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Co-administration of berberine and plant stanols synergistically reduces plasma cholesterol in rats

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Abstract

The objective of the present study was to determine the beneficial effects and the safety of oral administration of the combination of berberine (BBR) and plant stanols (PS) on plasma lipid profiles in male Sprague–Dawley rats. Four groups of animals were fed a cornstarch–casein–sucrose-based high-cholesterol (2%, w:w) and high-fat (27.5%) diet. Three treatment groups were supplemented with either BBR (100 mg kg^{-1} body weight d^{-1}), PS (1% in diet, w:w), or the combination of both (BBRPS). After 6 wk, animals were sacrificed and followed immediately with the collection of blood and organ samples. Lipid analysis revealed that PS lowered plasma total cholesterol (T-C) by 18% ($p = 0.067$) and non-HDL-cholesterol (non-HDL-C) by 29% ($p = 0.013$) as compared with the control, while BBR had no effect on both T-C and non-HDL-C. The combination treatment of BBRPS reduced plasma T-C by 41% ($p = 0.0002$) and non-HDL-C by 59% ($p < 0.0001$) compared to the control group. BBR reduced plasma TG levels by 31% at a marginal significance relative to the control ($p = 0.054$), whereas PS had no effect. BBRPS showed an additive effect of BBR and PS on plasma TAG. PS and BBRPS both decreased liver cholesterol ($p = 0.0027$ and 0.0002, respectively). BBR and PS, either alone or in combination, did not show any toxic effects as assessed by plasma concentration of hepatic biochemical parameters. These results demonstrate that BBR and PS, when combined, synergistically lower plasma cholesterol levels and significantly reduce liver cholesterol, without the observation of any toxic effects.

Keywords: Berberine; Plant stanols; Plasma cholesterol; Plasma triacylglyceride; Liver cholesterol; Toxicity; Rats

1. Introduction

Cardiovascular disease is the major cause of morbidity and mortality in Western population [1]. Abundant evidence in humans demonstrates that elevated total cholesterol (T-C) and LDL-cholesterol (LDL-C), together with decreased HDL-cholesterol (HDL-C) are primary risk factors that are positively associated with the development and progression of atherosclerosis and coronary heart disease [2–7]. Elevated plasma triacylglycerides (TAG) is an independent risk factor for developing cardiovascular disease [8–11]. Therefore, the development of strategies that reduce both cholesterol and TAG levels has generated a considerable interest to combat hyperlipidemia-associated cardiovascular disease.

The level of circulating cholesterol is a function of cholesterol input less its output, which is determined collectively by multiple pathways involved in cholesterol metabolism, including absorption, synthesis, clearance and excretion.

Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; BBR, berberine chloride; BBRPS, combination of BBR and plant stanols; BW, body weight; CT, control; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; non-HDL-C, non-HDL-cholesterol (VLDL-cholesterol + intermediate density lipoprotein cholesterol + LDL-cholesterol); PS, plant stanols; T-C, total cholesterol; TAG, triacylglycerides.

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Alterations in any one of these pathways affect cholesterol homeostasis resulting in changes in plasma cholesterol levels [12–14]. A higher efficacy would be expected if multiple pathways were altered favourably toward decreases of cholesterol input and/or increases of cholesterol output/excretion. Drug therapy, especially the use of statins, is highly beneficial to the hyperlipidemic populations through lowering blood cholesterol and to a lesser extent, lowering TAG levels [15,16]. However, severe side effects are known to be associated with the use of the statin class of lipid-lowering medications [14,17,18] and in addition, a subgroup of the population responds poorly to statin drugs [19]. With the advent of functional foods and nutraceuticals, an increasing number of patients with moderately elevated blood lipids, or clinical hyperlipidemia, are now searching for natural products or diet interventions, as alternatives to the use of cholesterol-lowering drugs, to optimize their blood lipid levels. Phytosterols and their derivatives are the most widely used natural products for lowering blood cholesterol [12,20–22]. Phytosterols lower plasma cholesterol by inhibiting intestinal cholesterol absorption [12,23–25]. However, the cholesterol-lowering efficacy of phytosterols is low compared to cholesterol drugs [15,16]. In addition, phytosterols have no effect on plasma TAG levels [12,20–22]. Therefore, it is important to develop novel natural products or formulations that possess higher cholesterol-lowering efficiencies and are also able to reduce plasma TAG as well.

Recently, increased interest in understanding the cardioprotective effect of plant alkaloid, berberine (BBR) has arisen, especially in terms of its lipid-lowering properties. Previous studies demonstrated that the cholesterol-lowering efficacy of BBR was higher than most of current natural products but moderate compared to statin drugs [13,26,27]. BBR lowers plasma cholesterol through upregulating LDL-receptor mediated cholesterol clearance [13]. In addition, BBR has also reported to reduce plasma TAG levels in humans and animals [13,26,27]. Because BBR and phytosterols reduce cholesterol via distinct mechanisms, it was hypothesized that the combination of BBR and PS would synergistically lower blood cholesterol. It was also expected that BBR and phytosterols would decrease plasma TAG levels when provided in combination. Because plant sterols, stanols, and their ester forms possess similar capacities to lower circulating cholesterol [23–25,28–37], in the present study we have chosen the stanol form of phytosterols (PS) to determine the effect of combined treatment of BBR and PS on plasma cholesterol and TAG concentrations in rats fed a high-fat and high-cholesterol diet. The safety/toxicity of BBR and PS was evaluated by hepatic biochemical parameters that are routinely used in basic toxicological research and the clinical diagnosis of drug/chemical-induced toxicity. The effect of BBR and PS either alone or in combination on liver cholesterol content and major organ weights relative to body weight was also investigated.

Table 1
Composition of control diet

Ingredient[a]	Amount (g/kg diet)
Casein	196.1
Corn starch	98.0
Sucrose	313.7
Oil	274.5
Cellulose	42.2
DL-methionine	4.9
AIN-93G mineral mix[b]	39.2
AIN-93-VX vitamin mix[b]	9.8
Choline bitartrate	2.0
Cholesterol	19.6
Butylated hydroxytoluene	0.2

[a] All food ingredients were purchased from MP Biomedicals (Solon, OH, USA).
[b] Composition of AIN-93G mineral mix and AIN-93-VX vitamin mix is the same as reported previously [70].

2. Materials and methods

2.1. Animals and diets

Forty male Sprague–Dawley rats (Charles River Laboratories, Montreal, Que., Canada), weighing 150–170 g, were housed individually in cages with a 12 h light cycle. Animals were fed regular rodent chow with free access to food and water. After 1 wk of adaptation, rats were weighed and randomly divided into four groups ($n = 10$). Control animals (CT) were given a semi-purified cornstarch–casein–sucrose based diet with 2% cholesterol and 27.5% fat in a form of mixture of beef tallow:sunflower oil (96:4, v:v; Table 1). The three treatment groups were fed the control diet supplemented with either BBR (100 mg kg^{-1} body weight (BW) d^{-1}), PS (1% in diet, w:w), or the combination of BBR and PS (BBRPS). BBR was dissolved in tap water and orally administered by gavage feeding twice a day. The control and PS groups were gavaged with a control vehicle of tap water. Diets were prepared weekly and stored at 4 °C. The purity of BBR was >98% (Sigma–Aldrich, Ont., Canada). The purity of PS was >92% (82% sitostanols and 10% campestanols; provided by the Forbes-Medi-Tech Inc., BC, Canada). The animal use and experimental procedures were approved by the Joint Animal Care and Research Ethics Committee of the National Research Council Canada—Institute for Nutrisciences and Health and the University of Prince Edward Island. The study was conducted in accordance with the guidelines of the Canadian Council on Animal Care.

After 6 wk on the experimental diets, rats were sacrificed and blood was collected into EDTA-tubes and immediately placed on ice. Plasma was separated by centrifugation and stored at −80 °C. The major organs were carefully dissected and weighed. Liver was frozen in liquid nitrogen and stored at −80 °C for the measurement of liver cholesterol and TAG content.

2.2. Analysis of plasma lipids

Plasma T-C, HDL-C and TAG were measured in triplicate by enzymatic methods using a Pointer 180 Analyzer (Pointe Scientific Inc., Canton, MI, USA). HDL-C was measured after the precipitation of apolipoprotein-B containing lipoproteins with dextran sulfate and magnesium chloride [38]. Because the Friedewald equation may not be applicable in rats, non-HDL-C (VLDL-cholesterol + intermediate density lipoprotein cholesterol + LDL-cholesterol) instead of LDL-C was used and calculated by subtracting HDL-C from T-C [39].

2.3. Analysis of liver lipids

Liver lipids were extracted using the method described previously [40]. Briefly, 0.5 g of liver was weighed into a 50 mL glass tube with addition of 15 mL methanol. The tubes were shaken at 55 °C for 15 min and added with 24 mL of hexane: chloroform (4:1, v:v) and 2 mL of water. After 15 min shaking, samples were separated by centrifugation and the supernatant was collected. The extraction was repeated three times and supernatants were pooled together. After drying up under nitrogen gas, the lipids were re-dissolved in ethanol and chloroform mixture (2:1). The total cholesterol and TAG were measured using commercial enzymatic kits (Wako Chemicals USA, Inc., Richmond, VA, USA).

2.4. Measurement of plasma concentration of hepatic biochemical parameters

The toxicity of BBR and PS was assessed by measuring plasma concentration of several blood biochemical parameters. These parameters include alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamic transpeptidase (GGT), total bilirubin, free bilirubin, conjugated bilirubin, and albumin. Plasma level of these enzymes, different forms of bilirubin, and albumin was analyzed using a Roche Cobas C501 biochemistry analyzer (Roche Diagnostics, Indianapolis, IN, USA) in the Atlantic Veterinary College—Diagnostic Services at the University of Prince Edward Island, Charlottetown, PE, Canada.

2.5. Data analysis

Statistical analyses were performed using SAS 9.0. One-way ANOVA was used to analyze the overall effect of treatments on plasma and liver lipids, relative organ weight to body weight, and the plasma concentration of hepatic biochemical parameters. When a significant treatment effect was obtained, the least squares means test was used to test differences between treatment groups. Relationships between plasma lipid concentrations and liver cholesterol and TAG were analyzed using Pearson's correlation coefficients. Significance level was set at $p < 0.05$. Data are presented as means ± S.E.M.

3. Results

3.1. Body weight and food intake

The food intake in each week was not affected by either treatment. The average food intake was 19.5, 18.1, 19.8, and 19.2 g d^{-1} during the first week and 20.5, 19.4, 21.2, and 20.7 g d^{-1} during the last week (wk 6) for the CT, BBR, PS, and BBRPS groups, respectively. Similarly, body weight did not differ among the four groups. The average body weight was 203.8, 202.7, 205.8, and 204.3 g at the beginning of treatment and 417.8, 423.0, 436.8, and 424.6 g after 6 wk of treatment for the CT, BBR, PS, and BBRPS groups, respectively.

3.2. Plasma lipid profiles

In comparison to the control, BBR did not affect either plasma T-C or non-HDL-C levels in rats after 6-wk of feeding a high-cholesterol and high-fat diet (Table 2). PS lowered plasma cholesterol by 18% ($p = 0.067$) and non-HDL-C by 29% ($p = 0.013$). Dramatic reductions were observed in both T-C and non-HDL-C after BBRPS supplementation. BBRPS decreased T-C by 41, 38, and 28% compared to the control ($p = 0.0002$), BBR ($p = 0.0009$), and PS ($p = 0.02$), respectively. BBRPS lowered non-HDL-C by 59, 57, and 41% as compared with the control ($p < 0.0001$), BBR ($p < 0.0001$), and PS ($p < 0.05$), respectively. The level of plasma HDL-C did not differ among the four groups. BBR reduced

Table 2
Effect of berberine and plant stanols alone and in combination on plasma lipids in rats

Treatment	Total cholesterol (mmol/L)	HDL-cholesterol (mmol/L)	Non-HDL-cholesterol (mmol/L)	Triacylglyceride (mmol/L)
CT	2.93 ± 0.19^{a}	0.75 ± 0.05	2.19 ± 0.17^{a}	2.21 ± 0.27^{a}
BBR	2.81 ± 0.25^{a}	0.71 ± 0.07	2.10 ± 0.18^{a}	1.53 ± 0.13^{b}
PS	2.41 ± 0.24^{b}	0.86 ± 0.08	1.55 ± 0.20^{b}	2.47 ± 0.34^{a}
BBRPS	1.74 ± 0.12^{c}	0.83 ± 0.06	0.91 ± 0.08^{c}	1.83 ± 0.22^{ab}

Data were analyzed using one-way ANOVA. Differences between treatment groups were further analyzed using the least squares means test after a significant treatment effect was detected. Values are means ± S.E.M. ($n = 10$). Values bearing different superscript letters (a, b, and c) are different ($p < 0.05$). CT, Control diet; BBR, the control diet supplemented with 100 mg kg^{-1} body weight d^{-1} of BBR; PS, the control diet supplemented with 1% (w:w) of plant stanols; BBRPS, combination of BBR (100 mg kg^{-1} body weight d^{-1}) and PS (1% plant stanols).

Table 3
Effect of berberine and plant stanols alone and in combination on liver cholesterol and triacylglyceride content in rats

Treatment	Cholesterol (mg/g)	Triacylglyceride (mg/g)
CT	48.79 ± 3.11[a]	93.2 ± 8.62
BBR	44.39 ± 3.61[a]	82.47 ± 6.66
PS	34.08 ± 3.19[b]	83.88 ± 10.86
BBRPS	29.54 ± 2.44[b]	92.69 ± 14.6

Data were analyzed using one-way ANOVA. Differences between treatment groups were further analyzed using the least squares means test when a significant treatment effect was obtained. Values are means ± S.E.M. ($n = 7$). Values bearing different superscript letters (a and b) are different ($p < 0.05$). For the description of treatments see Table 2.

plasma TAG levels by 31%, which differed from the control at a marginal significance level as compared with the control ($p = 0.054$). PS increased plasma TAG levels by 12% whereas the combination of BBR and PS (BBRPS) reduced TAG by 17%, which appeared to be the net sum of the effects induced by BBR and PS administered alone. However, all these effects were not different from the control as large variations within the treatment groups were observed.

3.3. Liver lipids

The effects of BBR and PS alone and in combination on liver cholesterol and TAG concentrations are presented in Table 3. As compared to the control, BBR did not affect cholesterol content in the liver although there were marginal reductions (−9%). PS lowered liver cholesterol by 30% ($p < 0.005$) compared to the control and 23% relative to BBR ($p < 0.05$). Further reductions were observed in animals treated with BBRPS. Rats supplemented with BBRPS had liver cholesterol that was 39% lower than controls ($p = 0.0002$) and 33% lower than those treated with BBR ($p < 0.003$). The cholesterol content of the liver was significantly correlated with plasma T-C ($r = 0.62$, $p < 0.05$) and non-HDL-C levels ($r = 0.69$, $p < 0.02$). The TAG content of the liver was not significantly affected by any treatment and not correlated with plasma TAG levels.

3.4. Relative weight of the major organs to body weight

Rats treated with BBRPS had significantly lower ($p < 0.05$) liver weights than control rats or those treated with BBR or PS. The liver weight relative to body weight was 4.4, 4.3, 4.2, and 3.8% for the CT, BBR, PS, and BBRPS groups, respectively. The relative spleen weight was also decreased ($p < 0.05$) after BBRPS treatment, which was 0.17, 0.17, 0.17, and 0.15% for the CT, BBR, PS, and BBRPS, respectively. The relative weight of heart and kidney was not affected by any treatment compared to the control, and ranged from 0.57 to 0.59% for kidney and 0.26 to 0.27% for the heart.

3.5. Plasma concentration of hepatic biochemical parameters

Plasma concentration of liver enzymes, ALT, AST, and ALP was not affected by BBR, PS, or BBRPS. The values were 41.0, 50.5, 41.9, and 44.2 U/L for ALT, 112.4, 115.2, 103.1, and 110.1 U/L for AST, and 1.4, 1.2, 0.9, and 1.5 U/L for ALP for the CT, BBR, PS, and BBRPS groups, respectively. GGT was undetectable in most samples. Similarly, none of the three treatments influenced the plasma levels of total bilirubin, free bilirubin, conjugated bilirubin, and albumin (data not shown).

4. Discussion

The cholesterol-lowering property of BBR was initially observed in atherosclerotic human aortic intimal cells [41] and later in rodents [13,26,42,43] and humans [13]. However, the current study did not reveal a significant effect of BBR on plasma cholesterol in rats fed a semi-synthetic, high-cholesterol and high-fat diet for 1.5 months. This discrepancy may have resulted from the differences in the diet composition, especially dietary cholesterol and fat levels, bile acid supplementation, and the time of BBR administration between the present study and previous studies [13,26,42,43]. It could also be due to the differences in cholesterol metabolism between humans and animal species [44–46]. In addition, it should be noted that the effect of BBR on blood cholesterol in rats was previously examined only in disease models such as diabetes or nephritis [42,43,47]. Response of plasma lipids to BBR treatment in these disease models may be different from that in healthy animal models.

In agreement with previous studies [21,24,25,30,31], dietary supplementation of PS significantly decreased plasma T-C and non-LDL-C levels. The most significant finding in the current study is that BBRPS dramatically reduced plasma T-C and non-HDL-C. This outcome appeared to be a result of a synergistic action of BBR and PS as the effect of the combined treatment was much greater than the sum of the net effect of BBR and PS alone. Similarly, liver cholesterol content was significantly reduced after the supplementation of PS or BBRPS. Reductions of liver cholesterol would result in a lesser amount of cholesterol secreted in the form of VLDL into circulation and thus lower plasma cholesterol. The decreased liver cholesterol content by PS supplementation might be a result of reduced cholesterol input resulting from the inhibition of intestinal cholesterol absorption [48] and/or increased output from enhanced biosynthesis of bile acids and secretion of cholesterol and bile salts [49,50]. BBR has been reported to have no effect on cholesterol synthesis but upregulates LDL-receptor mediated clearance, resulting in the reduction of plasma and liver cholesterol [13,26]. Therefore, it was hypothesized that the combined treatment of BBRPS would lower plasma and liver cholesterol synergistically by upregulating LDL-cholesterol clearance in the liver

and inhibiting cholesterol absorption in the intestine. However, because BBR did not affect plasma and liver cholesterol in the current study, we speculate that the combined treatment of BBRPS reduced cholesterol in the plasma and liver by inhibiting intestinal cholesterol absorption through a synergistic manner.

Supplementation of BBR reduced plasma TAG at a marginal significance level compared to the control. Reductions of plasma TAG by BBR were also observed in other studies in rats with diabetes and nephritis [42,43,47]. Results from several in vitro studies have demonstrated that BBR decreases TAG levels through regulating fatty acid metabolism toward the downregulation of adipogenesis [51–53]. BBR upregulates the phosphorylation of the AMP-activated protein kinase (AMPK), which phosphorylates and inactivates acetyl-CoA carboxylase (ACC) [51,52]. The inactivation of ACC results in the inhibition of fatty acid synthesis but the enhancement of fatty acid oxidation. In addition, BBR downregulates the expression of adipogenic enzymes and transcription factors [53]. These effects collectively lead to the inhibition of TAG synthesis and consequently the reduction of plasma TAG levels. A great body of evidence demonstrates that PS supplementation has no effect or tends to increase plasma TAG levels [21,24,25,29–32,54–57]. When BBR was co-administered with PS, an additive effect of BBR and PS on plasma TAG was observed. Therefore, the combined use of PS with BBR may reduce plasma TAG and/or combat the tendency of increase of plasma TAG induced by PS supplementation.

Measurement of hepatic biochemical parameters in plasma or serum has been widely used in basic toxicological research and clinical toxicity testing [58,59]. These parameters can provide highly valuable information in assessing not only the extent and severity of liver damage, but also the type of liver damage [58]. ALT and AST are the most commonly used indicators of liver (hepatocellular) damage [58–60]. BBR, PS, or BBRPS did not induce liver and other tissue damage as indicated by the insignificant effect of either treatment on the concentration of AST and ALT in the plasma. ALP and GGT are considered cholestatic-induction enzymes of hepatobiliary tissue and are widely employed to detect impaired bile flow [61]. Plasma levels of total bilirubin, free bilirubin, and conjugated bilirubin are used to detect cholestasis in combination with ALP and GGT. BBR, PS and BBRPS did not induce significant changes in the plasma concentration of ALP and GGT and any form of bilirubin measured. In addition, albumin is the major protein in the blood and is only synthesized by the liver. As such, it is often used to test liver function. BBR, PS, and BBRPS did not alter plasma concentration of albumin, suggesting that none of the treatments affected liver function. The results collectively suggest that supplementation of BBR and PS either alone or in combination for 6 wk is not toxic in rats under the experimental conditions employed in the present study.

Although liver TAG was unaffected, liver cholesterol was significantly reduced after BBRPS treatment, suggesting that the decrease of liver lipid storage contributed at least in part to the liver weight reduction by BBRPS. Similarly, a previous study demonstrated that feeding hamsters with a high-fat diet induced massive accumulation of lipids in hepatocytes and treatment of hamsters with BBR-containing extract from goldenseal significantly reduced lipid storage in the liver by decreasing both cholesterol and TAG [26]. The reduction of liver lipids is beneficial to liver function by restoring hepatocyte morphology and reduction of liver steatosis [26]. Published data indicated that BBR and PS, when administered alone, have beneficial effects on the immune function [62–66]. As it was not measured in the current study, it is not clear whether reduced spleen weight by the combined treatment of BBRPS influences the immune function.

A dose of 1% PS in the diet employed in the current study was used in several animal studies to determine the effect of PS on plasma cholesterol [57,67]. In humans, 1.8–2 g d^{-1} was recommended to lower blood cholesterol [12,68]. According to a recent publication on the dose translation from animal to human studies [69], 100 mg kg^{-1} BW d^{-1} of BBR in rats is equivalent to 16.7 mg kg^{-1} BW d^{-1} in humans and 1.2 g d^{-1} for a subject with 70 kg of body weight. In fact, a recent study demonstrated that BBR at a dose of 0.5 g twice a day significantly decreased plasma lipids and improved liver function in hypercholesterolemic subjects after a 3-month supplementation [13].

In conclusion, BBR alone was ineffective in lowering plasma cholesterol but reduced TAG levels in rats fed a high-fat and high-cholesterol diet. PS significantly decreased plasma cholesterol, especially non-HDL-C but had no effect on plasma TAG. The most interesting observation in the present study is that BBR and PS, when combined, markedly decreased plasma cholesterol through which appeared to be a synergistic action and produced an additive effect on plasma TAG reduction. The combination of BBR and PS also lowered liver cholesterol and liver weight. No toxic effects were observed after BBR, PS, or BBRPS treatments. Results of this study demonstrate that the combination of BBR and PS is potentially highly valuable in the field of the development of novel and efficacious natural products to effectively lower blood lipids, in particular, cholesterol without any apparently toxic effects.

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