

Original Article

Effect of berberine on a cellular model of non-alcoholic fatty liver disease

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Abstract: To investigate the effect of berberine (BBR) on non-alcoholic fatty liver disease (NAFLD), a cellular model of NAFLD based on HepG2 cells induced by oleic acid (OA) was used. The fat overload and triglyceride (TG) content quantities of the cellular model were documented by oil red O staining. The expression of the genes involved in lipogenesis, including farnesoid X receptor (FXR), sterol regulatory element-binding proteins-1c (SREBP-1c) and Fatty acid synthase (FAS) was assayed by real time PCR (RT-PCR) and Western-blot (WB). Results showed that BBR exerted no cytotoxicity on HepG2 cells, and BBR can significantly decrease the fat overload and TG content in OA-induced HepG2 cells. BBR increased the mRNA of FXR, but decreased the mRNA of SREBP-1c and FAS in a dose-dependent manner. In addition, BBR increased the protein level of FXR but decreased the SREBP-1c and FAS. Thus BBR exerted a possible therapeutic effect on lipid metabolism disorder in the OA-induced NAFLD cellular model by regulating the FXR/SREBP-1c/FAS pathway.

Keywords: Non-alcoholic fatty liver disease (NAFLD), berberine (BBR), HepG2 cell, farnesoid X receptor (FXR), sterol regulatory element-binding proteins-1c (SREBP-1c), fatty acid synthase (FAS)

Introduction

Non-Alcoholic Fatty Liver Disease (NAFLD) has been recognized as a major health issue which is currently a leading cause of chronic liver diseases. NAFLD is characterized by hepatic fat accumulation, insulin resistance and commonly impaired glucose and lipid metabolism [1, 2]. Patients with obesity and high plasma insulin levels exhibit an increase in the accumulation of non-esterified fatty acids (NEFA) in the liver. Consequently, fatty liver aggravates insulin resistance, glucose metabolic disorders, and other pathological conditions [3]. NAFLD has been regarded as a significant health problem affecting 20% to 30% of the general population, among which 5% to 20% develop liver cirrhosis during a 10-year period [4]. Several pharmacologic interventions have been attempted to treat NAFLD, but none of these strategies have shown an excellent curative effect [5, 6]. Many research efforts have focused on the development of drugs from natural products because of the serious adverse effects and limited effectiveness of currently available pharmacological therapies for “metabolic syndrome”.

Berberine (BBR), an active ingredient from natural plants, is the major pharmacological component of the Chinese herb *Coptis chinensis*. Recently, many clinical [7, 8] and laboratory [9, 10] studies reported that BBR exerts antidiabetic and antihyperlipidemic effects. Additionally, BBR has demonstrated biological activities and pharmacological effects in metabolic diseases such as NAFLD [11]. The proposed mechanisms of BBR include the inhibition of gluconeogenesis [12], as well as the stimulation of glycolysis [13]. However, the mechanisms underlying the effects of BBR on NAFLD remain elusive.

In the present study, we investigated the efficacy and safety of BBR on an oleic acid (OA)-induced NAFLD cellular model of HepG2 cells and explored the mechanisms of BBR's effect against this model. CCK8 assay showed that BBR exerted no significant cytotoxicity on HepG2 cells. BBR significantly decreased the fat overload and triglyceride (TG) content in the cytoplasm of OA-induced HepG2 cellular model, which implied a good effect of BBR on NAFLD. Real time PCR (RT-PCR) and Western-blot (WB) analyses showed that BBR increased the

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Table 1. Gene-specific primers used for Real-time RT-PCR

Gene	Forward	Reverse
FAS	5'-ctggctcagcacctctatcc-3'	5'-caggtgtccctgtgatcct-3'
FXR	5'-atcaaaggggatgagctgtg-3'	5'-tcccatctctttgcatttcc-3'
SREBP-1c	5'-aggtggaggacacactgacc-3'	5'-caggacagggcagaggaagac-3'
GAPDH	5'-gtcagtggtggacctgacct-3'	5'-tgctgtagccaattcgttg-3'

ing 10% FBS, 1% antibiotic-antimycotic solution, and 3.7 g/L sodium bicarbonate under an atmosphere of 5% CO₂ at 37°C with 95% humidity.

Protein extract and Western blot analysis

Cells were rinsed twice with ice-cold PBS, scraped with lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NaF, 1 µg/ml proteinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin), and subjected to WB analysis.

Oil red O staining

Cells in the different groups were pre-incubated with DMEM containing 10% BSA for 24 h. Cells were exposed to 1 mM Oleic acid for 24 h. Subsequently, cells were fixed with 4% paraformaldehyde after being washed by PBS twice for 30 min at room temperature (RT). The cells were stained with oil red O buffer (0.5% oil-red buffer of isopropanol and ddH₂O, 1:1) for 30 min. Up to 60% isopropanol was used as wash buffer to reduce the background staining.

To quantify the oil red O content, isopropanol was added to each sample, which was shaken at room temperature for 5 min. The optical density of each 100 µL of isopropanol-extracted sample was read using a spectrophotometer at 510 nm.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted with Trizol Reagent (Invitrogen, Carlsbad, USA), in accordance with the manufacturer's instructions. Total RNA (1 µg) was reverse transcribed using an oligo (dT) 16 primer to generate cDNA. Real-time PCR was used to measure the mRNA expression levels of FAS, SREBP-1c, FXR and GAPDH. For real-time PCR, the cDNA and primers were prepared using a SYBR Green qPCR SuperMix Kit (Invitrogen), as described in the instruction manual. The sequences of the primers are listed in **Table 1**. The amplification conditions were described as follows: 95°C for 5 min for initial denaturation, followed by 40 amplification cycles of denaturation at 95°C for 15 s and

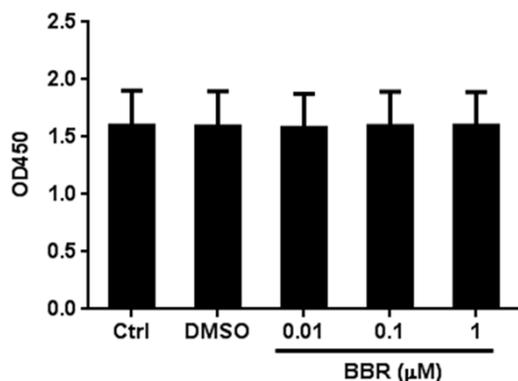


Figure 1. Effect of berberine (BBR) on HepG2 cells. CCK8 assay was used to detect the cytotoxicity of BBR after incubation for 24 h. Three groups of BBR with different concentrations: 0.01, 0.1 and 1 µM. The values are expressed as means ± standard deviation (n = 5). No significant difference was observed in all five groups.

expression of farnesoid X receptor (FXR), but decreased the expression of Sterol regulatory element-binding proteins-1c (SREBP-1c) and Fatty acid synthase (FAS). These results suggest that BBR can regulate the FXR/SREBP-1c/FAS pathway in the NAFLD model.

Materials and methods

Materials

BBR (Sigma-Aldrich, St Louis, MO, USA), oleic acid (Sigma-Aldrich, St Louis, MO, USA), qPCR mix (Promega, Madison, WI), and Cell Counting Kit 8 (Dojindo Co., Kumamoto, Japan), as well as anti-FXR antibody (Abcam, Cat#ab51970), anti-SREBP-1c antibody (Abcam, Cat#ab3259), and anti-FAS antibody (Abcam, Cat#ab15285) were used in this study.

Cell culture

The HepG2 cell line was provided by Prof. Nanping Wang of Beijing Medical University. HepG2 cells were cultured with DMEM contain-

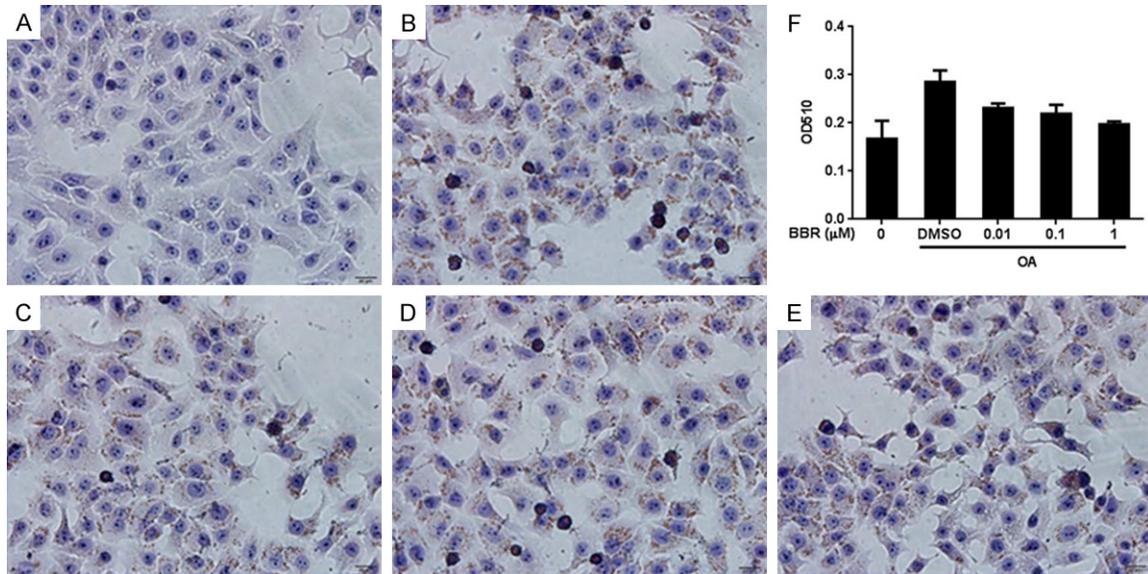


Figure 2. Oil red O staining of oleic acid (OA)-induced HepG2 cells incubated with BBR. A. Control cells without OA and BBR. B. OA-induced HepG2 cells incubated with DMSO. C. OA-induced HepG2 cells incubated with 1 μM BBR. D. OA-induced HepG2 cells incubated with 0.1 μM BBR. E. OA-induced HepG2 cells incubated with 0.01 μM BBR. BBR incubated for 24 h. These pictures depict one of the three representative results. F. Quantification of positive oil red O staining. The values are expressed as means ± standard deviation (n = 3). *P<0.01, comparison between the two groups.

combined annealing and extension at 60°C for 30 s. Real-time quantitative PCR was performed on ABI PRISM® 7500 Sequence Detection System (Applied Biosystems).

Statistical analysis

All values are expressed as mean ± S.E. Comparison between two mean values was evaluated by an unpaired Student's two-tailed *t*-test, whereas that between three or more groups was evaluated by one-way analysis of variance followed by Bonferroni's post hoc analysis. Statistical significance was accepted at P<0.05.

Results

BBR exerts no cytotoxicity on HepG2 cells

To ensure the safety of BBR on HepG2 cells, we performed CCK8 assay to detect whether BBR exerted cytotoxicity on HepG2 cells. BBR was diluted in DMSO, and DMSO was used as control to exclude the function of DMSO in HepG2 cells. **Figure 1** shows that no significant difference existed between the DMSO group and the three BBR groups. We used three different BBR concentrations in the experiments and no cyto-

toxicity effect was detected, even in the highest concentration group.

BBR decreased the fat overload and TG content in the HepG2 cellular model.

OA was used to induce fat and TG in the cytoplasm of the HepG2 cellular model via oil red O staining. **Figure 2A** and **2B** show that the HepG2 cellular model was successfully established because more positive staining was observed in **Figure 2B** than in **Figure 2A**. **Figure 2C-E** present the staining lipid of HepG2 cells incubated with different BBR concentrations. These results indicated that the staining area in the 1 μM group is significantly smaller than that in the 0.01 μM group.

To illustrate the effect of BBR on lipid quantity, the positive staining on cells was quantified by a spectrophotometer, as shown in **Figure 2F**. These data showed a significant increase in the OA group, which is similar to that of **Figure 2A** and **Figure 2B**. Furthermore, the quantities of fat overload and TG content of the 1 μM BBR group were less than those of the 0.1 μM and 0.01 μM groups, showing the same tendency in **Figure 2**. These results suggested that BBR can decrease the fat and TG content in the

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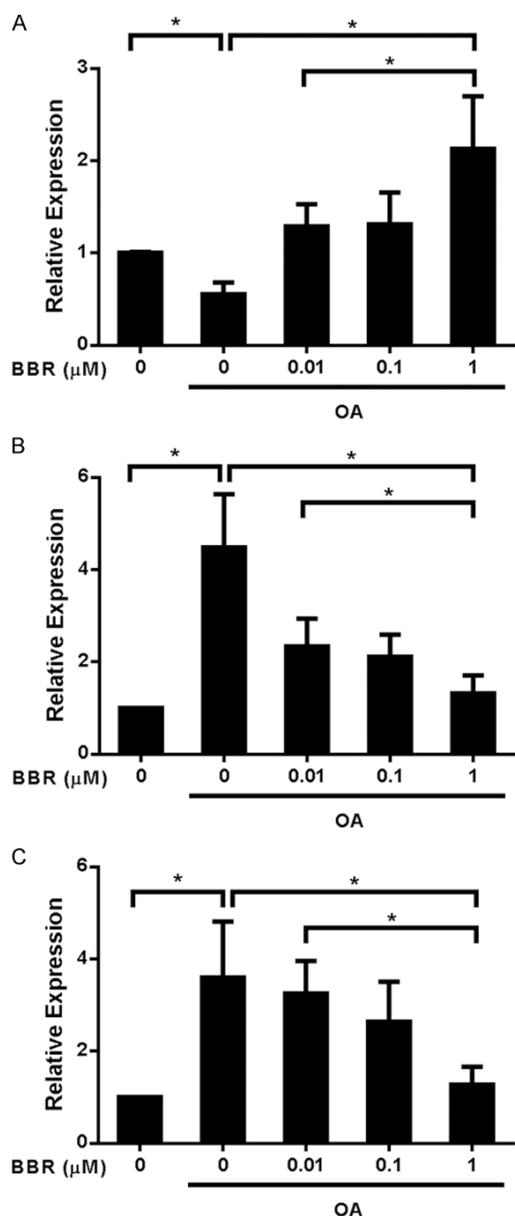


Figure 3. Effect of BBR on mRNA of FXR, SREBP-1c and FAS. A. Relative expression of FXR. B. Relative expression of SREBP-1c. C. Relative expression of FAS. Three groups of BBR with different concentrations: 0.01, 0.1 and 1 μM. The values are expressed as means ± standard deviation (n = 3). *P<0.01, comparison between the two groups.

cytoplasm, and this ingredient may be considered an efficient drug to treat NAFLD.

BBR regulates the FXR/SREBP-1c/FAS pathway to attenuate NAFLD

To understand the BBR mechanisms involved in the pathogenesis of NAFLD, we investigated

the transcriptional factors, farnesoid X receptor (FXR) and lipogenic genes, Fatty acid synthase (FAS), which played important roles in NAFLD [14, 15]. The mRNA levels of both FXR and FAS were first detected, as shown in **Figure 3A**. The relative expression of FXR in the OA group significantly decreased, suggesting that FXR can inhibit the lipogenesis which is also reported [16]. In the three other groups with different BBR concentrations, the high concentration of BBR resulted in increased expression of FXR; therefore, BBR can regulate the mRNA level of FXR. **Figure 3B** and **3C** show the relative expression of SREBP-1c and FAS, the mRNA level of the OA group was higher than that of the control, and the high BBR concentration decreased the expression of SREBP-1c and FAS, hence suggesting that BBR can inhibit the SREBP-1c and FAS expression. Moreover, both SREBP-1c and FAS were higher in NAFLD than in normal cells, which showed a similar tendency with our results [17].

WB was used to detect the protein levels of FXR, SREBP-1c, and FAS as shown in **Figure 4**. The tendency of FXR, SREBP-1c, and FAS was similar to that in **Figure 3**, which is a good evidence to illustrate the mechanisms of BBR in the pathogenesis of NAFLD. FAS can be regulated by SREBP-1c, and SREBP-1c can be regulated by FXR. FXR activation can inhibit the expression of SREBP-1c and FAS. These results suggested that BBR can regulate the FXR/SREBP-1c/FAS pathway to attenuate NAFLD.

Discussion

This study showed the effect of BBR on liver cells with fatty degeneration and HepG2 cells induced by oleic acid. First, BBR exerted no cytotoxicity on HepG2 cells at the concentration of 1 μM. BBR can also decrease the fat overload and TG content in the cytoplasm of HepG2 cells. This finding suggests that BBR is an efficient drug to treat NAFLD. To illustrate the mechanisms underlying this phenomenon, FXR and FAS, which are important in NAFLD, were detected at the mRNA and protein levels. Our results showed that BBR can promote the FXR expression but inhibit the FAS expression. FAS can be regulated by FXR, and our results suggest that FXR is a target of BBR in NAFLD.

FXR had shown a considerable effect on lipogenesis. Hepatic lipogenesis is mainly regulat-

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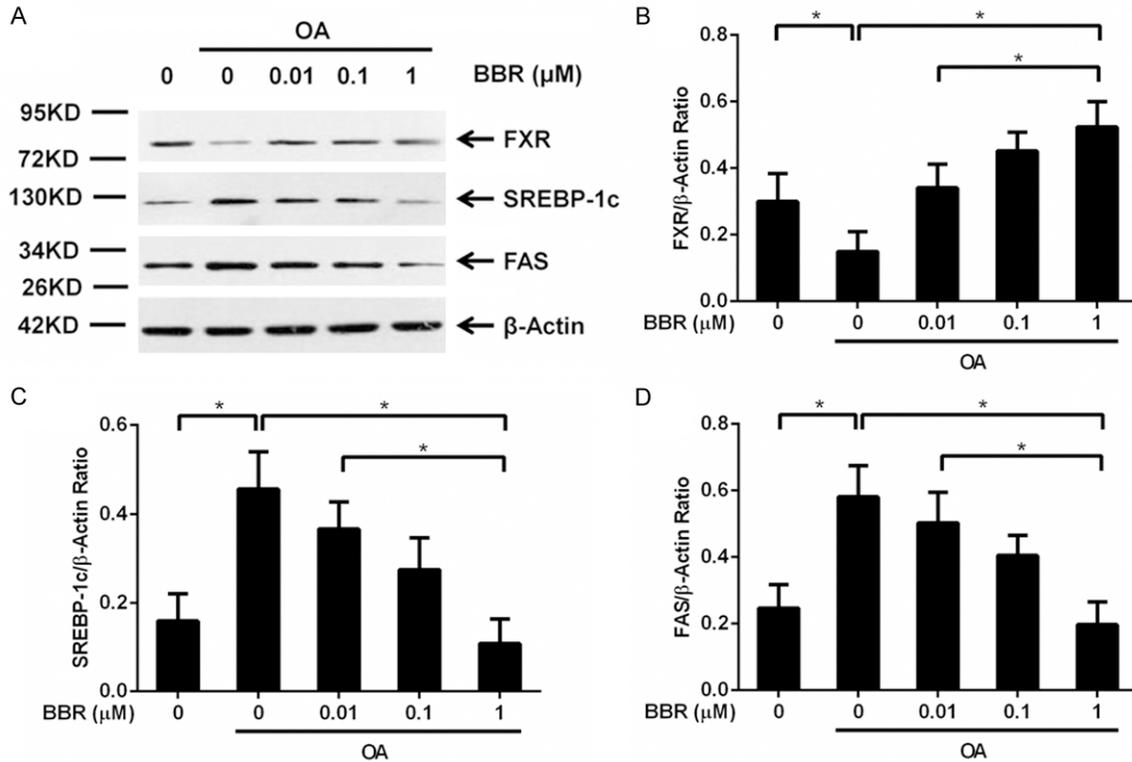


Figure 4. Effect of BBR on the protein levels of FXR, SREBP-1c and FAS. A. WB results of FXR, SREBP-1c, and FAS. B. Ratio of gray scanning of FXR/ β -actin. C. Ratio of gray scanning of SREBP-1c/ β -actin. D. Ratio of gray scanning of FAS/ β -actin. Three groups of BBR with different concentrations: 0.01, 0.1 and 1 μ M as shown in A, which is one of the three representative results. The values are expressed as means \pm standard deviation ($n = 3$). * $P < 0.01$, comparison between the two groups.

ed by sterol regulatory element binding protein 1c (SREBP-1c), which is known as the master regulator of both the lipid biosynthesis and the expression of several genes involved in lipogenesis [18]. FXR activation can inhibit the expression of SREBP-1c and its target enzymes, including FAS [19]. In the present study, FXR decreased in the HepG2 model but increased after BBR was added. In addition, the expression level of FXR changed along with the BBR concentration. An increase in the BBR concentration also increased the expression of FXR. These results suggest that FXR is a target of BBR in attenuating NAFLD.

Sterol regulatory element-binding proteins-1c (SREBP-1c) is responsible for the synthesis and uptake of fatty acids, cholesterol, and TG. As a transcription factor, SREBP-1c directly activates the expression of more than 30 genes, including FAS, which catalyzes the formation of long-chain fatty acids from acetyl-CoA, malonyl-CoA, and NADPH [20]. SREBP-1c increases

lipogenesis at the transcriptional level, and its expression is upregulated by FXR. Moreover, the role of SREBP-1c in de novo lipogenesis and NAFLD pathogenesis is well acknowledged and has been suggested as a potential therapeutic target [21]. Thus, the FXR/SREBP-1c/FAS signaling may contribute to the development of NAFLD [22, 23]. Our results exhibited that BBR dose-dependently decreased SREBP-1c and its downstream key gene FAS, as well as the corresponding mRNA and protein expression in the NAFLD model.

In conclusion, our study provides evidence of the BBR mechanisms in the NAFLD model based on OA-induced HepG2 cells. BBR can significantly decrease the fat overload and TG content in the OA-induced HepG2 cells, and this activity is important in NAFLD. The underlying mechanism for the beneficial effects of BBR was illustrated by RT-PCR and WB. BBR can regulate the FXR/SREBP-1c/FAS pathway, promote the expression of FXR and inhibit the

expression of SREBP-1c and FAS. Therefore, BBR would be an efficient drug in patients with NAFLD in the future.

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Disclosure of conflict of interest

None.

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References

- [1] de Alwis NM and Day CP. Non-alcoholic fatty liver disease: the mist gradually clears. *J Hepatol* 2008; 48 Suppl 1: S104-112.
- [2] Fon Tacer K and Rozman D. Nonalcoholic Fatty liver disease: focus on lipoprotein and lipid deregulation. *J Lipids* 2011; 2011: 783976.
- [3] Nagle CA, Klett EL and Coleman RA. Hepatic triacylglycerol accumulation and insulin resistance. *J Lipid Res* 2009; 50 Suppl: S74-79.
- [4] Farrell GC and Larter CZ. Nonalcoholic fatty liver disease: from steatosis to cirrhosis. *Hepatology* 2006; 43: S99-s112.
- [5] Abd El-Kader SM and El-Den Ashmawy EM. Non-alcoholic fatty liver disease: The diagnosis and management. *World J Hepatol* 2015; 7: 846-858.
- [6] Uhl P, Fricker G, Haberkorn U and Mier W. Current status in the therapy of liver diseases. *Int J Mol Sci* 2014; 15: 7500-7512.
- [7] Yin J, Xing H and Ye J. Efficacy of berberine in patients with type 2 diabetes mellitus. *Metabolism* 2008; 57: 712-717.
- [8] Kong W, Wei J, Abidi P, Lin M, Inaba S, Li C, Wang Y, Wang Z, Si S, Pan H, Wang S, Wu J, Wang Y, Li Z, Liu J and Jiang JD. Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. *Nat Med* 2004; 10: 1344-1351.
- [9] Cameron J, Ranheim T, Kulseth MA, Leren TP and Berge KE. Berberine decreases PCSK9 expression in HepG2 cells. *Atherosclerosis* 2008; 201: 266-273.
- [10] Lu SS, Yu YL, Zhu HJ, Liu XD, Liu L, Liu YW, Wang P, Xie L and Wang GJ. Berberine promotes glucagon-like peptide-1 (7-36) amide secretion in streptozotocin-induced diabetic rats. *J Endocrinol* 2009; 200: 159-165.
- [11] Liu Y, Zhang L, Song H and Ji G. Update on berberine in nonalcoholic Fatty liver disease. *Evid Based Complement Alternat Med* 2013; 2013: 308134.
- [12] Xia X, Yan J, Shen Y, Tang K, Yin J, Zhang Y, Yang D, Liang H, Ye J and Weng J. Berberine improves glucose metabolism in diabetic rats by inhibition of hepatic gluconeogenesis. *PLoS One* 2011; 6: e16556.
- [13] Yin J, Gao Z, Liu D, Liu Z and Ye J. Berberine improves glucose metabolism through induction of glycolysis. *Am J Physiol Endocrinol Metab* 2008; 294: E148-156.
- [14] Hwang KA, Hwang YJ, Kim GR and Choe JS. Extracts from *Aralia elata* (Miq) Seem alleviate hepatosteatosis via improving hepatic insulin sensitivity. *BMC Complement Altern Med* 2015; 15: 347.
- [15] Aguilar-Olivos NE, Carrillo-Cordova D, Oriahernandez J, Sanchez-Valle V, Ponciano-Rodriguez G, Ramirez-Jaramillo M, Chable-Montero F, Chavez-Tapia NC, Uribe M and Mendez-Sanchez N. The nuclear receptor FXR, but not LXR, up-regulates bile acid transporter expression in non-alcoholic fatty liver disease. *Ann Hepatol* 2015; 14: 487-493.
- [16] Carr RM and Reid AE. FXR agonists as therapeutic agents for non-alcoholic fatty liver disease. *Curr Atheroscler Rep* 2015; 17: 500.
- [17] Basaranoglu M and Ormeci N. Nonalcoholic fatty liver disease: diagnosis, pathogenesis, and management. *Turk J Gastroenterol* 2014; 25: 127-132.
- [18] Xiong X, Wang X, Lu Y, Wang E, Zhang Z, Yang J, Zhang H and Li X. Hepatic steatosis exacerbated by endoplasmic reticulum stress-mediated downregulation of FXR in aging mice. *J Hepatol* 2014; 60: 847-854.
- [19] Li X, Li Y, Yang W, Xiao C, Fu S, Deng Q, Ding H, Wang Z, Liu G and Li X. SREBP-1c overexpression induces triglycerides accumulation through increasing lipid synthesis and decreasing lipid oxidation and VLDL assembly in bovine hepatocytes. *J Steroid Biochem Mol Biol* 2014; 143: 174-182.
- [20] Kojima T, Hashimoto Y, Ohbora A, Kato T, Nakamura N, Fukui M, Lee MS, Kim JS, Cho SM, Lee SO, Kim SH and Lee HJ. Fermented *Rhus verniciflua* Stokes Extract Exerts an Antihepatic Lipogenic Effect in Oleic-Acid-Induced HepG2 Cells via Upregulation of AMP-Activated Protein Kinase. *Liver Int* 2015; 63: 7270-7276.
- [21] Ferre P and Foufelle F. Hepatic steatosis: a role for de novo lipogenesis and the transcription factor SREBP-1c. *Diabetes Obes Metab* 2010; 12 Suppl 2: 83-92.
- [22] Ai ZL, Zhu CH, Min M, Wang J, Lan CH, Fan LL, Sun WJ and Chen DF. The role of hepatic liver X

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- receptor alpha- and sterol regulatory element binding protein-1c-mediated lipid disorder in the pathogenesis of non-alcoholic steatohepatitis in rats. *J Int Med Res* 2011; 39:
- [23] Wang LL, Zhang ZC, Hassan W, Li Y, Liu J and Shang J. Amelioration of free fatty acid-induced fatty liver by quercetin-3-O-beta-D-glucuronide through modulation of peroxisome proliferator-activated receptor-alpha/sterol regulatory element-binding protein-1c signaling. *Hepatol Res* 2016; 46: 225-38.