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Inhibited fatty acid β-oxidation impairs stress resistance ability in Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Energy metabolism plays important roles in stress resistance and immunity in mammals, however, such functions have not been established in fish. In the present study, Nile tilapia (*Oreochromis niloticus*) was fed with mildronate, an inhibitor of mitochondrial fatty acid (FA) β-oxidation, for six weeks subsequently challenged with *Aeromonas hydrophila* and ammonia nitrogen exposure. Mildronate treatment reduced significantly L-carnitine concentration and mitochondrial FA β-oxidation efficiency, while it increased lipid accumulation in liver. The fish with inhibited hepatic FA catabolism had lower survival rate when exposed to *Aeromonas hydrophila* and ammonia nitrogen. Moreover, fish fed mildronate supplemented diet had lower immune enzymes activities and anti-inflammatory cytokine genes expressions, but had higher pro-inflammatory cytokine genes expressions. However, the oxidative stress-related biochemical indexes were not significantly affected by mildronate treatment. Taken together, inhibited mitochondrial FA β-oxidation impaired stress resistance ability in Nile tilapia mainly through inhibiting immune functions and triggering inflammation. This is the first study showing the regulatory effects of lipid catabolism on stress resistance and immune functions in fish.

1. Introduction

Immunity reactions and resistance to environmental stress, such as low temperature, have been verified as energy-consuming processes in mammals [1–3]. Mitochondria not only play indispensable roles in supplying energy in animal cells, but also are essential in the process of animal immunity and stress resistance. In mammals, a clear correlation between mitochondrial dysfunction or injury and immune function decline has been established [4,5]. Fish, as the largest vertebrate group, also face a number of stresses in water, especially in intensive aquaculture systems, in which pathogens and environmental stress factors, such as ammonia nitrogen, have been the main restrictions for aquaculture development. Presumably, energy metabolism also plays essential roles in stress resistance in fish as has been indicated in mammals. However, the correlation between energy metabolism and stress resistance has not been well established in fish.

In energy metabolism, mitochondrial fatty acid (FA) β-oxidation is an essential pathway in ATP production. In the β-oxidation process, the entry of long chain fatty acids (LCFAs), in the form of acyl-carnitine into mitochondrial matrix by using carnitine palmitoyltransferase 1 (CPT 1) is regarded as the speed-limiting step of the whole biochemical process [6,7]. In this process, L-carnitine as a carrier of LCFAs, is essential for the fatty acid β-oxidation, thus L-carnitine has been proved to possess regulatory functions in energy metabolism in human and other animals [8–10]. Moreover, some mammalian studies have indicated that dietary L-carnitine supplementation improves immunity by increasing mitochondrial FA β-oxidation [11,12], suggesting mitochondrial FA β-oxidation is an important process in stress resistance in mammals. In fish, some studies have shown that the addition of L-carnitine in feeds can

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improve the immune function [13] and the resistance to ammonia nitrogen [14]. However, studies from other fish species have indicated that dietary L-carnitine supplementation does not produce significant physiological effects [15,16]. These contradictions hinder the understanding between lipid catabolism and stress resistance ability in fish. Similar to mammals, fish can synthesize carnitine such that the supplemental effects of dietary L-carnitine might be shadowed by sufficient endogenous L-carnitine. Accordingly, dietary supplementation of L-carnitine might not be an ideal method to investigate the effects of mitochondrial FA β-oxidation in stress resistance in fish without inhibiting endogenous carnitine synthesis.

Mildronate is an inhibitor of L-carnitine biosynthesis by gamma-butyrobetaine (GBB) hydroxylase and acts as a competitive inhibitor of renal carnitine reabsorption, thus, its use has caused reduced FA β-oxidation in mammals [17,18]. At present, mildronate is considered an anti-ischemic drug which improves peripheral circulation in patients with chronic heart failure [19]. In addition, mildronate is also commonly used in mammalian studies to investigate the effects of mitochondrial FA β-oxidation in different metabolic syndromes owing to its efficient L-carnitine lowering effects and low toxicity [20–22]. Similarly, based on its L-carnitine lessening ability and harmless effects, mildronate is a potential useful tool for investigating the effects of mitochondrial FA β-oxidation in stress resistance in fish.

Nile tilapia (*Oreochromis niloticus*) is an important aquaculture species worldwide. It is a good fish research model, because it not only grows faster and has higher resistance to diseases and toxic stress [23], but also its whole genomic information is available [24]. In the present study, juvenile Nile tilapia were fed with mildronate supplemented diets for six weeks. Afterwards, fish were exposed to pathogenic bacteria, *Aeromonas hydrophila* and ammonia nitrogen to scrutinize the stress resistance ability of the mildronate-treated fish. The aim was to establish the possible relationship between mitochondrial FA β-oxidation and stress resistance ability in Nile tilapia. Growth performance, carnitine content, mitochondrial FA β-oxidation activities, and other immunology-related biochemical and molecular biological indices were measured in both fish groups. Our study indicated that mildronate decreased significantly the endogenous L-carnitine concentration and mitochondrial FA β-oxidation activities, and subsequently reduced significantly the resistance ability of Nile tilapia against *Aeromonas hydrophila* and ammonia nitrogen. To the best of our knowledge, this is the first report establishing the correlation between lipid catabolism and stress resistance in fish.

2. Materials and methods

All experiments were conducted under the Guidance of the Care and Use of Laboratory Animals in China. This research was approved by the Committee on the Ethics of Animal Experiments of East China Normal University.

2.1. Fish, diets and experimental design

Juvenile Nile tilapia were obtained from Aquaculture Genetics and Breeding Technology center at Shanghai Ocean University (Shanghai, China). Before experiments, all fish were acclimated in three 200-L tanks for two weeks. During this period, fish were hand-fed using a commercial diet (Chengdu, China) with protein and lipid content of ≥33% and ≥5%, respectively. After acclimation, three hundred healthy juvenile Nile tilapia with comparable similar initial mean weight (1.97 ± 0.12 g) were selected and randomly distributed into two groups (three replicates per group, fifty fish per replicate): control group and mildronate group. The trial lasted for six weeks.

In order to ensure complete intake of mildronate, every morning during feeding trial, the overnight-fasted fish in the two groups were first hand-fed with small wheat flour-dough particles at 1% of their average body weight (BW) without (control group) or with mildronate (mildronate group). Afterwards, all fish were hand-fed thrice per day with the same basic diet at 8:00, 14:00 and 20:00 h. The feeding rate of the basic diet was 3% BW. The mildronate intake was set as 1000 mg/kg BW per day, which was determined by using a pre-experiment to guarantee efficient L-carnitine lowering effects (data not shown). Moreover, this value is also a dose commonly used in mammalian studies [21,25–28]. The formulations of the basal diet and wheat flour-dough particles and their composition are listed in Table 1. The diets were made as described previously [29]. The basic diet contained 370 g/kg protein and 50 g/kg fat. Casein and gelatin were used as protein sources while soybean oil was used as the lipid source.

2.2. Fish rearing and sampling

During the 6-week trial, fish in each tank were supplied with compressed air via air-stones from air pumps at a 10 h/14 h light/dark cycle. Water temperature, dissolved oxygen, pH and total ammonia nitrogen were maintained at ranges from 26 to 28 °C, 4.8–6.4 mg/L, 7.5 to 7.9 and < 0.02 mg/L, respectively. The weight of fish was recorded after every two weeks, and the feeding amount was adjusted accordingly. At the end of trial, all fish were fasted overnight, and ten fish from each group were anesthetized with MS-222 (20 mg/L) and sacrificed for tissue samples collection. Another six fish from each group were anesthetized and kept at −80 °C for body composition analysis. Blood was collected from the caudal vein into syringes and centrifuged (1500 rpm, 15 min). The resulting serum was immediately frozen at −80 °C for further

Table 1
Formulation of the basic diet and wheat flour-dough particles and their proximate analysis.

Diet component (g/kg)	
Casein	300
Gelation	70
Soybean	50
Corn starch	300
Vitamin premix[a]	15
Mineral premix[b]	45
Carboxy methyl cellulose (CMC)	30
Cellulose	182.75
Choline chloride	5
Dimethyl-β-propiothetin (DMPT)	2
Butylated hydroxytoluene (BHT)	0.25
Proximate composition (g/kg)	
Total protein	370
Total lipid	50
Total carbohydrate	300
Wheat flour component (100 g)	
Protein (g)	12.2
Lipid (g)	1.5
Carbohydrate (g)	70

[a] Vitamin premix, (mg or IU/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.

[b] Mineral premix, (g/kg): 314.0 g $CaCO_3$; 469.3 KH_2PO_4; 147.4 g $MgSO_4 \cdot 7H_2O$; 49.8 g NaCl; 10.9 g Fe(II) gluconate; 3.12 g $MnSO_4 \cdot H_2O$; 4.67 g $ZnSO_4 \cdot 7H_2O$; 0.62 g $CuSO_4 \cdot 5H_2O$; 0.16 g kJ; 0.08 g $CoCl_2 \cdot 6H_2O$; 0.06 g NH_4 molybdate; 0.02 g $NaSeO_3$.

analysis. Liver tissues were collected from dissected fish and immediately frozen in liquid nitrogen and kept at −80 °C for latter measurements of molecular and biochemical indices.

Weight gain, feed conversion ratio, protein efficiency, hepatosomatic index, mesenteric fat index and spleen index were calculated using the following formulae:

Weight gain = 100 × (Final fish weight-Initial fish weight)/Initial fish weight

Feed conversion ratio = Feed intake/(Final fish weight-Initial fish weight)

Protein efficiency = (Protein amount in final fish body-protein amount in initial fish body)/Net dietary protein intake

Hepatosomatic index = 100 × (Liver weight/body weight)

Mesenteric fat index = 100 × (Mesenteric fat weight/body weight)

Spleen index = 100 × (Spleen weight/body weight)

2.3. Biochemical parameters measurements

Crude protein was determined by the Kjeldahl method using a semi-automatic Kjeldahl System after acid digestion [30]. The lipid contents in liver and whole fish body were extracted using chloroform/methanol (2:1, *v/v*). Hepatic triglyceride (TG) and malondialdehyde (MDA) were assessed by using commercial kits (Jiancheng Biotech Co. China) based on manufacturer's instructions. The activities of lysozyme (LSZ), acid phosphatase (ACP), alkaline phosphatase (AKP), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) in serum or liver were assessed by using commercial kits (Jiancheng Biotech Co. China) according to manufacturer's instructions. The carnitine concentration in liver samples was detected by using liquid chromatography-tandem mass spectrometry analysis (LC-MS/MS) as described in our previous study [29].

2.4. Mitochondrial and peroxisomal [1-^{14}C] palmitate oxidation in liver homogenates

At the end of the feeding trial, liver tissues from four fish collected in each group were weighed and homogenized (1:40, *w/v*) in ice-cold 0.25M-sucrose medium containing 2 mM-ethylene glycol-bis-N,N,N′,N′-tetraacetic acid, (EGTA) and 10 mM-Tris-Cl with a pH of 7.4. The homogenate samples were used for immediate measurements of mitochondrial and peroxisomal [1-^{14}C] palmitate β-oxidation. The palmitate oxidation rate was determined as described previously [31]. The total palmitate β-oxidation reaction was initiated by adding 100 μM palmitate (supplemented with 1 μCi [1-^{14}C], specific activity 60 Ci mmol^{-1}) bound to fatty-acid-free bovine serum albumin (BSA). After 30 min of incubation, the samples were treated with 10% (w/v) perchloric acid, which precipitated proteins. To measure peroxisomal palmitate oxidation, the mitochondrial β-oxidation activity was inhibited by pre-incubating the samples by using 10 μM rotenone and 250 μM potassium cyanide (KCN). The rate of total or peroxisomal palmitate oxidation was calculated from the radioactivity of the acid-soluble products. The rate of mitochondrial palmitate oxidation was expressed as the difference between the total palmitate oxidation (without inhibitors) and the peroxisomal oxidation rate. The final reaction media were filtered using Millipore filters (0.45 μm pore size) and the filtrate containing the acid-soluble products (ASP, the short metabolites from FA oxidation) was mixed with Ultima Gold XR (Packard) for radioactivity measurements.

2.5. Isolation of RNA, synthesis of cDNA and quantitative real-time PCR (qRT-PCR)

Total RNA from liver tissues was isolated by using a Tri Pure Reagent (Aidlab, China) according to the manufacturer's protocol. The quality and quantity of total RNA were tested by using NANODROP 2000 Spectrophotometer (Thermo, USA). First-strand cDNA was synthesized using a PrimerScript™ RT reagent Kit with a gDNA Eraser (Perfect Real Time) (Takara, Japan) by using S1000TM Thermal Cycler (Bio-Rad, USA) following the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis for genes were performed by mixing 1 μl synthesized cDNA, 1.6 μl forward and reverse primers specific for genes, 10 μl 2 × Ultra SYBR Mixture (CWbio, China), and 7.4 μl nuclease-free water, and run in the CFX Connect Real-Time System (Bio-Rad) according to the manufacturer's protocol. Elongation factor 1 alpha (EF1α) and β-actin were used as reference genes. To ensure specificity on intended genes, the primers for qRT-PCR were designed to span an intron. The primers sequences used for the qRT-PCR analysis of genes are listed in Table 2. During the study, the qRT-PCR efficiency was between 98% and 102% and the correlation coefficient was above 0.97 for each gene. Each qRT-PCR run was performed in triplicate and negative controls (no cDNA) were conducted. The relative expressions of genes were calculated by using the $2^{-\Delta Ct}$ method, thereof, $\Delta Ct = Ct_{target} - (Ct_{EF1\alpha} + Ct_{\beta\text{-actin}})/2$.

2.6. Histological analysis

Liver tissues were fixed with 4% paraformaldehyde and embedded in paraffin as described before [32]. Sections of 5 μm thickness were stained using the Harris hematoxylin–eosin (HE staining) mixture and examined under a light microscope.

2.7. Stress tests

2.7.1. Aeromonas hydrophila challenge test

Aeromonas hydrophila inoculum was prepared by cultivating in Luria Broth (LB) at 37 °C for 14 h with constant shaking (250 rpm). Bacteria were re-suspended in sterilized phosphate-buffered saline (PBS, pH 7.4) to obtain a density of 4.2 × 10^8 CFU/ml. After the feeding trial, the experimental fish were randomly divided into two groups (three replicates/group, fifteen fish/replicate). All fish were intraperitoneally injected with *A. hydrophila* (50 μl, containing 2.1 × 10^7 CFU/fish). At the same time, fifteen fish in each group were randomly selected and were injected with 50 μl sterilized phosphate-buffered saline (PBS, pH 7.4) as the control. After injection, all fish were returned into their respective tanks and mortality was recorded every 4 h for twenty-four days. A fish was considered dead when the gill operculum stopped moving.

2.7.2. Ammonia nitrogen exposure test

Before the stress test, the LC50 of ammonia nitrogen was evaluated as previous described [33,34], and the value obtained was 400 mg/L. Ammonia solutions were prepared by adding ammonium chloride (NH_4Cl) to freshwater until the desired concentration (400 mg/L) was attained. During the experimental period, the water temperature and pH were 26 ± 0.5 °C and 7.8, respectively. Thirty fish with relatively similar sizes were chosen from the two groups used in the feeding trial, and were randomly allocated in the ammonia nitrogen exposure test (two groups) and clean water was used as a control. Fifty percentage by volume of the water in the experimental tanks was replaced by an equal amount of water containing 400 mg/L of ammonia nitrogen after every 24 h while water in the control fish group was similarly replaced using clean

Table 2
Primers used for the analysis of mRNA genes expressions by qRT-PCR.

Gene name		Sequences (5′ to 3′)	GenBank NO.
CPT-1α	F	TTTCCAGGCCTCCTTACCCA	XM_003440552
	R	TTGTACTGCTCATTGTCCAGCAGA	
CPT-1β	F	AAGGGACGTTACTTCAAGGTG	GQ395696
	R	TCCGACTTGTCTGCCAAGAT	
PPAPα	F	CTGATAAAGCTTCGGGCTTCCA	KF871430
	R	CGCTCACACTTATCATACTCCAGCT	
FAS	F	TCATCCAGCAGTTCACTGGCATT	GU433188
	R	TGATTAGGTCCACGGCCACA	
SREBP-1c	F	TGCAGCAGAGAGACTGTATCCGA	XM_005457771
	R	ACTGCCCTGAATGTGTTCAGACA	
TNFα	F	CAGAAGCACTAAAGGCGAAGAACA	NM_001279533
	R	TTCTAGATGGATGGCTGCCTTG	
IL-8	F	CTGTGAAGGCATGGGTGTGGAG	XM_019359413.1
	R	TCGCAGTGGGAGTTGGGAAGAA	
TGF-β1	F	AAGAGGAGGAGGAATACTTTGCCA	XM_003459454
	R	GAAGCTCATTGAGATGACTTTGGG	
IL-10	F	CAGCAGCAGGAGCATCAGCATT	XM_005469373.3
	R	CACAGGAGGACGGTCTGAGAAGT	
HSP70	F	TGCCTTTGTCCAGACCGTAG	XM_003448890.4
	R	GTGTCCAACGCTGTCATCAC	
CYP1A	F	CGTCGTCGTCTCTGTTGCC	FJ389918.2
	R	CATCGTCGTGGTGGTCATAGC	
EF1α	F	ATCAAGAAGATCGGCTACAACCCT	KJ123689
	R	ATCCCTTGAACCAGCTCATCTTGT	
β-actin	F	AGCCTTCCTTCCTTGGTATGGAAT	KJ126772
	R	TGTTGGCGTACAGGTCCTTACG	

water. Fish survival rate in each tank was recorded every 4 h for fifteen days. During the experiment, no feed was supplied to the tanks, and detected dead fish whose gill operculum stopped moving were removed from the tank, counted and recorded.

2.8. Statistical analysis

All results are expressed as mean ± SEM (n = 6–8). Statistical significant differences ($p < 0.05$ or $p < 0.01$) of measured variables between control and mildronate groups were evaluated using the independent samples *t*-test. Statistical analyses were performed using the SPSS Statistics 19.0 software (IBM, Chicago, IL, USA) for windows.

3. Results

3.1. Mildronate retarded growth performance and increased liver weight

Mildronate treatment decreased significantly the growth performance and feed efficiency of Nile tilapia (Fig. 1A–C). It increased significantly the relatively liver weight of the treated fish (Fig. 1D), but did not affect mesenteric fat tissue and spleen weights (Fig. 1E and F). These results indicate that mildronate might have adverse effects on growth performance and liver is one of the main target organs for mildronate treatment in Nile tilapia.

3.2. Mildronate decreased hepatic L-carnitine content, inhibited mitochondria FA β-oxidation and induced hepatic fat deposition

Mildronate treatment decreased significantly the carnitine concentration in Nile tilapia liver (Fig. 2A). Correspondingly, the *in vitro* total FA β-oxidation (including mitochondrial plus peroxisomal β-oxidation capacity) tended to decrease and the mitochondrial β-oxidation capacity decreased significantly in the liver homogenate of mildronate group when exogenous L-carnitine was absent in the *in vitro* reaction medium (Fig. 2B). When the exogenous L-carnitine was added in the *in vitro* reaction medium, the total and mitochondrial β-oxidation capacities were both increased significantly (Fig. 2B). Different from mitochondrial β-oxidation, the peroxisomal β-oxidation capacity was not affected significantly by mildronate (Fig. 2B). Although the fat and protein contents of whole fish body were not affected (Fig. 2C and D), the hepatic histological assay clearly indicated substantial empty spaces corresponding to fat droplets in the mildronate-treated fish liver, showing excess hepatic fat accumulation (Fig. 2E). Mildronate treatment also increased significantly the triglyceride concentration in serum (Fig. 2F).

3.3. Mildronate increased mitochondrial FA β-oxidation related genes expressions in Nile tilapia

The mRNA levels of some genes related to lipid catabolism (CPT-1α, CPT-1β and PPARα) and lipogenesis (FAS and SREBP-1c) are presented in Fig. 3. The results indicated that mildronate mainly increased significantly the mRNA expression of CPT-1, which is the speed-limiting enzyme in β-oxidation and SREBP-1c.

3.4. The inhibited FA β-oxidation weakened stress resistance ability in Nile tilapia

In order to test whether the mildronate-induced FA β-oxidation inhibition would affect the stress resistance ability of Nile tilapia, the fish fed mildronate supplemented and basic diets for six weeks were challenged by either injecting them with *Aeromonas hydrophila* or exposing them to high concentration of ammonia nitrogen. The results clearly indicated that under both stress situations, the survival rates of mildronate group were significantly lower than control group (Fig. 4), indicating that, the mildronate-induced FA β-oxidation inhibition markedly decreased the stress resistance ability of Nile tilapia.

3.5. Mildronate suppressed immunity and elicited inflammation in Nile tilapia

The lowered stress resistance ability is frequently related to

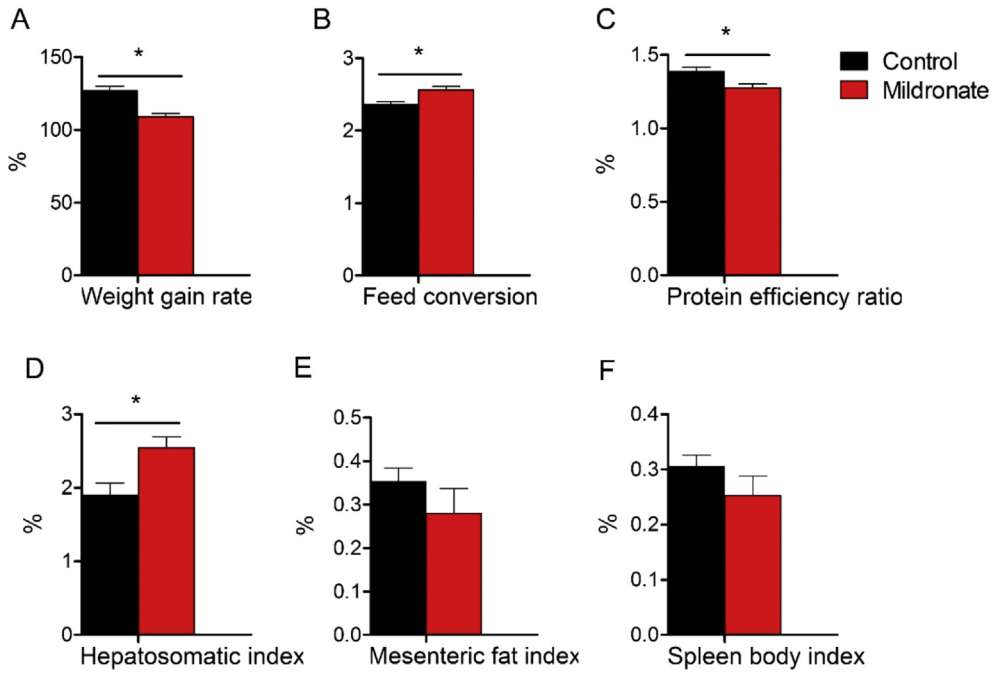

Fig. 1. Growth performance, feed utilization and organ weights in two groups. (A) Weight gain; (B) Feed conversion ratio; (C) Protein efficiency ratio; (D) Hepatosomatic index; (E) Mesenteric fat index; (F) Spleen body index. All values are means ± SEM (n = 8). Values with * statistically differ at $p < 0.05$.

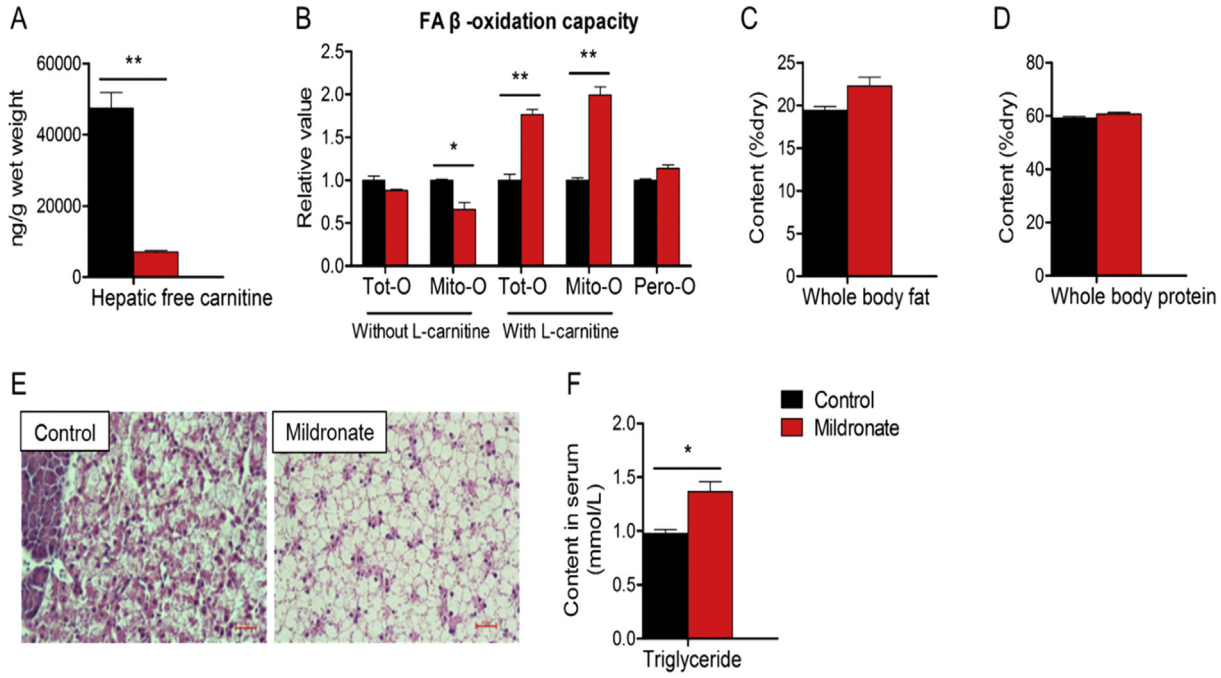

Fig. 2. The hepatic L-carnitine content, FA β-oxidation and lipid deposition in two groups. (A) Hepatic carnitine concentration; (B) FA β-oxidation capacity; (C) Whole body fat content; (D) Whole body protein content; (E) Histological characteristics of liver (20x); (F) Serum triglyceride (TG). Tot-O, total FA β-oxidation. Mito-O, mitochondrial FA β-oxidation. Pero-O, peroxisomal β-oxidation. All values are means ± SEM (n = 6). Values with × and ** statistically differ at $p < 0.05$ and $p < 0.01$, respectively.

impaired immune function and higher inflammation, thus some immune and inflammatory indices were measured. The results indicated that compared to control group, the serum activities of lysozyme (LSZ) and alkaline phosphatase (AKP) and liver acid phosphatase (ACP) in mildronate group decreased significantly (Fig. 5A–C). Similarly, serum acid phosphatase (ACP) and liver alkaline phosphatase (AKP) also decreased but not significantly (Fig. 5B and C). In contrast, the mRNA expressions of pro-inflammatory cytokines TNFα and IL-8 in liver of Nile tilapia treated with mildronate were significantly higher than those in the control group (Fig. 5D). Furthermore, the expressions of anti-inflammatory cytokines TGFβ-1 and IL-10 were significantly lower in the mildronate group compared to the control. Meanwhile, the expression of heat shock protein 70 (HSP70), an intracellular

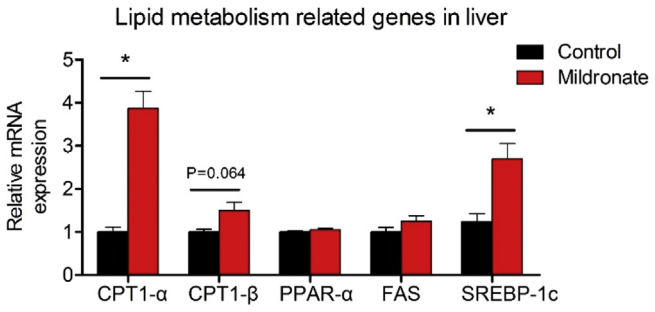

Fig. 3. Transcriptional changes of lipid metabolism related genes in liver. All values are means ± SEM (n = 8). Values with * statistically differ at $p < 0.05$.

stress signaling molecule activating immune cells to produce pro-inflammatory cytokines, was also increased significantly in the mildronate group. These results indicated that the immune functions were lowered and inflammation was increased in the mildronate-treated Nile tilapia.

3.6. Mildronate had marginal effects on oxidative stress and detoxification in Nile tilapia

Oxidative stress is commonly observed in animals suffering from environmental stress, thus some oxidative stress-related indices were measured (Fig. 6). The results indicated that except for the activity of glutathione peroxidase (GPx) in liver which was

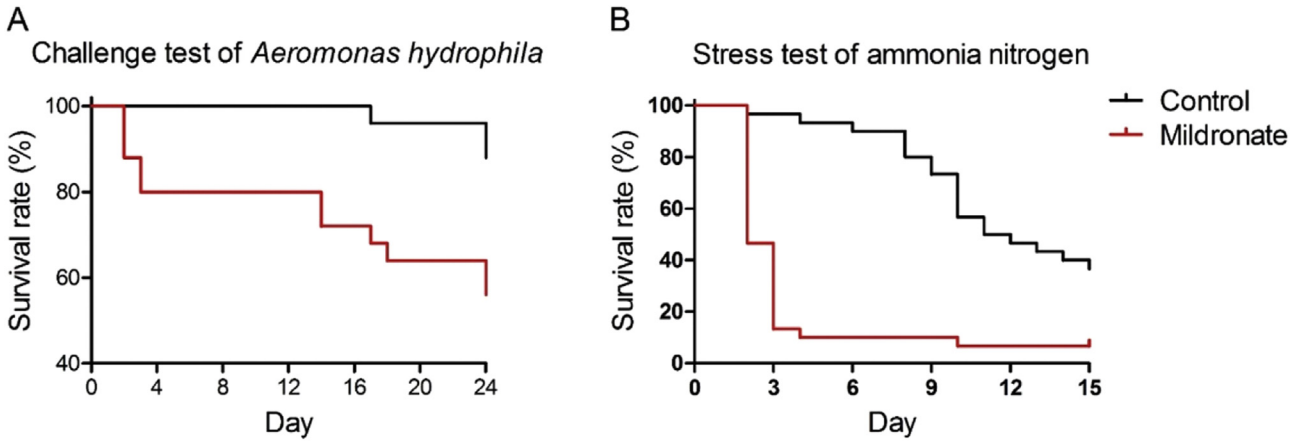

Fig. 4. The effect of inhibited FA β-oxidation on stress resistance ability in tilapia. (A) *Aeromonas hydrophila* challenge test; (B) ammonia nitrogen stress test.

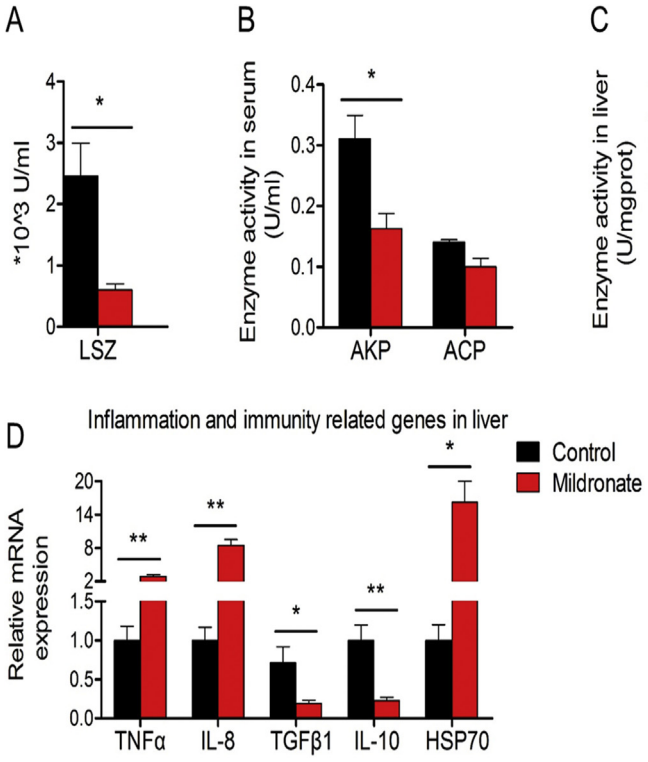

Fig. 5. Immunity and inflammation related enzyme activities and gene expressions in two groups. (A) Serum lysozyme (LSZ); (B) Serum alkaline phosphatase (AKP) and acid phosphatase (ACP); (C) Hepatic AKP, ACP; (D) The mRNA expressions related to inflammation (tumor necrosis factor α (TNF-α), interleukin 8 (IL-8), transforming growth factor beta 1 (TGFβ-1) and interleukin 10 (IL-10) and immunity (heat shock protein 70 (HSP70) in liver. All values are means ± SEM (n = 6). Values with × and ** statistically differ at $p < 0.05$ and $p < 0.01$, respectively.

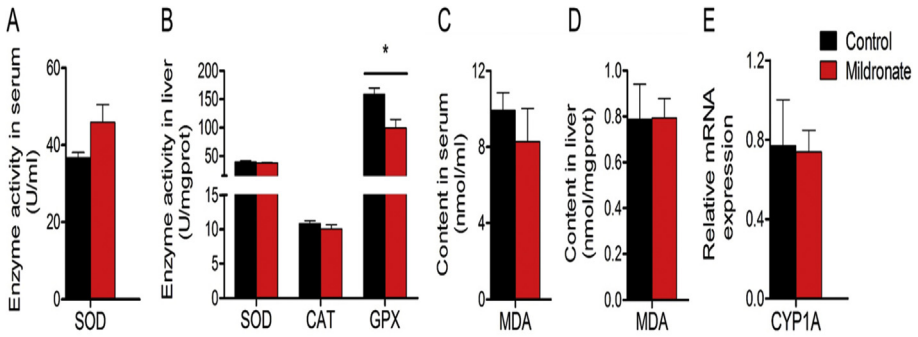

Fig. 6. Oxidative stress and detoxification related enzyme activities and gene expressions in two groups. (A) Serum superoxide dismutase (SOD); (B) Hepatic SOD, catalase (CAT), glutathione peroxidase (GPx); (C) Serum malondialdehyde (MDA); (D) Hepatic MDA; (E) Hepatic gene expression of cytochrome P450 (CYP1A). All values are means ± SEM (n = 6). Values with *statistically differ at $p < 0.05$.

inhibited significantly by mildronate treatment, no differences were obtained in the concentrations of malondialdehyde (MDA) and the activities of superoxide dismutase (SOD) in serum and liver, and the activity of catalase (CAT) in liver between the two groups, suggesting mildronate did not cause considerable oxidative stress. Of note, the mRNA expression of cytochrome P450 (CYP1A), a marker gene of detoxification, was comparable between the two groups, indicating mildronate does not affect the detoxification ability in Nile tilapia.

4. Discussion

4.1. Inhibited L-carnitine synthesis lowers mitochondrial FA β-oxidation

The importance of L-carnitine for mitochondrial FA β-oxidation is beyond doubt [10,35]. The results showed that mildronate-induced decrease in carnitine concentration in Nile tilapia significantly inhibited mitochondrial FA β-oxidation activities, but did not affect peroxisomal FA β-oxidation. In mammals, mitochondrial, but not peroxisomal FA β-oxidation is carnitine-dependent [36,37]. However, in some mammalian studies, mildronate-induced decrease in carnitine concentration lead to increased peroxisomal FA β-oxidation as a compensatory response to the decreased mitochondrial FA β-oxidation [38,39]. In the present study, the peroxisomal FA β-oxidation activity was not affected by the decreased carnitine concentration, suggesting that the peroxisomal FA β-oxidation in Nile tilapia is not as sensitive as in mammals. Nevertheless, our present study confirmed that carnitine is also necessary for Nile tilapia mitochondrial FA β-oxidation as has been established in mammals. Considering mitochondrial FA β-oxidation not only is the main pathway in lipid catabolism [40], but also contributes a large portion in energy supply in animals [41,42], it is reasonable that mildronate-treated Nile tilapia in the present study had reduced growth rate and feed utilization (Fig. 1A–C) and accumulated lipid in organs (Fig. 2E and F). Notably, in the *in vitro* assay, the presence of exogenous L-carnitine increased the activity of mitochondrial FA β-oxidation in the mildronate-treated group (Fig. 2B). This is because of the insufficient endogenous L-carnitine in the mildronate-treated Nile tilapia, which caused a compensatory increase in mitochondrial FA β-oxidation-related enzymes' activities or protein concentrations. Thus, when exogenous L-carnitine was added in the *in vitro* reaction medium, the final FA β-oxidation efficiency was increased substantially. This was also supported by the mRNA expressions results in which the two subtypes of CPT-1 were both increased significantly in the mildronate group, suggesting the low-carnitine status increased the genes expressions of mitochondrial FA β-oxidation-related enzymes.

4.2. Inhibited FA β-oxidation lowers stress resistance in Nile tilapia through impairing immune functions and triggering inflammation

Establishing the correlation between energy metabolism and stress resistance in Nile tilapia was the main purpose of the present study. The results showed that inhibited FA β-oxidation reduced significantly the survival rate of Nile tilapia when exposed to *Aeromonas hydrophila* and ammonia nitrogen, indicating that, the lowered lipid catabolism impairs stress resistance. Moreover, the reduced stress resistance was related to the decreased activities of some immune enzymes (LSZ, AKP, ACP) (Fig. 5A–C), decreased mRNA expressions of anti-inflammatory cytokines genes (TGFβ-1, IL-10), and increased mRNA expressions of pro-inflammatory cytokines genes (TNF-α, IL-8, HSP70). This is for the first time, our present study indicates that inhibited mitochondrial FA β-oxidation impairs immune functions and triggers inflammation process in fish. In other mammalian studies, some recent work have indicated that abnormal cellular nutrient metabolism cause immune response dysfunctions in immune cells, and that different immune cells have distinct preferences to utilize glucose or fatty acids as the main fuel sources [43]. Etomoxir, an agent that blocks mitochondrial FA β-oxidation at CPT-1 [44], has been proved to reduce proliferation and promote apoptosis of abnormally-dividing CD8 T cells in patients after bone marrow transplantation [45]. Moreover, a recent study has also demonstrated that mitochondrial dynamics controls T cell fate through metabolic programming [46]. Although the mechanisms and effects of reprograming metabolism immune functions have not been extensively studied in fish, our study at least provide a clue that lipid metabolism is tightly correlated to stress resistance through changing immunity and inflammatory progress in Nile tilapia.

4.3. Inhibited FA β-oxidation enhances lipid accumulation but does not induce oxidative stress

Previous studies have indicated that excess lipid accumulation in organs causes lipotoxicity subsequently results in high oxidative stress [47], which impairs normal immune functions and also induces inflammation [48]. In the present study, the inhibited mitochondrial FA β-oxidation caused lipid increase in liver and serum. However, except liver glutathione peroxidase activity which was lowered by mildronate treatment, the SOD activity and MDA concentration in serum and liver and CAT in liver were comparable between two groups. This indicates that mildronate treatment did

not induce severe oxidative stress and might not cause high lipotoxicity in Nile tilapia, supporting its harmless results obtained in mammalian studies. Particularly, in some previous fish nutrition studies, high-fat diet (HFD) also increased expressions of inflammation-related genes and decreased resistance ability of treated fish to pathogens [49,50]. Such results are normally explained based on oxidative stress aspects induced by HFD [49]. However, our present results suggest that HFD feeding-induced low stress resistance ability in Nile tilapia is also likely to be correlated to impaired mitochondrial FA β-oxidation because it had been verified previously in long-term HFD feeding in some fish [51]. Owing to the fact that, mitochondria are a common target of many stress factors, including pathogens, environmental factors and unbalanced diets, the importance of mitochondria-related metabolism in stress resistance in fish should be emphasized. On the other hand, improving mitochondrial metabolism, such as FA β-oxidation, may be a promising strategy to increase stress resistance in fish through promoting immune functions and suppressing inflammation.

5. Conclusion

The correlations between energy metabolism, stress resistance, immunity and inflammation in fish have not been well established. Our present study indicated that limited carnitine synthesis inhibited mitochondrial FA β-oxidation and decreased resistance to pathogens and ammonia nitrogen in Nile tilapia through impairing immune functions and triggering inflammation, but did not induce oxidative stress. This indicates the importance of mitochondria-related metabolism in stress resistance in Nile tilapia.

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