 ± 30.93	365.82 ± 66.83
SGR (%/d)	2.93 ± 0.01	3.01 ± 0.06	3.01 ± 0.13	2.97 ± 0.12	2.91 ± 0.30

Note: values are means ± SE ($n = 3$). Abbreviations: IBW: initial body weight; FBW: final body weight; WGR: weight gain rate; SGR: specific growth rate.

Table 4: Effect of choline levels in high-lipid diets on serum biochemical indices of hybrid grouper.

Items	D1	D2	D3	D4	D5
TG (mmol/L)	0.13 ± 0.01^{a}	0.14 ± 0.01^{a}	0.16 ± 0.01^{ab}	0.18 ± 0.02^{b}	0.16 ± 0.02^{ab}
TC (mmol/L)	2.19 ± 0.07^{a}	2.23 ± 0.12^{a}	2.23 ± 0.20^{a}	3.06 ± 0.21^{b}	2.60 ± 0.22^{ab}
LDL (mmol/L)	0.41 ± 0.01^{a}	0.51 ± 0.07^{a}	0.60 ± 0.02^{a}	1.16 ± 0.13^{b}	1.04 ± 0.08^{b}
HDL (mmol/L)	2.23 ± 0.30^{a}	2.52 ± 0.53^{a}	2.78 ± 0.41^{a}	3.79 ± 0.63^{b}	3.06 ± 0.96^{a}

Note: values are means ± SE ($n = 3$). The difference of superscript letters in the same line is significantly different ($P < 0.05$). Abbreviations: TG: triglyceride; TC: total cholesterol; HDL: high-density lipoprotein; LDL: low-density lipoprotein.

Table 5: Effect of choline levels in high-lipid diets on the biochemical indices and lipid metabolism enzyme activities in the liver of hybrid grouper.

Items	D1	D2	D3	D4	D5
TG (pmol/mg. pro)	11.18 ± 0.56^{b}	10.41 ± 0.65^{b}	7.89 ± 0.57^{a}	9.34 ± 0.30^{ab}	11.26 ± 0.29^{b}
TC (μmol/mg. pro)	11.16 ± 0.56^{c}	9.94 ± 0.66^{bc}	8.34 ± 0.53^{ab}	7.87 ± 0.75^{a}	8.15 ± 0.62^{ab}
LPL (mU/mg. pro)	332.99 ± 47.35^{a}	414.39 ± 27.43^{ab}	503.07 ± 49.49^{b}	454.06 ± 39.67^{ab}	431.87 ± 66.13^{ab}
HL (U/mg. pro)	40.62 ± 2.46^{a}	44.69 ± 2.75^{a}	46.84 ± 0.94^{a}	55.43 ± 2.02^{b}	46.76 ± 2.47^{a}
FAS (mU/mg. pro)	1225.6 ± 37.14^{a}	1533.7 ± 28.13^{b}	2711.16 ± 24.87^{c}	1471.43 ± 59.06^{b}	1250.66 ± 53.06^{a}
ATGL (mIU/mg. pro)	264.93 ± 9.43^{a}	301.07 ± 14.43^{ab}	406.07 ± 7.36^{c}	421.05 ± 9.89^{c}	348.76 ± 11.12^{b}
VLDL (μmol/mg. pro)	15.07 ± 0.71^{b}	14.78 ± 0.73^{b}	12.57 ± 1.18^{ab}	11.51 ± 1.27^{a}	13.13 ± 0.68^{ab}

Note: values are means ± SE ($n = 3$). The difference of superscript letters in the same line is significantly different ($P < 0.05$). Abbreviations: TG: triglyceride; TC: total cholesterol; LPL: lipoprotein lipase; HL: hepatic lipase; FAS: fatty acid synthetase; ATGL: adipose triglyceride lipase; VLDL: very low-density lipoprotein.

4. Discussion

It has been widely shown that choline has a growth-promoting effect, and its mechanism of action is mainly due to the ability of choline to provide active methyl groups, which are involved in the anabolism of phospholipids, carnitine, and other substances with important physiological effects [31, 32]. The addition of choline significantly promoted the growth and protein utilization of juvenile gibel carp (*Carassius auratus gibelio*) [33]. Dietary choline levels significantly increased the survival, weight gain, and feed utilization of cobia (*Rachycentron canadum*) [32] and yellow catfish (*Pelteobagrus fulvidraco*) [34]. In addition, when choline in feed exceeded 7500 mg/kg, the growth of yellow perch (*Perca flavescens*) was not affected by choline levels Twibell and Brown [21]. However, in the present study, FW, WGR, and SGR tended to increase and then decrease with increasing choline concentration, but there was no significant difference. This is in agreement with the findings of red drum (*Sciaenops ocellatus*) [35], giant grouper (*Epinephelus lanceolatus* (Bloch)) [36], Nile tilapia (*Oreochromis niloticus*) [37], and black seabream (*Acanthopagrus schlegelii*) Jin et al. [14]. And the reason for this difference may be that the lipid content of feed meets the requirement of grouper or the 8-week breeding cycle is not long enough. In addition, differences in fish species and growth stages may also be a cause [38, 39]. Besides, few studies have been conducted on the effects of dietary choline on fish fed with high-lipid diets. However, although the high-lipid feed with choline addition did not affect the growth performance of hybrid grouper, the present study showed that dietary choline had a significant effect on lipid deposition and lipid metabolism, which provides insight into possible regulatory mechanisms.

Choline prevents the abnormal accumulation of lipid in the liver and promotes the oxidative utilization of fatty acids in the liver, reduces lipid formation, and has the function of regulating body lipid deposition, so it is called a lipotropic factor [40]. Blood lipids are the general term for all lipids in the blood, including TG, TC, phospholipids, and free fatty

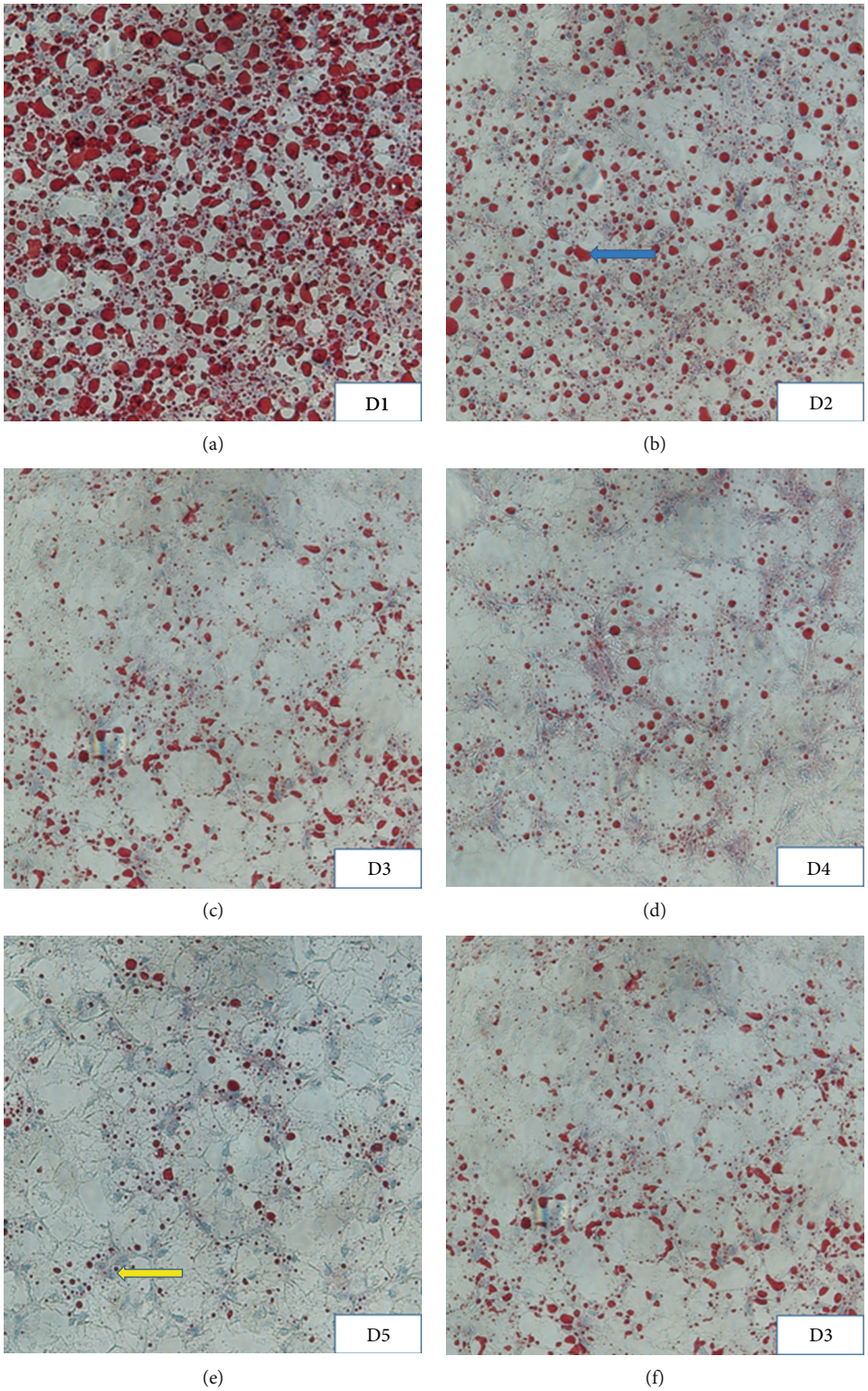

Figure 1: The effect of choline levels in high-lipid diets on the histological structure of the liver in hybrid grouper (400x). Notes: the red nearly spherical areas are lipid droplets (blue arrow); the small blue spots are the nucleus (yellow arrow). Lipid accumulation was quantified by measuring the intensity of the stained oil droplets. Values were presented as means ± SEM of three replicates, and mean values with unlike letters indicate significant differences ($P < 0.05$) (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

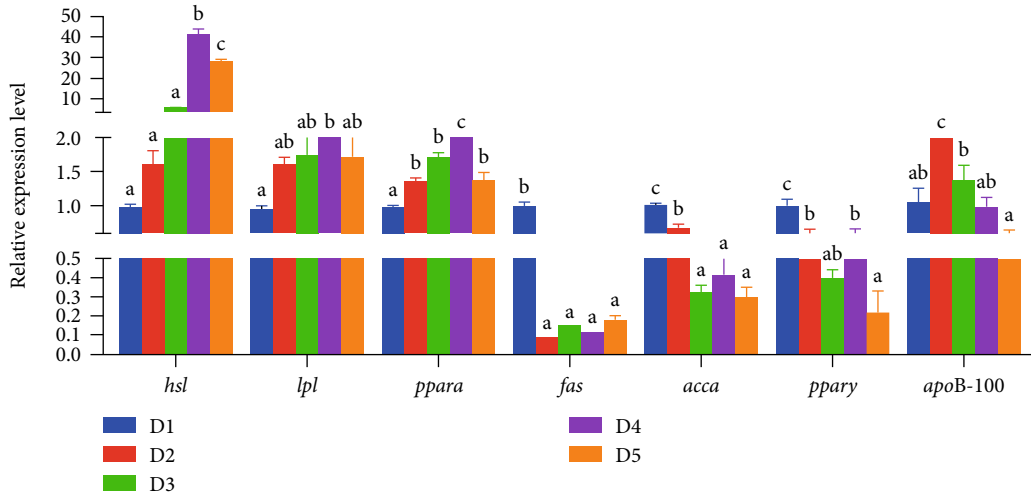

Figure 2: The effect of choline levels in high-lipid diets on relative expressions of lipid metabolism-related genes of the liver in hybrid grouper. Notes: values are means ± SE ($n = 3$). Different letters assigned to the bars represent significant differences ($P < 0.05$). *hsl*: hormone sensitive lipase; *lpl*: lipoprotein lipase; *pparα*: peroxisome proliferator-activated receptor α; *fas*: fatty acid synthetase; *accα*: acetyl-CoA carboxylase α; pparγ: peroxisome proliferator-activated receptor γ; *apo*B-100: *apolipoprotein* B-100.

acids [41]. Although they represent only a small fraction of the total lipids in body, they all need to pass through the bloodstream and travel to the tissues and therefore reflect the lipid metabolism in body [42]. Studies have shown that lack of choline in feed causes lower serum TG and TC of hybrid tilapia [20] and white sturgeon (*Acipenser transmontanus*) [43]. Probably due to the important contribution of choline to phospholipid synthesis [44], HDL and LDL reflect the lipid catabolism and transport in the animals' body [45]. HDL reduces excess cholesterol in blood by transporting cholesterol from the animal's body back to the liver in the form of HDL-C, which is converted and metabolized by the liver. In addition, HDL has been shown to play a growth-promoting role in fish and mammalian, but the mechanism is not clear [46], while LDL transports endogenous cholesterol synthesized by the liver to the body tissues as LDL-C [47]. As carrier proteins of lipid, changes in the content of HDL and LDL were positively correlated with changes in TG. When choline content in feed increased, the HDL activity in serum of gibel carp also increased [33]. In this experiment, as the concentration of choline in feed increased, serum TG, TC, HDL, and LDL showed a trend of first increasing and then decreasing, and D4 was higher than the remaining groups. It can be inferred that when choline was lacking in high-lipid diet, the lipid utilization efficiency of fish was low, and when choline in diet met the demand, the lipid utilization efficiency of fish was significantly increased and the organism metabolism was accelerated.

It is suggested that the effect of choline in reducing liver lipid content in fish may be achieved through the direct or indirect involvement of choline in hepatic lipid transport [48]. TG is the main component of hepatic lipids of fish, and it is transported by VLDL between the liver and serum [49]. The synthesis of VLDL is closely related to the phosphatidylcholine content. In the present experiment, choline addition significantly decreased TG and TC content in the liver, which was contrary to the results in serum. Similar results were obtained in different species, such as channel catfish (*Ictalurus punctatus*) (Wilson & Poe 1988) and lake trout (*Salvelinus namaycush*) [17]. And VLDL showed the same trend as TG in the liver. This suggests that choline addition may promote the transport of lipid from the liver to serum, thus reducing liver lipid deposition.

HL and LPL are two key enzymes involved in lipid degradation in the fish liver, together known as total lipase. HL is synthesized in hepatocytes and acts as a ligand to facilitate the entry of low-density lipoproteins and chylomicrons into the hepatocytes and is directly involved in the reversal of HDL cholesterol and HDL residue. LPL is the key enzyme that catalyzes the hydrolysis of triglycerides in chylomicrons and very low-density lipoproteins and plays a key role in the regulation of lipid metabolism Chen et al. [50]. Therefore, LPL activity in various tissues serves as an assessment of the catabolic utilization or storage of ingested feed lipid in the organism [51]. LPL abnormalities could easily lead to atherosclerosis, obesity, diabetes, Alzheimer's disease, and other diseases [52]. Reports show that increased HL and LPL activities promote lipid catabolism in the liver [53, 54]. Adipose triglyceride lipase (ATGL) is another key lipase identified in recent studies to initiate lipid mobilization. It specifically hydrolyzes the first ester bond of TG and is considered to be the rate-limiting enzyme in the TG hydrolysis process. It was found that a high-lipid diet significantly reduced the expression of ATGL mRNA in large yellow croaker (*Pseudosciaena crocea* R.) [55]. In this experiment, we can observe that with the increase in choline concentration, LPL, HL, and ATGL all showed a trend of increasing and then decreasing, which indicates that choline can improve the decomposition of lipid in the liver and reduce lipid deposition. In addition, the rise in FAS activity may be a response mechanism of the body to the accelerated lipid metabolism.

The above results indicate that choline can regulate lipid metabolism in the body in a direct or indirect manner. Lipid deposition in animals depends on the level of serum

triglycerides, and the amount of serum triglyceride depends on the synthesis in the liver. Therefore, the contents of liver lipid and serum triglyceride are interrelated and affect each other.

Oil red staining is able to judge the degree of steatosis and is a useful tool for histopathological diagnosis [56]. Oil red staining section is used to observe the amount of lipid in cells by the extent of stained lipid droplets [57], which is mainly present in the form of triglycerides in liver lipid [58]. In this study, we could observe that when choline is not added to the feed, the lipid droplets in the liver not only rise in number but also occupy a large area; the number and area of lipid droplets in the liver decreased significantly as the concentration of choline in feed increased ($P < 0.05$). This indicates that choline can reduce liver lipid deposition by decreasing the levels of TG and TC in the liver, which is consistent with the changes in the levels of TG and TC in the liver described above.

To further explore the mechanism of dietary choline in regulating lipid metabolism, we analyzed the relative expression of some hepatic genes involved in lipogenesis (*fas, accα, pparγ*), lipolysis (*hsl*, *lpl*, and *pparα*), and lipid transport (*apo*B-100). The results of this study can provide data to support the regulation of lipid metabolism by choline at the molecular level.

De novo lipogenesis is a major source of FA in hepatocytes, which is regulated by two rate-liming enzymes ACC and FAS [59]. FAS is the key enzyme for adipogenesis from scratch, and it is sensitive to both nutritional and hormonal regulation (Moustaid et al. 1996). Fatty acid (FA) oxidation is essential in hepatic lipid metabolism, especially for animals fed high-lipid diets (Du et al. 2006). When dietary lipid intake exceeds the ability of hepatocytes to oxidize FAs, large amounts of triglycerides are synthesized and deposited in vesicles, leading to steatosis and even causing fatty liver [10]. In the present study, choline addition significantly reduced *fas* expression compared to D1, while enzyme activity results showed an increase in FAS, suggesting that downregulation of *fas* was too weak to affect enzyme activity or that *fas* expression may rose after transcription [60]. Acetyl-CoA carboxylase α (ACCα) is a cytosolic enzyme that controls the production of malonyl-CoA and thus plays an important role in the biosynthesis of long-chain fatty acids [61]; PPARγ is considered to regulate adipocyte differentiation, fatty acid storage, and glucose metabolism [62]. The upregulated *acc* and *fas* were also observed in medaka fed with high-lipid diet [63]. In the present experiment, the expression of *accα* as well as *pparγ* decreased significantly with increasing choline levels, suggesting a decrease in lipid production in the liver which is similar to the juvenile black seabream (*Acanthopagrus schlegelii*) [14]. HSL is a key enzyme in lipolysis and plays a major role in the metabolism of the body by hydrolyzing TG and TC [64, 65]. LPL catalyzes the breakdown of protein-related triglycerides to glycerol and free fatty acids, which plays a key role in the metabolism of triglycerides [66]. *pparα* as a nuclear receptor is activated by fatty acids and regulates the transcription of numerous gene-encoding enzymes in fatty acid oxidation, such as CPT1 in mitochondria and CYP2E1 in extra mitochondria; it reduces the synthesis of TG, free FA, and VLDL mainly by regulating the oxidation and transport processes of FA metabolism-related genes, including FAβ (Yuan et al.). In the present study, choline significantly upregulated the expression of *hsl*, *lpl*, and *pparα*, suggesting that choline could reduce the amount of lipid deposition in the liver by reducing lipid synthesis and promoting lipid catabolism. There are very few studies on the effect of choline on the expression of genes related to lipid metabolism. The known studies showed that the addition of choline to high-lipid diets significantly upregulated the expression of *ppar* and *hsl* and inhibited the expression of *fas* in juvenile black seabream [16], while in the study of yellow catfish, it was found that the addition of choline to diets upregulated the expression level of *lpl* (Zhi et al., 2016). In addition, a study on betaine, which is closely related to choline, found that the addition of 1.2% betaine to high-lipid diets significantly downregulated the expression of *fas* and promoted the expression of *pparα* [67]. Lipid transport is another factor that affects lipid hemostasis. ApoB-100 is a major component of VLDL and LDL-C, which is involved in the assembly of VLDL and is important for the outward transport of lipids and cholesterol in the liver [68]. In the present experiment, the expression of *apo*B-100 tended to increase and then decrease with increasing choline levels, indicating that the outward transport of hepatic lipid first increased and then decreased, which may be related to the significant upregulation of lipolytic genes. Some studies have also shown that dietary bile acid enhanced the *apo*B-100 gene expression in juvenile largemouth bass (*Micropterus salmoides*) fed with high lipid [69]. Hence, we conclude that dietary choline supplementation can reduce lipid deposition and alleviate hepatic steatosis through the regulation of lipid metabolism by upregulating lipolysis and lipid transport and downregulating lipogenesis pathway gene expression levels.

5. Conclusion

In summary, choline can improve the utilization of high-lipid feeds in grouper by inhibiting *fas*, *accα*, and *pparγ* expression, upregulating *hsl*, *lpl*, *ppara*, and apoB-100 expression, regulating lipid metabolism-related enzyme activity, reducing abnormal lipid deposition, and promoting lipolysis. And the mechanism of regulation needs to be further investigated.

Data Availability

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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