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# Berberine inhibits cholesterol absorption in rats through multi-mechanisms

Yanwen Wang[a,b,*], Xin Yi[a,b], Khadija Ghanam[a,b], Shuocheng Zhang[a], Tiantian Zhao[a], and Xuemei Zhu[a]

[a]Aquatic and Crop Resource Development, National Research Council of Canada, Charlottetown, PE, Canada

[b]Department of Biomedical Sciences, University of Prince Edward Island, Charlottetown, PE, Canada

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*Corresponding author. Aquatic and Crop Resource Development, National Research Council of Canada, Charlottetown, PE, Canada C1A 4P3. Tel: +1 902 566 7953, Fax: +1 902 569 4289. Email address: yanwen.wang@nrc.ca (Y. Wang).

**Abstract**

*Objective.* The objective was to determine the mechanisms of action of berberine (BBR) on cholesterol homeostasis using *in vivo* and *in vitro* models.

*Methods.* Male Sprague-Dawley rats were fed the AIN-93G diet (normal control), or modified AIN-93G diet containing 28% fat, 2% cholesterol and 0.5% cholic acid with treatment of 0 (atherogenic control), 50, 100, and 150 mg/kg˙d of BBR, respectively by gavaging in water for 8 weeks. Cholesterol absorption rate was measured with the dual stable isotope ratio method, and plasma lipids determined using the enzymatic methods. Gene and protein expressions of Acyl-coenzyme A:cholesterol acyltransferase-2 were analysed *in vivo* and *in vitro*. Cholesterol micellarization, uptake and permeability were determined *in vitro*.

*Results.* Rats on the atherogenic diet showed significantly hypercholesterolemic characteristics compared to normal control rats. Treatment with BBR in rats on the atherogenic diet reduced plasma total cholesterol and nonHDL cholesterol levels by 29-33% and 31-41%, respectively, with no significant differences being observed among the three doses. The fractional dietary cholesterol absorption rate was decreased by 40-51%. Rats fed the atherogenic diet showed lower plasma triacylglycerol levels, and no changes were observed after the BBR treatment. BBR interfered with cholesterol micellarization, decreased cholesterol uptake by Caco-2 cells and permeability through Caco-2 monolayer. BBR also inhibited the gene and protein expressions of acyl-coenzyme A cholesterol acyltransferease-2 in the small intestine and Caco-2 cells.

*Conclusion.* BBR lowered blood cholesterol levels at least in part through inhibiting the intestinal absorption and further by interfering with intraluminal cholesterol micellarization and decreasing enterocyte cholesterol uptake and secretion.

Highlights:

1. Berberine lowers plasma total cholesterol and nonHDL cholesterol levels in rats.
2. Berberine inhibits the intestinal cholesterol absorption.
3. Berberine interferes with cholesterol micellarization.
4. Berberine reduces cholesterol uptake by Caco-2 cell and permeability through Caco-2 monolayer.
5. Berberine downregulates acyl-coenzyme A cholesterol acyltransferease-2 expression.

**Keywords:** Acyl-coenzyme A:cholesterol acyltransferase-2, berberine, Caco-2, cholesterol absorption, cholesterol micellarization, rat

**Abbreviations:** ABCG, ATP-binding cassette sub-family G; AC, atherogenic control; ACAT, Acyl-coenzyme A cholesterol acyltrasnferase; BBR, berberine; EMEM, Eagle's Minimum Essential Medium; IRMS, HBSS, Hank's buffered salt solution; isotope ratio mass spectrometry; LDL-C, LDL cholesterol; LDLR, LDL receptor; NC, normal control; nonHDL-C, nonHDL cholesterol, NPC1L1, Niemann-pick C1-like 1; RIPA, radioimmunoprecipitation assay; TAG, triacylglycerol; TBST, tris-buffered saline and Tween 20; T-C, total cholesterol.

## 1. Introduction

Berberine (BBR) is a plant alkaloid, which is the principal bioactive compound of *Coptis Chinensis* and many other medicinal plants. BBR possesses a wide range of biological and pharmacological functions. It has been used for thousands of years in traditional Chinese medicine to treat various diseases, such as infectious diseases and gastrointestinal disorders, without apparent side-effects being reported from home remedies and clinical uses [1, 2]. The cholesterol-lowering effect of BBR was reported back to the 1980's, with an observation that BBR lowered the intracellular cholesterol in cultured human aortic intimal cells [3]. However, this potential health benefit had not been paid serious attention until 2004 when BBR was found to markedly lower blood cholesterol levels *in vivo* in humans and hamsters [4]. Since then, several studies have repeatedly demonstrated the lowering effects of BBR on blood total cholesterol (T-C), LDL cholesterol (LDL-C), or nonHDL cholesterol (nonHDL-C) [5-10], whereas the mechanism of action remains to be further elucidated [4, 5].

In 2004, BBR was reported to increase LDL receptor (LDLR) gene expression by stabilizing LDLR mRNA [4]. Following this report, the same groups conducted additional studies predominantly in HepG2 cells, with similar effects being observed [8, 11-13]. In addition to LDLR-mediated LDL cholesterol clearance in the liver, several other mechanisms are involved in cholesterol homeostasis, including cholesterol absorption, cholesterol biosynthesis, cholesterol secretion, bile acid synthesis and secretion. Nevertheless, the effects of BBR on these processes have not been reported. Amongst these metabolic pathways, cholesterol absorption plays a very important role [14, 15]. It is well-known that cholesterol absorption is collectively controlled by multiple factors or processes in the small intestine. These factors include cholesterol micellarization in the intestinal lumen[16], the expression of sterol transporters [5,

17, 18], cholesterol uptake, and the expression and activity of enzymes that catalyze the esterification of free cholesterol in the enterocytes [19].

Cholesterol micellarization determines the amount of cholesterol that can be transported across the 'unstirred' water layer to the apical side of enterocytes for absorption [16]. Cholesterol is then taken up by the enterocytes. Apart from the passive penetration, cholesterol uptake by enterocyte also involves the active transport through sterol transporters such as Niemann-Pick C1 Like 1 (NPC1L1) [20] and ATP-binding cassette, subfamily G, member 5 (ABCG5) and 8 (ABCG8) [21, 22]. The expression of these transporters, together with passive penetration, mediates the dynamic flux of cholesterol at the apical side membrane of the enterocytes and thus the net amount of cholesterol that is taken up by the enterocytes. Prior to absorption, cholesterol esters of dietary and biliary sources are hydrolyzed to free cholesterol, which is the predominant form being absorbed into the enterocytes. Nevertheless, after entering the enterocytes free cholesterol needs to be converted back to esters because cholesterol is secreted out predominantly as ester form from the basolateral side membrane of enterocytes into the lymphatics and then the general circulation, and ultimately to be delivered to the liver. This step is catalyzed by acetyl-coenzyme A cholesterol acyltransferase (ACAT), primarily the ACAT2 isoform [19, 23]. Our previous study showed that BBR lowered blood cholesterol through a mechanism that is independent of the expression of sterol transporters NPC1L1, ABCG5, and ABCG8 [5]. Therefore, in the present study we investigated the mechanism of action of BBR on cholesterol metabolism in diet-induced hypercholesterolemic rats with a primary focus on the intestinal absorption and further on cholesterol micellization prior to absorption and the apical side uptake and basolateral side secretion of the enterocytes during the absorption.

## 2. Materials and Methods

### *2.1. Animals and diets*

Sixty male Sprague-Dawley rats (Charles River Laboratories, Montreal, QC, Canada), were housed one per cage with a 12 h light:dark cycle and fed a regular rodent chow. After 1 week of adaptation, rats were randomly divided into 5 groups (n = 12/group). One group was fed a semi-purified cornstarch-casein-sucrose-based AIN-93G diet and used as the normal control (NC). The other four groups were fed an atherogenic diet, which was the NC diet modified to contain 28% fat in a form of mixture of beef tallow and sunflower oil (4:1, w:w), 2% cholesterol and 0.5% cholic acid to induce hyperlipidemia [24]. One group was used as a hypercholesterolemic/atherogenic control (AC) and the other three were treated, twice a day, by gavage feeding with 50, 100, and 150 mg/kg·d of BBR in water, respectively. The control rats were gavaged with water only at the same volume as for the treatment groups. The purity of BBR (chloride salt) was > 98% (Sigma-Aldrich, ON, Canada). The animal use and experimental procedures were approved by the Joint Animal Care and Research Ethics Committee of the National Research Council Canada in Charlottetown and the University of Prince Edward Island. The study was conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Rats were treated for 8 weeks to obtain the stable treatment effect. Seventy-two hours prior to sacrifice, they were injected through tail vein with 0.4 mg $^{13}$C-cholesterol (99.5 atom% $^{13}$C, CDN Isotopes Inc., Quebec) in 1.0 mL Intralipid (20%, Baxter International Inc., Deerfield, IL), followed immediately by intragastric administration of 1.5 mg $^{18}$O-cholesterol (80.0 atom% $^{18}$O, CDN Isotopes Inc., Quebec) suspended in 1.0 mL of equal amounts of coconut, olive and sunflower oil-mix as reported previously [5]. At the end, rats were anaesthetized with isoflurane

inhalation. Blood was collected from cardiac puncture into EDTA-tubes and placed on ice. Plasma and red blood cells were separated by centrifugation and stored at -80°C for later analysis of plasma lipids and the determination of cholesterol absorption. Liver and small intestine were dissected, weighed and immediately frozen in liquid nitrogen and stored at -80°C for the measurement of protein expression.

*2.2. Analysis of plasma lipids*

After 2 weeks of storage at -80°C, plasma T-C, HDL cholesterol (HDL-C) and triacylglycerol (TAG) were measured in triplicate by enzymatic methods using a Pointe-180 Analyzer (Pointe Scientific Inc., Canton, MI). HDL-C was measured after the precipitation of apolipoprotein-B containing lipoproteins with dextran sulfate and magnesium chloride. Because the Friedewald equation may not be applicable in rats [25], nonHDL-C (very low density lipoprotein cholesterol + intermediate density lipoprotein cholesterol + LDL-C) instead of LDL-C was used and calculated by subtracting HDL-C from T-C [26].

*2.3. Measurement of fractional dietary cholesterol absorption*

The fractional dietary cholesterol absorption rate was measured using the dual isotope ratio mass spectrometry (IRMS) method as described previously [5]. Briefly, total lipids were extracted from 0.5 g of red blood cells and cleaned by saponification with 0.5 mol/L methanolic KOH [26]. The enrichment of $^{13}C$ in free cholesterol was analyzed in duplicate using an online GC/combustion/isotope ratio mass spectrometer (GC/C/IRMS), which is a DELTA$^{plus}$ XP IRMS coupled with an Agilent 6890 GC unit via a Conflow-III interface (Finnigan Mat, Bremen, Germany). The reference gas for $CO_2$ was injected via the Conflow-III interface. The enrichment

of $^{18}O$ was analyzed by GC/pyrolysis/IRMS (GC/P/IRMS). The CO reference gas was injected through the dual inlet system. The enrichments of both isotopes in free cholesterol are expressed in per mil ($^{0}/_{00}$) relative to PeeDee Belemnite (PDB), the limestone standard of the National Bureau of Standards (NBS). The percent cholesterol absorption was calculated using the ratio of orally ingested $^{18}O$- to intravenously administered $^{13}C$-cholesterol as described elsewhere [27].

*2.4.Cholesterol micellarization*

A solution was made in 50 mL tubes by mixing 1 mL of 264 mmol/L NaCl solution in water and methanol mixture at a ratio of 1:9 (v/v), 200 µL of 6 mmol/L phosphotidylcholine in methanol, 92.6 µL of monoolein, 40 µL of 50 mmol/L oleic acid in methanol, 400 µL of 5 mmol/L cholesterol in methanol and different amounts of 1 mmol/L BBR in methanol. The solution was dried under nitrogen stream, re-dissolved in 2 mL of 6.6 mmol/L sodium taurocholate solution, and incubated at 37°C for 1 h. Following 15 min of sonication, the solution was kept overnight at 37°C. The cholesterol content was measured as for the blood lipid analysis. The solutions were then ultracentrifuged at 28,000 RPM at 37°C for 1 h. The supernatant (micelles) was measured for the cholesterol concentration. The cholesterol micellarization was calculated by comparing the difference of cholesterol concentrations between micelles and the solution prior to ultracentrifugation. It is well established that plant stanols interfere with cholesterol incorporation into micelles [28], and thus used as the positive control to validate the cholesterol micellarization method.

*2.5. Cholesterol uptake in Caco-2 cells*

Caco-2 cells were grown in the Eagle's Minimum Essential Medium (EMEM) supplemented with penicillin and streptomycin and 20% FBS. At the passage number of 25-28, the cells were seeded onto 96-well plates at a density of 5 x $10^5$ cells/$cm^2$. After 20 days incubation, the cells were treated with BBR at indicated concentrations for 20 h. Then, fluorescent 22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol (NBD-cholesterol; Life Technologies Inc., Burlington, ON) was added and incubated for additional 2 h. Then, the cells were washed twice with cold PBS to stop cholesterol uptake and read for the fluorescence intensity at Ex/Em = 470/535 nm on a Varioskan Flash spectral scanning multimode plate reader (Thermo Fisher Scientific, Waltham, MA). Cholesterol uptake was also estimated by measuring the absolute amount of intracellular cholesterol using a commercial cholesterol assay kit (Biovision Inc., Milpitas, CA). Briefly, following 20 h of BBR treatment as mentioned above, the cells were washed with cold PBS, scratched and collected into glass tubes. Lipids were extracted and analyzed for cholesterol concentration with the cholesterol kit. The results are presented as the fold of control (0 μmol/L of BBR). The assay conditions were optimized for the dose of NBD-cholesterol and incubation time, and further validated using ezetimibe as the positive control (supplemental information - validation of cholesterol uptake assay).

*2.6. Cholesterol permeability through Caco-2 monolayer*

Caco-2 cells were seeded on the inserts of 24-well transwell microplates. The medium containing 20% FBS was added to both the apical (upper) and basolateral (lower) chambers. The cells were incubated for 21-28 d to allow fully differentiation and formation of a tight junction monolayer. The integrity of monolayer was confirmed by measuring trans-epithelial electrical resistance (TEER) prior to the permeation assay and conducting a leaking test with the Lucifer yellow

assay immediately post the assay. At the beginning of the experiment, both apical and basolateral chambers of the transwell were incubated with the indicated concentrations of BBR. After 20 h of incubation, the monolayer was washed with Hank's buffered salt solution (HBSS) in both chambers. The apical chamber was then treated with HBSS buffer containing 15 μg/mL of cholesterol and HBSS was added in the basolateral chamber. The samples were collected from both the apical and basolateral chambers after 2 h of incubation and analyzed for total cholesterol and free cholesterol contents using the Amplex Red cholesterol assay kit (Life Technologies Inc., Burlington, ON). Cholesteryl ester was calculated by subtracting free cholesterol from the total cholesterol. The results are expressed as fold of the control (0 μg/mL of BBR).

*2.7. ACAT1/2 protein expressions in Caco-2 cells*

Caco-2 cells were maintained in EMEM supplemented with penicillin and streptomycin and 20% FBS. For the experiment, the cells were seeded onto 6-well plates and the medium was changed every other day until fully differentiated. Then, the cells were treated with BBR at concentrations of 0, 5, 10, and/or 15 μg/mL. After indicated time of incubation, medium was removed and cells were washed twice with PBS and then 100 μL of radioimmunoprecipitation assay (RIPA) lysis buffer were added. The cells were scraped and transferred into 1.5 mL tubes, which were kept on ice for 30 min. Cells were broken by passing through a 21G syringe for 3 times and sonicated for 5 min in an ice water bath. After another 30 min on ice, the suspension was centrifuged at 12,000 *g* for 10 min at 4°C. The supernatant was transferred into a new tube on ice and the protein concentration was determined with the BCA assay. A gel with resolving gel (10%) and stacking gel (4%) was prepared. Protein samples were run on the gel and then transferred onto nitrocellulose membrane (Amersham Biosciences UK Limited) using a semi-dry transfer

apparatus (Bio-Rad Laboratories Canada Ltd., Mississauga, ON). The membrane was blocked for 1 h at room temperature using 5% skim-milk in a mixture of tris-buffered saline and Tween 20 (TBST). The membrane was rinsed twice with TBST, incubated with indicated primary antibodies ACAT1 (1:1000, a rabbit monoclonal antibody made against a synthetic peptide corresponding to residues on the C-terminus of human ACAT1, Abcam) or ACAT2 (1:1500, a mouse monoclonal antibody made against N-terminal amino acids of human ACAT2 protein, Abcam) diluted in blocking buffer for 1 h at room temperature, washed 3 times with 10 min each in TBST. The membrane was incubated with secondary antibodies diluted at 1:3000 or 1:1000 (Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L); Peroxidase-AffiniPure Donkey Anti-Mouse IgG (H+L), Jackson ImmunoResearch Laboratories, Inc.) in blocking buffer for 1 h at room temperature, washed 3 times with TBST. The target protein bands were revealed by BM chemiluminescence blotting substrate (POD, Roche Diagnostics) and visualized by a Bio-Rad ChemiDoc imaging system. The intensity of the specific blots was estimated using Bio-Rad Quantity One software, normalized against β-actin and expressed as fold of the control (0 µg/mL of BBR).

*2.8.Western blot analysis of ACAT2 in small intestine*

Approximately 150 mg of small intestine (jejunum) was homogenized in 800 µL of RIPA lysis buffer (Upstate, Temecula, CA) containing SIGMA*FAST* protease inhibitors (Sigma-Aldrich, St-Louis, MI). The samples were centrifuged at 17,000 g at 4°C for 15 min. Total protein concentration in the supernatants was determined using the BCA protein assay kit (Thermo Scientific, Rockford, IL) with BSA (Sigma-Aldrich, St-Louis, MI) as a standard. An aliquot (50 µg) of sample proteins was boiled in sample buffer containing 5% β-mercaptoethanol for 5 min

and loaded onto 8% SDS-PAGE gels. Following electrophoresis separation, proteins were transferred onto nitrocellulose membrane. The membranes were developed in the presence of primary and secondary antibodies and the intensity of blots were analyzed as described elsewhere [5]. The primary antibodies against ACAT2 (1:1500) or β-actin (1:1000 dilution) (Santa Cruz Biotechnology Inc. Santa Cruz, CA) and the second antibodies of goat anti-rabbit IgG-HRP (1:5000 dilution, Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) were used. The protein expression was normalized against β-actin and expressed as fold of the AC control.

*2.9. ACAT2 gene expression in small intestine and Caco-2 cells*

To further understand how protein expression was altered by BBR, mRNA expression of ACAT2 in the small intestine was measured using the method reported previously [5]. For the gene expression of ACAT2 in Caco-2 cells, the cells were seeded in 6-well plates and cultured for differentiation in EMEM medium containing 20% FBS for 10 d. The differentiated cells were treated with various concentrations of BBR in EMEM medium containing 0.5% lipoprotein deficient serum for 8 h. The cells were harvested and total RNA was extracted with Trizol reagent (Life technologies Inc., Burlington, ON). The extracted RNA was converted to cDNA and further analyzed for ACAT2 mRNA levels using the same method as for the tissue gene expression [5]. The results are expressed as fold of the NC in the small intestine or fold of the control (0 μg/mL of BBR) in Caco-2 cells.

*2.10. Statistical analysis*

Difference between the NC and AC groups was analyzed using the Student's t-test to determine whether the AC group was hypercholesterolemic and altered cholesterol metabolism. Then, one-way ANOVA was used to analyze the treatment effects of BBR on body weight (repeated measures), food intake (repeated measures), plasma lipids, cholesterol absorption rate, cholesterol micellarization, and protein expression in the small intestine and intestinal cell line Caco-2. The results of BBR on ACAT protein expression in Caco-2 cells following different treatment time were analyzed using two-way ANOVA. When a significant treatment effect was obtained, differences between treatments were determined by pairwise comparisons using the least squares means test. Relationships between plasma cholesterol concentrations and intestinal cholesterol absorption rates were analyzed using Pearson's correlation coefficients. Significance level was set at $p < 0.05$. All analyses were performed using SAS software, version 9.2 (SAS Institute, North Carolina, USA). Data are presented as means ± S.E.M. The statistical power is over 80% for all the parameters of the animal study.

## 3. Results

### *3.1. BBR has no effects on food intake and body weight*

All animals completed the 8-week treatment and did not show any abnormal behaviors. There were no toxic signs observed during the sacrifice in all organs and tissues, in agreement with our previous study using the same animal model that was treated with 100 mg/kg·d of BBR [7]. In line with dietary energy densities, food intake was higher in the NC group than the AC group in every week, and there were no differences among the AC and three treatment groups (supplemental Table 1). The AC group started to show higher ($p < 0.05$) body weights than the NC group in week 7 and remained the difference in week 8 (supplemental Table 2). In

comparison to the AC group, BBR did not show a significant effect on body weight and there were no differences among the three doses.

*3.2. BBR lowers plasma total cholesterol and nonHDL cholesterol levels*

After 8 weeks, rats on the AC diet showed remarkable increases in the plasma T-C and nonHDL-C but decreases in HDL-C levels, demonstrating the typical characteristics of hypercholesterolemia (Fig. 1A). The conclusion of BBR in the AC diet showed a significant effect of reversing the diet-induced hypercholesterolemia. Supplementation of BBR lowered plasma T-C by 29% ($p < 0.01$), 33% ($p = 0.003$), and 33% ($p = 0.002$) and nonHDL-C by 31% ($p = 0.024$), 41% ($p = 0.003$), and 38% ($p = 0.004$), respectively at the doses of 50, 100, and 150 mg/ as compared with the AC group. There were no significant differences among the three BBR doses. BBR did not affect HDL-C levels ($p > 0.05$). The AC group showed lower ($p < 0.05$) TAG levels than the NC group, and no effect of BBR was observed.

*3.3. BBR inhibits fractional dietary cholesterol absorption rate*

The intestinal cholesterol absorption is one of key metabolic pathways determining the circulating cholesterol levels and is important in understanding the mechanisms of action of BBR on cholesterol metabolism. As shown in Fig. 1B, the cholesterol absorption rate of the NC rats was only 16%, but with addition of 0.5% cholic acid, it was increased to approximately 60% ($p < 0.0001$). As a result, the plasma T-C levels were increased ($p < 0.0001$) to 2.5-fold of the NC group. Interestingly, BBR at doses of 50, 100, or 150 mg/kg˙d reduced ($p < 0.0001$) cholesterol absorption relative to the AC control. There were strong correlations between cholesterol absorption rates and plasma T-C ($p = 0.009$) or nonHDL-C ($p = 0.018$) levels, providing further

support to the notion that the inhibition of cholesterol absorption by BBR played a significant role in lowering blood cholesterol levels.

*3.4. BBR decreases cholesterol micellarization*

Following fat digestion and de-esterification of esters, free cholesterol is incorporated into mixed micelles in the intestinal lumen prior to delivering to the absorptive epithelium of small intestine. This step determines the amount of cholesterol available for the uptake at the apical side of the enterocyte. The *in vitro* cholesterol micellarization tests revealed that our positive control, plant stanols, reduced ($p < 0.0001$) cholesterol micellarization at a concentration as low as 0.15 µmol/L. The maximal inhibition (52%) was reached at 0.2 µmol/L of plant stanols ($p < 0.0001$; Fig. 2A). Similarly, BBR reduced ($p < 0.005$) the amount of cholesterol incorporated into micelles in a dose-dependent manner. The maximum reduction (32%) was seen at 10 µmol/L, ($p < 0.0001$, Fig. 2B).

*3.5. BBR reduces cholesterol uptake by Caco-2 cells*

The effect of BBR on cholesterol uptake was performed in Caco-2 cells with two different methods. In the first method, a fluorescent NBD-cholesterol was used as a tracer. The amount of cholesterol taken up by Caco-2 cells was estimated by measuring the fluorescent intensity of intracellular NBD-cholesterol (Fig. 3A). It was found that cholesterol uptake was significantly lower in cells treated with 100 µg/mL of BBR for 20 h and further decreased with the increases of BBR concentration. To confirm this observation, the intracellular cholesterol content was measured directly with a cholesterol assay kit. Similar results were obtained and a significant effect was seen at the dose of 50 µg/mL (Fig. 3B).

*3.6. BBR down-regulates ACAT2 protein expression in Caco-2 cells*

Treatment with BBR at doses of 5, 10, or 15 μg/mL for 21 h markedly reduced ($p < 0.005$) ACAT2 protein levels in Caco-2 cells. There were no significant differences among the three doses. BBR did not affect ACAT1 protein expression (Fig. 4A). In a separate study, Caco-2 cells were treated for 6 h or 24 h with 15 μg/mL of BBR to determine if the treatment efficacy increased with time. Again, BBR reduced ($p < 0.0001$) ACAT2 expression but did not alter ACAT1 protein levels (Fig. 4B). A stronger effect was observed after 24 h compared to 6 h of the treatment.

*3.7. BBR decreases ACAT2 protein expressions in rat small intestine*

To confirm the observed *in vitro* effect of BBR on ACAT2 protein expression, rat small intestines were analysed. Similar to the findings in Caco-2 cells, BBR at doses of 50 and 100 mg/kg˙d decreased ACAT2 protein expression, with no difference being observed between the two doses (Fig. 5). The dose of 150 mg/kg˙d did not show a significant effect. The reason for this is unclear but could be attributed to the large variations observed within groups, the impact of polymorphisms among the rats, or the U-shaped dose response [29].

*3.8.Effect of BBR on ACAT2 gene expression in rat small intestine and Caco-2 cells*

The mRNA levels of ACAT2 in the small intestine were not significantly different between the AC and NC groups, but decreased ($p < 0.05$) by BBR compared to the AC group (Fig. 6A). Similarly, BBR dose-dependently lowered ACAT2 mRNA levels in Caco-2 cells (Fig. 6B).

*3.9. BBR reduces cholesterol permeability through Caco-2 monolayer*

To verify the effect of BBR on ACAT2 expression, we performed a permeability assay using Caco-2 monolayer. BBR decreased the concentration of total cholesterol in the medium of the basolateral chamber while showing no effect on free cholesterol (Fig 7A, B and C). It is interesting to note that cholesterol ester in the medium of the basolateral chamber was decreased by BBR in a dose-dependent manner and in a similar pattern with the changes of total cholesterol concentration. As no cholesterol esters were added in the apical chamber, esters detected in the basolateral chamber medium must be the product of esterification of free cholesterol within the enterocytes. Thus, the results indicate that cholesterol esterification in Caco-2 cells was inhibited by BBR, in accordance with the decreased protein expression of ACAT2.

## 4. Discussion

Rats have a low cholesterol absorption rate compared to humans. Thus, the inclusion of cholic acid in a cholesterol diet has been widely used to improve cholesterol absorption and induce hypercholesterolemia in rats for studying human cholesterol metabolism [30-32]. In agreement with these previous reports, rats fed the diet without cholic acid had a very low cholesterol absorption rate, approximately 16%. When 0.5% cholic acid was added to the diet, cholesterol absorption rate was increased to about 60%, becoming close to that in humans and hamsters [14, 27]. More importantly, the cholesterol profiles of rats without supplementation of cholic acid were quite different from that of humans and by contrast became similar when cholic acid was provided [14]. Using this rat model, we demonstrated that BBR significantly inhibited the intestinal cholesterol absorption, leading to the large decreases of plasma total and nonHDL cholesterol levels. An indirect support has been provided by a recent study showing that BBR

promoted the excretion of neutral sterols [33]. Strong correlations between cholesterol absorption rates and plasma total or nonHDL cholesterol levels provide further support that BBR lowered blood cholesterol levels through inhibiting the intestinal absorption. This is another mechanism that is distinct from the previously reported enhancement of LDLR-mediated liver LDL-C clearance [13].

The small intestine is the only organ responsible for the absorption of dietary and biliary cholesterol, leaving the unabsorbed cholesterol to be excreted in feces and together contributing to the body cholesterol homeostasis [14, 22, 34, 35]. Following intraluminal hydrolysis, free cholesterol is first incorporated into micelles and then transported across the 'unstirred' water layer to the brush border membrane of small intestine. This process has been demonstrated to be essential for the intestinal cholesterol absorption [16]. Thus, cholesterol micellarization assays have been developed and used, together with other methods, to study cholesterol absorption [36], particularly a product or compound that potentially disrupts cholesterol solubilisation in micelles [28]. It is well documented that plant stanols/sterols inhibit cholesterol absorption by interfering with cholesterol incorporation into micelles [14, 36, 37]. Therefore, in the present study we used plant stanols as a positive control to validate the cholesterol micellarization assay, which was further used to assess the effect of BBR. It was found that in the presence of BBR, the content of cholesterol in micelles was significantly decreased. This effect could be explained by the physical-chemical properties of BBR molecule. With both hydrophobic and hydrophilic binding sites, BBR can interact with the corresponding molecules of micelles. The bindings of BBR to hydrophobic and hydrophilic molecules of micelles lead to the formation of agglomerates and subsequently reduce the capacity of micelles to incorporate cholesterol [38, 39]. This property has in return been used in recent years to improve the bioavailability of BBR in mice and rats by

mixing BBR in different emulsions or other similar solutions [40, 41]. Micellar size may affect the diffusion rate of micelles through the unstirred water layer and thus cholesterol absorption [42]. This potential effect was not measured in the present study.

Once delivered to the epithelium of the small intestine, free cholesterol penetrates into the enterocytes from the apical side, esterified, and then secreted out from the basolateral side into the lymphatic system, further into the blood stream and ultimately delivered to the liver. The uptake of cholesterol by enterocyte is determined by the passive penetration and active transport, in addition to the availability of cholesterol to the brush border membrane as mentioned above. In the *in vitro* assay, disruption of cholesterol micellarization less likely occurred as micelles were not used, but instead cholesterol was dissolved in small quantity of ethanol and then mixed in the culture medium. In addition, there was not an unstirred water layer surrounding the cultured Caco-2 cells. The sterol transporters NPC1L1 and ABCG5/8 are not involved in the cholesterol-lowering effect of BBR in hamsters [5]. Thus, the reduction of cholesterol uptake in Caco-2 cells by BBR might be a result of interference with the passive penetration and/or the effect on other sterol transporters and proteins involved in the intraluminal cholesterol influx and efflux. Similar to the effect on micelles, the dual binding properties of BBR could interact with molecules on the enterocyte membranes, resulting in a change of cell membrane properties. This in turn reduced its permeability, leading to a decrease of cholesterol uptake. However, further experiments are warranted to determine how BBR affects cholesterol uptake in the enterocyte.

Following uptake into the enterocyte, the re-esterification of free cholesterol becomes a key step because cholesterol is secreted out from the basolateral side in a form of esters [19]. This step is catalyzed by ACAT. Several studies have shown that inhibition of ACAT expression decreases cholesterol absorption and plasma cholesterol levels [19, 43]. In mammals, two ACAT

genes have been identified, which are referred to ACAT1 and ACAT2 [19]. ACAT1 is a ubiquitous protein and functions in converting cellular cholesterol into cholesteryl ester in response to intracellular cholesterol abundance. ACAT2 is expressed only in hepatocytes and enterocytes and appears to provide cholesteryl esters for transport in lipoproteins. In line with their differential functions, the deletion of ACAT2 is consistently atheroprotective whereas deletion of ACAT1 is varyingly problematic [44-46]. BBR decreased ACAT2 protein expression while having no effect on ACAT1. Further experiments demonstrated that BBR decreased ACAT2 protein expression by downregulating its gene transcription. These results suggest that BBR might be an ACAT2 specific inhibitor in the enterocyte.

To further verify the effect of BBR on ACAT2 protein expression, we have conducted an *in vitro* permeability assay using Caco-2 monolayer. This assay has long been used to predict the *in vivo* absorption and bioavailability of a compound. It was observed that the amount of cholesterol esters in the medium of basolateral chamber was significantly decreased in the presence of BBR and the effect was dose-dependent. A similar effect was seen on the permeability of total cholesterol while no effect was found on free cholesterol. It is indicated by the results that cholesterol esterification in Caco-2 cells was inhibited by BBR, in agreement with the reduced gene and protein expressions of ACAT2 in both the Caco-2 cells and small intestine.

As there were no significant differences of cholesterol-lowering efficacy among the three BBR doses, a lower dose than 50 mg/kg·d might be equally effective. Thus, a dose optimization study should be conducted prior to clinical application. According to the dose translation from animals to humans [47], the dosage of 50 mg/kg·d in rats is equivalent to 567 mg/d for a 70 kg adult, which is highly achievable. Various dosages were used in different *in vitro* assays. For the

micellarization assay, a series of dosage was applied. The results were presented only for those that showed significant effects and a few higher concentrations that showed no further improvement. For cell-based assays, cell toxicity was performed first and then treatment concentrations were chosen within the non-toxic range. These concentrations are physiologically relevant in rats as estimated on the dosages of BBR and water consumption [48], the primary dilutor in the gastro-intestinal lumen, and the bioavailability of BBR [49]. However, a pharmacokinetic study is required to determine whether the concentrations used in the present studies are physiologically relevant.

In human subjects, cholesterol absorption is partially determined by genetic traits and increased body weights were also reported to lower cholesterol absorption efficiency due to increased biosynthesis [5]. In the current study, there was not a huge difference in the body weights of rats fed the AC and NC diets and no significant correlation was found between the body weights and cholesterol absorption rates in rats of these two diet groups. It was suggested that if there was any impact of body weight on cholesterol absorption, the effect might be minimal and confound with the effects of dietary cholesterol and bile acid supplementation. Moreover, the NC was used to determine whether rats fed the AC diet significantly increased blood cholesterol levels and thus validated the experimental model. The primary focus was to determine the efficacy and mechanisms of action of BBR on cholesterol metabolism by comparing among the AC and three treatment groups. The significant difference in blood lipid levels but no significant difference in body weights occurred among the AC and three treatment groups provided meaningful determinations on the mechanism of action of BBR in cholesterol absorption and homeostasis.

The present study investigated the intestinal cholesterol absorption rate, which was

measured in a biology system using the plasma dual isotope ratio method. This method was well-established and cross-validated against several other methods that have long been used to quantitatively measure cholesterol absorption [50]. The rate of cholesterol absorption in a biology system is a result of comprehensive effects of multiple steps involved in the absorption process and a reliable parameter for the effect of a given compound on cholesterol absorption *in vivo*. Further *in vitro* and *in vivo* experiments on these steps using biochemical and molecular approaches consistently supported the inhibitory effect of BBR on cholesterol absorption. The effect of BBR on cholesterol micellarization and absorption could be further evaluated *in vivo* by measuring cholesterol content in the intestinal lumen micelles and lymphatic chylomicrons, respectively, which become the limitations of the present study.

In conclusion, BBR lowered blood cholesterol levels in diet-induced hypercholesterolemic rats at least in part through inhibiting intestinal cholesterol absorption. BBR is an inhibitor of intraluminal cholesterol micellarization and cholesterol uptake by enterocytes. BBR is also an inhibitor of ACAT2 gene and protein expressions in the small intestine, leading to the reduction of cholesterol esterification and secretion, i.e., permeability through the Caco-2 monolayer. In rats, the NC diet is better than the AC plus BBR. However, due to the differences in diet composition and cholesterol metabolism between humans and rats, it is not possible to conclude that the NC diet is a better approach than the AC plus BBR in humans. Generally, the NC diet does not apply in humans, as people, even vegetarians, eat diets containing cholesterol and have high cholesterol absorption rates. This study provides important information for better understanding the mechanism of action of BBR on cholesterol metabolism and homeostasis. Together with previously published data, the results of this study demonstrate that BBR is a promising lipid-lowering agent that may be further developed into a function

ingredient for hyperlipidemic subjects and those who want to maintain healthy lipid profiles.

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**Disclosure statement**

The authors declare that there is no conflict of interests.

**Author contributions**

Y. Wang designed the experiments, assisted animal study, analyzed data, and wrote the manuscript; Y. Xin conducted animal experiment and analyzed blood lipids; K. Ghanam conducted stable isotope analysis for the determination of cholesterol absorption; S. Zhang conducted the experiments for the measurement of gene and protein expressions of ACAT1/2 in rat small intestine tissues and Caco-2 cells; X. Zhu performed the experiments for the determination of cholesterol micellarization; and T. Zhao measured cholesterol uptake and permeability in Caco-2 cells.

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**Figure legends**

**Fig. 1 - Effect of berberine on the plasma lipids (A) and fractional dietary cholesterol absorption rate (B) in rats fed a high-cholesterol and high-fat diet for 8 weeks. NC, normal control; AC, atherogenic control; B50, B100, and B150, rats fed the AC diet supplemented twice a day by gavage feeding with 50, 100, and 150 mg/kg˙d of berberine in water, respectively. Difference between the NC and AC was analyzed using the student's *t*-test. Treatment effect was analyzed using one-way ANOVA and differences among the AC and three treatment groups were determined by pairwise comparisons using the least squares means test. Results are presented as means ±S.E.M (n = 12). $^{*}$Different from the NC group ($p < 0.05$); $^{\dagger}$different from the AC; $^{\ddagger}$different from the AC and B50, $p < 0.05$.**

**Fig. 2 - Effect of BBR on cholesterol micellarization (A). Plant stanols were used to validate the assay (B). Data were analyzed using one-way ANOVA and differences among the various concentrations were determined by pairwise comparisons using the least squares means test. Results are means ± S.E.M (n = 4-7). $^{*}p < 0.0001$ vs. control (0 µmol/L of plant stanols or BBR).**

**Fig. 3 - Effect of berberine on cholesterol uptake in Caco-2 cells. The uptake of cholesterol was quantitated using a fluorescent NBD-cholesterol tracer (A) or directly measured using the Biovision cholesterol assay kits (B). Data were analyzed using one-way ANOVA followed by pairwise comparisons using the least squares means test. The results are**

**presented as means ± S.E.M (n=3). In Fig. 3A, [*]different from 0 and 50 µg/mL; [†]different from 0, 50, and 100 µg/mL, respectively and in Fig. 3B, [*]different from 0 µg/mL, $p < 0.05$.**

**Fig. 4 - Effect of BBR on ACAT1/2 protein expressions in Caco-2 cells treated with different concentrations of BBR for 21 h (A) or treated with 0 and 15 µg/mL of BBR, respectively for 6 or 24 h (B). CT, control of 0 µg/mL of BBR; B5, B10, and B15, treatments with 5, 10, and 15 µg/mL of BBR, respectively. Data were analyzed using one-way ANOVA (Fig. 4A) or two-way ANOVA (Fig. 4B) followed by pairwise comparisons using the least squares means test. Results are presented as means ± S.E.M (n=3). [*]different from the CT, $p < 0.05$.**

**Fig. 5 - Effect of BBR on ACAT2 protein expression in the small intestine of rats fed an atherogenic high-cholesterol and high-fat diet for 8 weeks. AC, atherogenic control; B50, B100, and B150, rats fed the AC diet supplemented twice a day by gavage feeding with 50, 100, and 150 mg/ kg·d of BBR in water, respectively. Data were analyzed using one-way ANOVA followed by pairwise comparisons using the least squares means test. Results are means ± S.E.M (n=6). [*]different from the AC, $p < 0.05$.**

**Fig. 6 - Effect of BBR on the mRNA expression of ACAT2 in the small intestine (A) and Caco-2 cells (B). NC, normal control; AC, atherogenic control; B50, B100, and B150, rats fed the AC diet supplemented with 50, 100, and 150 mg/kg·d of berberine in water, respectively by gavage feeding twice a day. The treatment effects were analyzed using one-way ANOVA followed by pairwise comparisons using the least squares means test. The**

**difference between the AC and NC was determined using the student's *t*-test. The results are presented as means ± S.E.M (n=3). [*]different from the AC group in Fig. 6A and the control of 0 µg/mL in Fig. 6B, $p < 0.05$.**

**Fig. 7 - Effect of BBR on the concentration of total cholesterol (A), free cholesterol (B), and cholesterol esters (C) in the medium of basolateral chamber determined *in vitro* by the cholesterol permeability assay in Caco-2 cells. Data were analyzed using one-way ANOVA followed by pairwise comparisons using the least squares means test. The results are presented as means ± S.E.M (n=4). In Fig. 7A, [&]different from the control of 0 µg/mL; [†]different from 0 µg/mL and 10 µg/mL, respectively and in Fig. 7C, [*]different from the control of 0 µg/mL and 10 µg/mL; [†]different from all other concentrations, $p < 0.05$.**

Figure

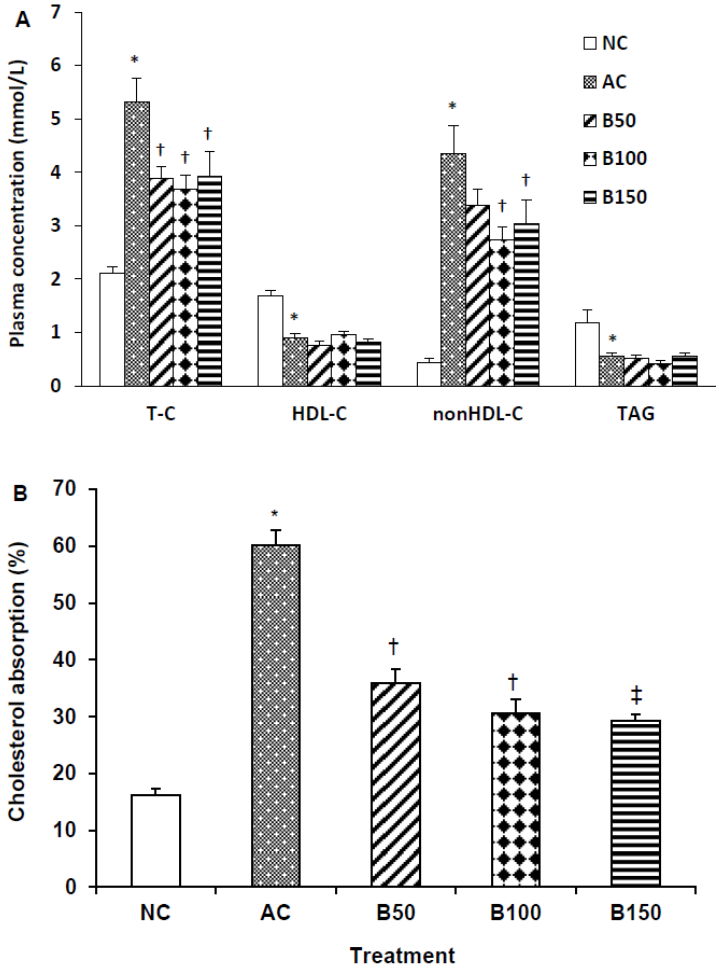


Figure. 1

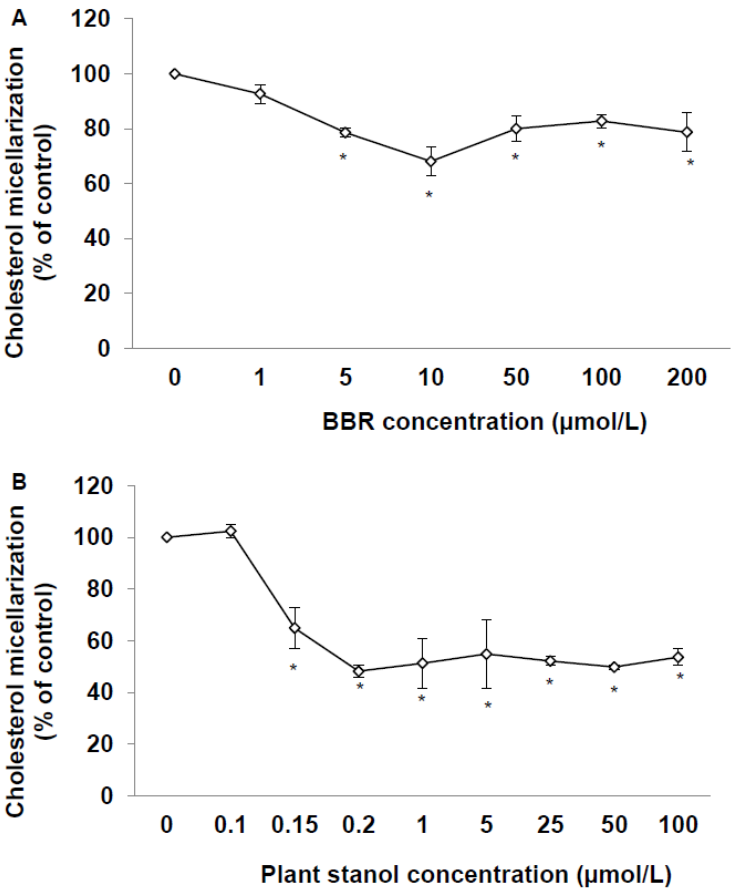


Figure 2

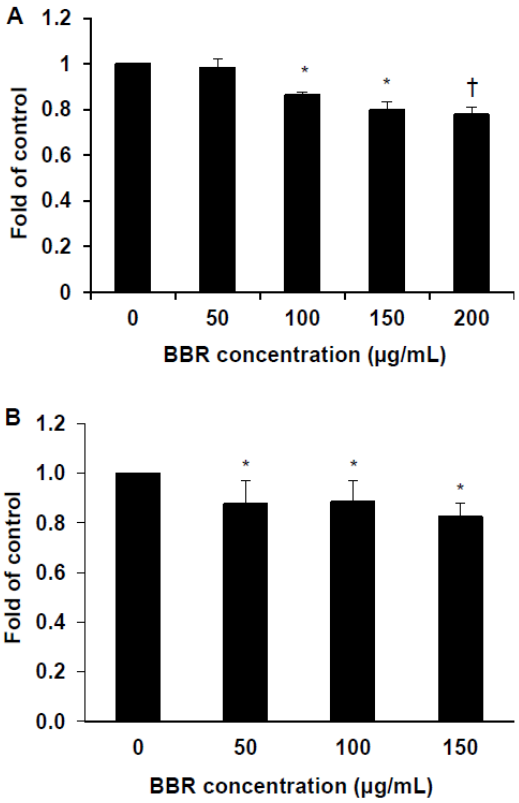


Figure 3

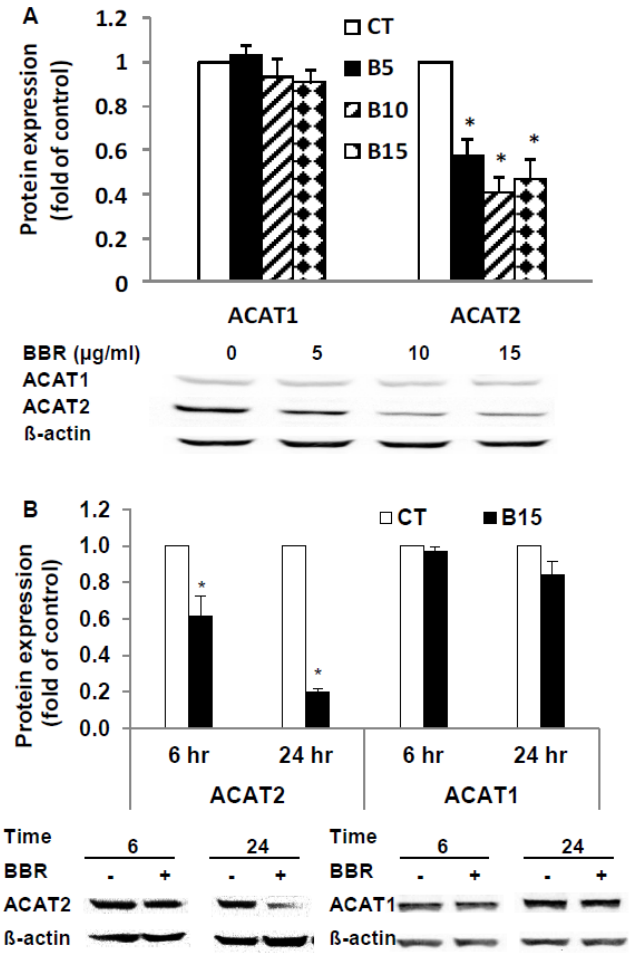


Figure 4

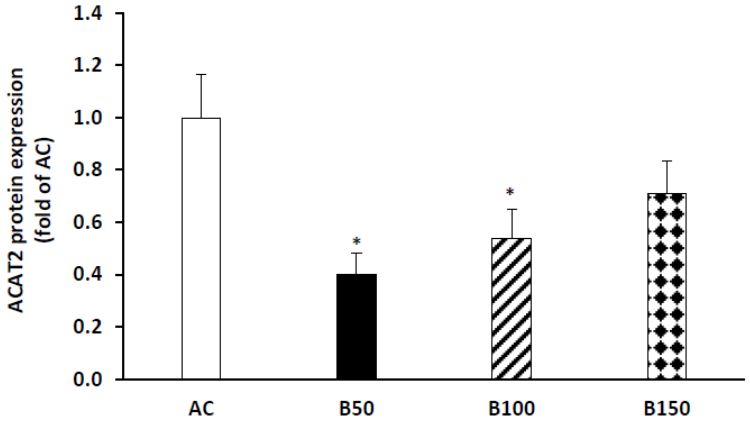


Figure 5

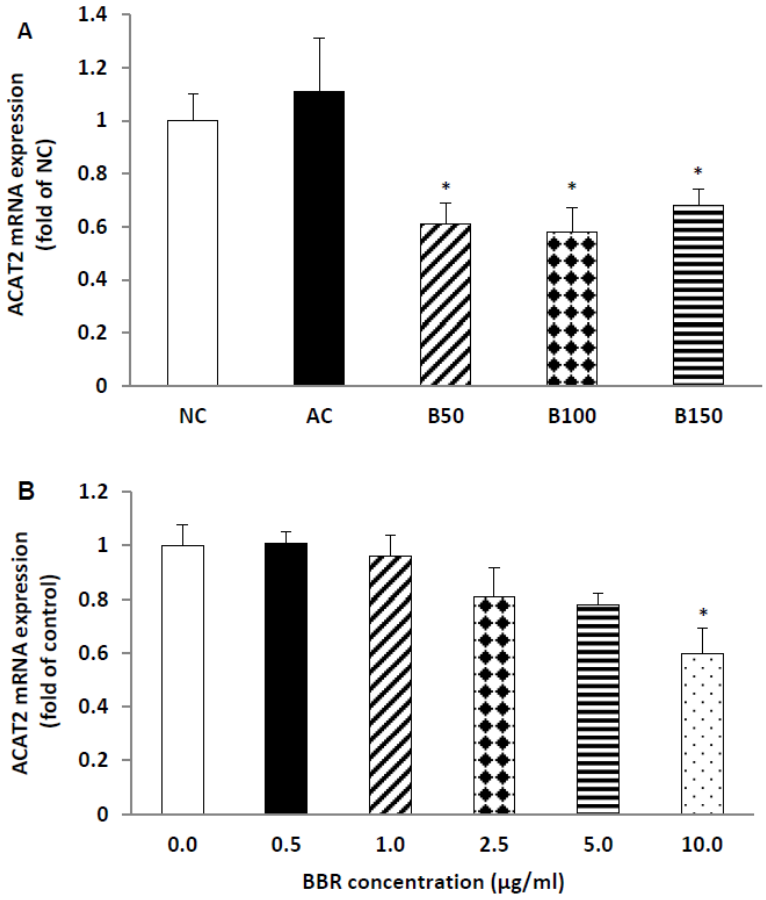


Figure 6

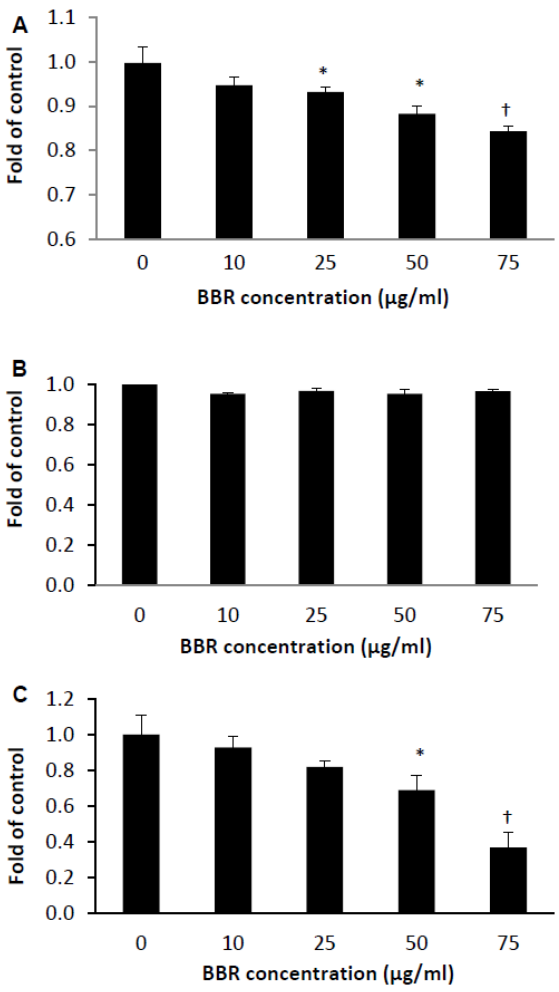


Figure 7