Berberine Improves High-Fat Diet Induced Atherosclerosis and Hepatic Steatosis in Apoe$^{-/-}$ Mice by Down-Regulating PCSK9 via ERK1/2 Pathway

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Research

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Abstract

Background: Berberine (BBR) is a kind of alkaloid derived from Chinese herbal medicine, which has multiple pharmacological activities including anti-atherosclerosis (AS). However, the mechanism underlying the role of BBR in modulating lipid metabolic disorders is not fully clear. The aim of the present study was to investigate the beneficial effects of BBR on AS in ApoE$^{-/-}$ mice and its potential mechanisms.

Methods: Eight-week old ApoE$^{-/-}$ mice with high-fat diet (HFD) and wild type mice were administered eitherBBR (50mg/kg/d and 100mg/kg/d, respectively) or equivoluminal saline. After the 16-week treatment, the blood was collected for lipid evaluation, and aorta and liver were obtained from the mice for hematoxylin-eosin (HE) staining, oil red O staining and Western blotting. HepG2 Cells were treated by BBR (0, 5, 25, and 50 μg/ml) for 24 hours. Real-time PCR or Western blotting was used to examine the expression levels of proprotein convertase subtilisin/kexin type 9 (PCSK9), LDL receptor (LDLR), ATP-binding cassette transporter A1(ABCA1), ATP-binding cassette transporter G1(ABCG1) and scavenger receptor class B type I(SR-BI).

Results: BBR significantly decreased serum total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL) cholesterol (LDL-C) and increased high-density lipoprotein cholesterol (HDL-C) level in ApoE$^{-/-}$ mice fed with HFD. Moreover, BBR markedly reduced aorta atherosclerotic plaque, ameliorated lipid deposition in the liver in vivo. BBR could also promote intracellular cholesterol efflux and regulate LDLR and PCSK9 expression via the ERK1/2 pathway in HepG2 cells.

Conclusions: BBR could improve lipid metabolism, decrease aorta AS and hepatic lipid accumulation in ApoE$^{-/-}$ mice fed with HFD, which was associated with down-regulation of PCSK9 through ERK1/2 pathway.

Introduction

Atherosclerosis (AS) is a complex chronic inflammatory and metabolic disease in which aberrant inflammatory responses and dysregulation of lipid metabolism in the arterial walls at predisposed sites plays an important role from the initiation to progression and eventually rupture of the atherosclerotic plaque[1, 2]. Atherosclerosis and its complications considerably cause increased morbidity and mortality worldwide and account for almost a third of the deaths in the world [3]. Till now, dyslipidemia, mainly presented as elevated low-density lipoprotein (LDL) cholesterol (LDL-C) level and inflammationhave been considered as two important risk factors of AS. A large number of evidences clearly indicated that a reduction in the circulating levels of total cholesterol (TC) and LDL-Ccould reduce the risk for AS. In addition, it has been demonstrated that inflammation is a key process for the initiation of AS in the early stage; circulating monocytes in the blood adhere to the endothelium and migrate into the subendothelial space [4]. Macrophages would be activated and oxidized lipoprotein particles are deposited under endothelial cells. Later, an inflammatory response cascade occurs as a result of endothelium damage.

Increased production of pro-inflammatory mediators takes part in the initiation and development of AS[5]. Although the big progress has been made in the prevention and treatment of AS during past several decades, atherosclerotic cardiovascular disease (ASCVD) remains a main cause for human's mortality all over the world due to an unsatisfactory status for AS interventions [6], suggesting a big space for the improvement.

A large number of previous studies have shown that traditional Chinese medicine appears an alternativestrategy in the prevention and treatment of AS [7, 8]. Berberine (BBR) is an isoquinoline alkaloid isolated from coptischineses[9]. Publishedinvestigations have revealed that BBRisan effective traditional Chinese herbeal in treating many disorders, such as bacterial infection, diabetes, hypertension, and obesity et al [10-13]. Our previous study found that BBR significantly reduced body weight gain and improved lipid profile in high fat diet-fed rat [14]. More interestingly, data also showed that BBR could improve the dyslipidemia in both animal and human studies, resulting in beneficial effects on AS [15, 16]. However, the exact mechanism regarding the impact of BBR on dyslipidemia and AS has not been fully examined. Hence, the aim of the present study was to investigate the beneficial effects of BBR on lipid and AS in$ApoE^{-/-}$ mice and its potential mechanisms.

Methods

1.1 Animals and regents

Male C57BL/6J mice (n=7, 8 weeks old) and male $ApoE^{-/-}$ mice (n=35, 8 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Berberine was purchased from Sigma-Aldrich (St. Louis, MO, USA). The human hepatoma cell line, HepG2, obtained from Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China), U0126 (ERK1/2 inhibitor) was purchased from Cell Signaling Technology (Beverly, MA). Anti-PCSK9, Anti-LDLR and GAPDH antibodies were obtained from Abcam (Cambridge, UK). Antibodies against phospho-ERK1/2, total ERK1/2, were purchased from Cell Signaling Technology (Beverly, MA).

1.2 Animal treatment

All animals were maintained in an air-conditioned environment with a controlled temperature at 22 ± 2 °C and 50–60% relative humidity under a 12:12 h light/dark cycle. After an adaptation period of a week, all mice were divided into the following groups: group 1 (wild typeC57BL/6J mice, normal diet), group 2 ($ApoE^{-/-}$ mice, normal diet), group 3 ($ApoE^{-/-}$ mice, high fat diet), group 4 ($ApoE^{-/-}$ mice, high fat diet, and treatment with low dose berberine of 50mg/kg/d),and group 5 ($ApoE^{-/-}$ mice, high fat diet, and treatment with low dose berberine of 100 mg/kg/d), group 6 ($ApoE^{-/-}$ mice, high fat diet , and treatment with atorvastatin of 20mg/k/d).After 16-week treatment,mice were euthanized using 1% sodium pentobarbital(50 mg/kg) after a 4-hour fast. The eyeballs were removedand blood samples were collected.The subsequent serumwas used to determine blood lipid parameters.The left ventricle was perfused with 4% neutral paraformaldehyde for 1 hour, then the abdominal cavity was opened and the

liver, spleen and kidney were removed in turn, and the chest cavity was opened, the sternum was removed, the heart was exposed and the heart and aorta were removed.

1.3 Serum lipids analysis

According to the manufacturer's instructions, serum was prepared from each blood sample by centrifugation at 3500 rpm for 10 min. Serum TC, blood glucose, triglyceride (TG), LDL-C and high density lipoprotein cholesterol (HDL-C) were examined by the automatic biochemistry analyser (Hitachi 917, Tokyo, Japan).

1.4 Hematoxylin–eosin staining

Mouse liver specimens were processed according to a standard HE staining technique [17]. Briefly, liver tissues were fixed by 10% neutral formalin, dehydrated in ethanol, and then embedded. Subsequently, liver sections (4 μm) were stained with HE for pathological changes under an optical microscope.

1.5 Oil red O staining

Mouse liver tissues were immediately snap-frozen in liquid nitrogen and placed in OCT cryostat embedding compound (Tissue-Tek, Torrance, CA, USA). Frozen liver sections (8 μm) were stained with oil red O according to previous report [18], and the intracellularlipid droplets were observed andassessed by bright-field microscopy(Leica, Wetzlar, Germany).

1.6 Real time quantitative PCR (qRT-PCR) assay

SYBR green quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect mRNA levels of PCSK9, LDLR, ABCA1, ABCG1, SR-B1. The Trizol method was used to extract total RNA from mouse liver tissue. RNA yield and purity was confirmed by measuring the ratio of the absorbance at 260 nm and 280 nm. cDNA was synthesized using the SuperScript III First-Strand Synthesis System. The qRT-PCR reaction, containing target genes and SYBR Green PCR master mix, was performed on a Bio-Rad CFX connect real-time system (Bio-Rad, USA). The qRT-PCR, containing target genes and SYBR Green PCR master mix, was carried out on a Bio-Rad CFX connect real-time system (Bio Rad, USA) at95°C for 3min, cycled at 95°C for 10s,56°C for 30s and 72 °C for 30 s for 42 cycles. Melt curves were performed from 56.0 °C to 95.0 °C with intervals at 0.5 °C for 5 s. Relative RNA levels were determined by analyzing the changes in SYBR Green fluorescence by the $2^{-\Delta\Delta CT}$ method according to the manufacturer's instructions. GAPDH was amplified in parallel and the results were used for normalization. The PCR product was confirmed by gel electrophoresis on a 2% agarose gel stained with ethidium bromide. Purity of amplified PCR products was determined by melting point analysis using ICycler software. Experiments were performed in triplicate.

1.7 Western blot

Total proteins were extracted from liver (100 mg) derived from ApoE$^{-/-}$mice. Liver were homogenized in 1 ml of RIPA lysis buffer containing protease inhibitors and vortexed on ice for 30 min. After centrifugation at 12,000×g for 20 min, the supernatant, containing total protein extract, was collected, and protein concentrations were determined by the bicinchoninic acid (BCA) method. Cytosolic proteins were extracted using cytosol protein kits. Equal amounts of protein (20–40 μg) were electrophoresed on 8–12% SDS-PAGE gels for 2 h at 90 V and electrotransferred onto PVDF membranes at 125 mA for 1–2 h. The membranes were blocked with 5% non-fat dry milk in 20 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20 (Tris-Buffered Saline and Tween 20, TBST) for 1 h at room temperature, then membranes were incubated overnight at 4 °C with one of the following primary antibodies: anti-proprotein convertase subtilisin/kexin type 9 (PCSK9), anti-LDLrecepyor (LDLR), anti-ABCA1, anti-ABCG1, anti-SR-BI. After washing with TBST, membranes were incubated with secondary antibodies derived from goat for 1.5 h at room temperature. The results of Western blots were analyzed by the Image J program. The expression of each protein was normalized to the corresponding GAPDH.

1.8 Statistical analysis

GraphPad Prism 7 software was used for data analysis. Results are expressed as mean ± SEM. $p<0.05$ was considered statistically significant, and $p<0.01$ was considered extremely significant.

Results

1.1 BBR improved lipid metabolism in the serum

After BBR treatment for 16 weeks, we collected serum and analyzed the indicators, such as TC, TG, LDL-C and HDL-C. As shown in Fig. 1, the plasma TC, TG and LDL-C of ApoE$^{-/-}$ mice fed with normal diet(ND) and high fat diet (HFD), especially HFD group, were significantly higher than wild type mice. Low dose BBR (50mg/kg/d) and high dose BBR (100mg/kg/d) significantly decreased serum TC, TG and LDL-C levels and increased HDL-C level in ApoE$^{-/-}$ mice.

1.2 Effect of BBR based therapy on aortapathology

Our data demonstratedthat the general state of the aorta and found that ApoE$^{-/-}$ mice fed with ND and HFD have plaques in the aorta, the HFD group was more significant. Low dose BBR (50mg/kg/d) and high dose BBR (100mg/kg/d) could dramatically reduce the number of plaques (Fig. 2).

Furthermore, the HE staining of aorta showed that there were lipid foam like macrophages and vacuolated fibroid cells in the aorta in ApoE$^{-/-}$ mice fed with ND and HFD, especially in HFD group(Fig. 3). At the same time, oil red o showed that the lipid droplets in ApoE$^{-/-}$ mice fed with ND and HFD were significantly increased compared with the wild type mice. Then our date demonstrated that low dose BBR (50mg/kg/d) and high dose BBR (100mg/kg/d) could improve aortic lesion in ApoE$^{-/-}$ mice fed with HFD.

1.3 BBR reduced lipid accumulation in the liver.

Our data by HE staining and oil red O in liver demonstrated that the fatty degeneration was found in liver section of ApoE$^{-/-}$ mice fed with ND and HFD (Fig. 4). The lipid droplets and lipid deposition were observed in the HFD group and its pathological changes were more significant than those in the ND group. Low dose BBR (50mg/kg/d) and high dose BBR (100mg/kg/d) could reduce lipid deposition in liver tissue compared with ApoE$^{-/-}$ mice fed with HFD in a dose-dependent manner.

1.4 Effect of BBR based therapy on lipid metabolism-related genes and proteins

Compared with wild-type mice, the expressions of LDLR, ABCA1, ABCG1 and SR-B1 in the liver of ApoE$^{-/-}$ mice fed with ND and HFD were significantly lower. Low dose BBR (50mg/kg/d) and high dose BBR (100mg/kg/d) could increase the expression of LDLR ,ABCA1, ABCG1, SR-B1 and in liver of ApoE$^{-/-}$ mice, and decrease the level of PCSK9 (Fig. 5).

1.5 BBRdecreased PCSK9 expression by activating the ERK1/2 pathway

HepG2 cells were treated with BBR (0, 5, 25 and 50 μg/ml) for24h. Subsequently, we determined whether BBR could affect the expression of PCSK9 and LDLR in HepG2, and found that BBR exhibited the potential effects on the up-regulation of LDLR expression, which was accompanied by a steady decline of PCSK9 level(Fig. 6a).

We next examined the possible involvement of MAPK/ERK1/2 pathway in BBR-decreased PCSK9 expression. ERK1/2 is one member of MAPKKs family, which is an important kinase involved in many kinds of biological physiological process. Therefore, we investigated whether BBR could stimulate intracellular ERK1/2 phosphorylation events. After serum-starvation for 12 hours, HepG2 cells were stimulated with BBR (0, 5, 25 and 50 μg/ml) 24h. Activation of ERK1/2 was analyzed by Western blotting using anti-phospho-ERK1/2 antibody. Data showed that exposure of HepG2 cells to BBR enhanced the level of ERK1/2 phosphorylation in a dose-dependent manner, which started at concentration of 5 μg/ml (Fig. 6a). Subsequently, the results suggested that the decrease in PCSK9 expression caused by BBR was abolished by the ERK1/2 inhibitor U0126 (50 μM), indicating that an ERK1/2 pathway might be involved in such effect (Fig. 6b). Briefly, BBR decreased PCSK9 expression in HepG2 cells through activating the MAPK/ERK1/2 signal pathway.

Discussion

In this study, we investigated the effect of BBR on hepatic steatosis andAS in ApoE$^{-/-}$ mice and its potential mechanisms. The main findings covered: 1) BBR couldimprove lipid metabolism, presented as lowering TC, TG, and LDL-C in ApoE$^{-/-}$ mice fed with HFD; 2) significant reduction of the formation of hepatic steatosis (liver lipid deposition)and aortic atheroma were found in BBR-treated animals compared that in control ones; 3) Regarding the mechanisms, data suggested that BBR can significantly enhance the expression of cholesterol reverse transport related genes in liver and down-regulate PCSK9 expression in vitro, which was associated with the activation of MAPK/ERK1/2 signaling pathway. Our findings may

help to explain the beneficial effects of BBR on dyslipidema, liver lipid depositionand atherosclerotic plaque formation.

AS is a multifactorial, long-lasting and chronic processin humans, which is calculated by year. Consequently, animal models in whichmore rapid changes occur can be useful for the study ofthis process. Among these animal models, ApoE$^{-/-}$ mice is cheap, easily productive, and reliable, which give insight into thehuman process. It has been demonstrated that ApoE$^{-/-}$ mice show impaired clearingof plasma lipoproteins and develop AS in ashort time, and hence they are an excellent model inwhich to assess the impact of dietary factors, pharmacological therapy and developing new drugs[19].Previous studies have suggestedthat the levels of TC, TG and LDL-Cwere significantly higher in HFD-fed ApoE$^{-/-}$ mice than in WT mice, as well as that atherosclerotic plaques were obvious in the aortic arch, thoracic and abdominal aorta regions in HFD-fed ApoE$^{-/-}$ mice [20].In our current study, results showed that serum lipid levels including TC, LDL-C and TG in ApoE$^{-/-}$ mice fed with HFD were significantly higher than those fed with ND in ApoE$^{-/-}$ mice and WT mice. Additionally, the serum HDL-C level in ApoE$^{-/-}$ mice fed with HFD was lower than other groups. More importantly, a marked formation of atheromatous plaque and significant lipid accumulation were also stably found in ApoE$^{-/-}$ mice fed with HFD, indicating that this model may be suitable for further study on the impact of BBR on these pathophsyiologic changes during the development of AS.

The effects of BBR on improving lipid metabolism and anti-AS have widely been studied in recent years. Data suggested that BBRexerted protective effects against AS bymodulating various pathological and physiological process, which is definitely associated with lipid modification presented as the decrease in serum TC, TG, and LDL-C levels [21].Similarly, BBR also could reduce serum TC, TG, and LDL-C levels in hyperlipidemic mice[20]. In addition, these results were supported by two meta-analyses, which also reported increased HDL-C levels after BBR treatment [15, 22]. In the present study, our results showed that low dose BBR (50mg/kg/d) and high dose BBR (100mg/kg/d) could significantly decrease the levels of serum TC, TG and LDL-C, while increase the concentration of HDL-C in ApoE$^{-/-}$ mice fed with HFD, resulting in a significant attenuation of the formation of aortic atheroma in a dose-dependent manner.

PCSK9 is a keyregulator of cholesterol homeostasis that controls LDLR density on the surface of hepatocytes. The best known function of PCSK9is the post-translational regulation of LDLR in hepatocytes,representing the major route for LDL-Cclearancefrom the blood circulation [23]. The overall trend in in vitro and in vivo findings has been infavor of a PCSK9-lowering effect for BBR that could justifythe lipid-lowering activity of this nutraceutical through enhancedLDLR density on the surface of hepatocytes [24, 25]. In vitro studies showed that both PCSK9 and HNF1a proteinlevels were decreased in BBR-treated HepG2 cells [26]. In this ApoE$^{-/-}$ mice fed with HFD, our data indicated that BBRcould significantly decrease PCSK9 expression both in ApoE$^{-/-}$ mice fed with HFD and HepG2 cells, the underlying mechanisms might through the activation of the MAPK/ERK1/2 signaling pathway, which might provide additional information regarding the potential role of BBR in anti-AS process besides of its

anti-inflammatory action[27, 28], reducing oxidative stress [6, 29, 30], and enhancingcholesterol efflux [31-33], which was summarized in Fig. 7.

Another finding in our study is that BBR could markedly reduce hepatic steatosis, which was in agreement with previous studies.Zhu et al demonstratedthat BBR plus lifestyle intervention more effectively reducedliver fat content than lifestyle intervention alone and exhibited a trendof toward lower liver fat content compared with pioglitazone pluslifestyle intervention in a clinical trial[34].An animal study suggested that BBR attenuated nonalcoholic hepatic steatosis through the AMPK-SREBP-1c-SCD1 pathway [35]. Other investigation performed by Sun et al indicated thatBBRattenuated hepatic steatosis andenhanced energy expenditure in micebyinducing autophagy and fibroblast growthfactor 21 [36]. In addition, BBR improved lipidmetabolism and glucogenesis in nonalcoholic fatty liver disease (NAFLD) rat model, as well as obviously attenuated the ectopic liver fataccumulation [37]. In our present study, the data showed that BBR could significantly reduce liver fat depositionin $ApoE^{-/-}$ mice fed with HFD, which may be an explanation why BBR can exert a beneficial effecton NAFLD.

Conclusions

In conclusion, the present study showed that BBR could significantly ameliorate the extent of HFD-induced AS in $ApoE^{-/-}$ mice, improve the lipid profiles and hepatic fat accumulation, which appears associated with the down-regulation of PCSK9 mediated by the MAPK/ERK1/2 signal pathway, suggesting that more study is needed to further examine the potential mechanisms with regard to the impact of BBB on AS.

Abbreviations

BBR: Berberine; AS: atherosclerosis; ASCVD: atherosclerotic cardiovascular disease; ND: normal diet; HFD: high-fat diet; PCSK9: proprotein convertase subtilisin/kexin type 9; LDLR: LDL receptor; TC: total cholesterol; TG: triglyceride; LDL-C: low-density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; ABCA1: ATP-binding cassette transporter; ABCG1: ATP-binding cassette transporter G1; SR-BI: scavenger receptor class B type I; HepG2: human hepatoma cell.

Declarations

Ethics approval and consent to participate

The studies were approved by Fuwai Hospital ethics committees, in accordance with the Helsinki Declaration.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

Ma CY, Shi XY and Wu YR completed the project, analyzed the data, and wrotethe manuscript. Xu RX and Li JJ established the study, interpreted the data,and contributed to reviewing/editing the manuscript. Zhang Y, Qu HL, Guo YL and Tang YD contributedto assay and analyzing the data. All authors read and approved the finalmanuscript.

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Figures

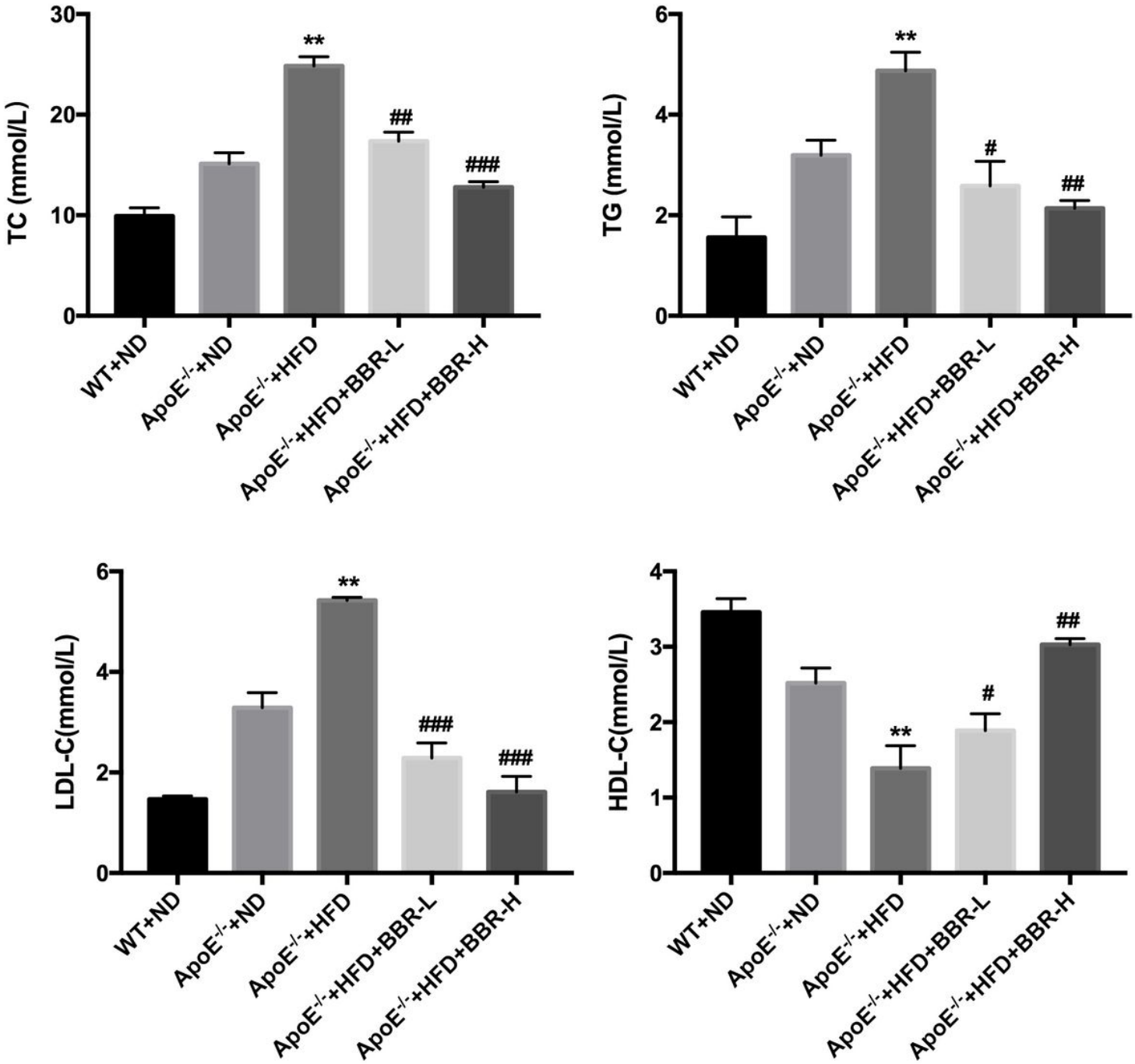

Figure 1

Effects of Berberine on serum lipid profile (TC, TG, LDL-C and HDL-C) in WT mice and ApoE-/- mice.**p<0.01:ApoE-/-+HFDvs ApoE-/-+ND.#p<0.05,##p<0.01, ###p<0.001: ApoE-/-+HFD+BBR-LorApoE-/-+HFD+BBR-H vs ApoE-/-+HFD.

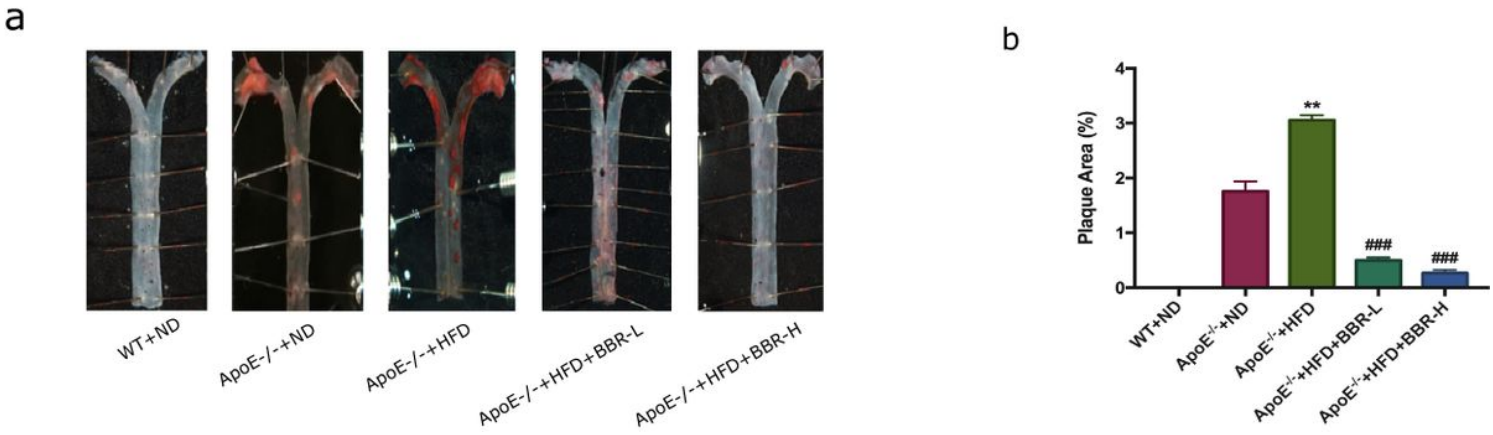

Figure 2

Berberine could alleviate aortic lesion in ApoE-/- mice fed with HFD.**p<0.01:ApoE-/-+HFDvs ApoE-/-+ND.###p<0.001: ApoE-/-+HFD+BBR-L, ApoE-/-+HFD+BBR-H vs ApoE-/-+HFD.

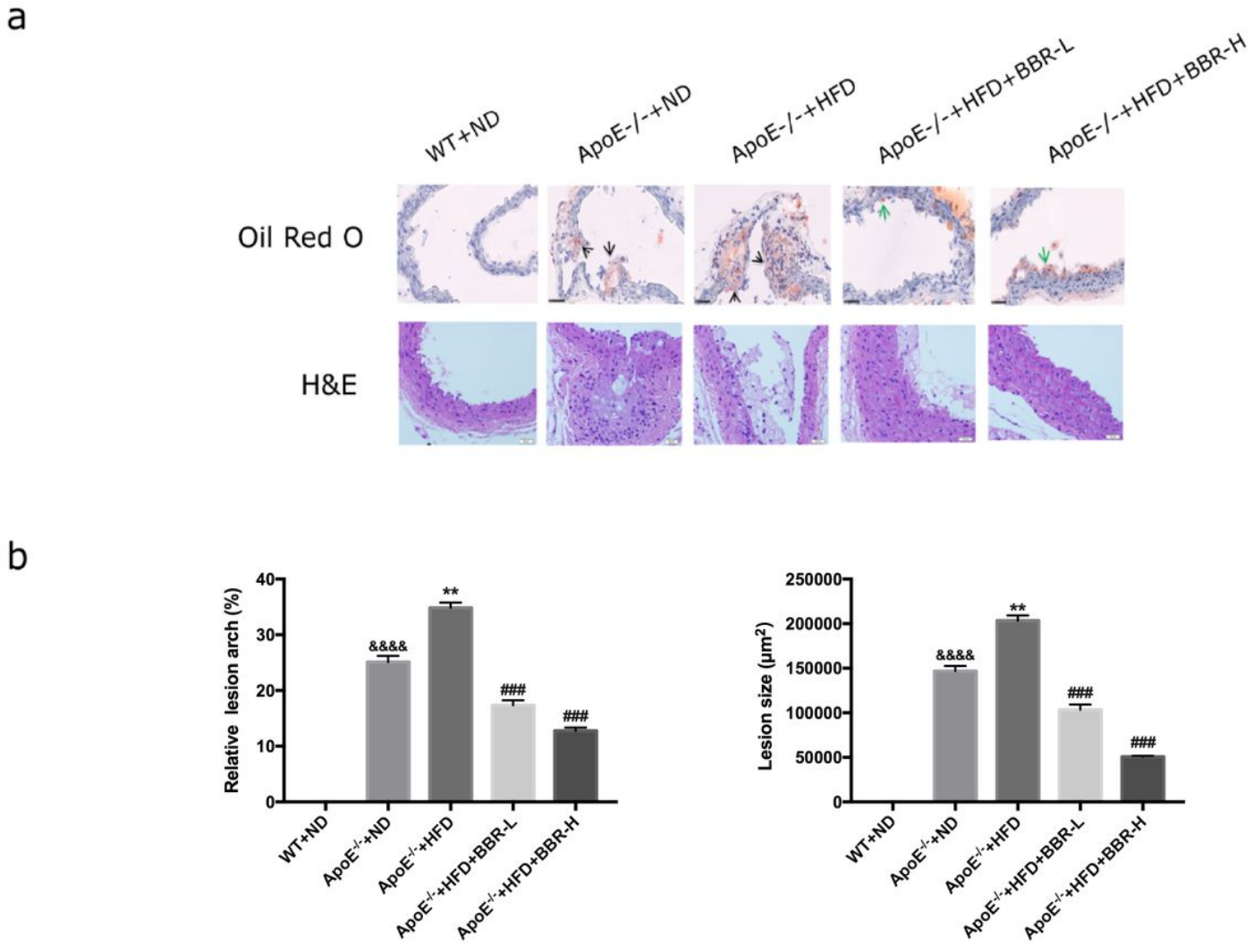

Figure 3

Effects of Berberine on the formation of atherosclerotic lesions in mice. (a) Representative hematoxylin and eosin (H&E)-stained and Oil Red O results cross-sections of aorta in mice (magnication, x40). (b) Quantitative analysis of H&E-staining and Oil Red O results. **p<0.01:ApoE-/-+HFDvs ApoE-/-+ND.###p<0.001: ApoE-/-+HFD+BBR-L, ApoE-/-+HFD+BBR-H vs ApoE-/-+HFD.

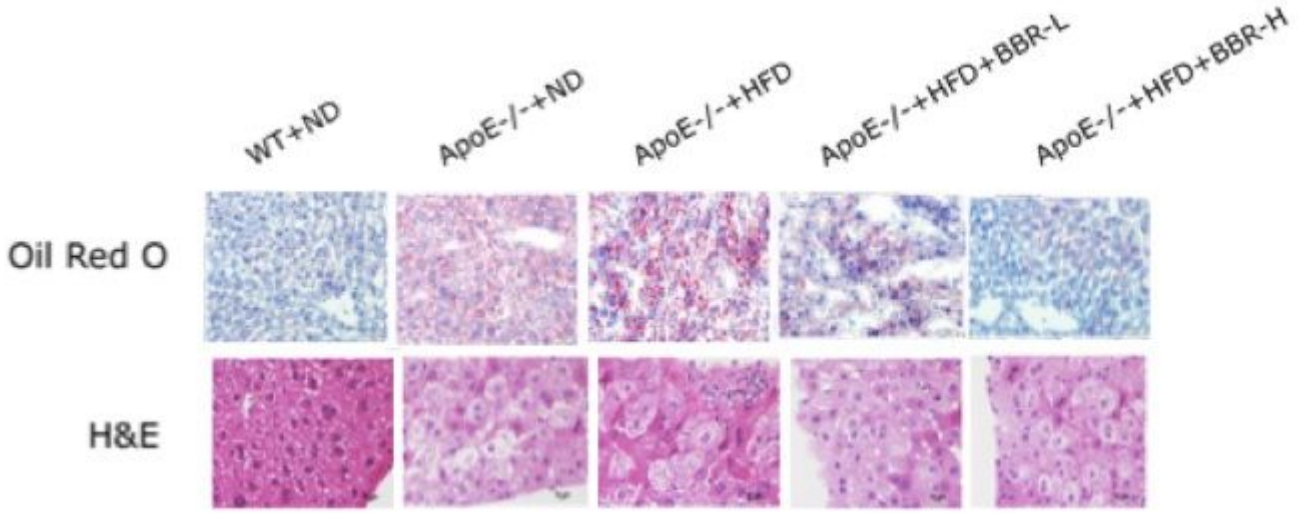

Figure 4

Berberine decreasedhepatic steatosis in ApoE-/- mice fed with HFD staining with Oil Red O or H&E.

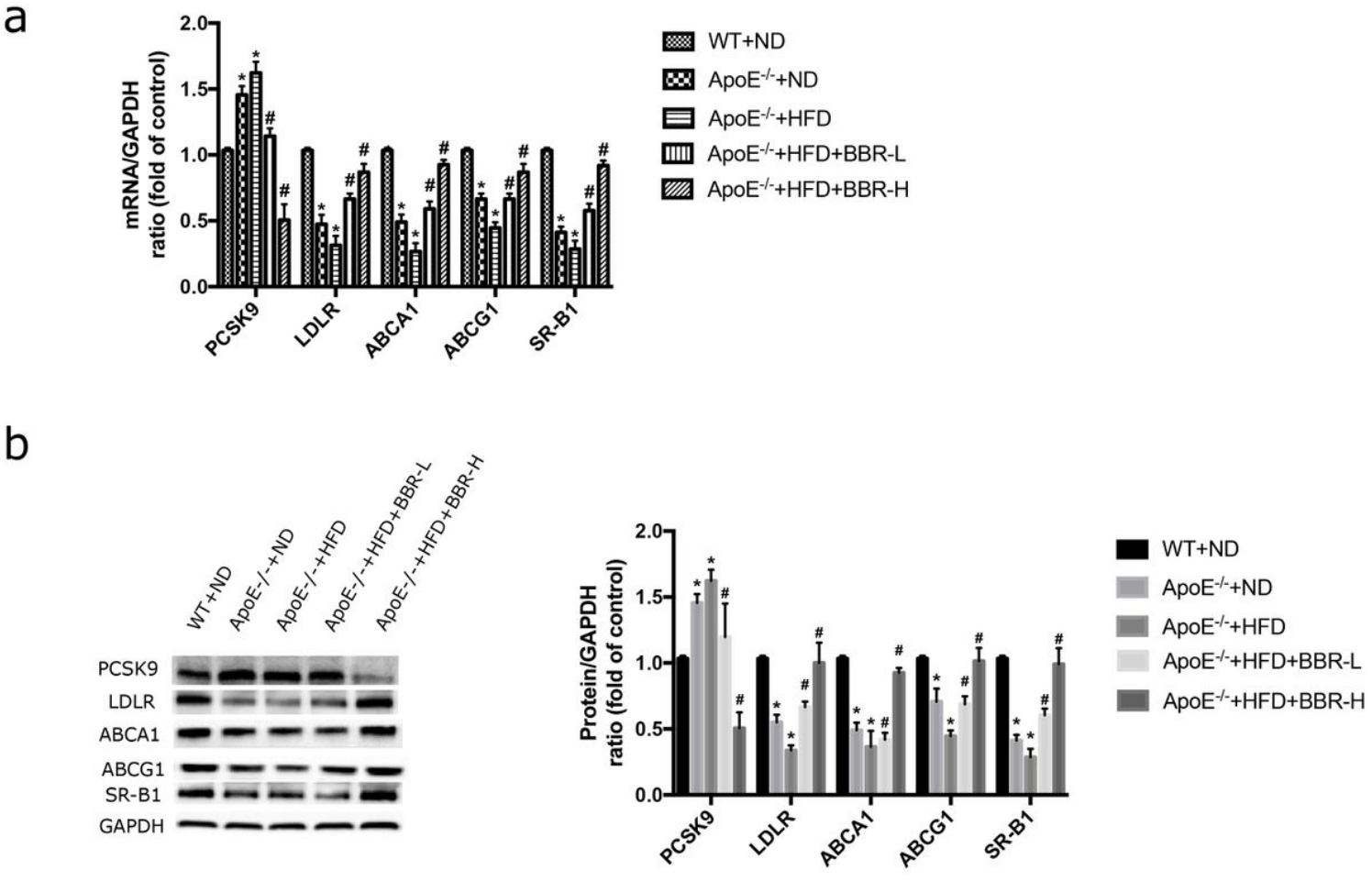

Figure 5

Effects of Berberine on PCSK9, LDLR, ABCA1, ABCG1 and SR-B1 gene (a) and protein (b) expressions in livers of WT mice and ApoE-/- mice. *p<0.05:ApoE-/-+HFDvs WT+ND orApoE-/-+ND. #p<0.05:ApoE-/-+HFD+BBR-L orApoE-/-+HFD+BBR-H vs ApoE-/-+HFD.

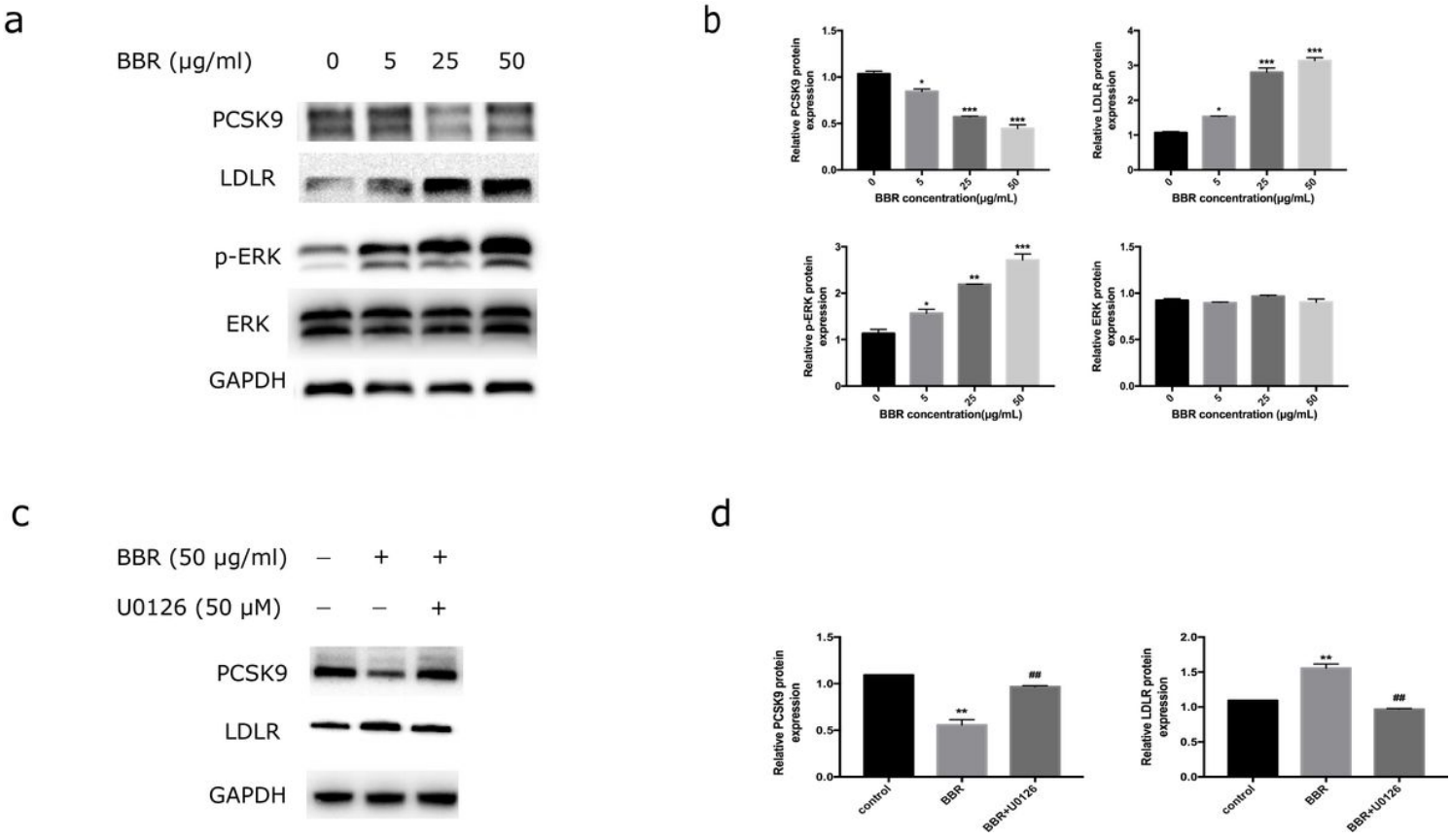

Figure 6

Effects of Berberine on PCSK9, p-ERK and ERK protein expressions in HepG2 cells(a and b).U0126, the ERK1/2 inhibitor, attenuated the decreased PCSK9 expression induced by Berberine in HepG2 cells(c and d).*p<0.05, **p<0.01, ***p<0.001: BBR vs control.##p<0.01:BBR +U0126 vs BBR.

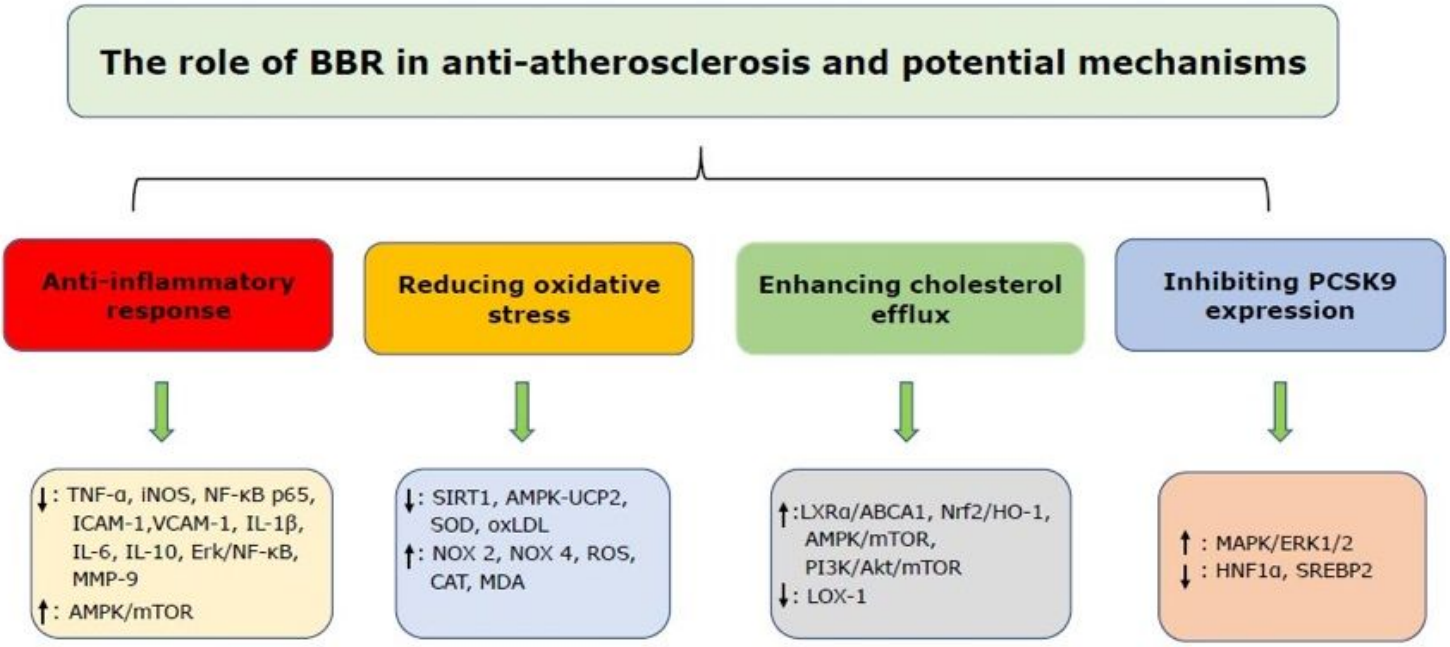

Figure 7

The role of BBR in anti-atherosclerosis such as reducing inflammatory response and oxidative stress, enhancing cholesterol efflux, and inhibiting PCSK9 expression as well as the potential mechanisms.