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Reduced fatty acid β-oxidation improves glucose catabolism and liver health in Nile tilapia (*Oreochromis niloticus*) juveniles fed a high-starch diet

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ABSTRACT

Fish are poor users of dietary carbohydrates and often display prolonged hyperglycemia and fat deposition after feeding high digestible carbohydrate diets. Recently, fatty acid β-oxidation (FAO) inhibition has been reported to increase glucose oxidation in fish. Therefore, this study tested the assumption that the inhibition of FAO with mildronate (MD, a carnitine synthesis inhibitor) might also increase glucose utilization and alleviate adverse effects induced by high starch diet (HSD) in Nile tilapia, *Oreochromis niloticus*. Nile tilapia juveniles (6.13 ± 0.11 g) were cultured in nine 200-L tanks (30 fish per tank) and divided into three groups (three tanks per group). The fish were fed twice a day (9:00 and 18:30) at 4% body weight by using a normal starch diet (NSD, 30% corn starch), a HSD (45% corn starch), or a HSD supplemented with MD (25 g/kg of diet, HSD + MD) for eight weeks. These three feeds contained approximately 35.8% protein and 6.4% lipid. The fish each tank were weighed every two weeks, and the feeding amount was adjusted accordingly. After the feeding trial, the fish fed on HSD showed higher hepatosomatic index (HSI), visceral somatic index (VSI), serum triglyceride concentration and whole-body and tissue (liver and muscle) lipid contents than those fed on NSD. The fish fed on HSD also had higher relative area of vacuolation in the liver, hepatic malondialdehyde (MDA) content, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in the serum than those fed on NSD. Moreover, the fish fed on HSD increased serum glucose and insulin concentrations, and hepatic lactate, pyruvate and glycogen contents, but reduced whole-body protein content and dietary protein utilization than those fed on NSD, indicating that HSD induced fat deposition, liver damage, glucose intolerance and lowered protein-sparing effect. However, the fish fed on HSD + MD decreased hepatic carnitine content and FAO activity, attenuated the indexes related to fat deposition and liver damage, improved blood glucose clearance and whole-body protein deposition than those fed on HSD, suggesting that the adverse effects caused by HSD were reversed after FAO inhibition. Furthermore, the fish fed on HSD down-regulated the expression of genes associated with glucose uptake, glycolysis, FAO process, and lipolysis compared to those fed on HSD + MD and NSD, yet up-regulated lipogenic and proteolytic genes. These data suggested that inhibition of FAO improved glucose utilization and alleviated the HSD-induced adverse effects in Nile tilapia. This work demonstrates that, modifying mitochondrial FAO activity regulates the ability of fish to adapt to HSD intake through remodeling energy homeostasis. Our study provides new insights into improving carbohydrate utilization in aquatic animals.

1. Introduction

Carbohydrates are considered as the most economical source of energy for aquaculture due to their abundance and relatively low cost (Kamalam et al., 2017). High digestible carbohydrate diets are currently applied extensively in fish feeds for omnivorous (Boonanuntanasarn et al., 2018) and herbivorous (Tan et al., 2009) to reduce the use of dietary protein, resulting in a protein-sparing effect for growth in some

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fish species (Kamalam et al., 2017). However, unlike mammals, teleost are generally characterized by a limited capacity to utilize efficiently carbohydrates for energy and are thus regarded as "glucose intolerant" (Polakof et al., 2012; Wilson, 1994). Indeed, many fish display prolonged hyperglycemia after feeding high digestible carbohydrate diets (Boonanuntanasarn et al., 2018; Jin et al., 2014; Kamalam et al., 2017; Pérez-Jiménez et al., 2015; Polakof et al., 2011; Tan et al., 2009). Moreover, intake of high carbohydrate diets causes excess fat deposition in tissues and whole-body in farmed fish, consequently induce retarded growth (Boonanuntanasarn et al., 2018; Liu et al., 2018; Xu et al., 2017; Xu et al., 2018). The low capacity of carbohydrate utilization in fish has been ascribed partly to the imbalance between glucose consumption (glycolysis) and production (gluconeogenesis), owing to insulin resistance (Hemre et al., 2002; Kamalam et al., 2017). Therefore, finding effective approaches to improve glucose utilization and reduce adverse effects caused by feeding high digestible carbohydrate diets in fish is a critical research topic for fish nutritionists.

Mitochondrial fatty acid β-oxidation (FAO) is a major lipid catabolism pathway in mammals and plays a pivotal role in maintaining energy homeostasis (Houten et al., 2016; Lee et al., 2017; Morrow et al., 2017; Vandanmagsar et al., 2016; Wicks et al., 2015). It has been pointed out that inhibited mitochondrial FAO triggers a systemic compensatory metabolic response that protects mice against high-energy diet-induced obesity and glucose intolerance (Lee et al., 2015; Lee et al., 2016; Lee et al., 2017; Zhang et al., 2010). Evidently, inhibited FAO after mildronate (MD, a carnitine synthesis inhibitor) treatment elevated insulin sensitivity and glucose utilization by facilitating glucose uptake and glycolysis (Liepinsh et al., 2008, 2009a). In our recent fish studies, dietary MD supplementation also reduced endogenous carnitine content and inhibited mitochondrial FAO activity in zebrafish (*Danio rerio*) (Li et al., 2018) and Nile tilapia (*Oreochromis niloticus*) (Li et al., 2020a, 2020b). In these studies, carbohydrate utilization was improved through enhanced glucose uptake and glycolysis and reduced gluconeogenesis and glycogenesis (Li et al., 2018; Li et al., 2020a). In addition, inhibition of FAO also increased whole-body and muscle protein contents, owing to the activation of the mechanistic target of rapamycin (mTOR) signaling pathway and decreased amino acid catabolism and proteolysis (Li et al., 2018; Li et al., 2020a). These preliminary results indicate that inhibited FAO in fish fed on a normal diet alters nutrient metabolism by increasing glucose utilization and protein deposition as compensative mechanisms. Therefore, we hypothesized that FAO inhibition might also play beneficial effects on glucose utilization and alleviate the adverse effects induced by feeding fish with high starch diet (HSD).

Tilapia generally require 30 to 35% starch for optimum growth, thus 45% starch diet has been commonly called "high-starch diet" (Wang et al., 2005; Boonanuntanasarn et al., 2018). In this study, Nile tilapia juveniles were fed with a normal starch diet (NSD, 30% corn starch), HSD (45% corn starch), or HSD supplemented with MD (25 g/kg of diet, HSD + MD) for eight weeks. The aim was to evaluate the effects of inhibited FAO on glucose utilization, fat deposition, and protein metabolism in fish fed on HSD. Afterwards, the growth performance, biochemical parameters and expression genes involved in energy metabolism were analyzed. This is the first study to investigate the potential regulatory role of mitochondrial FAO inhibition in glucose utilization and nutrient metabolism in fish fed on HSD. The data generated enhance our understanding of energy homeostasis regulation among nutrients in fish.

2. Materials and methods

This study was approved by the Committee on the Ethics of Animal Experiments of East China Normal University (Approval ID: F20190101).

2.1. Experimental fish, diets, and design

All juvenile Nile tilapia were obtained from Yiqian aquafarm (Guangzhou, China). Before the feeding trial, the fish were acclimated in five 200-L tanks at 28 °C under a 12 h/12 h light /dark cycle for two weeks. During this period, the fish were fed by using a commercial diet containing ≥35% protein and ≥ 5% lipid (Tongwei, Co. Ltd., Chengdu, China). Three experimental diets [normal starch diet (NSD, 30% corn starch), a high-starch diet (HSD, 45% corn starch), and a HSD supplemented with MD (Chengdu Micxy Chemical Co. LTD, China) (HSD + MD)] were formulated for use in the present study (Table 1). The dose for dietary MD was 25 g/kg based on our previous studies in Nile tilapia (Li et al., 2020a, 2020b; Pan et al., 2017). The diets contained approximately 35.8% protein and 6.4% lipid and were made as described previously in our study (Lu et al., 2019). Casein and gelatin were used as protein sources, soybean oil was used as the lipid source while corn starch was the carbohydrate source. After acclimation, 24 fish with relatively similar weights (a pooled sample from four fish mixture, six replicates) were collected and stored at −20 °C for initial whole-body protein content required for calculation of protein productive value (PPV). Then, 270 healthy Nile tilapia (6.13 ± 0.11 g, bulk weighed) were randomly allocated into nine tanks (30 fish per tank) in a recirculating aquaculture system. The fish were divided into three groups (three replicates for each group) and fed twice a day (9:00 and 18:30) at 4% body weight by using the NSD, HSD, or HSD + MD for eight weeks. The feed consumption and fish weight in each tank were recorded daily and every two weeks, respectively. During the feeding trial, all feeds were eaten completely every day. The total feed consumption was used for calculation of feed conversion ratio (FCR), protein efficiency ratio (PER) and protein productive value (PPV). During the study period, the water temperature ranged from 26 to 28 °C, dissolved oxygen from 4.8 to 6.4 mg/L and pH from 7.5 to 7.9, while ammonia nitrogen was <0.02 mg/L.

2.2. Sample collection

At the end of the feeding experiment all fish survived, and fish in

Table 1
The formulation and proximate composition of the experimental diets.

Ingredients (g/kg)	NSD	HSD	HSD + MD
Casein	300	300	300
Gelatin	75	75	75
Soybean oil	70	70	70
Corn starch	300	450	450
Mildronate	0	0	25
Vitamin premix[1]	10	10	10
Mineral premix[2]	20	20	20
$Ca(H_2PO_4)_2$	10	10	10
Carboxy methyl cellulose	30	30	30
Cellulose	177.75	27.75	2.75
Choline chloride	5	5	5
Dimethyl-β-propiothetin	2	2	2
Butylated hydroxytoluene	0.25	0.25	0.25
Proximate composition (dry matter basis, %)			
Dry matter	90.2	90.0	90.0
Total protein	35.8	35.7	35.7
Total lipid	6.4	6.4	6.4
Ash	5.3	5.4	5.4

[1] Vitamin premix, (mg/kg): 500,000 I.U. (international units) Vitamin A, 50,000 I.U. Vitamin D3, 2500 mg Vitamin E, 1000 mg Vitamin K3, 5000 mg Vitamin B1, 5000 mg Vitamin B2, 5000 mg Vitamin B6, 5000 μg Vitamin B12, 25,000 mg Inositol, 10,000 mg Pantothenic acid, 100,000 mg Cholin, 25,000 mg Niacin, 1000 mg Folic acid, 250 mg Biotin, 10,000 mg Vitamin C.

[2] Mineral premix, (g/kg): 314.0 g $CaCO_3$; 469.3 g KH_2PO_4; 147.4 g $MgSO_4.7H_2O$; 49.8 g NaCl; 10.9 g Fe (II) gluconate; 3.12 g $MnSO_4.H_2O$; 4.67 g $ZnSO_4.7H_2O$; 0.62 g $CuSO_4.5H_2O$; 0.16 g KI; 0.08 g $CoCl_2.6H_2O$; 0.06 g NH4 molybdate; 0.02 g $NaSeO_3$.

each tank were fasted overnight, counted and bulk weighed for determination of final body weight. Nine fish from each treatment (three fish per replicate) were randomly sampled and euthanized with 20 mg/L MS-222 (Western Chemicals, Inc., Ferndale, Washington) for blood collection. The blood was drawn from the caudal vein of individual fish by using 2 mL syringes (Klmedical, China) and centrifuged at 4 °C by 1500 rpm/min for 15 min. The obtained serum samples were stored at −80 °C for biochemical analysis. Afterwards, the nine fish (3 fish per tank) were individually weighed and dissected for collecting liver and muscle samples. The viscera and liver samples were weighed for organ indices analysis ($n = 3$, the mean value of three samples per tank was calculated as a replicate). The liver and muscle samples from each fish were placed into four separate tubes and instantly frozen in liquid nitrogen, and then stored at −80 °C for biochemical analysis, RNA isolation, and histological analysis. Another six fish from each treatment (two fish per tank) were sacrificed as described above and stored at −20 °C for body proximate composition analysis ($n = 3$, the mean value of two samples per tank was calculated as a replicate). The weight gain (WG), survival rate, FCR, hepatosomatic index (HSI), and visceral somatic index (VSI) were calculated by using the following formulae:

WG (%) = 100 × (final body weight - initial body weight) / initial body weight.

Survival rate (%) = 100 × (final fish number / initial fish number).

FCR = Total dry feed fed in each tank / (total wet weight gained by fish in each tank).

HSI (%) = 100 × liver weight / individual fish body weight.

VSI (%) = 100 × viscera weight / individual fish body weight.

2.3. The proximate composition analysis of whole-body fish

Six initial and final fish samples per treatment were analyzed for whole-body proximate composition. The moisture content was analyzed by drying samples to a constant weight at 105 °C. The total protein was determined by measuring N of the samples using a semi-automatic Kjeldahl System (FOSS, Sweden). The total lipid was extracted and determined by using chloroform/methanol (2:1, *v:v*) method (Folch et al., 1957). The ash content of diets was determined after combustion at 550 °C for 6 h in a muffle furnace (Nabertherm, Bremen, Germany). The values of PER and PPV ($n = 3$, the mean value of two samples per tank was calculated as a replicate) were calculated by using the following formulae:

PER (%) = 100 × weight gain / total protein intake.

PPV (%) = 100 × (final body protein - initial body protein) / total protein intake.

2.4. Biochemical parameters measurement

We assessed biochemical parameters from nine fish in each treatment by using specific commercial assay kits (Nanjing Jiancheng Biotech Co., Ltd., China) according to the manufacturer's protocols. Therefore, the concentrations of triglyceride (TG) (GPO-PAP method, 510 nm) and glucose (Glucose oxidase method, 505 nm) and the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Reitman-Frankel method, 510 nm) were analyzed in the serum. Similarly, the contents of malondialdehyde (MDA) (TBA method, 532 nm) in the serum and liver, lactate (Lactate dehydrogenase catalysis method, 530 nm) and pyruvate (Colorimetric method, 505 nm) in the serum, liver and muscle were also measured. Furthermore, we determined the glycogen contents (Anthranone method, 620 nm) of liver and muscle.

The insulin concentration in the serum was measured by using enzyme-linked immune sorbent assay (ELISA) reaction (565 nm) using an Ultra Sensitive Fish Insulin ELISA Kit (R&D Systems Biotechnology Co., Ltd., USA) following the instructions. The absorbance of all biochemical parameters were measured on a microplate reader (BioTek Instruments, Inc., USA).

2.5. Carnitine content determination

Six parts of fish liver and muscle samples from each treatment ($n = 6$) were cut, weighed, and then homogenized by using double distilled water (1:10, *w:v*). The carnitine content was determined as described in our previous study (Li et al., 2017). In brief, the homogenates were centrifuged at 2000 rpm/min for 15 min. Then, each 100 μL of homogenate sample was mixed directly with 60 μL double distilled water. The protein in samples was precipitated by adding 200 μL cold acetonitrile solution containing 1 μg/mL carbachol, and then the samples were centrifuged at 16,900 ×*g* for 20 min. A 1 μL of supernatant was taken for liquid chromatography-tandem mass spectrometry analysis (LC-MS/MS) analysis. The analysis was conducted in multiple reactions monitoring mode, and carnitine monitoring was with the transitions of *m/z* 162.0 → 103.0.

2.6. The [1–^{14}C]-palmitic acid oxidation assay

Another six parts of fish liver and muscle samples from each treatment ($n = 6$) were cut, weighed, and then homogenized (1:40 and 1:20, *w:v*) in ice-cold 0.25 M sucrose medium with pH 7.4 (containing 2 mM EGTA and 10 mM Tris-HCl). The homogenates were used for the [1–^{14}C]-palmitic acid (PerkinElmer, USA) oxidation assay as described previously in our studies (Li et al., 2018; Ning et al., 2016). The radioactivity was measured by using a Tri-Carb 4910TR Liquid Scintillation Analyzer (PerkinElmer, USA).

2.7. Glucose tolerance test

The glucose tolerance test (GTT) was performed as described before in our laboratory (Liu et al., 2018; Li et al., 2020a). After the feeding experiment, 36 fish (six fish per treatment, $n = 6$) were fasted overnight and then intraperitoneal injected with 500 mg D-glucose (Sigma, Germany) per kg body weight (20% glucose dissolved in 0.85% NaCl). Six fish from each treatment were then sampled after 0, 0.5, 1, 3, 6, and 12 h glucose injection, blood drawn and liver tissues collected as described above. The concentrations of glucose and insulin in the serum and glycogen contents in the liver were detected by using specific commercial assay kits as mentioned above.

2.8. Real-time quantitative PCR (qPCR) analysis

Nine liver and muscle samples from each treatment were collected for total RNA isolation by using a Tri Pure Reagent (Aidlab, China). The quality and quantity of total RNA were tested by using NANODROP 2000 Spectrophotometer (ThermoFisher, USA). All RNA samples had satisfactory quality of 1.9 to 2.0 for the 260/280 absorbance ratio. The cDNA synthesis was conducted with the S1000TM Thermal Cycler (Bio-Rad, USA) by using a PrimerScript™ RT Master Mix Kit (Takara, Japan). The qPCR reaction was conducted by using a CFX96 real-time PCR system (Bio-Rad, USA) as described before in our laboratory (Li et al., 2020b). The reaction conditions for qPCR were 94 °C for 2 min, 40 cycles of 94 °C for 10 s, 60 °C for 15 s and 72 °C for 20 s. The qPCR efficiency was between 98% and 105%. The details of gene primers are shown in Supplementary Table 1. The elongation factor 1 alpha (EF1α) and β-actin were used as housekeeping genes. The relative mRNA expression was estimated by the $2^{-\Delta\Delta Ct}$ method.

2.9. Histological analysis

Small pieces of six fish livers (two fish from each tank) from each treatment were immediately fixed in 4% paraformaldehyde solution. The samples were further dehydrated in 70%, 80%, 90%, 95% and 100% ethanol, equilibrated in xylene, and embedded in paraffin as described previously (Betancor et al., 2015). Then, the embedded tissue block was fixed on the slicer and cut into thin slices of 5 to 8 μm thickness. The liver

slices (three sections from each fish) of 5 μm thickness were subjected to the Harris hematoxylin and eosin (H & E) staining and examined on a microscope (BX51, Olympus, Japan). The sections were assessed for anomalies in structure (the relative area of vacuolation in liver) using the ImageJ software.

2.10. Statistical analysis

All data are shown as means ± SEM (standard error of the mean). The data were tested for normality and the homogeneity of variances by using Shapiro-Wilk test and Levene's test, respectively. The two-tailed independent *t*-test was used to evaluate the significant differences ($p < 0.05$) on measured parameters between the two groups (NSD *vs* HSD, HSD *vs* HSD + MD). All statistical analyses were performed using the SPSS Statistics 23.0 software (IBM, Michigan Avenue, USA).

3. Results

3.1. The effects of dietary HSD and MD on growth, organ indices and proximate composition of Nile tilapia

After the feeding trial, the fish survival rate (data not shown), WG (Fig. 1A), and FCR (Fig. B) did not vary among the three experimental treatments ($p > 0.05$). The fish fed on HSD had higher HSI (Fig. 1C) and VSI (Fig. 1D) values than those fed on NSD and HSD + MD ($p < 0.05$). The fish fed on HSD increased the total lipid content (Fig. 1E), but decreased total protein content in the whole-body (Fig. 1F) compared to the fish fed on NSD and HSD + MD ($p < 0.05$). In addition, the fish fed on HSD decreased significantly the PER and PPV values (Fig. 1G) compared to those fed on NSD and HSD + MD ($p < 0.05$). These results suggest that dietary MD supplementation attenuated the HSD-induced lipid accumulation and promoted protein deposition in Nile tilapia.

3.2. The effects of dietary HSD and MD on carnitine content, FAO efficiency and fat deposition in Nile tilapia

The fish fed on HSD had higher levels of hepatic carnitine content (Fig. 2A) and FAO efficiency (Fig. 2B) compared to the fish fed on NSD and HSD + MD ($p < 0.05$). However, these indexes were not affected significantly in the muscle among the three treatments ($p > 0.05$; Fig. 2A and B). The fish fed on HSD increased significantly the TG level in the serum (Fig. 2C), and the contents of total lipid in the liver and muscle (Fig. 2D) than the fish fed on NSD and HSD + MD ($p < 0.05$). Besides, the histomorphological analysis showed that the fish fed on HSD had higher relative area of vacuolation in the liver than the fish fed on NSD and HSD + MD (Fig. 2E). Feeding the fish with HSD + MD decreased significantly the AST (Fig. 3A) and ALT (Fig. 3B) enzyme activities in the serum than fish fed on the HSD ($p < 0.05$). Moreover, the fish fed on HSD had higher hepatic MDA concentration than those fed on NSD and HSD + MD ($p < 0.05$; Fig. 3C). The MDA content in the serum had no apparent changes among the three treatments ($p > 0.05$; Fig. 3D).

3.3. The effects of dietary HSD and MD on glucose utilization and insulin sensitivity in Nile tilapia

The fish fed on HSD had higher concentrations of glucose (Fig. 4A) and insulin (Fig. 4B) in the serum compared to the fish fed on NSD and HSD + MD ($p < 0.05$). However, lactate (Fig. 4C) and pyruvate (Fig. 4D) contents in the serum were not statistically affected among the three treatments ($p > 0.05$). The fish fed on HSD had higher serum glucose concentration after glucose injection compared to the fish fed on NSD ($p < 0.05$; Fig. 4E), while those fed on HSD + MD had a significant faster glucose clearance capacity (Fig. 4E) with lower insulin levels (Fig. 4F) compared to the fish fed on HSD during the GTT ($p < 0.05$). Additionally, the fish fed on HSD + MD decreased hepatic glycogen content compared to those fed on HSD after 6-h glucose injection ($p < 0.05$; Fig. 4G). Moreover, the fish fed on HSD had higher hepatic lactate (Fig. 4H), pyruvate (Fig. 4I), and glycogen (Fig. 4J) contents compared to those fed on NSD and HSD + MD ($p < 0.05$). The lactate (Fig. 4H) and glycogen (Fig. 4J) contents in the muscle did not vary significantly among the three treatments ($p > 0.05$). However, the fish fed on HSD and HSD + MD had lower pyruvate content than those fed on the NSD ($p < 0.05$; Fig. 4I). These findings demonstrate that the inhibited hepatic FAO alleviated HSD-induced insulin resistance and promoted glucose utilization in Nile tilapia.

3.4. The effects of dietary HSD and MD on mRNA expression of genes related to lipid, glucose and protein metabolism in the liver of Nile tilapia

The fish fed on HSD down-regulated the expressions of carnitine palmitoyltransferase 1b (CPT-1b) and adipose triglyceride lipase (ATGL), but up-regulated the expression of fatty acid synthase (FAS)

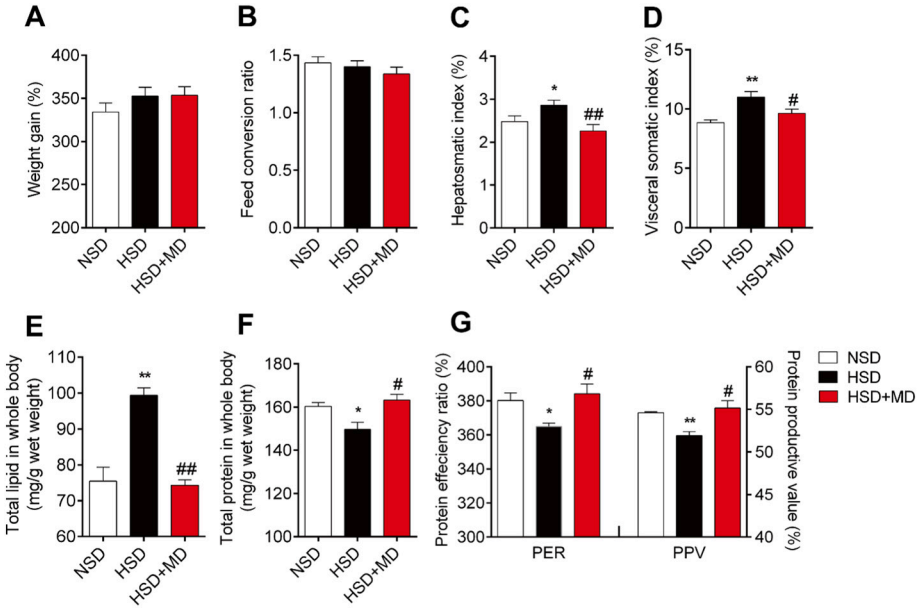

Fig. 1. Effects of dietary HSD and MD on growth performance, feed utilization, organ indices and body composition in Nile tilapia. (A) Weight gain ($n = 3$); (B) Feed conversion ratio ($n = 3$); (C) Hepaticsomatic index ($n = 3$); (D) Visceral somatic index ($n = 3$); (E) Total lipid in whole body ($n = 3$). (F) Total protein in whole body ($n = 3$). (G) Protein efficiency ratio (PER) and protein productive value (PPV) ($n = 3$). Data are represented as mean ± SEM. $*p < 0.05$, $**p < 0.01$ significance between NSD and HSD groups, $\#p < 0.05$, $\#\#p < 0.01$ significance between HSD and HSD + MD groups.

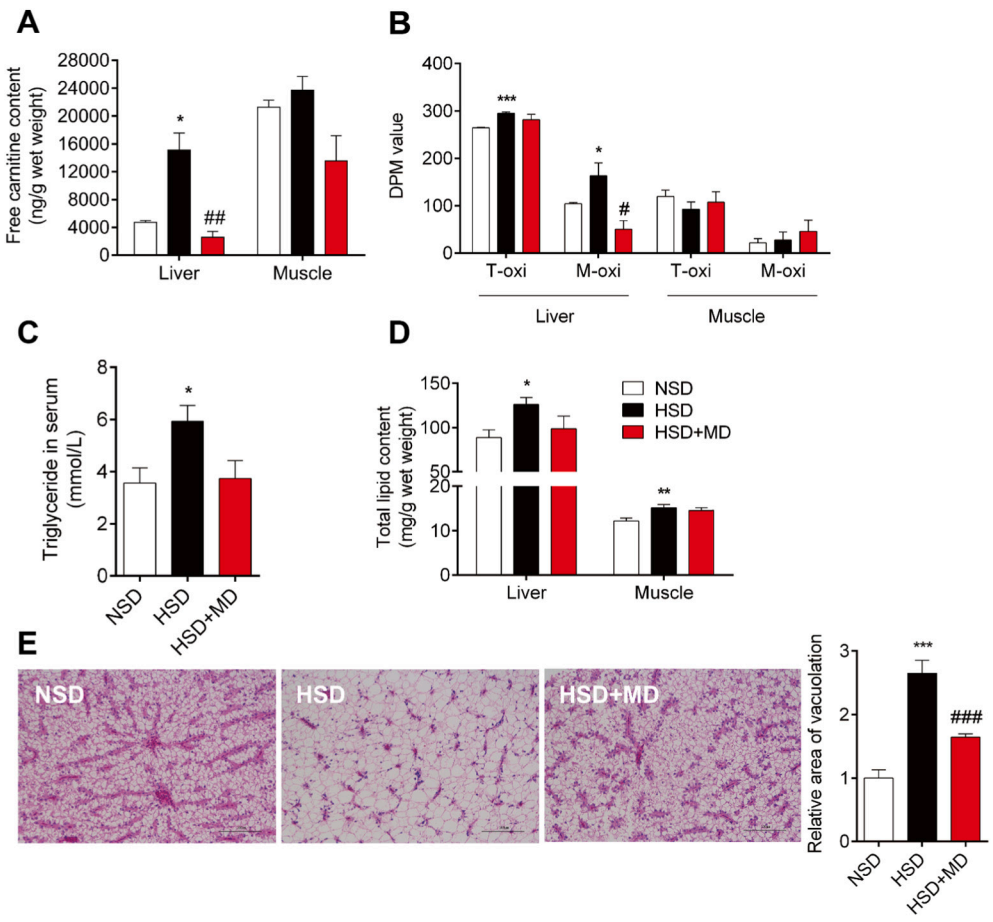

Fig. 2. Effects of dietary HSD and MD on carnitine content, fatty acid β-oxidation and fat deposition in Nile tilapia. (A) Carnitine contents in liver and muscle ($n = 6$); (B) Palmitate oxidation efficiency in liver and muscle ($n = 6$); (C) Serum triglyceride content ($n = 9$); (D) Total lipid content in liver and muscle ($n = 9$); (E) H & E staining in liver tissue section. Photomicrographs (×200) and scale bars (100 μm), the column graphs in the right of pictures represent the relative vacuolation area of liver tissue ($n = 6$). Data are represented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significance between NSD and HSD groups, $\#p < 0.05$, $\#\#p < 0.01$ significance between HSD and HSD + MD groups.

genes compared to the fish fed on NSD ($p < 0.05$; Fig. 5A). The fish fed on HSD + MD increased the expression of CPT-1a, CPT-1b, hormone-sensitive lipase (HSL) and ATGL genes, but decreased the expression of PPARα, acetyl-CoA carboxylase α (ACCα) and FAS genes compared to those fed on HSD ($p < 0.05$; Fig. 5A). The expression of genes related to glucose metabolism, including insulin receptor (IR), glucose transporter 2 (Glut2), glucokinase (GK), pyruvate dehydrogenase 1a (PDH1a), and glucose-6-phosphatase (G6Pase) showed no obvious changes between the fish fed on NSD and HSD ($p > 0.05$; Fig. 5B). However, the fish fed on HSD decreased the expression of pyruvate kinase (PK) than those fed on the NSD ($p < 0.05$; Fig. 5B). Moreover, feeding fish with HCD + MD increased the expression of IR, Glut2, GK, PK, and PDH1a genes compared to the fish fed on HSD ($p < 0.05$; Fig. 5B). The fish fed on HSD enhanced remarkably aminopeptidase N (APN) but decreased mTOR expression than those fed on NSD ($p < 0.05$; Fig. 5C), while the fish fed HSD + MD down-regulated the expression of asparagine synthetase (ASNS) and APN compared to the fish fed on HSD ($p < 0.05$; Fig. 5C). These results suggest that the inhibited hepatic FAO changed the transcriptional expression of genes involved in nutrient metabolism in Nile tilapia.

4. Discussion

4.1. Inhibited FAO alleviates the HSD-induced glucose intolerance in fish through enhancing insulin sensitivity and glucose utilization

Dietary carbohydrate contributes to energy supply of fish (Hemre et al., 2002). Currently, carbohydrate-enriched diets are widely utilized in aquaculture (Polakof et al., 2012; Xu et al., 2018). However, fish usually show persistent hyperglycemia after glucose loading or intake of high digestible carbohydrate diets (Boonanuntanasarn et al., 2018; Jin et al., 2014; Kamalam et al., 2017; Ma et al., 2020; Qiang et al., 2016). In the present study, the high values of serum glucose and insulin obtained in the Nile tilapia fed on HSD compared with NSD, indicate a hyperglycemia and hyperinsulin state induced by high carbohydrate intake. In turn, the increased blood glucose stimulated insulin synthesis and secretion (Kamalam et al., 2017), which subsequently accelerated glucose disposal in peripheral insulin target tissues through enhancing glucose uptake, glycolysis, and glycogenesis coupled with suppressed gluconeogenesis (Polakof et al., 2012). During the glucose tolerance test, the Nile tilapia fed on HSD showed lower glucose clearance compared with the NSD-fed fish, owing to no significant change of serum insulin and reduced hepatic IR expression. This indicates that the HSD intake impaired insulin sensitivity in tissues of the experimental fish. Glucose is absorbed from the blood by the liver tissue with the assistance of glucose transporter GLUT (Enes et al., 2009). The lack of significant change in hepatic Glut2 gene in the fish fed on NSD and HSD, suggests a lower glucose uptake capacity in hepatocytes of fish subjected to a long-term HSD intake. Consistently, feeding high-carbohydrate diets increased glucose and insulin levels in the plasma but decreased the expression of insulin receptor substrate 1 (IRS1) and glucose transporters in tissues of blunt snout bream (Xu et al., 2018) and Nile tilapia (Boonanuntanasarn et al., 2018). These results suggest that long-term intake of high digestible carbohydrate diet impairs insulin sensitivity, which decreases glucose clearance, thereby resulting in hyperglycemia and hyperinsulin state in fish.

High level of blood insulin usually induces dephosphorylation of glycogen phosphorylase (GPase) and glycogen synthase (GSase) by decreasing adenylyl cyclase activities and intracellular cAMP formations, thereby inhibiting glycogen degradation (Moon, 2001). We thus

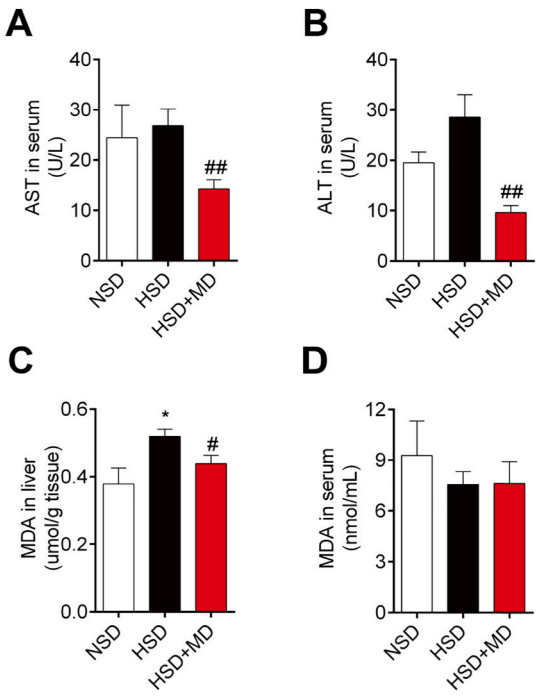

Fig. 3. Effects of dietary HSD and MD on liver damage of Nile tilapia. (A and B) Enzyme activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum ($n = 9$); (C and D) Malondialdehyde (MDA) contents in liver and serum ($n = 9$). Data are represented as mean ± SEM. $*p < 0.05$ significance between NSD and HSD groups, $\#p < 0.05$, $\#\#p < 0.01$ significance between HSD and HSD + MD groups.

found higher hepatic glycogen in the fish fed on HSD, indicating increased glucose storage in the liver, but presumably insufficient to avoid hyperglycemia, as reported previously in many fish species (Boonanuntanasarn et al., 2018; Ma et al., 2020; Moreira et al., 2008; Xu et al., 2017; Xu et al., 2018). Accordingly, these data suggest that the intake of high digestible carbohydrate diet promotes glycogenesis in fish, leading to increased glucose storage as glycogen in the liver. Moreover, the liver of most fish species can regulate glucose storage, through distinct control of hepatic glucose oxidation (glycolysis) and production (gluconeogenesis) (Enes et al., 2009). In the present study, hepatic transcriptions of glycolytic genes (GK and PK) were remarkably down-regulated after feeding Nile tilapia with HSD, suggesting reduced glycolysis, consistent with results on blunt snout bream (Xu et al., 2018) and zebrafish (Ma et al., 2020). We found no significant change in gluconeogenic gene (G6Pase) in the Nile tilapia fed on HSD, suggesting limited influence of HSD on gluconeogenesis in the fish. Therefore, the possible reasons for the prolonged hyperglycemia and glycogen deposition in fish fed on high digestible carbohydrate diet include impaired insulin sensitivity and molecular adaptation of glucose use in the liver, leading to relatively lower glucose uptake and oxidation in peripheral tissues.

Accumulating evidences suggest that, MD serves as an effective inhibitor of mitochondrial FAO due to its endogenous carnitine synthesis suppression in mammals (Liepinsh et al., 2009a, 2009b) and fish (Li et al., 2018; Li et al., 2020a, 2020b; Pan et al., 2017), and plays a critical role in lowering hyperglycemia (Liepinsh et al., 2009a). In the present study, the Nile tilapia fish fed on dietary HSD + MD reduced significantly hepatic carnitine content and mitochondrial FAO, confirming the low carnitine-induced FAO inhibition. Meanwhile, the MD-induced FAO inhibition reduced significantly glucose and insulin levels in the serum, and hepatic lactate and pyruvate contents compared to the fish fed on HSD, indicating increased insulin sensitivity and glucose utilization. Previous studies confirmed that mice and fish treatment with MD reduced blood glucose and insulin concentrations through improved insulin sensitivity, leading to increased glucose uptake and glycolysis accompanied by depression of gluconeogenesis (Li et al., 2020a; Liepinsh et al., 2011). Accordingly, after glucose administration, the Nile tilapia fed on HSD + MD showed faster blood glucose clearance than those fed on HSD, accompanied by a lowered insulin level, confirming again that the inhibition of FAO alleviated HSD-induced glucose intolerance in fish similar to mammals (Lee et al., 2017). Moreover, hepatic transcription of IR was significantly up-regulated in the Nile tilapia fed on HSD + MD. The elevated expression of IR has been implicated in the increase of cellular glucose uptake (Nevado et al., 2006). Accordingly, we obtained higher hepatic expression of glucose transporter Glut2 in the Nile tilapia fed on HSD + MD than those fed on HSD. In hepatocytes, excess glucose is degraded through the glycolysis, which is characterized by the enhanced expression of glycolytic genes (GK and PK) (Enes et al., 2009). In the present study, the GK, PK, and PDH1a genes were upregulated in the Nile tilapia fed on HSD + MD, suggesting that the inhibited FAO accelerated glucose oxidation in the liver. On the contrary, our recent studies showed that increased FAO, either through activating peroxisome proliferator-activated receptor α (PPARα) (Ning et al., 2016) or increasing endogenous L-carnitine concentration (Li et al., 2017; Li et al., 2019), suppressed glucose utilization in fish. Furthermore, PPARα deficiency or low carnitine-mediated FAO inhibition initiated the adenosine 5′-monophosphate-activated protein kinase (AMPK) signal, and consequently improved insulin sensitivity and glucose utilization *via* protein kinase B (AKT) activation-induced energy homeostasis regulation in fish (Li et al., 2020a, 2020c) and mammals (Vandanmagsar et al., 2016; Wicks et al., 2015). All these results indicate that inhibited FAO alleviated the HSD-induced glucose intolerance through enhanced insulin sensitivity and glucose utilization in Nile tilapia. These results provide strong evidence that FAO activity is required to mediate glucose homeostasis of fish fed on HSD.

4.2. *Inhibited FAO alleviates the HSD-induced lipid accumulation and promotes protein deposition in fish through improving glucose utilization*

Prolonged feeding of high digestible carbohydrate diet induces energy imbalance, which leads to excess fat accumulation and prevalence of metabolic diseases due to negative effects on protein deposition in fish (Luo et al., 2020). In the present study, feeding Nile tilapia with HSD did not influence growth and feed utilization, but increased lipid accumulation evidenced by higher organ indexes (HSI and VSI) and whole-body lipid content. Furthermore, the Nile tilapia fed on HSD in the present study increased fat contents in the liver and muscle, and had severe vacuolation in the liver, similar to previous results in different fish species fed on high digestible carbohydrate diet (Boonanuntanasarn et al., 2018; Luo et al., 2020; Pérez-Jiménez et al., 2015; Rawles et al., 2008; Tan et al., 2009; Xu et al., 2017; Xu et al., 2018). These results suggest that fish might increase lipid accumulation in whole body, muscle and liver following HSD intake.

The liver is regarded as the main organ for lipid storage in fish (Rawles et al., 2008). Lipid accumulation in hepatocytes makes them more susceptible to injury by oxidative stress and inflammatory cytokines, whose production in the liver is triggered by the lipid peroxidation products (Gorden et al., 2011). Accordingly, the hepatic MDA (one of the lipid peroxidation products) was increased dramatically in the Nile tilapia following the HSD intake, accompanied by the elevated AST and ALT enzymes in the serum, which were interpreted as damage to the liver, indicating the development of hepatic damage induced by lipid accumulation after excess starch intake. It has been proposed that, excess carbohydrate intake in fish is usually converted to fat through increasing hepatic lipogenic activity (Polakof et al., 2011; Qiang et al., 2016; Rawles et al., 2008). Actually, the liver has high enzymic activities of several major pathways, including lipogenesis and glycolysis (Postic et al., 2004). One route of the metabolites produced by hepatic glycolysis is to provide carbons for the *de novo* lipogenesis (Postic et al., 2004),

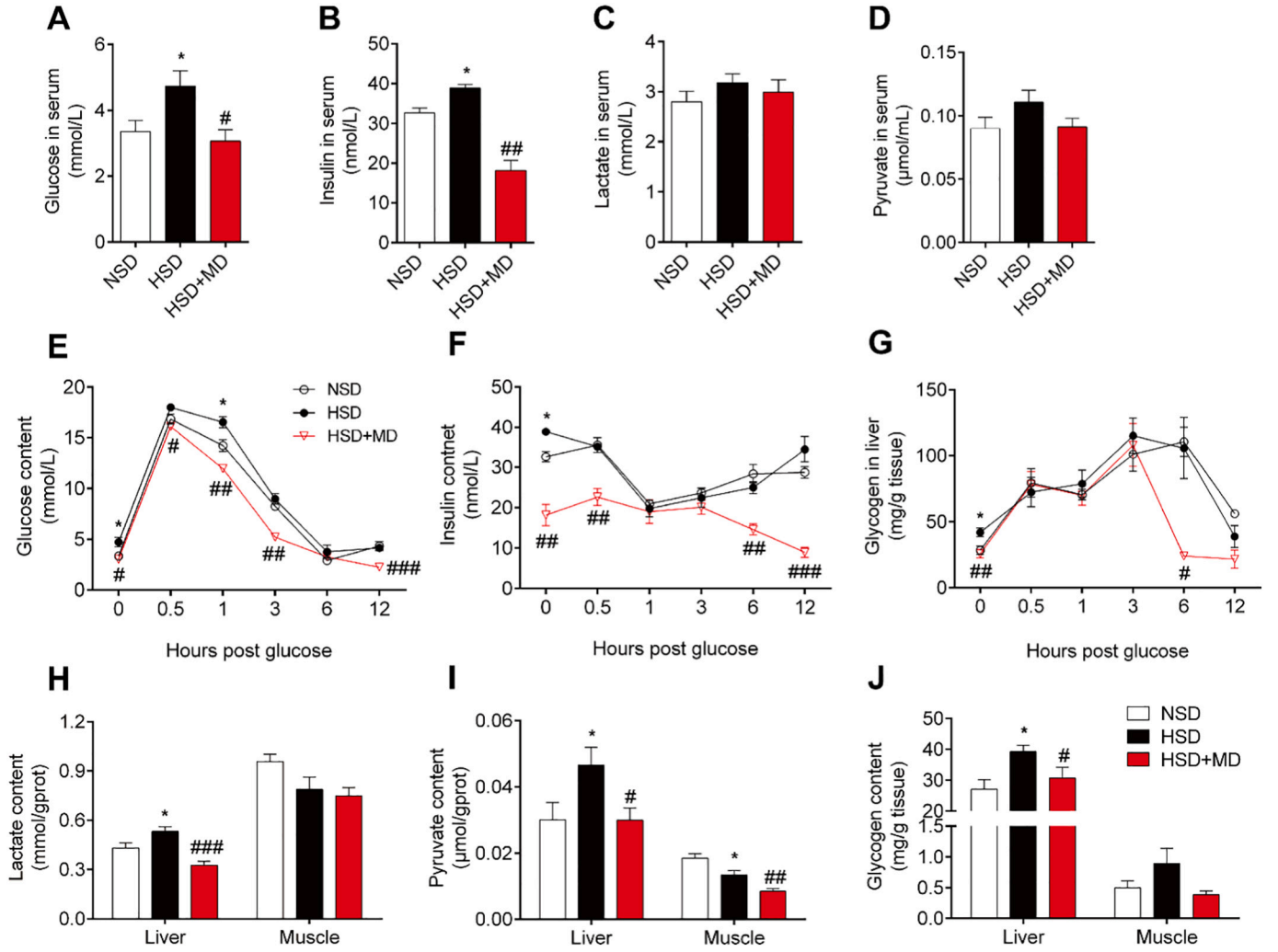

Fig. 4. Effects of dietary HSD and MD on glucose utilization in Nile tilapia. (A and B) Serum glucose (A) and insulin (B) concentrations ($n = 9$); (C and D) Serum lactate (C) and pyruvate (D) contents ($n = 9$); (*E*-G) Serum glucose (E) and insulin (F) concentrations, and hepatic glycogen content (G) in the intraperitoneal glucose tolerance test (GTT) ($n = 6$); (H-J) Lactate (H), pyruvate (I) and glycogen (J) contents in liver and muscle ($n = 9$). Data are represented as mean ± SEM. $^*p < 0.05$ significance between NSD and HSD groups, $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ significance between HSD and HSD + MD groups.

thus the high insulin concentration and glucose intermediate metabolites can stimulate hepatic lipogenesis, reflecting the enhanced transcriptions of ACCα and FAS genes (Girard et al., 1997; Polakof et al., 2011). In the present study, the Nile tilapia fed on HSD had higher levels of insulin in the serum and hepatic lactate and pyruvate, which increase the rate of *de novo* lipogenesis, as reported previously (Rawles et al., 2008). Accordingly, the hepatic mRNA of ACCα and FAS genes were up-regulated in the Nile tilapia fed on the HSD, suggesting the activation of lipogenesis. This is similar to a previous study in Nile tilapia fed on HSD (Luo et al., 2020), in which ACCα and FAS were increased in the liver and adipose tissue, indicating improved *de novo* lipogenesis. In addition to lipogenesis, lipid catabolism, including FAO and lipolysis, also play important roles in mediating lipid homeostasis. In the present study, hepatic transcriptions of CPT-1b and ATGL genes were both decreased in the Nile tilapia fed on HSD, as reported in previous studies (Jin et al., 2014; Luo et al., 2020; Xu et al., 2017). These data indicate that HSD also decreased lipid catabolism in fish, which also contributed to the fat deposition. Therefore, the fat deposition induced by feeding Nile tilapia with HSD was not only caused by increased lipogenesis but also by decreased lipid catabolism. On the contrary, after feeding HSD + MD, the lipid contents in serum, liver, and muscle of the Nile tilapia were decreased. Accordingly, the HSD-induced liver damage and oxidative stress were also significantly alleviated by the MD supplementation. More importantly, the intermediate metabolites (lactate and pyruvate) from glucose were reduced remarkably in the Nile tilapia tissues fed on HSD + MD, owing to the improved insulin sensitivity and glucose utilization. This possibly decreased largely the lipogenesis from glucose and also suggested that the fat deposition caused by feeding fish with HSD was sourced from glucose. It is worth noting that the Nile tilapia fed on the HSD + MD, up-regulated significantly the hepatic transcriptions such as CPT-1a, CPT-1b, HSL, and ATGL genes. These results suggest the stimulation of compensatory metabolic responses *via* increased lipid catabolism genes, consistent with our previous results on fish fed on normal diets containing MD (Li et al., 2018; Li et al., 2020a; Pan et al., 2017). Moreover, the Nile tilapia fed on HSD + MD decreased lipogenic genes (ACCα and FAS). Of note, the feeding Nile tilapia with HSD increased lipid synthesis, but decreased lipid catabolism as discussed above. Therefore, although MD supplementation inhibited lipid oxidation, it still reduced the HSD-induced lipogenesis through increased glucose utilization, which resulted in lower lipid accumulation for the Nile tilapia fed on HSD + MD than those fed on HSD. Together, these findings suggest that high carbohydrate intake initiated a shift from using carbohydrate as fuel to the synthesis of lipids, whereas the FAO inhibition accelerated glucose utilization, thereby attenuated fat deposition in fish.

In some fish nutrition studies, the optimal inclusion of dietary

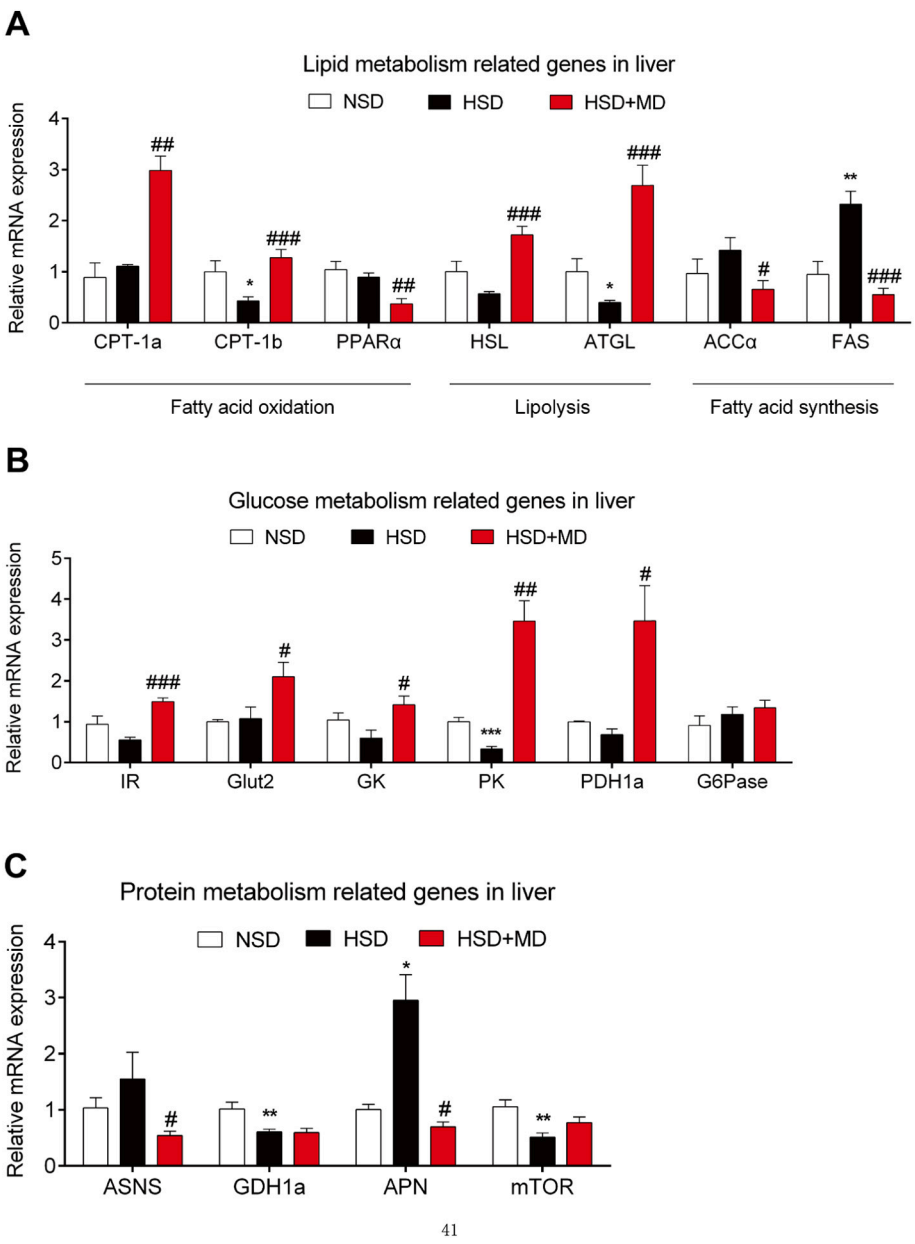

Fig. 5. Effects of dietary HSD and MD on the mRNA expressions of genes involved in lipid, glucose and protein metabolism in liver of Nile tilapia. (A) The mRNA expression of lipid metabolism-related genes; (B) The mRNA expression of glucose metabolism-related genes; (C) The mRNA expression of protein metabolism-related genes. CPT-1a, carnitine palmitoyltransferase 1a; CPT-1b, carnitine palmitoyltransferase 1b; PPARα, peroxisome proliferator activated receptor α; HSL, hormone-sensitive lipase; ATGL, adipose triglyceride lipase; ACCα, acetyl-CoA carboxylase α; FAS, fatty acid synthase; IR, insulin receptor; Glut2, glucose transporter 2; GK, glucokinase; PK, pyruvate kinase; PDH1a, pyruvate dehydrogenase 1a; G6Pase, glucose-6-phosphatase; ASNS, asparagine synthetase; GDH1a, glutamate dehydrogenase 1a; APN, aminopeptidase N; mTOR, mammalian target of rapamycin. Data are represented as mean ± SEM ($n = 9$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ significance between NSD and HSD groups, #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ significance between HSD and HSD + MD groups.

carbohydrates plays "protein-sparing effect", which increases protein retention by conserving some protein for energy generation (Kamalam et al., 2017). However, our present results found that high carbohydrate intake decreased significantly the PER and PPV in Nile tilapia, and had lower total protein in the whole fish body. In addition, the Nile tilapia fed on HSD decreased protein synthesis evidenced by the mTOR downregulation, but increased proteolysis manifested by the upregulation of the APN gene. These data suggest that Nile tilapia had a limited ability to utilize excess dietary carbohydrates. However, the MD supplementation elevated the whole-body protein content of Nile tilapia fed on HSD, accompanied by the elevated PER and PPV. In our recent study, we found that inhibited mitochondrial FAO increased glucose utilization and promoted protein deposition in Nile tilapia through the activation of the mTOR signaling pathway and the suppression of the GCN2 signaling pathway (Li et al., 2020a). These findings suggest that FAO inhibition promoted protein deposition owing to the increased energy supply from glucose oxidation.

Generally, during energy production from glucose, the glucose carbon must enter the Krebs cycle as pyruvate, followed by a conversion to acetyl-CoA for the complete oxidation for fuels (Rawles et al., 2008). Our results showed that feeding Nile tilapia with HSD + MD decreased pyruvate content in both liver and muscle, indicating increased oxidation of glucose into the Krebs cycle for energy production to save protein catabolism. Accordingly, the expression of the genes involved in protein breakdown (ASNS and APN) were also decreased in the Nile tilapia fed on HSD + MD. Similarly, in our previous study, the zebrafish treated with MD reduced the expression of the genes associated with amino acid degradation and increased mTOR expression (Li et al., 2018). These results indicate that inhibited FAO may promote protein-sparing effect from carbohydrate through increasing glucose-sourced energy supply in the fish fed on HSD.

5. Conclusion

The present study demonstrated that the FAO inhibition promotes glucose utilization and alleviates the adverse effects induced by feeding HSD in Nile tilapia *via* stimulating glucose uptake and glycolysis, coupled with the depression of *de novo* lipogenesis. Moreover, the

inhibited FAO-induced glucose oxidation in the fish fed on HSD reduces protein catabolism and increases protein synthesis, resulting in elevated protein deposition. This study indicates that the alteration of mitochondrial FAO activity remodels the metabolic balance of different nutrients. Our study highlights the possible improvement of carbohydrate utilization through energy homeostasis regulation in fish fed on high digestible carbohydrate diets.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.736392.

Declaration of Competing Interest

Authors declare that they have no conflict of interest in this work.

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