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 1×10^6 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 PPARα, CPT I, AOX, PGC-1α 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 become 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; Gélineau 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 (Jäger 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 (Jørgensen 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

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

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

111 **2. Materials and methods**

112 2.1. Experimental fish

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

120 2.2. Isolation of hepatocytes

121 Prior to isolation of hepatocytes, fish were anesthetized with MS-222 (tricaine
122 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 mm^3) 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 1×10^6 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

147 Frankel (1957).

148 2.5. Gene expression

149 Total RNA was extracted from the hepatocytes using RNAiso Plus (Takara Co. Ltd,
150 Japan) according to the protocol provided by the manufacturer. Isolated RNA was
151 quantified using the NanoDrop ND-2000 spectrophotometer, and its integrity was
152 confirmed by agarose gel electrophoresis. RNA samples were treated by RQ1 RNase-
153 Free DNase prior to RT-PCR (Takara Co. Ltd, Japan) to avoid genomic DNA
154 amplification. cDNA was generated from 500 ng DNase-treated RNA using ExScript^{TM}
155 RT-PCR kit (Takara Co. Ltd, Japan), and the mixture consisted of 500 ng RNA, 2 µl
156 buffer (5×), 0.5 µl dNTP mixture (10 mM each), 0.25 µl RNase inhibitor (40 U μl^{-1}),
157 0.5 µl dT-AP primer (50 mM), 0.25 µl ExScript^{TM} RTase (200 U μl^{-1}), and total volume
158 made up to 10 µl with DEPC-treated H_2O. The reaction conditions were as follows:
159 42 °C for 40 min, 90 °C for 2 min, and 4 °C thereafter.

160 Real-time PCR was employed to determine mRNA levels based on the SYBR®
161 Green I fluorescence kit (Takara Co. Ltd, Japan). Primer characteristics used for real-
162 time PCR are listed in Table 1, according to the MIQE Guidelines (Bustin et al., 2011).
163 Real-time PCR was performed in a Mini Option real-time detector (BIO-RAD, USA).
164 The fluorescent quantitative PCR reaction solution consisted of 12.5 µl SYBR® premix
165 Ex Taq^{TM} (2×), 0.5 µl PCR forward primer (10 µM), 0.5 µl PCR reverse primer (10
166 µM), 2.0 µl RT reaction (cDNA solution), and 9.5 µl dH_2O. The reaction conditions
167 were as follows: 95 °C for 3 min followed by 45 cycles consisting of 95 °C for 10 s and
168 60 °C for 20 s. The fluorescent flux was then recorded, and the reaction continued at
169 72 °C for 3 min. The dissolution rate was measured between 65 and 90 °C. Each
170 increase of 0.2 °C was maintained for 1 s, and the fluorescent flux was recorded. All
171 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 ($2^{-\Delta\Delta Ct}$) 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 ×*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 μg 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-AMPKα (Cell Signaling Technology, USA) and antiphospho-AMPKα (#2535, Cell Signaling Technology, USA) at 4 °C overnight. After washing, membranes were incubated with anti-rabbit secondary antibody. Bands were visualized by an electro-chemiluminescence (ECL) system (GE Healthcare, Buckinghamshire, UK) and quantified by the densitometry band analysis tool in ImageJ 1.44p.

196 2.7. Mitochondria

197 The activities of respiratory chain complexes (I, II, III) and citrate synthase were
198 determined using commercial kits (Nanjing JianCheng Bioengineering Institute, China)
199 as described previously (Lu et al., 2017). Thiobarbituric-acid-reactive substances
200 assays were performed with a malondialdehyde (MDA) kit (Nanjing JianCheng
201 Bioengineering Institute) as described by Rueda-Jasso et al. (2004). Mito tracker green
202 was used for mitochondrial labeling. Hepatocytes were incubated in phenol red-free
203 Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal calf serum and
204 labeled with 80 nM Mito Tracker Green FM (Beyotime C1048-50μg; Nantong, China),
205 and incubated for 30 min at 28 °C, in a humidified atmosphere, 5% CO_2 in air,
206 subsequently washed with DMEM medium, and examined and photographed by
207 fluorescence microscopy (450-490 nm excitation light, 520 nm barrier filter; Olympus).

208 2.8. Fatty acid oxidation rate

209 Mitochondrial and peroxisomal β-oxidation of hepatocytes were determined using
210 radiolabelled [1-^{14}C] palmitate (16:0) as a substrate, as described previously (Lu et al.,
211 2014b). Palmitate oxidation rates were measured at 28 °C using two media as described
212 by Frøyland et al. (1995), the first allowing the total (mitochondrial and peroxisomal)
213 activities to occur (13.2 mM HEPES [pH 7.3], 16.5 mM $MgCl_2$, 82.5 mM KCl, 13.2
214 mM dithiothreitol, 6.6 mM ADP, 0.2 mM NAD^+, 100 mM-CoA and 0.7 mM EDTA),
215 the second allowing the peroxisomal activity only (the medium only differing by the
216 presence of 73 mM antimycin and 10 mM rotenone to block the mitochondrial
217 respiratory chain). Palmitate oxidation was measured with 115 μM [1-^{14}C] palmitate
218 supplemented with 1.2 mM L-carnitine. The samples were incubated for 60 min at
219 28 °C, then reactions were stopped by addition of 1.5 M KOH; fatty acid-free bovine
220 serum albumin (BSA, 100 mg ml^{-1}) was added to the suspension in order to bind

221 unoxidized substrates and then 4 M $HClO_4$ was added to precipitate unoxidized
222 substrates bound to BSA. The total solution was then centrifuged at 1880 $\times g$ for 15
223 min. Aliquots of 200 µl were transferred to a scintillation tube containing 4 ml of liquid
224 scintillation cocktail and assayed for radioactivity in a LS6500 liquid scintillation
225 analyzer (Beckman, USA).

226 2.9. Oxidative status

227 Cell suspensions were incubated at 37 °C for 30 min with 10 µM 2',7'
228 dichlorofluorescein diacetate (DCFH/DA, Nanjing Jiancheng Bioengineering Institute,
229 China). To measure the intracellular reactive oxygen species (ROS), the fluorescence
230 of DCF was excited by a 15mW laser tuned to 488 nm and the emitted fluorescence
231 was measured with 530/30 band pass filter in a FACScalibur Becton Dickinson flow
232 cytometer. The conditions for data acquisition and data analysis were established using
233 negative and positive controls with the CellQuest Program of Becton Dickinson and
234 these conditions were maintained during all the experiments. Total superoxide
235 dismutase (SOD) activity was measured using a commercial kit (Nanjing Jiancheng
236 Bioengineering Institute, China) according to Nakano (1990). Glutathione peroxidase
237 (GPX) activity was measured using the method of Dabas et al. (2012). Thiobarbituric-
238 acid-reactive substances assays were performed with amalondialdehyde (MDA) kit
239 (Nanjing Jiancheng Bioengineering Institute, China) as described by Rueda-Jasso et al.
240 (2004).

241 2.10. Statistical analysis

242 Data were analyzed by one-way ANOVA using the SPSS 16.0 for Windows.
243 Duncan's test was used for the multiple comparisons. The level of significance was set
244 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 PPARα, PPARβ, CPT I, AOX, PGC-1α, PGC-1β, ACC2, TFAM, FAS and SREBP-1C are presented in Fig. 4. The results showed that metformin supplementation significantly

up-regulated the expression of PPARα, CPT I, AOX, PGC-1α 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 PPARβ and PGC-1β 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

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

295 **4. Discussion**

296 Metformin has long been used as an anti-diabetic drug capable of modulating
297 muscle and liver metabolism and controlling hyperglycemia in mammals (Alengrin,
298 1995; Lin et al., 2000; Panserat et al., 2009; Zhou et al., 2001). These functions have
299 also been investigated in fish and the results have revealed the mammalian-like effects
300 of metformin (Magnoni et al., 2012; Polakof et al., 2011a; Xu et al., 2018). It has been
301 suggested that metformin exerts its therapeutic effects through activation of AMPK
302 (Zou et al., 2004) which is linked to reduced glucose production and facilitated fatty
303 acid oxidation in hepatocytes (Zhou et al., 2001). In addition, studies in humans showed
304 that metformin can ameliorate liver dysfunction in patients with fatty liver (Garinis et
305 al., 2010). Lipid accumulation has been reported in chronic metabolic diseases such as
306 obesity and type 2 diabetes, and it has been demonstrated that AMPK activation inhibits
307 excessive nutrient-induced hepatic lipid accumulation (Li et al., 2014). To our
308 knowledge, this is the first report to evaluate the regulatory effects of metformin on
309 lipid metabolism in fish with a particular emphasis on its efficacy in AMPK activation.

310 The results achieved for TG and TC concentrations in hepatocytes demonstrated
311 clearly that metformin could attenuate lipid accumulation in fish hepatocytes subjected
312 to “high-fat” via supplementation with oleic acid. In agreement with our results, Xu et
313 al. (2018) showed that metformin supplementation in both low and high carbohydrate
314 diets for blunt snout bream resulted in reduced lipid accumulation in liver and adipose
315 tissues and decreased plasma triglyceride concentration. The authors attributed these
316 results to increased fatty acid oxidation and/or inhibited fatty acid synthesis through
317 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

343 statistically significant in the case of ALT activity.

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

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

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

378 ACC has two different isoforms, ACC1 and ACC2, with the only difference
379 between the two forms being the presence of an extra NH_2-terminal extension of 146
380 amino acids in ACC2, localizing the enzyme to mitochondria (Abu-Elheiga et al., 1995,
381 2000). Such localization has been suggested to be related to the regulatory effects of
382 ACC2 on fatty acid oxidation, as malonyl-CoA is produced in close proximity to CPT
383 I (Iverson et al., 1990). The results of early experiments in mammals showed that
384 AMPK inhibits ACC2 activity by phosphorylating at Ser-221 (Ahu-Fjheiga and Wakil,
385 1997). It has been shown that ACC2 exhibits similar phosphorylation in fish and these
386 serine residues are conserved (Cheng et al., 2011; He et al., 2014). Although in the
387 current study malonyl-CoA content was not measured, the expression of ACC2 gene in
388 liver was down-regulated by metformin indicating that inhibition of ACC2 by AMPK
389 activation contributed to increased CPT I activity and fatty acid β-oxidation. Moreover,
390 it has been reported that expression of CPT I mRNA is influenced by PPARs as CPT I
391 has a PPAR responsive component (Rao and Reddy, 2001). All the mammalian isotypes
392 of PPAR have also been recognized in several fish species although their function has

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

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

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

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

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

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

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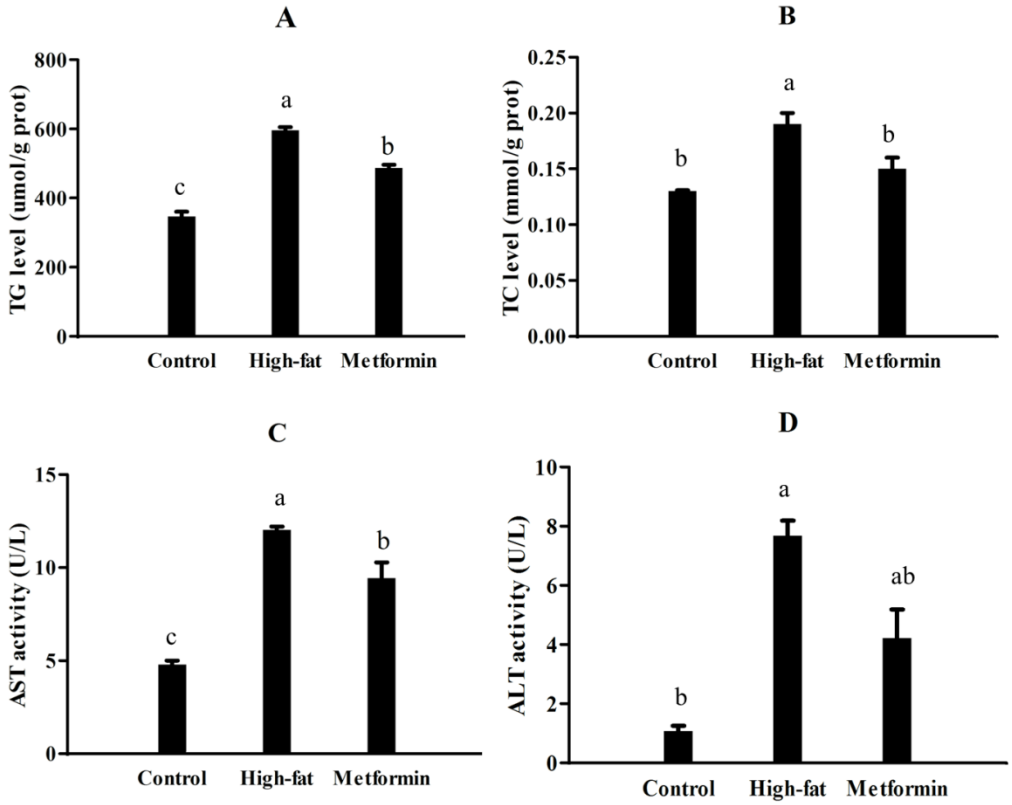


765

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 ($P < 0.05$).

770

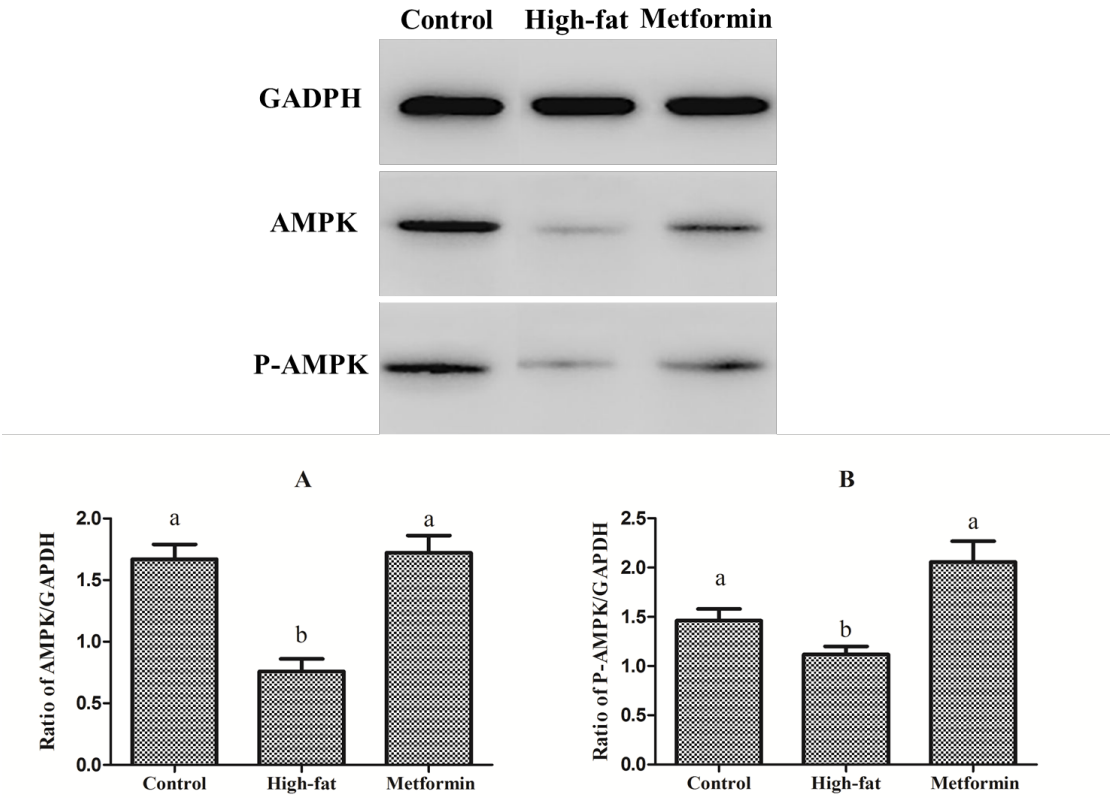


Fig.2. AMPK (A) and phospho-AMPK (B) expression levels as determined by Western blot in primary hepatocytes of blunt snout bream. Bars with different letters are significantly different ($P < 0.05$).

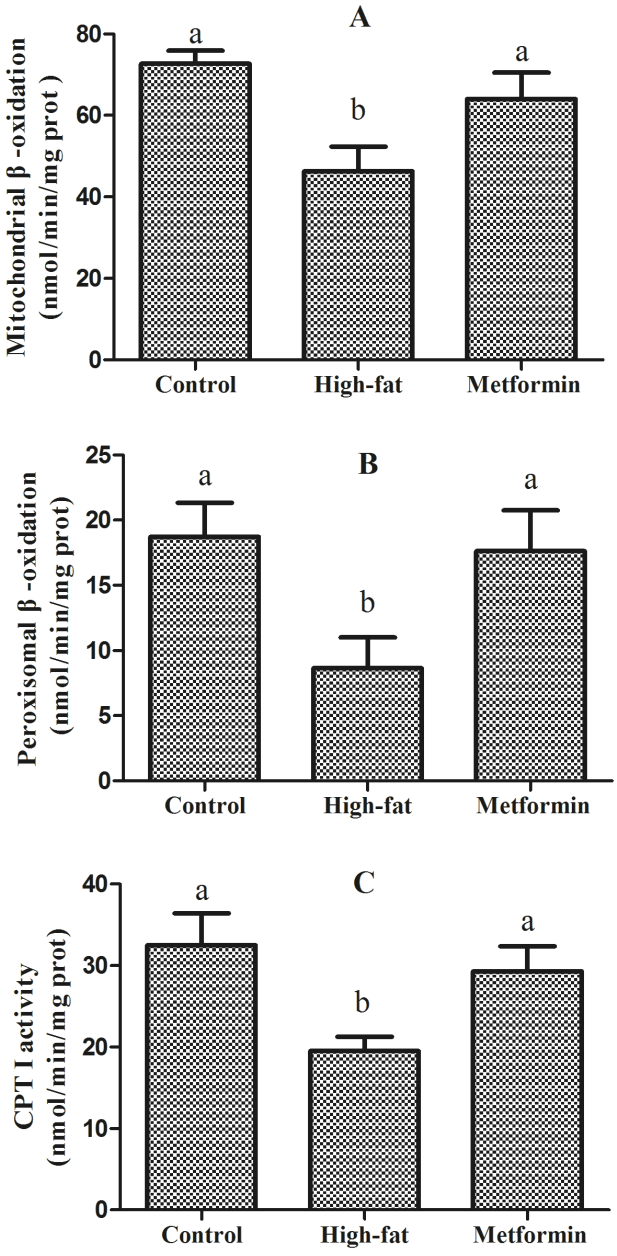
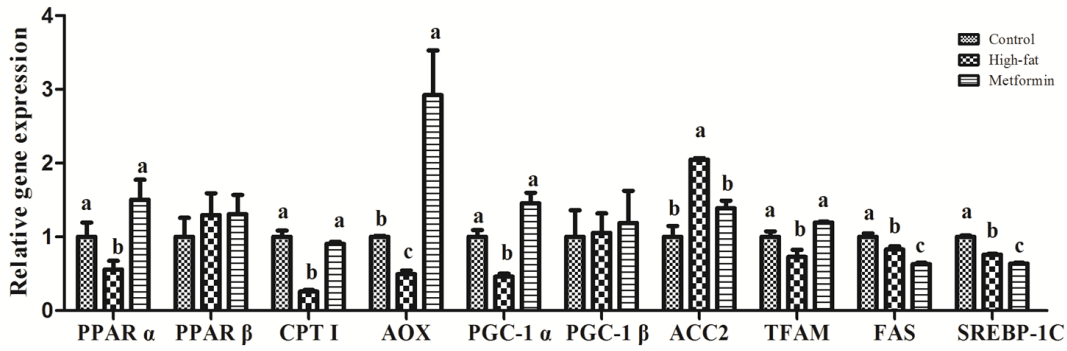


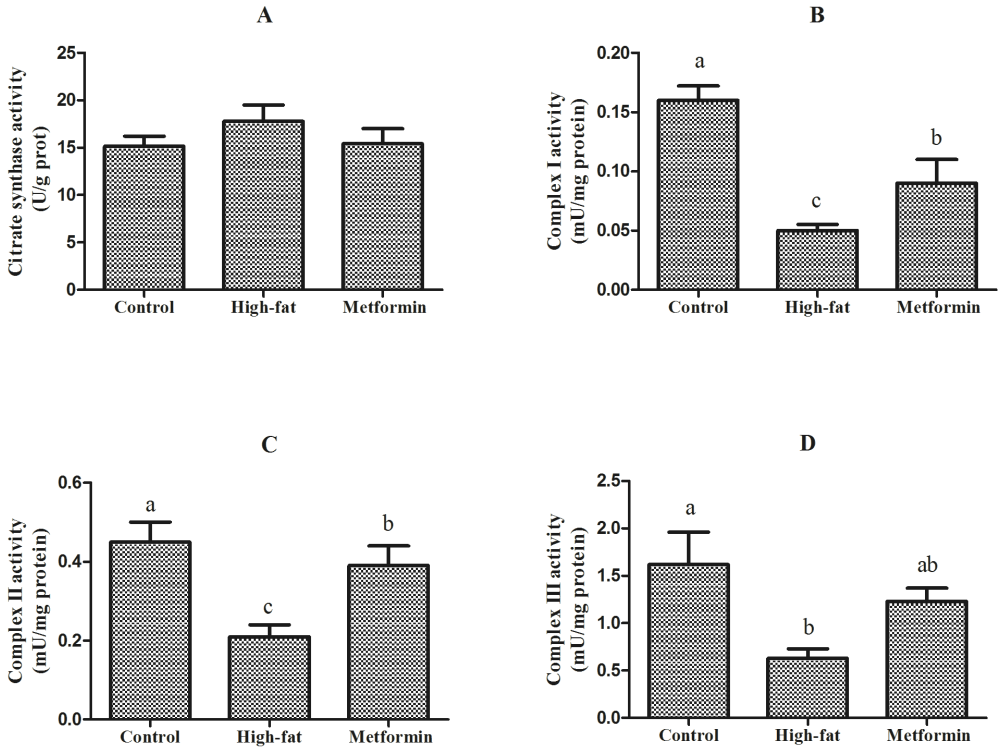
Fig.3. Mitochondrial (A) and peroxisomal (B) β-oxidation, and CPT I (C) activity in primary hepatocytes of blunt snout bream. Bars with different letters are significantly different ($P < 0.05$).



822

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

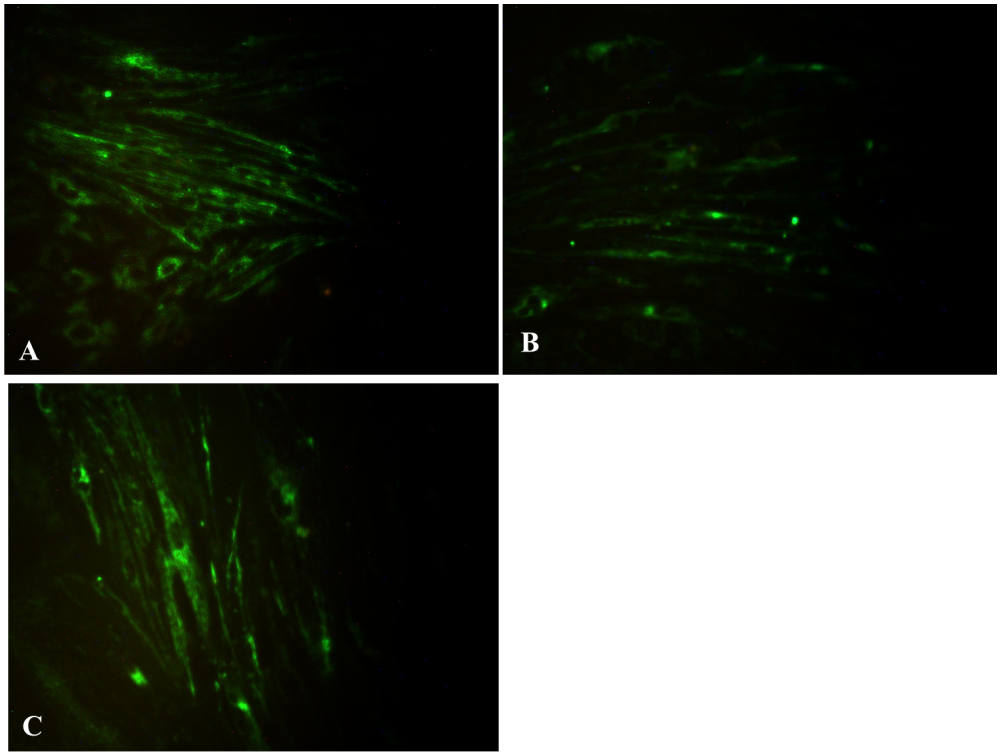
825



826

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

830



831

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

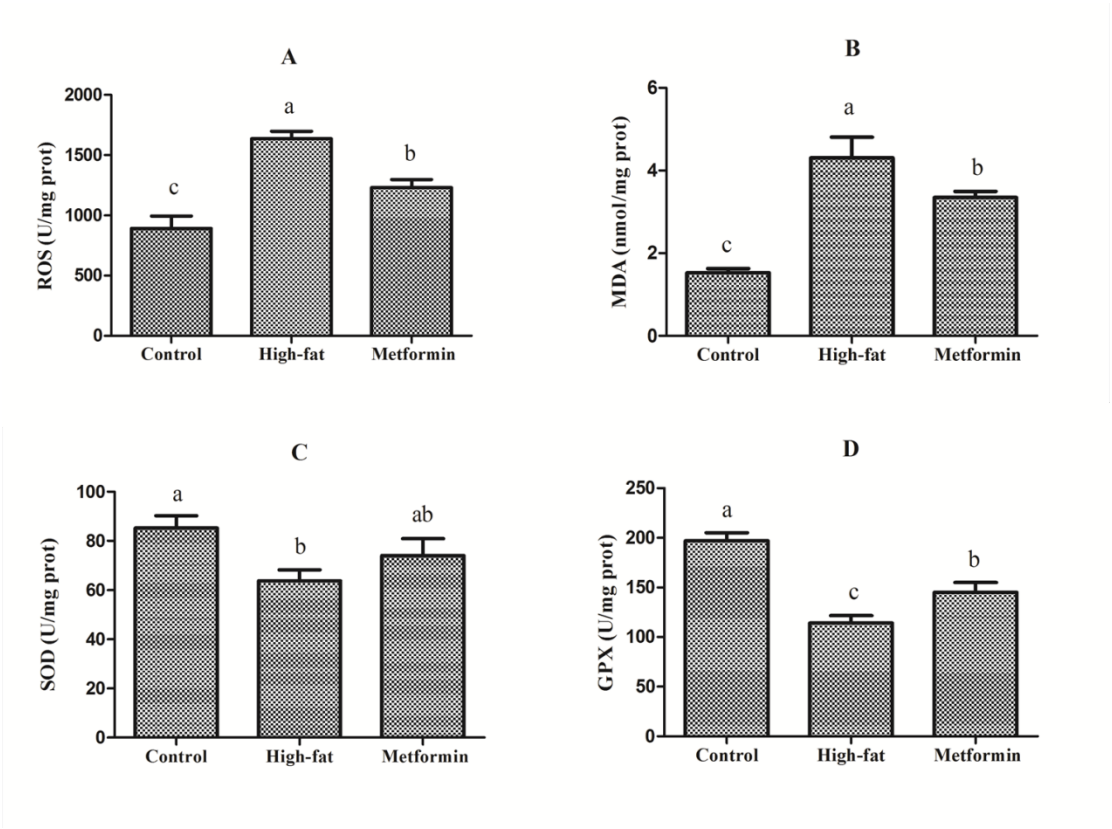


Fig.7. Concentrations of reactive oxygen species (ROS: A) and malondialdehyde (MDA: B), and activities of superoxide dismutase (SOD: C) and glutathione peroxidase (GPX: D) in primary hepatocytes of blunt snout bream. Bars with different letters are significantly different ($P < 0.05$).

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
PPARα	GAGGAACCGAAACAAGTGCCAATA	GCTCAGTCACCGTCTCAACC	60
PPARβ	GGACTCACTATGGCAGGCAGAA	CACTGGCAGCGGTAGAAGACAT	60
CPT I	TACTTCCAAAGCGGTGAG	AGAGGTATTGTCCGAGCC	60
AOX	GCTCAACCCTGGCATACT	TCATCACACCCATTCGCT	60
PGC-1α	TGCCCTCGGTTCATTGTC	GATTTCTGATTGGTCGCTGTA	60
PGC-1β	CTCTAAGGGTGAATCGCAACG	TCCTCCGCCACTTCCACAT	60
ACC2	CGGAGTTATCAAGCCAAGAGC	ACAGCAGTCGCCGCAAA	60
TFAM	CTTTGGTATCCAGGGAGCAGT	GTTGAATCGCATCCAGTCGT	60
FAS	TTGTTCCTCATCCACCCC	TGCCTCAAGCACTCCACG	60
SREBP-1C	AGAACAGAGGAGTGCGAGAT	CCGCTGCCTAGTTTGATG	60

PPARα: Peroxisome proliferator-activated receptor α

PPARβ: Peroxisome proliferator-activated receptor β

CPT I: Carnitine palmitoyltransferase I

AOX :Acyl-Co A Oxidase

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

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

ACC2: Acetyl CoA carboxylase 2

TFAM: Mitochondrial transcription factor A

FAS: Fatty acid synthesis

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

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