

Metformin attenuates lipid accumulation in hepatocytes of blunt snout bream (*Megalobrama amblycephala*) via activation of AMP-activated protein kinase

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Abstract

Currently, there is a trend to use high-fat diets in intensive aquaculture that is accompanied with incidence of fatty liver when dietary lipid level surpasses an upper limit. So, it is necessary to develop appropriate strategies to reduce the risk of fatty liver in commercial fish farming. Studies in mammals have revealed a correlation between fatty liver and AMP-activated protein kinase (AMPK) activity, which has been recognized as a key modulator of lipid metabolism. Considering the frequent occurrence of fatty liver in blunt snout bream farming, an *in-vitro* study was designed to evaluate the efficiency of metformin, as a stimulator of AMPK, in activation of AMPK and its subsequent effects on lipid metabolism in primary hepatocytes. Fish hepatocytes were seeded at a density of $1 \times 10^6 \text{ ml}^{-1}$ in 6-well tissue culture plates and treated with three different media including: 1) Leibovitz's L-15 medium [L15] as control, 2) high-fat medium [L15+400 μM oleic acid], and 3) metformin medium [L15+400 μM oleic acid+200 μM metformin]. After 48 h of culture, the cells and supernatant were collected for analysis. The results showed significant ($P < 0.05$) enhancement of cell triglyceride and total cholesterol concentrations in the high-fat medium group over control, and metformin addition significantly reduced the values. Also, the high-fat medium group exhibited significantly higher aspartate aminotransferase activity than both control and metformin groups. The lowest AMPK and phospho-AMPK protein expression was found in the high-fat medium group while metformin addition significantly up-regulated their expression levels. Mitochondrial and peroxisomal oxidation rates in the high-fat medium group were significantly lower than control while similar oxidation rates were observed for metformin treated and control groups. The high-fat medium group showed significantly lower CPT I activity than control, and metformin inclusion increased the activity. Expression of genes

associated with lipid metabolism such as PPAR α , CPT I, AOX, PGC-1 α and TFAM was suppressed in the high-fat medium group, and metformin supplementation up-regulated their expression levels. The opposite trend was true for the expression of ACC2 gene. Also, the results showed down-regulation of FAS and SREBP-1C genes in the high-fat medium group, and metformin addition resulted in further reduction of their expression level. The lowest activities of mitochondrial complexes (I-III) were found in the high-fat medium group and metformin prevented high-fat-induced reduction of mitochondrial complexes activity. Notably increased concentrations of reactive oxygen species and malondialdehyde were found in the high-fat medium group, and metformin treatment reduced their concentrations. Moreover, metformin group exhibited higher glutathione peroxidase activity than the high-fat medium group. The findings in this study showed clearly that metformin activated AMPK in blunt snout bream hepatocytes, which contributed to enhanced lipid metabolism and attenuated lipid deposition in the cells incubated with high-fat medium.

Key words: blunt snout bream; fatty liver; metformin; AMP-activated protein kinase; lipid metabolism

1. Introduction

It is well known that adequate levels of non-protein energy sources should be incorporated in fish diet in order to maximize protein utilization for growth (Wilson and Halver, 1986). Dietary lipids have been extensively used as a source of concentrated energy for saving protein and increasing feed efficiency in economical fish farming practices (Boujard et al., 2004; Hillestad et al., 1998). Use of high-fat (high energy) diets has become a common practice in the aquaculture industry since the discovery of the protein sparing effects of lipids by Lee and Putnam (1973). However, it became apparent that dietary lipid content could be increased up to a certain level but, thereafter, undesirable impacts could be achieved such as reduced growth performance and unwanted lipid accumulation that can subsequently result in hyperlipidemia, fatty liver, and lipid peroxidation (Du et al., 2005, 2008; Ji et al., 2011; Jin et al., 2013; Li et al., 2012; Lu et al., 2013a). Fatty liver, which is characterized by excessive triglyceride accumulation in hepatocytes (Bolla et al., 2011; Lu et al., 2013), is not only a sign of wasted dietary energy but also has detrimental effects on fish health (Lu et al., 2013b; Nanton et al., 2003). Poor growth performance and high mortality caused by fatty liver have been reported in several farmed fish species leading to substantial economic losses (Du et al., 2014). Accordingly, it is vital to recognize the nutritional factors and mechanisms involved in the development of fatty liver.

The complex metabolic regulation systems in animals enable them to adapt to nutritional modifications (Soengas, 2014). As such, in most animals increasing dietary lipid is accompanied with alterations in lipid metabolism including suppression of

lipogenic enzymes (Clarke and Hembree, 1990; G lineau et al., 2001; Hillgartner et al., 1995) and increased fatty acid oxidation (Kim et al., 2004). Therefore, an appropriate approach for cost effective fish production could be developing strategies to inhibit excessive fat deposition in liver, and instead increase the utilization of dietary lipid to promote edible biomass production. Research in mammals has revealed a correlation between fatty liver and AMP-activated protein kinase (AMPK) activity (You and Rogers, 2009). It has been demonstrated that AMPK plays a key role in regulating lipid metabolism, and is now known as a metabolic master switch for modification of cellular energy charge (J ger et al., 2007). Stimulation of AMPK leads to suppression of anabolic processes such as fatty acid and cholesterol synthesis, and activation of catabolic processes like fatty acid oxidation (Fryer and Carling, 2005; Hardie et al., 2006; Kahn et al., 2005). For instance, AMPK phosphorylates acetyl-CoA-carboxylase-2 (ACC-2) leading to reduction of malonyl-CoA (an inhibitor of carnitine palmitoyltransferase I, CPT I), which subsequently results in increased fatty acid oxidation in mitochondria (Merrill et al., 1997; Vavvas et al., 1997). Knockout of the AMPK gene is linked to higher incidence of obesity and fatty liver (Viollet et al., 2003). On the other hand, its over-expression attenuates fatty liver through activation of enzymes associated with fatty acid oxidation (J rgensen et al., 2007; Winder et al., 2000). Thus, AMPK has received attention as a novel target for treatment of fatty liver in mammals (Lin et al., 2007). Although the existence of AMPK has been reported in several fish species including blunt snout bream (*Megalobrama amblycephala*) (Xu et al., 2017), rainbow trout (*Oncorhynchus mykiss*) (Polakof et al., 2011b) and turbot

(*Scophthalmus maximus*) (Zeng et al., 2016), its role in regulating fish lipid metabolism has not yet been investigated.

Blunt snout bream is an herbivorous freshwater fish native to China, and has been a favored aquaculture species in China due to its fast growth, tender flesh and high disease resistance. However, occurrence of fatty liver has been a common issue when reared in captivity due to its lower hepatosomatic index compared to other farmed fish species (Lu et al., 2014a). Metformin has been identified as one of the most commonly known agents for activation of AMPK and its potency in treatment of fatty liver has been established in mammals. To better understand the role of AMPK in regulating lipid metabolism in fish, effects of metformin on AMPK activation, lipid metabolism and lipid accumulation were investigated in primary hepatocytes of blunt snout bream.

2. Materials and methods

2.1. Experimental fish

Blunt snout breams (averaging 100 g) were purchased from a private farm and transported to the aquaculture laboratory of Jimei University. The fish were stocked into a 1000-L tank supplied with aerated fresh water in a recirculating system and fed twice daily (8:30 and 16:30) with a commercial diet (Tongwei, Suzhou, China) (35% protein and 5% lipid) for two weeks. The average water temperature was 28±1.5 °C and the photoperiod was maintained on a 12:12 light:dark schedule. The fish were fasted for 24 h prior to sampling.

2.2. Isolation of hepatocytes

Prior to isolation of hepatocytes, fish were anesthetized with MS-222 (tricaine methanesulfonate; Sigma, USA) (100 mg l⁻¹) and bled by cutting the gill arches. Then,

liver was rapidly isolated and washed several times in ice-cold phosphate buffered saline (PBS) containing antibiotic (100 IU ml⁻¹ penicillin G sodium and 100 IU ml⁻¹ streptomycin). After removal of PBS by sterile pipette, the samples were cut into small pieces (about 1 mm³) and digested with pancreatin at 28 °C for 30 min. Thereafter cell suspension was centrifuged at 500 ×g for 10 min and washed twice. The harvested cell pellets were re-suspended in Leibovitz's L-15 medium (L15 medium) (HyClone™, USA) with 15% fetal bovine serum (Biological Industries, USA) at a density of 1×10⁶ ml⁻¹. For each test three different fish were used and each time the livers were pooled to make a single sample.

2.3. Cell treatment

Two milliliter of isolated hepatocytes was seeded in each well of 6-well culture plates. After 24 h, all cells attached and cultured in 2 ml of the following media: control medium (L15), high-fat medium (L15+400 μM oleic acid), and metformin medium (L15+400 μM oleic acid+200 μM metformin). After 48 h, the cells and supernatant were collected for analysis. The supernatant was collected by sterile pipette. Then, cells were harvested by trypsinization (0.25% trypsin–EDTA) at 25 °C in 5 min. All the tests were performed in three replicates. Each replicate was made up by pooling six wells for Western blotting, and two wells for the rest of the tests.

2.4. Biochemical parameters

Cell triglyceride and total cholesterol (TC) concentrations were determined by colorimetric enzymatic methods using commercial kits (Beijing BHKT Clinical Reagent Co., Ltd, China) as described previously (Lu et al., 2016a). Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in supernatant were measured through enzymatic colorimetric methods according to Reitman and

Frankel (1957).

2.5. Gene expression

Total RNA was extracted from the hepatocytes using RNAiso Plus (Takara Co. Ltd, Japan) according to the protocol provided by the manufacturer. Isolated RNA was quantified using the NanoDrop ND-2000 spectrophotometer, and its integrity was confirmed by agarose gel electrophoresis. RNA samples were treated by RQ1 RNase-Free DNase prior to RT-PCR (Takara Co. Ltd, Japan) to avoid genomic DNA amplification. cDNA was generated from 500 ng DNase-treated RNA using ExScript™ RT-PCR kit (Takara Co. Ltd, Japan), and the mixture consisted of 500 ng RNA, 2 µl buffer (5×), 0.5 µl dNTP mixture (10 mM each), 0.25 µl RNase inhibitor (40 U µl⁻¹), 0.5 µl dT-AP primer (50 mM), 0.25 µl ExScript™ RTase (200 U µl⁻¹), and total volume made up to 10 µl with DEPC-treated H₂O. The reaction conditions were as follows: 42 °C for 40 min, 90 °C for 2 min, and 4 °C thereafter.

Real-time PCR was employed to determine mRNA levels based on the SYBR® Green I fluorescence kit (Takara Co. Ltd, Japan). Primer characteristics used for real-time PCR are listed in Table 1, according to the MIQE Guidelines (Bustin et al., 2011). Real-time PCR was performed in a Mini Option real-time detector (BIO-RAD, USA). The fluorescent quantitative PCR reaction solution consisted of 12.5 µl SYBR® premix Ex Taq™ (2×), 0.5 µl PCR forward primer (10 µM), 0.5 µl PCR reverse primer (10 µM), 2.0 µl RT reaction (cDNA solution), and 9.5 µl dH₂O. The reaction conditions were as follows: 95 °C for 3 min followed by 45 cycles consisting of 95 °C for 10 s and 60 °C for 20 s. The fluorescent flux was then recorded, and the reaction continued at 72 °C for 3 min. The dissolution rate was measured between 65 and 90 °C. Each increase of 0.2 °C was maintained for 1 s, and the fluorescent flux was recorded. All amplicons were initially separated by agarose gel electrophoresis to ensure that they

were of correct size. A dissociation curve was determined during the PCR program to make sure that specific products were obtained in each run. All reactions were performed in three technical replicates. The gene expression levels were normalized towards mean of the reference gene (β -actin). The gene expression was calculated by using the comparative ($2^{-\Delta\Delta C_t}$) method (Livak and Schmittgen, 2001).

2.6. Western blotting

Western blots were carried out following the protocols described by Lau and Richards (2011) with slight modifications. Briefly, cell pellets (about 10^8 cells) were lysed in ice-cold lysis buffer (Cell Signaling, Danvers, MA, USA) and centrifuged at $12000 \times g$ for 5 min, and then the resulting supernatants were stored at -80°C . Total protein was determined according to the methods outlined by Bradford (Bradford, 1976). Aliquots of each sample were added to an equal volume of SDS-sample buffer (Laemmli, 1970), boiled for 5 min, and $20\ \mu\text{g}$ of total protein was loaded into each well, separated by SDS-PAGE for 1-2 h at 100 V using a Mini-Protean system (BioRad, Spain) and transferred to a polyvinylidene fluoride (PVDF) membranes (Millipore, Massachusetts, USA). Subsequently, the membrane was blocked with blocking buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.6) containing 5% (w/v) non-fat dry milk for 1 h. The membrane was then incubated with rabbit polyclonal antibodies against GAPDH blots (Cell Signaling Technology, USA), anti-AMPK α (Cell Signaling Technology, USA) and antiphospho-AMPK α (#2535, Cell Signaling Technology, USA) at 4°C overnight. After washing, membranes were incubated with anti-rabbit secondary antibody. Bands were visualized by an electrochemiluminescence (ECL) system (GE Healthcare, Buckinghamshire, UK) and quantified by the densitometry band analysis tool in ImageJ 1.44p.

2.7. Mitochondria

The activities of respiratory chain complexes (I, II, III) and citrate synthase were determined using commercial kits (Nanjing JianCheng Bioengineering Institute, China) as described previously (Lu et al., 2017). Thiobarbituric-acid-reactive substances assays were performed with a malondialdehyde (MDA) kit (Nanjing JianCheng Bioengineering Institute) as described by Rueda-Jasso et al. (2004). Mito tracker green was used for mitochondrial labeling. Hepatocytes were incubated in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal calf serum and labeled with 80 nM Mito Tracker Green FM (Beyotime C1048-50 μ g; Nantong, China), and incubated for 30 min at 28 °C, in a humidified atmosphere, 5% CO₂ in air, subsequently washed with DMEM medium, and examined and photographed by fluorescence microscopy (450-490 nm excitation light, 520 nm barrier filter; Olympus).

2.8. Fatty acid oxidation rate

Mitochondrial and peroxisomal β -oxidation of hepatocytes were determined using radiolabelled [1-¹⁴C] palmitate (16:0) as a substrate, as described previously (Lu et al., 2014b). Palmitate oxidation rates were measured at 28 °C using two media as described by Frøyland et al. (1995), the first allowing the total (mitochondrial and peroxisomal) activities to occur (13.2 mM HEPES [pH 7.3], 16.5 mM MgCl₂, 82.5 mM KCl, 13.2 mM dithiothreitol, 6.6 mM ADP, 0.2 mM NAD⁺, 100 mM-CoA and 0.7 mM EDTA), the second allowing the peroxisomal activity only (the medium only differing by the presence of 73 mM antimycin and 10 mM rotenone to block the mitochondrial respiratory chain). Palmitate oxidation was measured with 115 μ M [1-¹⁴C] palmitate supplemented with 1.2 mM L-carnitine. The samples were incubated for 60 min at 28 °C, then reactions were stopped by addition of 1.5 M KOH; fatty acid-free bovine serum albumin (BSA, 100 mg ml⁻¹) was added to the suspension in order to bind

unoxidized substrates and then 4 M HClO₄ was added to precipitate unoxidized substrates bound to BSA. The total solution was then centrifuged at 1880 ×g for 15 min. Aliquots of 200 µl were transferred to a scintillation tube containing 4 ml of liquid scintillation cocktail and assayed for radioactivity in a LS6500 liquid scintillation analyzer (Beckman, USA).

2.9. Oxidative status

Cell suspensions were incubated at 37 °C for 30 min with 10 µM 2',7'-dichlorofluorescein diacetate (DCFH/DA, Nanjing Jiancheng Bioengineering Institute, China). To measure the intracellular reactive oxygen species (ROS), the fluorescence of DCF was excited by a 15mW laser tuned to 488 nm and the emitted fluorescence was measured with 530/30 band pass filter in a FACScalibur Becton Dickinson flow cytometer. The conditions for data acquisition and data analysis were established using negative and positive controls with the CellQuest Program of Becton Dickinson and these conditions were maintained during all the experiments. Total superoxide dismutase (SOD) activity was measured using a commercial kit (Nanjing Jiancheng Bioengineering Institute, China) according to Nakano (1990). Glutathione peroxidase (GPX) activity was measured using the method of Dabas et al. (2012). Thiobarbituric-acid-reactive substances assays were performed with malondialdehyde (MDA) kit (Nanjing Jiancheng Bioengineering Institute, China) as described by Rueda-Jasso et al. (2004).

2.10. Statistical analysis

Data were analyzed by one-way ANOVA using the SPSS 16.0 for Windows. Duncan's test was used for the multiple comparisons. The level of significance was set at $P < 0.05$. All data were presented as means ± SE.

3. Results

3.1. Cellular lipid accumulation

Hepatocytes cultured with high-fat medium had significantly ($P < 0.05$) higher TG and TC contents than the control group, and metformin addition to high-fat medium significantly reduced their values (Fig. 1A, B). Also, significantly higher AST activity was detected in the high-fat medium group and a significant reduction was obtained by metformin supplementation (Fig. 1C). A similar trend was observed for ALT activity although no significant differences were found between high-fat and metformin groups (Fig. 1D).

3.2. AMPK activity

Western blot analyses revealed significant reduction in expression of AMPK and phospho-AMPK proteins in the high-fat medium group, while metformin inclusion significantly increased their expression levels (Fig. 2).

3.3. Fatty acid β -oxidation

Both mitochondrial and peroxisomal fatty acid oxidation rates were significantly lower in the high-fat medium group than in the control, while their values were significantly improved by metformin addition and comparable values to those of control group were achieved (Fig. 3A, B). Similarly, CPT I activity was significantly lower in the high-fat medium group and this was reversed by supplementing metformin to the culture medium (Fig. 3C).

3.4. Expression of lipid metabolism-related genes

Expression of the genes associated with lipid metabolism including PPAR α , PPAR β , CPT I, AOX, PGC-1 α , PGC-1 β , ACC2, TFAM, FAS and SREBP-1C are presented in Fig. 4. The results showed that metformin supplementation significantly

up-regulated the expression of PPAR α , CPT I, AOX, PGC-1 α and TFAM compared to the high-fat medium group. An opposite trend was true in the case of ACC2 gene, where the high-fat medium treated group exhibited dramatically higher expression level of ACC2 than the other groups. Expression of FAS and SREBP-1C genes were down-regulated in high-fat medium group and a further reduction was observed by metformin addition. Expression of other investigated genes including PPAR β and PGC-1 β were unchanged.

3.5. Mitochondrial status

Activities of citrate synthase and mitochondrial complexes are presented in Fig. 5. Activities of mitochondrial complex I, II and III were significantly suppressed in high-fat medium group compared to control. Metformin supplementation significantly increased activities of mitochondrial complex I and II, however, the values were still significantly lower than those of the control group. A similar increase was observed for complex III activity but the difference was not significant. However, citrate synthase activity was not significantly influenced. Mito-Tracker Green, a fluorescent molecular probe with high affinity for mitochondrial membranes, was used to identify mitochondria in living cells. As shown in Fig. 6, the fluorescence intensity in the high-fat medium group was weaker than the other treatments.

3.6. Oxidative status

The results showed significant enhancement of ROS in the high-fat medium group and inclusion of metformin significantly reduced ROS concentration in cells incubated in high-fat medium (Fig. 7A). Likewise, significantly higher MDA concentration was detected in the high-fat treatment and an intermediary value was achieved with the metformin-treated group (Fig. 7B). High-fat medium showed significantly lower SOD

and GPX activities than control, and metformin addition resulted in significant enhancement of GPX activity and numeral increase of SOD activity (Fig. 7C, D).

4. Discussion

Metformin has long been used as an anti-diabetic drug capable of modulating muscle and liver metabolism and controlling hyperglycemia in mammals (Alengrin, 1995; Lin et al., 2000; Panserat et al., 2009; Zhou et al., 2001). These functions have also been investigated in fish and the results have revealed the mammalian-like effects of metformin (Magnoni et al., 2012; Polakof et al., 2011a; Xu et al., 2018). It has been suggested that metformin exerts its therapeutic effects through activation of AMPK (Zou et al., 2004) which is linked to reduced glucose production and facilitated fatty acid oxidation in hepatocytes (Zhou et al., 2001). In addition, studies in humans showed that metformin can ameliorate liver dysfunction in patients with fatty liver (Garinis et al., 2010). Lipid accumulation has been reported in chronic metabolic diseases such as obesity and type 2 diabetes, and it has been demonstrated that AMPK activation inhibits excessive nutrient-induced hepatic lipid accumulation (Li et al., 2014). To our knowledge, this is the first report to evaluate the regulatory effects of metformin on lipid metabolism in fish with a particular emphasis on its efficacy in AMPK activation.

The results achieved for TG and TC concentrations in hepatocytes demonstrated clearly that metformin could attenuate lipid accumulation in fish hepatocytes subjected to “high-fat” via supplementation with oleic acid. In agreement with our results, Xu et al. (2018) showed that metformin supplementation in both low and high carbohydrate diets for blunt snout bream resulted in reduced lipid accumulation in liver and adipose tissues and decreased plasma triglyceride concentration. The authors attributed these results to increased fatty acid oxidation and/or inhibited fatty acid synthesis through activation of AMPK that ultimately resulted in reduced lipid accumulation (Zang et al.,

2004). Also, Kim et al. (2010) reported that metformin lowered liver TG and TC contents and prevented fat accumulation in liver of mice fed a high-fat diet. These authors suggested that such effects of metformin were mediated through AMPK activation, which eventually results in decreased expression of sterol regulatory element binding protein-1C (SREBP-1C) and fatty acid synthase (FAS). Similarly, in the current study metformin treatment down-regulated the expression of both SREBP-1C and FAS genes. SREBPs play key roles in both lipogenesis and cholesterol homeostasis (Horton et al., 1998; Kim et al., 1998; Pai et al., 1998; Shimano et al., 1996). It is believed that SREBP-1 is particularly involved in activation of the genes that control fatty acid metabolism and *de novo* lipogenesis (Horton et al., 1998; Pai et al., 1998). Furthermore, it has been suggested that modulation of FAS by SREBP-1c is dependent on upstream stimulatory factors (Griffin et al., 2007; Latasa et al., 2003; Yoshikawa et al., 2001). SREBP-1c is the key modulator of hepatic triglyceride synthesis, making it a target for the inhibition and/or therapy of steatosis in hepatocytes.

AST and ALT levels are often used as indicators of liver disease including non-alcoholic fatty liver (Krakoff et al., 2010). The results of a study on mice showed enhancement of serum AST and ALT activities following administration of a high-fat diet, and metformin supplementation suppressed the activity of both enzymes (Kim et al., 2013). Krakoff et al. (2010) used serum ALT activity as a marker for non-alcoholic fatty liver disease (NAFLD) in humans, and their results showed a significant reduction of ALT activity in metformin-treated patients. Furthermore, it has been reported that metformin lowered aminotransferases level and decreased liver fat content in mice with NAFLD (Lin et al., 2000). Likewise, in the current study, the high-fat medium group showed significantly higher AST and ALT activities than control, and treatment with metformin led to reduced activity of both enzymes although the difference was not

statistically significant in the case of ALT activity.

The results of a recent *in vivo* study revealed the induction of AMPK phosphorylation by metformin administration in fish (Xu et al., 2018). It has been reported that metformin phosphorylates AMPK via stimulation of LKB1 kinase activity (Shaw et al., 2005). As such, the results of Western blot analysis in the present study showed clearly that metformin can up-regulate the expression of AMPK and P-AMPK proteins in hepatocytes. Similarly, Kim et al. (2010) showed that metformin enhanced AMPK and P-AMPK expression in mice compared to mice receiving a high-fat diet. However, some *in vitro* studies showed no significant alteration of AMPK phosphorylation in fish hepatocytes (Polakof et al., 2011b). These inconsistent results could be due to differences in the duration of treatment of hepatocytes with metformin; in the current study hepatocytes were treated with 200 μ M metformin for 48 h while Polakof et al. (2011b) incubated the rainbow trout hepatocytes with metformin for 16 h.

It has been shown that there is a correlation between liver fatty acid oxidation and fatty liver (Smith et al., 2016); where enhancement of oxidation rate reduced fatty liver (Perry et al., 2015; Stefanovic-Racic et al., 2008) while suppressed oxidation facilitated development of fatty liver (Zhang et al., 2007). AMPK, as a cellular “energy sensor”, plays a key role in lipid metabolism (Hardie and Sakamoto, 2006). Once fatty acids are taken up across the plasma membrane and activated to fatty acyl-CoA, they are either directed towards oxidation or storage. At this stage, AMPK plays a role by determining the fate of the absorbed fatty acids as it is known to phosphorylate and inactivate acetyl-CoA carboxylase (ACC) leading to reduced malonyl-CoA, which is an inhibitor of CPT I (McGarry et al., 1978; Saha and Ruderman, 2003). CPT I is considered as the mitochondrial gateway for fatty acid entry into the matrix, and is the main modulator

of hepatic mitochondrial β -oxidation flux (Bartlett and Eaton, 2004; Lu et al., 2016b). Attenuated β -oxidation capacity resulting from reduced catalytic efficiency of CPT I has been recognized as one of the main causes of fatty liver in fish (Lu et al., 2014b). Our previous research showed that reduction of fatty acid β -oxidation in blunt snout bream is closely linked to the occurrence of fatty liver (Lu et al., 2014b). In the current study, the metformin-treated group exhibited significantly higher AMPK protein expression level and β -oxidation capacity than the high-fat medium group indicating that metformin increased fatty acid oxidation in hepatocytes through AMPK activation. This notion was also supported by enhanced CPT I activity in the metformin-treated group over high-fat medium group.

ACC has two different isoforms, ACC1 and ACC2, with the only difference between the two forms being the presence of an extra NH_2 -terminal extension of 146 amino acids in ACC2, localizing the enzyme to mitochondria (Abu-Elheiga et al., 1995, 2000). Such localization has been suggested to be related to the regulatory effects of ACC2 on fatty acid oxidation, as malonyl-CoA is produced in close proximity to CPT I (Iverson et al., 1990). The results of early experiments in mammals showed that AMPK inhibits ACC2 activity by phosphorylating at Ser-221 (Ahu-Fjheiga and Wakil, 1997). It has been shown that ACC2 exhibits similar phosphorylation in fish and these serine residues are conserved (Cheng et al., 2011; He et al., 2014). Although in the current study malonyl-CoA content was not measured, the expression of ACC2 gene in liver was down-regulated by metformin indicating that inhibition of ACC2 by AMPK activation contributed to increased CPT I activity and fatty acid β -oxidation. Moreover, it has been reported that expression of CPT I mRNA is influenced by PPARs as CPT I has a PPAR responsive component (Rao and Reddy, 2001). All the mammalian isotypes of PPAR have also been recognized in several fish species although their function has

393 been shown to be different (Leaver et al., 2005). In fish, PPAR α activates lipid
394 catabolism through transcriptional control of target genes encoding enzymes involved
395 in peroxisomal and mitochondrial β -oxidation mainly in the liver (Michung, 2009). Up-
396 regulation of PPAR α was correlated with increased CPT I activity (Moraes et al., 2007).
397 Meanwhile, previous studies have revealed that AMPK activation is accompanied by
398 increased PPAR α expression (Baar, 2004; Lee et al., 2006). So, up-regulation of PPAR α
399 by AMPK activation in this study could be another contributing factor to enhanced CPT
400 I activity and fatty acid β -oxidation. Lu et al. (2014b) reported the down-regulation of
401 AOX gene expression in blunt snout bream following administration of a high-fat diet.
402 AOX is believed to catalyze the first rate-limiting step in peroxisomal β -oxidation
403 (Moraes et al., 2007). The authors attributed the reduced AOX activity to the decreased
404 peroxisomal β -oxidation. Likewise, in the current study both AOX gene expression and
405 β -oxidation rate decreased in the high-fat medium group.

406 In addition to CPT I activity, both quantity and quality of mitochondria are
407 considered as critical factors in determining β -oxidation capacity (Du et al., 2006;
408 Morash et al., 2008). There are several reports indicating that drastic decrease in
409 mitochondrial protein content of liver as well as the impairment of mitochondria leads
410 to reduced metabolic activity and oxidative capacity in fish (Du et al., 2006; Lu et al.,
411 2014b). In the present study, Mito-Tracker Green, which is a molecular probe with high
412 affinity for mitochondrial membranes, was used to identify mitochondria in the living
413 cells. The results demonstrated lower abundance of mitochondria in the high-fat
414 medium group compared to the other groups. This is consistent with previous findings
415 in fish that showed the administration of high-fat diets reduced mtDNA copies and
416 mitochondrial protein (Liao et al., 2016; Lu et al., 2014a). Furthermore, our results
417 indicated enhanced abundance of mitochondria following metformin supplementation.

This could be due the fact that AMPK is involved in mitochondrial biogenesis (Zong et al., 2002). In mammals, PGC-1 α is considered as a key regulator of mitochondrial biogenesis through stimulating the expression of mitochondrial transcriptional factor A (TFAM) (Lehman et al., 2000; Puigserver et al., 1998; Wu et al., 1999). AMPK activation has been identified as a prerequisite for increased expression of PGC-1 α (Zong et al., 2002). Accordingly, it could be suggested that AMPK activation by metformin in the present study up-regulated PGC-1 α expression leading to subsequent enhancement of mitochondrial biogenesis. We found a notable increase in expression of complex I, II and III by metformin inclusion. Although the precise underlying mechanism is still unclear, we suggest that this could be associated with enhanced mitochondrial biogenesis as newly generated mitochondria exhibit improved biological function.

There are numerous studies indicating that lipid accumulation in liver adversely affects liver function and induces oxidative stress in fish (Lu et al., 2016a). Since mitochondria are the main site of ROS formation, mitochondria dysfunction could be taken as one of the main causes of oxidative stress. Likewise, in the current study the high-fat medium group exhibited the highest concentrations of ROS and MDA and the lowest SOD and GPX activities, and metformin supplementation could reduce ROS and MDA concentrations and enhance GPX activity. It is believed that mitochondrial complex I is involved in scavenging ROS in the inner mitochondrial membrane (Bottje and Carstens, 2009), and that the lower activity of complex I results in over production of ROS (Lu et al., 2016a). Therefore, reduction of ROS and MDA concentrations by metformin in the present study could be attributed to enhanced complex I activity.

In conclusion, the findings in the present study showed that metformin activated AMPK in hepatocytes of blunt snout bream, and this was accompanied by enhanced

fatty acid β -oxidation via AMPK/ACC2/CPT I and AMPK/PPAR α /CPT I pathways. Furthermore, AMP activation up-regulated the expression of PGC-1 α and TFAM, which are involved in mitochondrial biogenesis. Furthermore, metformin decreased hepatic ROS and MDA concentrations via enhancing mitochondrial complexes activity. Overall, activation of AMPK by metformin could attenuate lipid accumulation and oxidative stress in hepatocytes mainly due to elevation of fatty acid β -oxidation and mitochondrial function.

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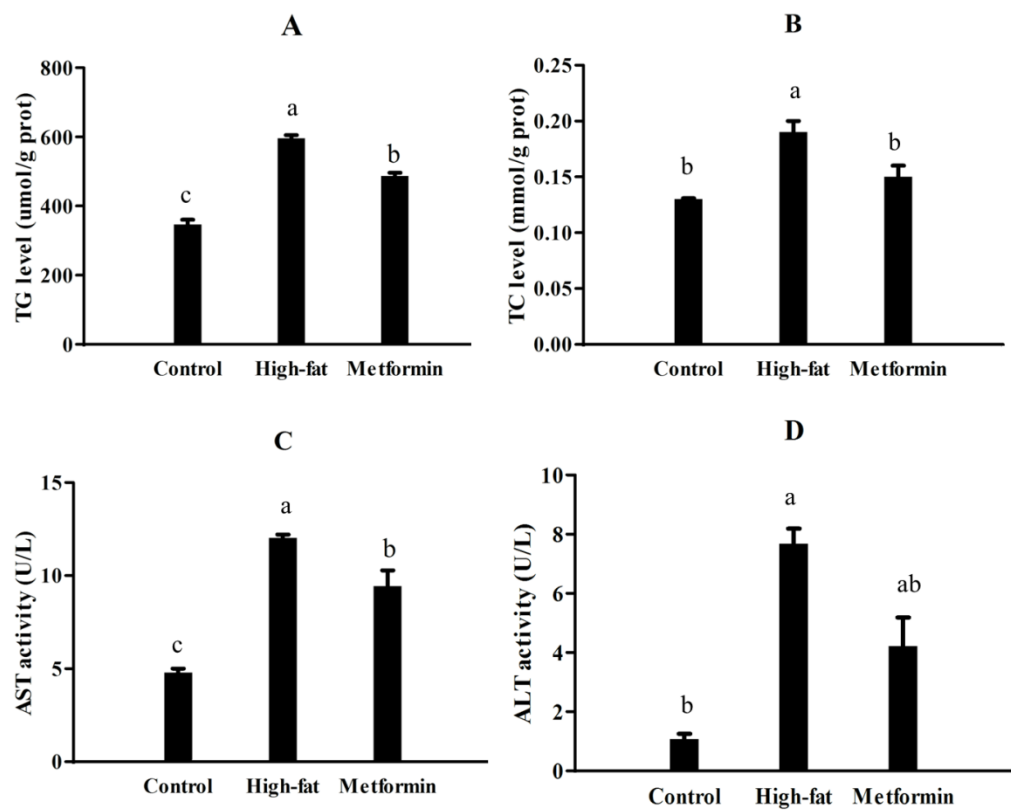
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766 **Fig.1.** Concentrations of triglyceride (TG: A) and total cholesterol (TC: B), and
767 activities of aspartate aminotransferase (AST: C) and alanine aminotransferase (ALT:
768 D) in primary hepatocytes of blunt snout bream. Bars with different letters are
769 significantly different ($P < 0.05$).
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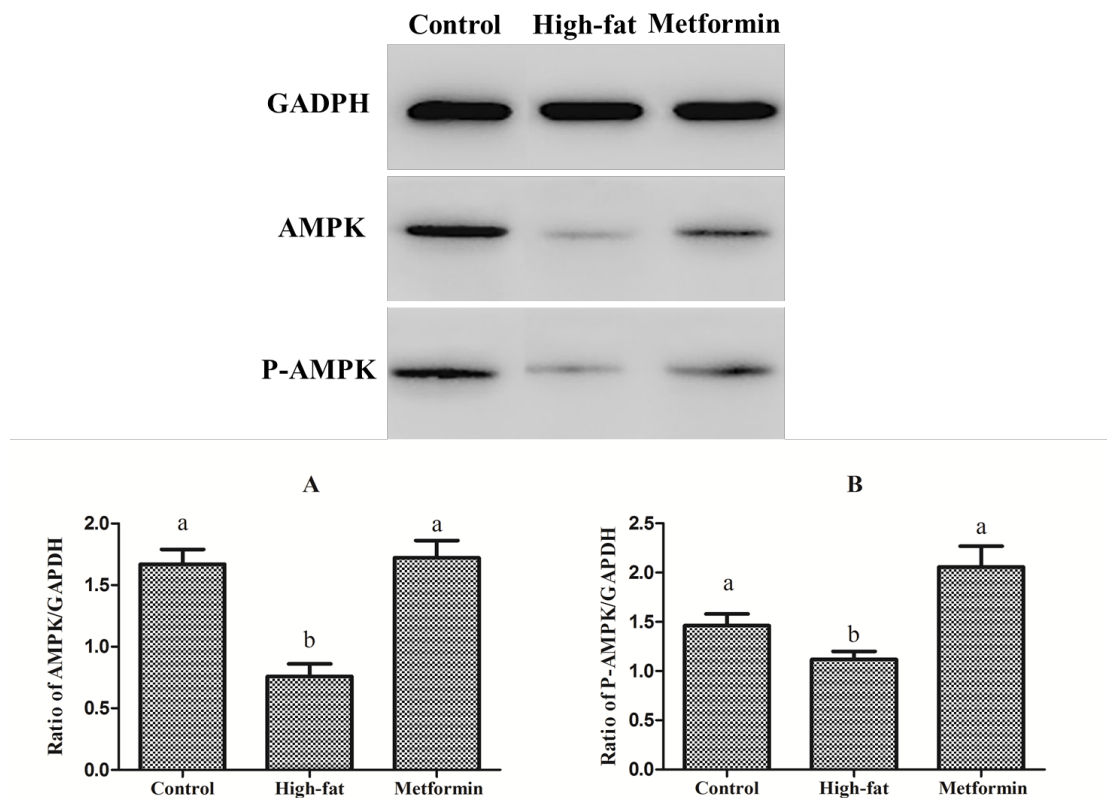


Fig.2. AMPK (A) and phospho-AMPK (B) expression levels as determined by Western blot in primary hepatocytes of blunt snout bream. Bars with different letters are significantly different ($P < 0.05$).

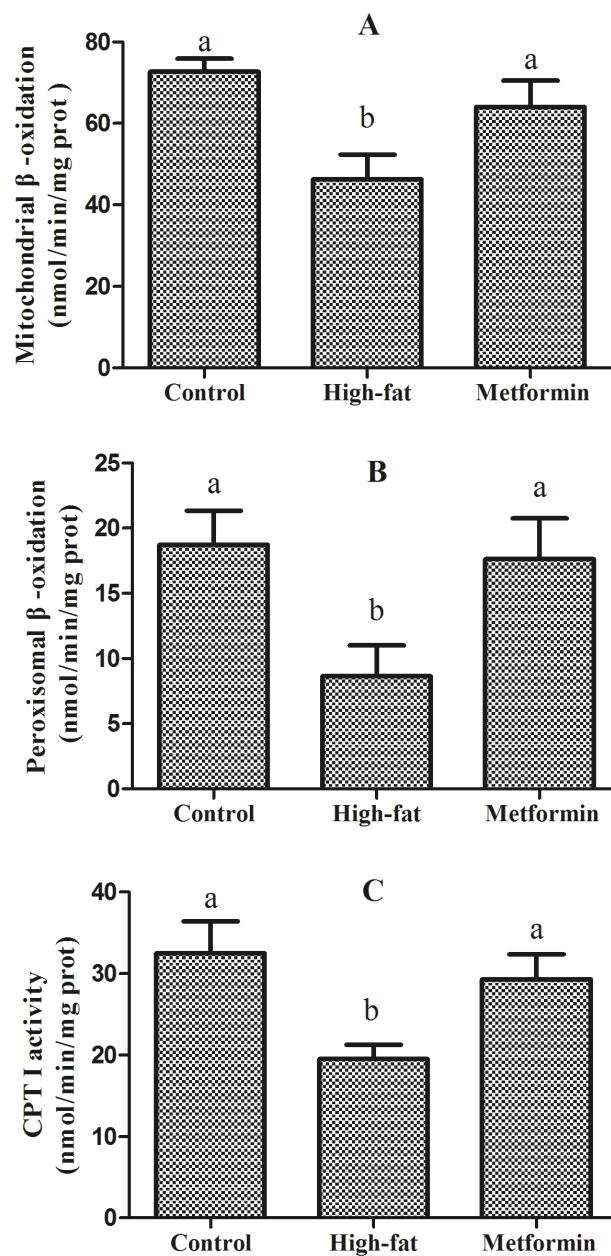


Fig.3. Mitochondrial (A) and peroxisomal (B) β -oxidation, and CPT I (C) activity in primary hepatocytes of blunt snout bream. Bars with different letters are significantly different ($P < 0.05$).

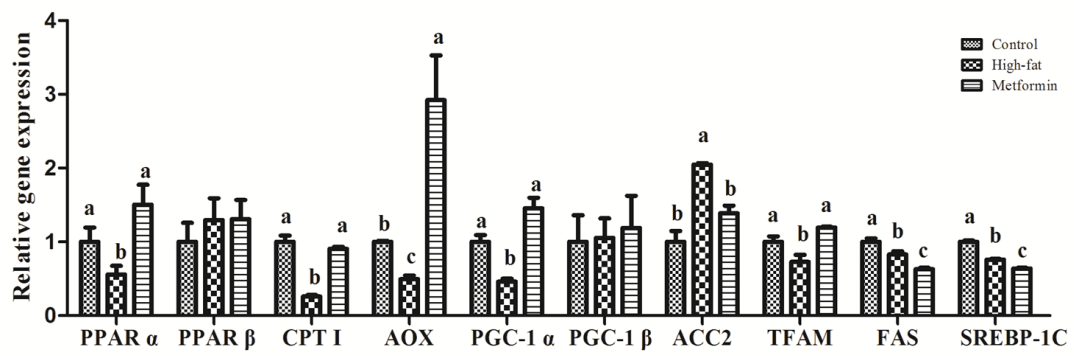


Fig.4. Relative expression of lipid metabolism related genes in primary hepatocytes of blunt snout bream. Bars with different letters are significantly different ($P < 0.05$).

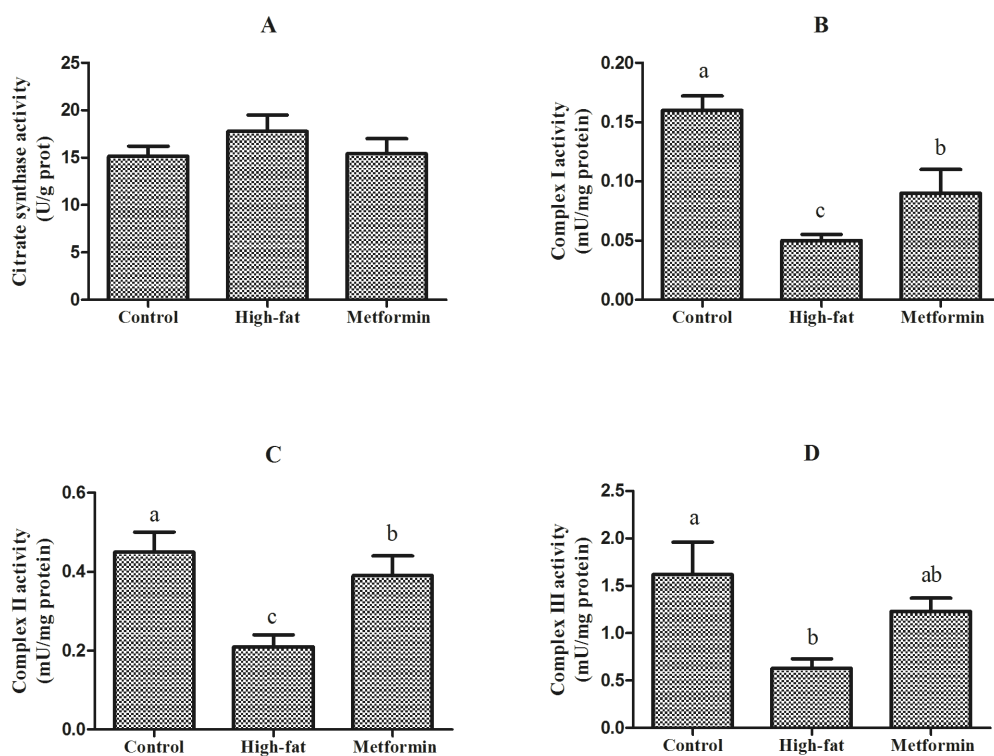


Fig.5. Activities of citrate synthase (A) and mitochondrial complexes (I: B, II: C, III: D) in primary hepatocytes of blunt snout bream. Bars with different letters are significantly different ($P < 0.05$).

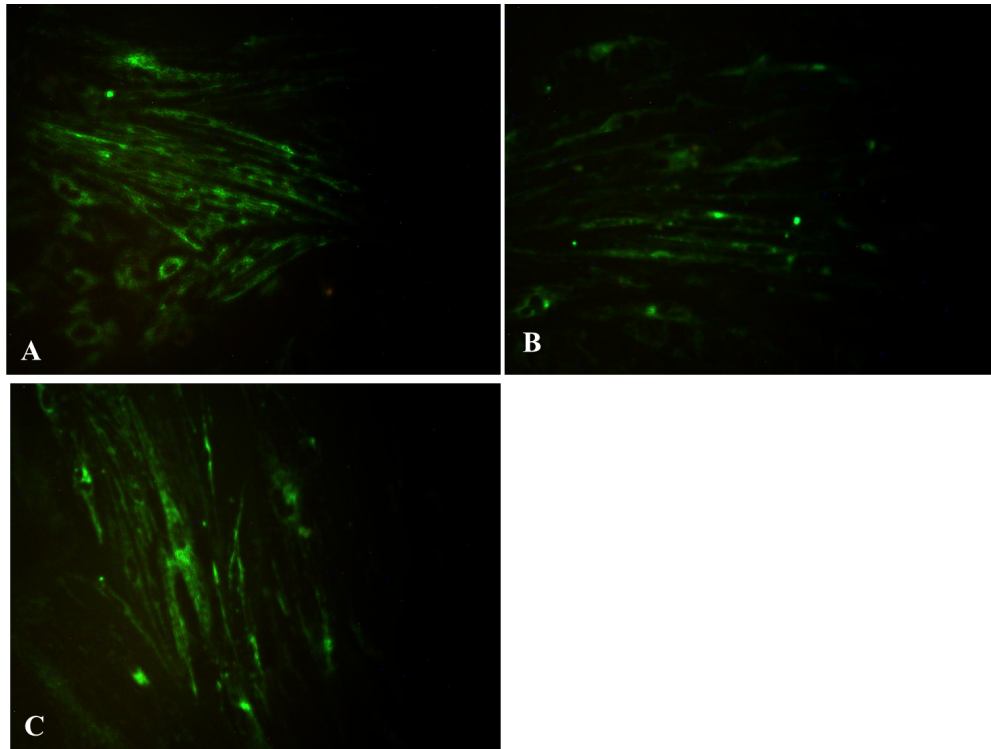
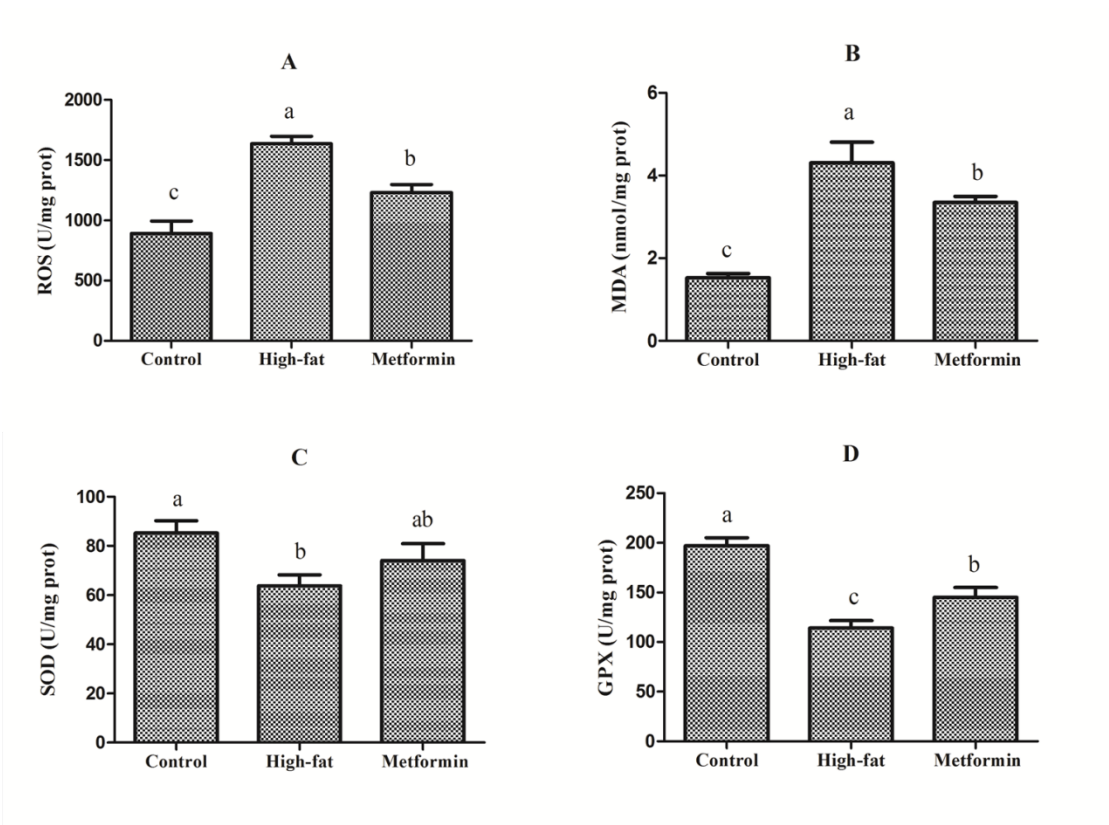


Fig.6. Mitochondria abundance identified by Mito-Tracker Green in hepatocytes of blunt snout bream (A: control, B: high-fat, C: metformin). The fluorescence intensity is indicative of mitochondria abundance.

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Fig.7. Concentrations of reactive oxygen species (ROS: A) and malondialdehyde (MDA: B), and activities of superoxide dismutase (SOD: C) and glutathione peroxidase (GPX: D) in primary hepatocytes of blunt snout bream. Bars with different letters are significantly different ($P < 0.05$).

843 **Table 1.** Sequences of primers used for RT-PCR in this study.

Target genes	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Annealing temperature (°C)
β -Actin	CGGACAGGTCATCACCATTG	CGCAAGACTCCATACCCAAGA	60
PPAR α	GAGGAACCGAAACAAGTGCCAATA	GCTCAGTCACCGTCTCAACC	60
PPAR β	GGACTCACTATGGCAGGCAGAA	CACTGGCAGCGGTAGAAGACAT	60
CPT I	TACTTCCAAAGCGGTGAG	AGAGGTATTGTCCGAGCC	60
AOX	GCTCAACCCTGGCATACT	TCATCACACCCATTCGCT	60
PGC-1 α	TGCCCTCGGTTCATTGTC	GATTTCTGATTGGTCGCTGTA	60
PGC-1 β	CTCTAAGGGTGAATCGCAACG	TCCTCCGCCACTTCCACAT	60
ACC2	CGGAGTTATCAAGCCAAGAGC	ACAGCAGTCGCCGCAAA	60
TFAM	CTTTGGTATCCAGGGAGCAGT	GTTGAATCGCATCCAGTCGT	60
FAS	TTGTTTCCTCATCCACCCC	TGCCTCAAGCACTCCACG	60
SREBP-1C	AGAACAGAGGAGTGCGAGAT	CCGCTGCCTAGTTTGATG	60

844 PPAR α : Peroxisome proliferator-activated receptor α

845 PPAR β : Peroxisome proliferator-activated receptor β

846 CPT I: Carnitine palmitoyltransferase I

847 AOX :Acyl-Co A Oxidase

848 PGC-1 α : Peroxisome proliferators activated receptor γ coactivator-1 α

849 PGC-1 β : Peroxisome proliferators activated receptor γ coactivator-1 β

850 ACC2: Acetyl CoA carboxylase 2

851 TFAM: Mitochondrial transcription factor A

852 FAS: Fatty acid synthesis

853 SREBP-1C: sterol regulatory element binding protein-1C

