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Berberine attenuates sodium palmitate-induced lipid accumulation, oxidative stress and apoptosis in grass carp(*Ctenopharyngodon idella*) hepatocyte in vitro

Shuo-Shuo Yang, Cheng-Bing Yu, Zhen Luo, Wen-Li Luo, Jing Zhang, Jian-Xiong Xu, Wei-Na Xu*

Shanghai Key Laboratory for Veterinary and Biotechnology, School of Agriculture and Biology, Shanghai Jiao Tong University, No.800 Dongchuan Road, Shanghai, 200240, People's Republic of China

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ABSTRACT

The objective of this work was to investigate the effect of berberine (BBR) on the Cell viability, lipid accumulation, apoptosis, cytochrome *c*, caspase-9 and caspase-3 in lipid accumulation-hepatocytes induced by sodium palmitate in vitro. The lipid accumulation-hepatocytes (induced by 0.5 mM sodium palmitate for 24 h) were treated with 5 μM berberine for 12 h. Then, the Cell viability, intracellular triglyceride (TG) content, lipid peroxide (LPO), malonaldehyde (MDA) content, cytochrome *c*, caspase-9, caspase-3 and apoptosis were detected. Sodium palmitate decreased Cell viability and increased intracellular TG content, lipid droplet accumulation, LPO and MDA concentrations, caused caspase-3 and caspase-9 activation, then led to apoptosis accompanied by cytochrome *c* release from mitochondria into the cytoplasm. Beberine could improve intracellular lipid droplet accumulation and oxidative stress, while reduce apoptosis induced by sodium palmitate.

1. Introduction

The dietary fat could not only provide energy in replacement of protein, but also provide the essential fatty acid to promote growth for fish. Raising the dietary fat level could decreased production costs. Therefore, the use of fat-rich feeds was the current trend in intensive aquaculture [1–4]. However, dietary fat level toward or over an upper limit often led to excessive lipid accumulation in the some organs or tissues of fish, especially in the liver [1,3,5]. The main form of lipids stored in lipid droplets is triglyceride (TG) [6].

Liver is the main organ of lipid synthesis in fish, in which 90% lipid was synthesized. The liver was the most active organ in the organism which played an important role in detoxification of environmental toxins and maintained the dynamic balance of amino acid-, carbohydrate- and fatty acid metabolism [7,8]. The excessive lipid accumulation in fish caused serious health problems and reduced harvest yields in farmer fish, which posed a great threat and caused considerable economic losses. Indeed, some reports showed that excessive lipid accumulation caused metabolic alterations, abnormal oxidative status and impairments of nutritional value and transformation yield, as well as affecting organoleptic and physical properties in fish [9–11]. To improve the situation of excessive lipid accumulation in fish, a series of functional feed additives had been developed. Tea Catechin,Algae, *Radix isatidis*, *Eucommia ulmoides* and pericarp had obvious effects on the enhancement of fish immunity [12,13] Recently, berberine (BBR), a kind of Chinese herbs, had received more attention to alleviate excessive lipid accumulation.

Berberine is a kind of isoquinoline alkaloid isolated from Chinese medicinal herb, such as *Coptidis Rhizoma*, *Coptis chinensis*, *Coptis teeta*, which had been used in traditional Chinese medicine (TCM) for centuries [14,15]. Studies revealed that BBR might be used for the improvement of chronic ailments or diseases including diabetes. Berberine had processed activity that exerted hepatoprotective, antioxidation, anti-inflammator, blood glucose-lowering, antiarrhythmic, lipid-lowering effects [16,17]. In later studies on fish, berberine attenuated liver damage induced by the high fat via the protection for mitochondria [18]. Furthermore, berberine to high fat diet could improve liver fat deposition and disorders. The lipid-lowering effects of berberine appeared to be mediated by activating genes related with fatty acid oxidation and decreasing genes for fatty acid uptake [19]. Our lab had reported that berberine as a functional feed additive significantly inhibited the progression of oxidative stress, reduced the apoptosis and enhanced the immunity of fish fed with high-fat diet [20–23].

However, all the above studies were experiments in vivo, lacking of experiments in vitro. Hepatocytes were the main parts of the liver. Some reports showed excessive nutrient intake and drug caused

* Corresponding author.
E-mail address: xuweina@sjtu.edu.cn (W.-N. Xu).

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hepatocyte lipid accumulation [24,25] to harm liver. The hepatocyte culture system in vitro had been used as a tool for xenobiotic metabolism and toxicity studies recently [26–28]. In mammals, some scholars had established the fatty liver model induced by free fatty acid via constructing the cell culture system and screened some functional components [29–31]. In fish, few scholars also established hepatocytes fat accumulation model induced by free fatty acid or damage model induced by carbon tetrachloride and explored the effect of Chinese herbs in vitro [7,32,33].

In this study, we established sodium palmitate-induced hepatocyte lipid accumulation model and explored the effects of berberine on lipid accumulation, oxidative stress and apoptosis. The data might be important to elucidate if berberine could alleviate hepatocytes lipid accumulation, since this widely used dietary supplement could be a useful modality for improvement fish lipid metabolism.

2. Materials and methods

2.1. Materials

The grass carp(*Ctenopharyngodon idella*) hepatocytes were obtained from China Center for Type Culture Collection (Wuhan, Hubei, China). MEMα, fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). Penicillin, streptomycin, DPBS, 0.25% trypsin (including EDTA) were purchased from thermo fisher scientific (Waltham, MA,UK). Berberine (purity > 90%), Oil Red O, sodium palmitate were purchased from Sigma-Aldrich(St.Louis, MO, USA). CCK-8 Assay Kit, BCA Protein Assay Kit, Triglyceride Assay Kit, LPO Assay Kit, MDA Assay Kit, Caspase-9 Activity Assay Kit, Caspase-3 Activity Assay Kit were purchased from Jian Cheng Bioengineering Institute (Nanjing, Jiangsu, China). Immunosorbent kit was obtained from ADL Co-Lab in USA. The mitochondria/cytosol Fractionation Kit Beyotime Inst. Biotech (Peking, China).

2.2. Cell culture and model establishment

The hepatocytes of grass carp were cultured using a previously described methods [26–28] with some modifications. Briefly, the hepatocytes were maintained in MEMα medium, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin in a humid atmosphere of 5% CO_2 at 28 °C. The hepatocytes were treated with different concentrations of sodium palmitate (SP, 0, 0.5,1,2 and 4 mM) for 6, 12 and 24 h, respectively [32–36]. Four replicates were performed for each treatment.

2.3. Cell treatments with berberine

Cell were seeded into cell culture plate for 24 h to 70–80% fusion and pretreated with SP according to 2.2 experiment result. Then cell were treated with 5 μM berberine(berberine was dissolved in 0.1% DMSO)for 12 h [37]. The cells incubated with only SP and without SP were taken as positive and negative controls respectively. 0.1% DMSO was also used as a control. Four replicates were performed for each treatment.

2.4. Cell viability assay

The cells were seeded on 96-well plates, after incubation with SP or BBR, the Cell viability was measured using CCK-8 method according to the manufacture's protocol [38]. In brief, 10 μl cck-8 solution was added to individual well and incubated 2 h in a humidified incubator of 5% CO_2 at 28 °C. Finally, the absorbance at 450 nm was measured using an automatic micro plate spectrophotometer (Bio-Rad, USA). The calculation formula was as follow: Cell viability (%) = (OD in treatment group/OD in control) × 100%

2.5. Oil Red O staining and intracellular triglyceride (TG) content assay

Lipid accumulation in cells was evaluated by Oil Red O staining and the measurement of triglyceride (TG) content. Intracellular lipid droplets were detected by Oil Red O Staining as described previously [39]. The cells were seeded on 6-well plates, after incubation with SP or BBR, cells were washed three times with DPBS and fixed with 10% formaldehyde for 30 min. After fixation, cells were washed 3 times with DPBS and stained with Oil Red O solution (60% Oil Red O stock solution and 40% distilled water) for 30 min at 28 °C. After staining, intracellular lipid droplets were observed using microscope (TE2000-E, Nikon).

The cells were seeded on 6-well plates, after treatment, cells were collected, and then centrifuged at 1, 000 rpm for 5 min. The cell pellet was resuspended in 1 mL of cold Lysis buffer for 30min. The cell lysates were used to measure TG level and protein content. Intracellular TG level was measured with GPO-POD method using Triglyceride Colorimetric Assay Kit (Nanjing, Jiangsu, China). The content of protein in each sample was determined by BCA Protein Assay Kit (Nanjing, Jiangsu, China). TG content was represented as μmol mg^{-1} cellular protein.

2.6. Intracellular MDA and LPO contents measurement

The cells were seeded on 6-well plates, after BBR treatment, cells were placed directly on ice and washed twice with cold DPBS and then were scraped into Lysis buffer collected the solution. The supernatant was obtained by centrifugation at 10, 000 × *g* for 10 min at 4 °C.The cell lysates were used to measure MDA, LPO content and protein content according to the instructions (Nanjing, Jiangsu, China). Lipid peroxide level was determined by measuring the formation of MDA using the method of Ohkawa et al. [40]. The MDA content was represented as with nmol mg^{-1} cellular protein and the LPO content was expressed with nmol mg^{-1} cellular protein [41].

2.7. Intracellular cytochrome c analysis

Cytochrome *c* in mitochondrial and cytosolic fractions was isolated using the mitochondria/cytosol Fractionation Kit (Beyotime, Nantong, China) [41]. After BBR treatment, cells were harvested and centrifuged at 800r at 4 °C for 10 min, the pellets combined with 0.2 mL of 20 mM N-2-hydroxyethylpiperazine-N0-20-ethanesulfonic acid (HEPES) buffer containing protease inhibitor (phenylmethanesulfonyl fluoride, 1 mmol L^{-1}), and disrupted using a glass tissue grinder. Homogenates were centrifuged at 800r at 4 °C for 10 min, the resulting supernatants transferred to 0.5 mL conical tubes, and further centrifuged at 10000r at 4 °C for 20 min. The final supernatants and pellets, containing the cytosolic and mitochondrial fractions, respectively, were analyzed for protein content [42]. Cytochrome *c* concentration were determined using ELISA Assay Kit (Nanjing Jiancheng, Jiangsu, China). The remaining steps were carried out according to the instructions supplied by the manufacturer of the assay kit. The absorbance of each microwell was read using 450 nm as the primary wavelength on an ELISA reader [43].

2.8. Caspase-9 and caspase-3 activity assay

Caspase-9 and Caspase-3 activities were measured by spectrophotometry using the Caspase 3/9 Activity Assay Kit (Nanjing Jiancheng, Jiangsu, China). Caspase 3 can catalyze the substrates Ac-DEVD-*p*NA (acetyl-asp-glu-val -Asp p-nitroanilide) to *p*NA (p-nitroaniline). Caspase 9 can catalyze the substrates Ac-LEHD-pNA(acetyl-Leu-Glu-His-Asp p-nitroanilide) to *p*NA (p-nitroaniline) which was strongly absorbed near 405 nm (lambda = 405 nm or 400 nm). After BBR treatment, 50 μL of cell lysates was mixed with 100 μL reaction buffer and 5 μL of caspase-3 substrate or caspase-9 substrate, incubated at

25 °C in the dark for 4 h, and the OD measured at 405 nm [42]. The calculation formula was as follow: Caspase-9/or Caspase-3 activity = measured OD value/control OD.

2.9. Cell apoptosis

Apoptotic cells were detected by Annexin V/FITC as described previously [44]. After BBR treatment, cell suspensions were collected and calculated. The cells (1×10^5/ml) were centrifuged at 1000r/min for 5 min, collected and suspended with 500 μl binding buffer. 500 μl cells suspension were stained by mixing with 5 μl each of Annexin V –FITC and PI and incubated at room temperature in the dark for 10min. Then the number of apoptotic cells was analyzed immediately using the flow cytometer.

2.10. Statistical analysis

All results were shown as the mean ± standard error (X ± SEM) and were analyzed using SPSS 20.0 statistics software. Data were subjected to one-way analysis of variance and Duncan's multiple range tests. $P < 0.05$ was considered to be significant statistically. According previous study, prior to statistical analysis, normality and homogeneity of variance were checked by using the Shapiro–Wilk test (Farrell & Rogers-Stewart 2006) and Levene's test (Brown & Forsythe 1974), respectively.

3. Result

3.1. Hepatocyte viability

As shown in Table 1, hepatocyte viability was significantly ($P < 0.05$) affected by sodium palmitate concentration. The hepatocyte viability significantly ($P < 0.05$) decreased with the concentration of sodium palmitate increased compared with the control. In addition, hepatocyte viabilities in 1, 2 and 4 mM sodium palmitate groups were significantly ($P < 0.05$) lower than that of in 0.5 mM group.

3.2. Hepatocyte lipid accumulation

As shown in Fig. 1, the hepatocytes in 0.5, 1, 2, 4 mM sodium palmitate groups were stained by Oil Red O dye compared with the control. As shown in Fig. 2, compared with the control, the intracellular TG content in 0.5, 1, and 2 mM sodium palmitate groups significantly ($P < 0.05$) increased, while that in 4 mM group significantly($P < 0.05$) decreased. When the concentration of sodium palmitate was 0.5 mM, intracellular triglyceride (TG) content was the most.

3.3. Berberine on sodium palmitate-induced hepatocyte viability

As shown in Fig. 3, compared with the control, the hepatocytes viabilities in DMSO and BBR groups had no significant($P > 0.05$) change, the hepatocytes viability in SP group significantly ($P < 0.05$) decreased. Compared with the SP group, the hepatocytes viability in SP + BBR group increased significantly ($P < 0.05$).

3.4. Berberine on sodium palmitate-induced hepatocyte lipid accumulation

As shown in Fig. 4, compared with the control group, the hepatocytes in SP group were stained obviously by Oil Red O solution. Compared with the SP group, the hepatocytes in SP + BBR group were stained slighter. At the same time, the intracellular TG content was measured quantitatively by TG Assay Kit. As shown in Fig. 5, compared with the control, the intracellular TG contents in SP group and SP + BBR group significantly ($P < 0.05$) increased, while TG content in BBR group significantly ($P < 0.05$) decreased. Compared with the SP group, intracellular TG content in SP + BBR group significantly ($P < 0.05$) decreased.

3.5. Berberine on sodium palmitate-induced hepatocyte LPO and MDA contents

As shown in Fig. 6, compared with the control, the intracellular LPO content in SP group significantly ($P < 0.05$) increased. Compared with the SP group, the LPO content in SP + BBR group significantly ($P < 0.05$) decreased. As shown in Fig. 7, the change of intracellular MDA content was same to LPO. Compared with the control, the MDA content in SP and SP + BBR groups significantly ($P < 0.05$) increased.

3.6. Berberine on sodium palmitate-induced hepatocyte apoptosis

As shown in Fig. 8, compared with the control, the levels of apoptosis in SP, BBR and SP + BBR groups significantly ($P < 0.05$) increased. Compared with the SP group, the levels of apoptosis in SP + BBR group significantly ($P < 0.05$) decreased.

3.7. Berberine on sodium palmitate-induced hepatocyte cytochrome c content

As shown in Fig. 9, compared with the control, the mitochondrial cytochrome *c* in SP group significantly ($P < 0.05$) decreased, while there were no no significant ($P > 0.05$) changes in DMSO, BBR and SP + BBR groups. The cytoplasm cytochrome *c* in SP, BBR and SP + BBR groups significantly ($P < 0.05$) increased. Compared with the SP group, the mitochondrial cytochrome *c* in SP + BBR group significantly ($P < 0.05$) increased, and the cytoplasm cytochrome *c* in SP + BBR group significantly ($P < 0.05$) decreased.

3.8. Berberine on sodium palmitate-induced hepatocyte caspase-9 and caspase-3 activity

As shown in Fig. 10, compared with the control, the intracellular caspase-9 and caspase-3 activities in SP, BBR and SP + BBR groups significantly ($P < 0.05$) increased. Compared with the SP group, caspase-9 and caspase-3 activities in SP + BBR group significantly ($P < 0.05$) decreased.

4. Discussion

In the present study, we had demonstrated that berberine (5 μM berberine) could alleviate cellular lipid metabolic disorder, including

Table 1
Sodium palmitate on grass carp hepatocyte activity.

T	T/C%				
	0 mM (control)	0.5 mM	1 mM	2 mM	4 mM
6 h	100.00 ± 0.00^a	72.36 ± 5.52^b	56.49 ± 3.72^c	53.33 ± 5.02^c	48.54 ± 2.48^c
12 h	100.00 ± 0.00^a	70.71 ± 6.10^b	61.45 ± 4.31^c	55.97 ± 4.66^c	43.35 ± 2.72^d
24 h	100.00 ± 0.00^a	77.58 ± 3.23^b	58.02 ± 4.23^c	54.50 ± 4.15^c	42.29 ± 3.85^d

Data were expressed with mean ± SEM (n = 4). Bars assigned with different lines were significantly ($P < 0.05$) different.

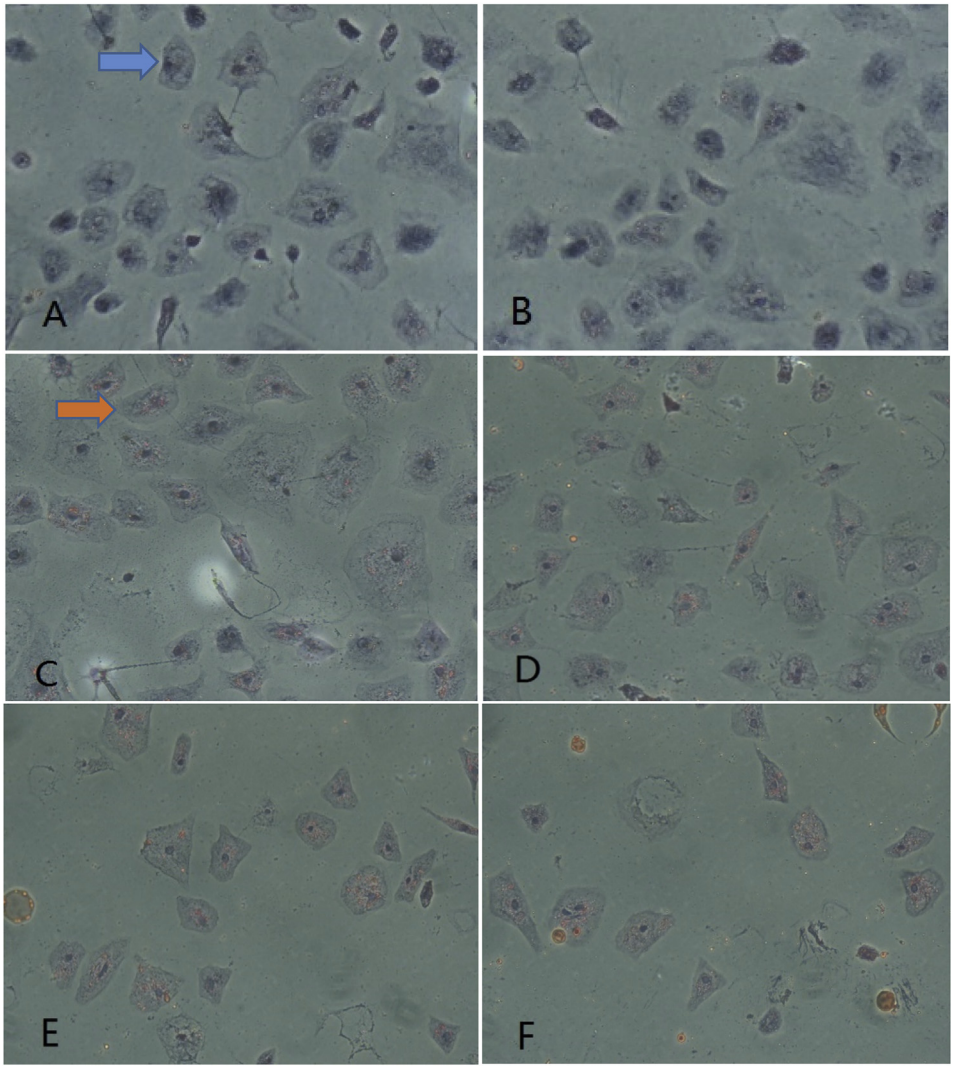


Fig. 1. Hepatocyte lipid accumulation.

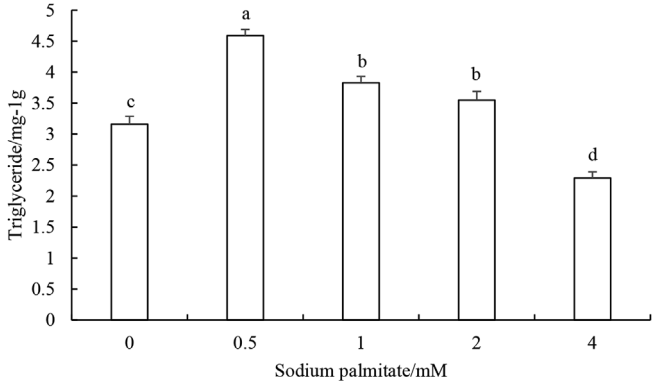


Fig. 2. S > odium palmitate > on hepatocyte TG content.

lipid peroxidation level and apoptosis in sodium palmitate-induced (0.5 mM, for 24 h) lipid accumulation hepatocytes in vitro. This was in agreement with our previous studies that had shown BBR played an important role in improvements high-fat diet-induced fish lipid metabolic disorder by reducing oxidative stress in vivo.

Firstly, in this study, lipid accumulation-hepatocytes model was established successfully. The hepatocyte viability decreased while intracellular TG content increased in a dose-dependent manner with the concentration of palmitic acid increased. The results were same to Hu et al. [45] and Jambor de Sousa et al. [46] studies. In this present study, the results indicated that 0.5 mM sodium palmitic increased hepatocytes TG content, MDA,LPO content, decreased Cell viability and induced apoptosis. According to the results of Cell viability and lipid accumulation, it suggested 0.5 mM sodium palmitate was used in this model of lipid accumulation in vitro. It was reported 0.5 mM paltimate acid could induce lipid accumulation in RIN-5F cell line [47]. Li et al. [48] used 0.25 mM palmitic acid to induce lipid accumulation in NIT-1 pancreatic β cells. Subsequently, 01 mM palmitate induced triglyceride accumulation in primary culture bovine satellite cells [49]. And Xiao et al. [35] used 0.4 mM palmitic acid to induce intracellular lipid accumulation in HepG2 cells. The different concentration of palmitic acid was used to induce lipid accumulation in cells, which might be due to the difference of cell types, sources and culture conditions. Most available studies demonstrate that fatty acid increased cell lipid accumulation [7,32,34], induced oxidative stress [50,51], decreased Cell viability [45,46] and caused apoptosis [34,45,52].

Recently, berberine as a regulating agent played an important role in attenuating PA-induced lipid accumulation [53–55], ameliorating fatty acid-induced oxidative stress [56] and reducing PA-induced apoptosis [57] of cells in vitro. Zhao et al. [48]reported that BBR could inhibit palmitic acid-induced lipid accumulation by decreasing lipogenesis and increasing lipid oxidation in NIT-1 pancreatic β cells. Berberine could alleviate lipid accumulation in endothelial cell induced by

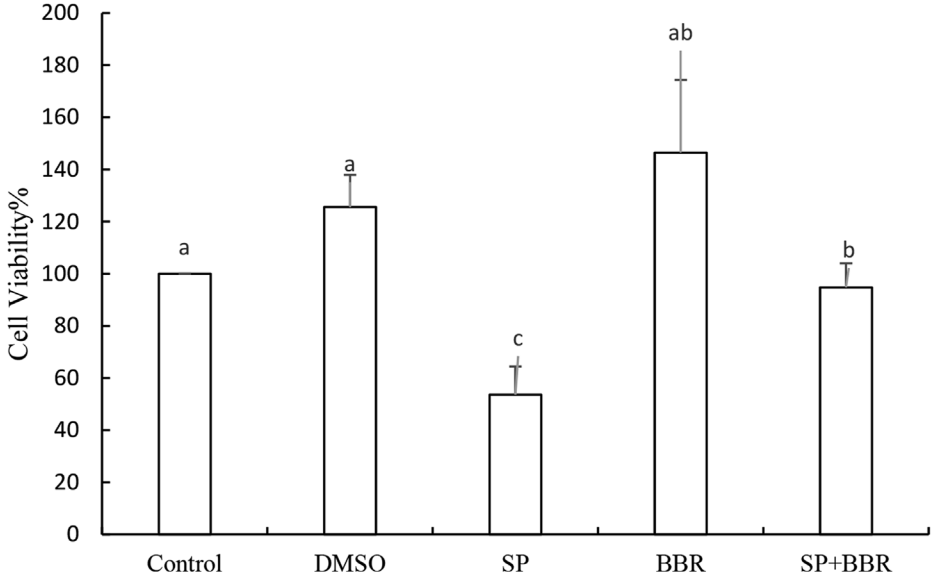


Fig. 3. Berberine on > sodium palmitate > -induced hepatocyte viability.

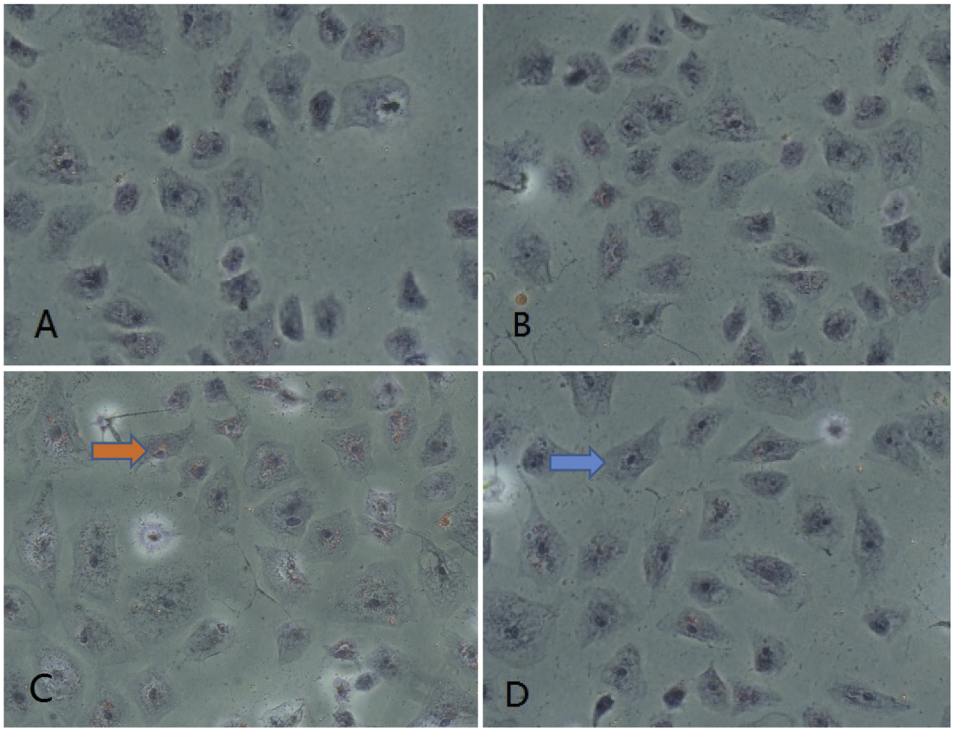


Fig. 4. Berberine on > sodium palmitate > -induced hepatocyte lipid accumulation.

oleic acid, which might be due to the activation of the AMPK-eNOS signaling pathway [58]. Therefore, we conclude that berberine plays a significant role in lipid accumulation within hepatocytes. Consistent with those data, this study demonstrates berberine could improve lipid accumulation in hepatocytes induced by 0.5 mM sodium palmitate. How did berberine decrease hepatocytes lipid accumulation?

Though, our previous study demonstrated that berberine as a functional feed additive significantly inhibited the progression of oxidative stress, reduced the apoptosis and enhanced the immunity of fish fed with high-fat diet [20–22,59]. Hasanein et al. had reported that berberine alleviated lipid peroxidation induced by lead acetate in rats [60]. Lee et al. suggested that berberine might improve colitis by inhibiting lipid peroxidation, enterobacterial growth and NF-κB activation [61]. Based on those, we inferred that berberine could regulate oxidative stress and immunity, improve cell function including Cell viability, cell apoptosis to ameliorate the metabolic properties of cells. Furthermore, we verified preliminarily via experiments in vitro. In this study, we found berberine could decrease lipid peroxidation level in hepatocytes. It was consistent with the above studies.

Apoptosis was one of major types of cell death, a key and active process to keep tissue homeostasis and to clear potentially harmful cells away in multicellular organisms [62], and inappropriate apoptosis was widely involved in the pathophysiology of diseases [63]. It was reported that free fatty acids induce JNK-dependent hepatocyte lipoapoptosis. Recently, many literature reported that palmitic acid could induce apoptosis of many kinds of cells, such as Huh-7 cells [64], AML

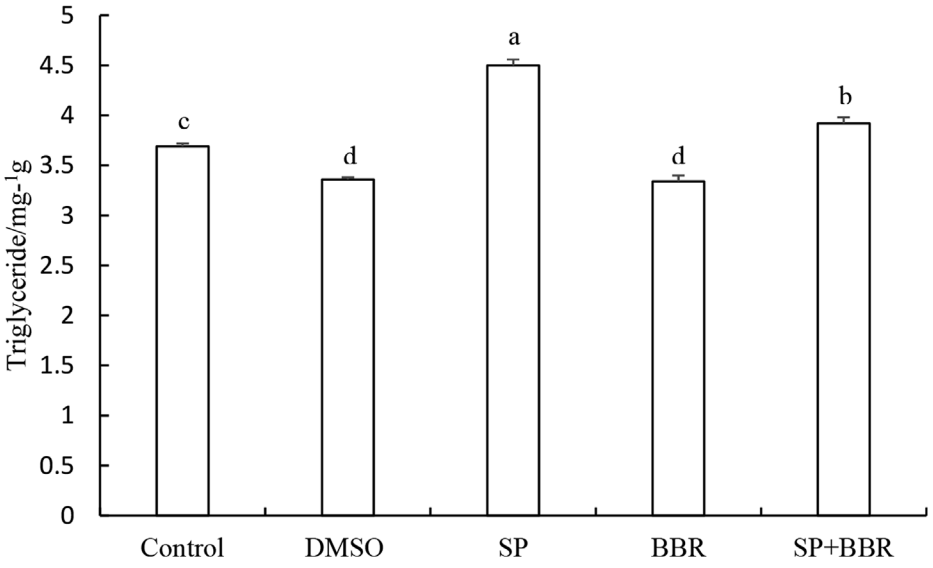


Fig. 5. Berberine on > sodium palmitate > -induced hepatocyte TG content.

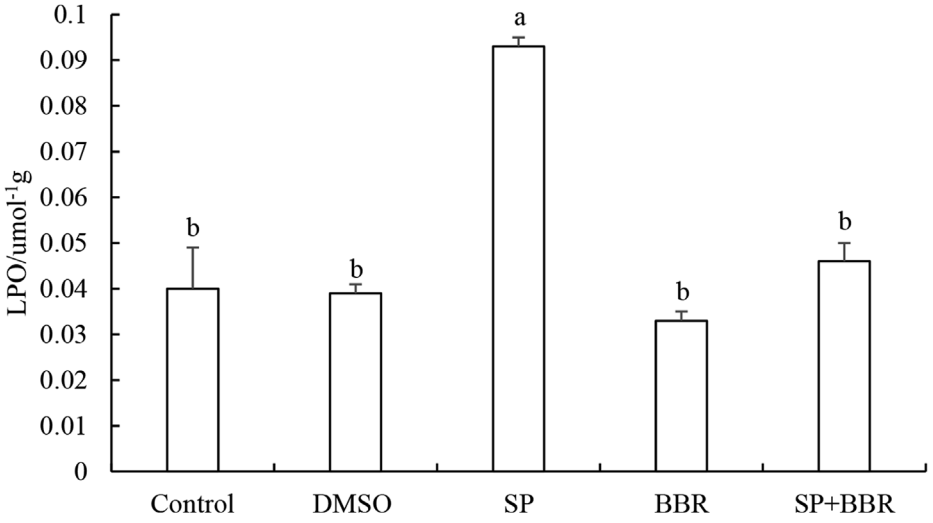


Fig. 6. Berberine on > sodium palmitate > -induced hepatocyte LPO content.

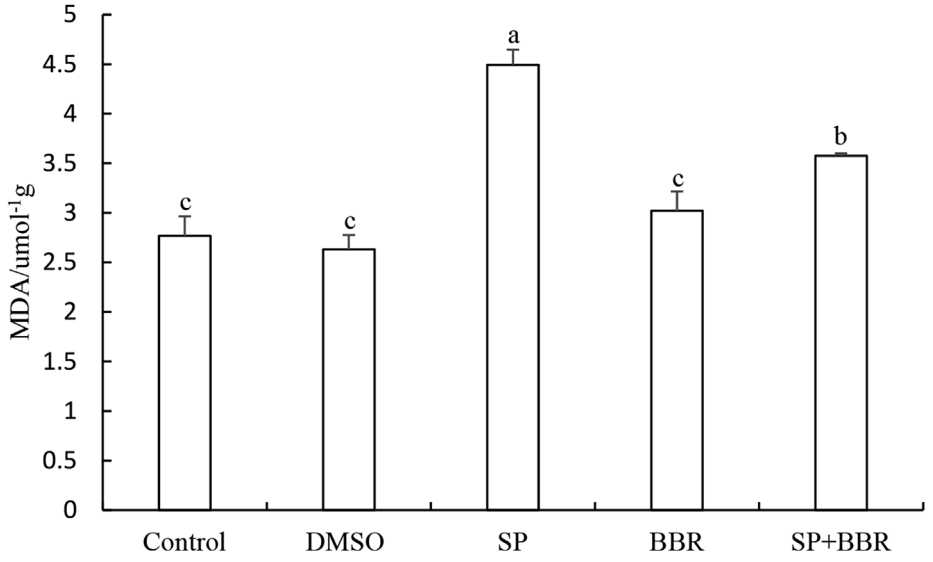
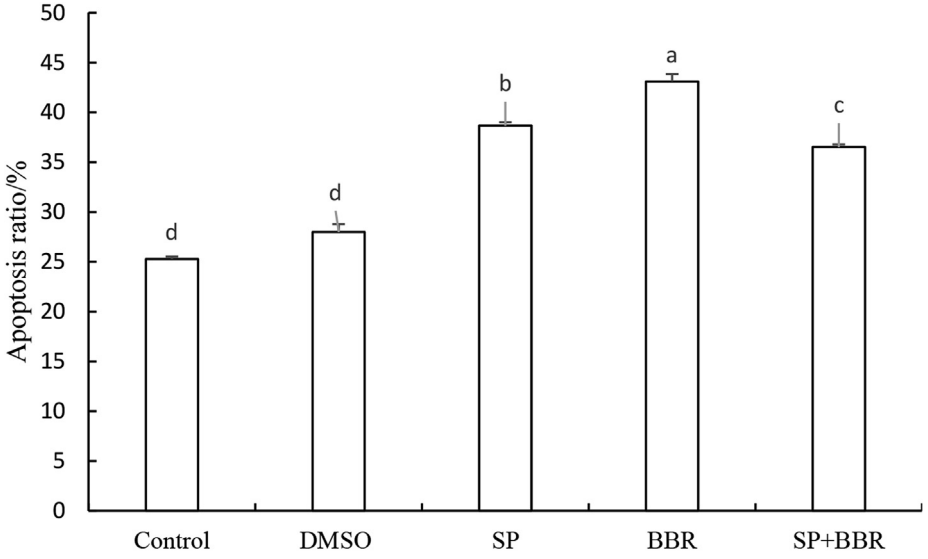


Fig. 7. Berberine on > sodium palmitate > -induced hepatocyte MDA content.



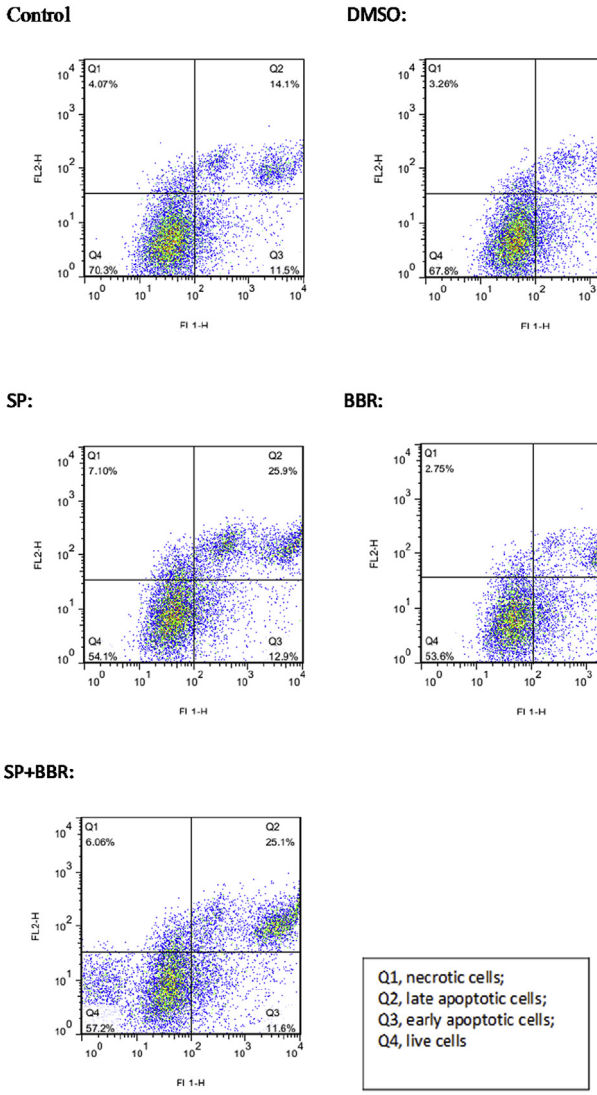


Fig. 8. Berberine on > sodium palmitate > -induced hepatocyte apoptosis.

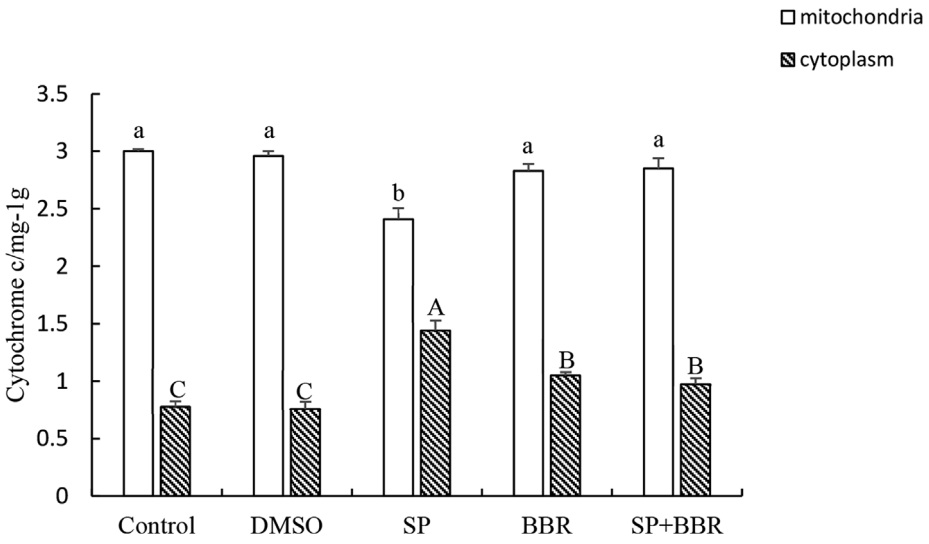


Fig. 9. Berberine on > sodium palmitate > -induced hepatocyte cytochrome c.

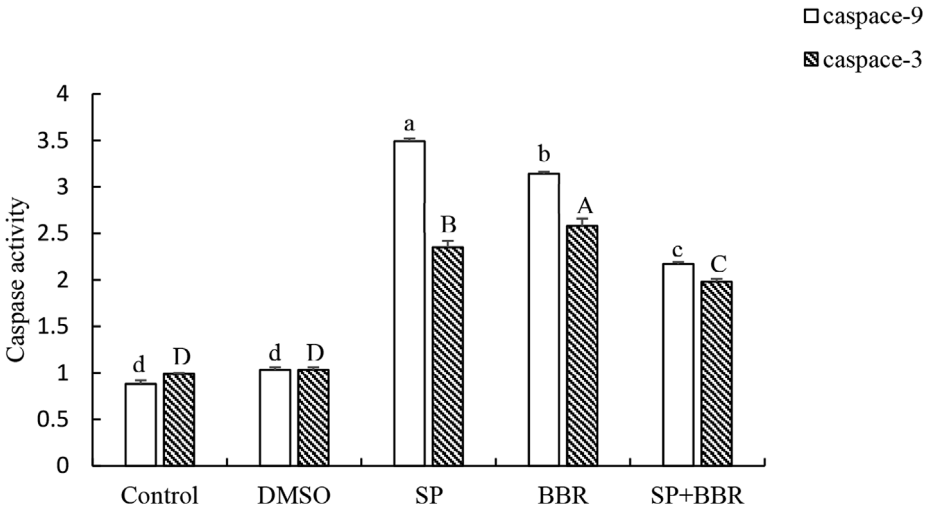


Fig. 10. Berberine on > sodium palmitate > -induced hepatocyte caspase-9 and caspase-3 activity.

12 cells [65], astrocytes [66], pancreatic NIT-1 beta cells [67], podocyte [52], cardiomyocyte [68], sertoli cell [69], and kidney tubular cells. In the present study, we demonstrated that sodium palmitate increased hepatocyte apoptosis which was consistent with the above reports. To understand the role of berberine on lipid accumulation-hepatocyte apoptosis, some parameters related to cell apoptosis were measured including apoptosis, cytochrome *c*, caspase-9 and caspase-3. The result indicated that berberine could significantly ($P < 0.05$) decreased cell apoptosis, the content of cytochrome *c* in cytoplasm, the activity of caspase-9 and caspase-3 in lipid accumulation-hepatocytes. It was reported that caspase-3 could be activated through the mitochondria, by the release of cytochrome *c*, which activated the apoptotic protease apoptotic protease-activating factor 1 (Apaf-1), leading to the activation of caspase-9 [70]. The result of the present study contributed to the overall understanding of the mechanism that berberine decrease cell apoptosis induced by sodium palmitate.

As a whole, the current study demonstrated that berberine could improve intracellular lipid droplet accumulation and oxidative stress, while reduce cell apoptosis induced by sodium palmitate in vitro. The result was consistent with our previous studies in vivo. These findings provided theoretical basis for that berberine could be considered a potential candidate to improve the excessive lipid accumulation in fish. How did berberine decrease hepatocytes lipid accumulation? What was the connection of berberine and immunity? Further studies were still needed to elucidate those using cell culture system in vitro.

Acknowledgments

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