Berberine enhances antidiabetic effects and attenuates untoward effects of canagliflozin in streptozotocin-induced diabetic mice

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Available online 20 Jul., 2016

[ABSTRACT] The present study aimed at determining whether berberine can enhance the antidiabetic effects and alleviate the adverse effects of canagliflozin in diabetes mellitus. Streptozotocin-induced diabetic mice were introduced, and the combined effects of berberine and canagliflozin on glucose metabolism and kidney functions were investigated. Our results showed that berberine combined with canagliflozin (BC) increased reduction of fasting and postprandial blood glucose, diet, and water intake compared with berberine or canagliflozin alone. Interestingly, BC showed greater decrease in blood urea nitrogen and creatinine levels and lower total urine glucose excretion than canagliflozin alone. In addition, BC showed increased phosphorylated 5' AMP-activated protein kinase (pAMPK) expression and decreased tumor necrosis factor alpha (TNFα) levels in kidneys, compared with berberine or canagliflozin alone. These results indicated that BC was a stronger antidiabetic than berberine or canagliflozin alone with less negative side effects on the kidneys in the diabetic mice. The antidiabetic effect was likely to be mediated by synergically promoting the expression of pAMPK and reducing the expression of TNFα in kidneys. The present study represented the first report that canagliflozin combined with berberine was a promising treatment for diabetes mellitus. The exact underlying mechanisms of action should be investigated in future studies.

[KEY WORDS] Canagliflozin; Berberine; Diabetes mellitus; AMP-activated protein kinase; Sodium-glucose cotransporter-2

Introduction

The incidence of hyperglycemia and diabetes increases globally, driven by population growth, aging, and increasing age-specific prevalence [1]. Diabetes mellitus is associated with classic diabetic syndromes and concomitant complications, such as cardiopathy, retinopathy, and nephropathy. These syndromes do not only cause severe physical pains and psychological distresses in patients but also bring about huge economic burdens to the patients, family and community. Hyperglycemia remains a main detriment and is the first complication required to be strictly controlled in the management of diabetes. Insulin supplement is one of the most effective methods to reduce hyperglycemic effects and normalize other syndromes of diabetes [2]. However, some patients with diabetes cannot tolerate this treatment [3], and long-term injection of exogenous insulin may cause inconvenience to some patients.

Canagliflozin (Pubchem CID: 24812758) is a newly developed treatment for type 2 diabetes whose mechanism of action is independent of insulin; instead, it involves the inhibition of sodium-glucose cotransporter-2 (SGLT2) in
kidneys, blocking the reabsorption of renal glucose \(^4\). Although canagliflozin alone shows effective hypoglycemic effects, the frequently reported adverse events still occur, such as female genital mycotic infections, urinary tract infections, increased urination, and severe renal impairment \(^5\). Therefore, the independent use of canagliflozin may complicate the treatment of diabetic nephropathy, a frequently occurring complication. Canagliflozin combined with metformin \(^6\), sulfonlurea \(^7\), insulin \(^8\), and pioglitazone \(^9\) is used to treat diabetes, demonstrating good tolerances and compatibilities. However, these combinations cannot completely attenuate the adverse effects of canagliflozin.

Berberine (Pubchem CID: CID 2353) is one of the isoquinoline alkaloids from a traditional Chinese medicine with broad-spectrum antimicrobial properties and antidiabetic activities \(^10-11\). Berberine seems to be able to improve kidney functions \(^12\). Therefore, we suspected that the combination of berberine and canagliflozin (BC) could enhance the antidiabetic properties with reduced adverse effects in diabetic individuals. In the present study, we investigated the synergic effects of BC on streptozotocin (STZ)-induced diabetic mice.

Materials and methods

Animals

Male 4-week-old NIH mice were purchased from Guangdong Medical Laboratory Animal Center, Foshan, China. The animals were housed in an environmentally controlled breeding room (temperature, 20 ± 2 °C; humidity, 60% ± 5%; dark/light cycle, 12 h/12h). The mice were fed with standard laboratory chow and water ad libitum. The experiment was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committee of Tsinghua University, Beijing, China. The protocol was approved by the Animal Welfare and Ethics Committee of Tsinghua University. All animals were fasted from 09 : 00 h to 15 : 00 h before the experiments.

Animal experimental procedures

Male NIH mice (weighing 18 ± 2 g) that were fasted for 24 h were used as diabetic models with intraperitoneal injection of 100 mg·kg\(^{-1}\) STZ (Sigma-Aldrich, St. Louis, MO, USA). One week after STZ injection, the diabetic animals were divided into five groups (\(n=10\)/group): normal and diabetic control mice, berberine (100 mg·kg\(^{-1}\)·d\(^{-1}\), Shanghai Biochempartner Co., Ltd., Shanghai, China), canagliflozin (100 mg·kg\(^{-1}\)·d\(^{-1}\), Shanghai Biochempartner Co., Ltd., Shanghai, China), and berberine combined with canagliflozin (BC, 100 mg·kg\(^{-1}\)·d\(^{-1}\) of canagliflozin plus 100 mg·kg\(^{-1}\)·d\(^{-1}\) of berberine). The drugs were freshly prepared and orally administered at 0.1 mL/10 g body weight twice a day. Normal and diabetic control mice were treated with identical volumes of distilled water. Diet and water intakes were periodically measured in metabolic cages within 24 h. Urinal excretions were periodically collected in metabolic cages overnight and then stored at −80 °C for further analysis. Blood glucose was assayed once a week. After 4 weeks of drug administration, the animals were fasted for 6 h and then subjected to slight anesthesia by intraperitoneal injection of 10% urethane (10 g·100 mL\(^{-1}\) in phosphate-buffered saline, PBS) at a dosage of 0.1 mL/10 g body weight. Blood samples were collected from the orbital venous plexus, and sera were instantly isolated for further biochemical assays. The animals were terminated by cervical dislocation, and kidneys were collected and weighed. The left kidneys were frozen transiently in liquid nitrogen and stored at −80 °C for Western blot and biochemical assays. The right kidneys were fixed in 10% formalin-containing PBS for immunohistochemistry (IHC) assays.

Oral glucose tolerance test (OGTT)

OGTT was conducted as previously described with slight modifications \(^13\). After 4 weeks of drug administration, six mice from each group were randomly selected and fasted for 6 h. Blood samples were collected from the tail veins to determine the blood glucose levels at 0, 0.5, 1, and 2 h after glucose (2.5 g·kg\(^{-1}\)) administration. Blood glucose concentration was analyzed using a blood glucose meter (ACCU-CHEK®, Roche, German). The area under the curve (AUC) of blood glucose and time was calculated as \(AUC_{0-2h} = [(G_{0h} + G_{0.5h})/2 + (G_{0.5h} + G_{1h})/2 + (G_{1h} + G_{2h})/2]\), where \(G\) is the blood glucose value.

Biochemical analysis

Blood glucose, triglycerides, total cholesterol, low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), urea nitrogen (BUN), and creatinine (Cre) levels were analyzed using common clinical diagnostic kits (Biosino Bio-technology and Science Inc., Beijing, China). Serum insulin level was assayed according to the method of ELISA (Westang Biotechnology Co., Ltd., Shanghai, China). Malondialdehyde (MDA) level and superoxide dismutase (SOD) activity in kidneys were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Western blot and IHC analyses

For Western blot analysis, the left kidneys of six randomly selected mice in each group were obtained. Briefly, 10% of the whole left kidney homogenates in PBS were centrifuged at 10 000 r·min\(^{-1}\) at 4 °C for 10 min. The supernatants were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween-20 (\(V/V\), PBST) for 1–2 h and incubated with primary antibody (5' AMP-activated protein kinase \(\alpha\), AMPK; phosphorylated AMPK, pAMPK; Glyceraldehyde 3-phosphate dehydrogenase, GAPDH; Cell Signaling TECHNOLOGY®) in 2.5% nonfat dry milk/PBST at 4 °C overnight. After rinsing thrice with PBST, the membranes were subjected to slight anesthesia by intraperitoneal injection of 10% urethane (10 g·100 mL\(^{-1}\) in phosphate-buffered saline, PBS) at a dosage of 0.1 mL/10 g body weight. Blood samples were collected from the orbital venous plexus, and sera were instantly isolated for further biochemical assays. The animals were terminated by cervical dislocation, and kidneys were collected and weighed. The left kidneys were frozen transiently in liquid nitrogen and stored at −80 °C for Western blot and biochemical assays. The right kidneys were fixed in 10% formalin-containing PBS for immunohistochemistry (IHC) assays.
were incubated with their corresponding secondary antibodies (Abcam®) conjugated with horseradish peroxidase (HRP) for 1–2 h at room temperature. After rinsing four times, the membranes were used to visualize the blots through chemiluminescence (KPL, Inc., Gaithersburg, MD, USA).

IHC analysis was performed as previously described with slight modifications [13]. Briefly, the right kidneys were fixed in 10% formalin-containing PBS for 3 days. Subsequently, the kidney tissues were embedded in paraffin and sectioned at 5-µm thicknesses. The tissues were deparaffinized and rehydrated, and antigen retrieval was conducted in water bath at 100 °C. The sections were blocked with 3% H2O2 and transferred to 10% goat serum in PBS. The sections were then incubated with anti-TNFα primary antibody (Santa Cruz Biotechnology®) at 4 °C overnight. Subsequently, the sections were incubated with HRP- conjugated secondary antibody and exposed to diaminobenzidine until a brown color appeared. Cell nuclei were counterstained with hematoxylin. After dehydration and stabilization with mounting medium, the stained sections were examined under a light microscope (ECLIPSE TE2000-E, Nikon Corporation, Tokyo, Japan) (100–200x). The intensity of the brown color (grey density values) was mainly confined in the renal glomeruli and analyzed using the Image J software (http://imagej.nih.gov/ij/).

Statistical analysis

The data were expressed as mean ± SD, and the mean values for the groups were compared using one-way ANOVA. Newman-Keuls test was used to determine the source of significant differences. *P < 0.05 indicated statistical significance.

Results

Effects of BC on body weight, diet, and water intake

The STZ-induced diabetic mice showed a significant decrease in body weight but a significant increase in dietary and water intake, compared with the normal controls (Fig. 1). Canagliflozin did not exert significant effects on body weight, compared with the vehicle control. However, berberine reduced the body weight of mice compared with the vehicle control. Although BC decreased the body weight on the 14th and 17th day of administration, no significant decrease was observed on the other days. In addition, the diabetic mice exhibited a significant increase in kidney weight per body weight (kidney index), compared with the normal controls. However, all the tested drugs inhibited the increase in kidney index, compared with the vehicle control. The administration of berberine or BC showed a significant decrease in the dietary and water intake of the diabetic mice, compared with the vehicle control. However, canagliflozin slightly attenuated dietary and water intakes, compared with the vehicle control. Compared with canagliflozin, BC significantly decreased dietary and water intakes.

![Graphs showing changes in body weight, kidney weight, diet, and water intake](image)

**Fig. 1** Change in A) body and B) kidney weight/body weight, and C) diet and D) water intake in mice. Normal, normal mice; DM, diabetic mice; BBR, berberine-treated diabetic mice; CAN, canagliflozin-treated diabetic mice; BBR + CAN, diabetic mice treated with the combination of berberine and canagliflozin. The data were expressed as mean ± SD, **P < 0.05 vs normal control; *P < 0.01 vs DM control; **P < 0.05 vs BBR + CAN**
Effects of BC on fasting blood glucose, insulin, and postprandial blood glucose levels

The diabetic mice exhibited significantly increased blood glucose levels compared with the normal controls (Fig. 2A). Canagliflozin or BC significantly reduced the fasting blood glucose (FBG) in STZ-induced diabetic mice within 4 weeks of administration. Berberine moderately attenuated the increase of FBG during the first, second, and third weeks, but this effect was weakened during the fourth week. BC seemed to demonstrate a stronger reducing effect on FBG than canagliflozin at the 3rd and 4th week. Serum insulin concentration was significantly reduced in the STZ-induced diabetic mice, compared with the normal controls (Fig. 2B). However, berberine alone did not cause any change in the insulin level compared with the vehicle control, whereas canagliflozin further reduced the serum insulin levels, compared with the vehicle control. BC did not increase the insulin levels in diabetic mice but showed a significant effect, compared with canagliflozin alone.

Effects of BC on serum lipid levels

The diabetic mice showed a moderate increase in serum triglycerides and LDL-c, which were attenuated by the administration of berberine or BC (Figs. 3A and 3B). However, no significant change in the total serum cholesterol level was observed between any groups (Fig. 3C). In addition, canagliflozin or BC significantly increased serum HDL-c levels in diabetic mice, compared with the vehicle control (Fig. 3D). In particular, BC significantly decreased the serum LDL-c levels, compared with canagliflozin alone.

Effects of BC on urine volume and total urine glucose excretion, serum BUN, and Cre levels.

The diabetic mice showed a significant increase in urine excretion and total urine glucose excretion, compared with normal controls (Figs. 4A and 4B). Berberine or BC significantly attenuated these parameters in diabetic mice.
compared with the vehicle control, but canagliflozin did not show any effect. In addition, the diabetic mice showed a significant increase in serum BUN and Cre levels, compared with normal controls (Figs. 4C and 4D). All the drugs significantly reduced the serum BUN levels, but BC caused greater reduction than canagliflozin alone. BC significantly

Fig. 3 Changes in serum A) total cholesterol (TC), B) LDL-c, C) triglycerides (TG), and D) HDL-c levels in mice after oral administration of drugs. Normal, normal mice; DM, diabetic mice; BBR, berberine-treated diabetic mice; CAN, canagliflozin-treated diabetic mice; BBR+CAN, diabetic mice treated with the combination of berberine and canagliflozin. Data were expressed as mean ± SD, *P < 0.05, **P < 0.05 vs normal control; *P < 0.05, **P < 0.01 vs DM control; $$$P < 0.05 vs BBR + CAN

Fig. 4 Changes in A) urine excretion volumes, B) total urine glucose excretion, and serum C) BUN and D) Cre levels in mice after oral administration of drugs. Normal, normal mice; DM, diabetic mice; BBR, berberine-treated diabetic mice; CAN, canagliflozin-treated diabetic mice; BBR+CAN, diabetic mice treated with the combination of berberine and canagliflozin. Data were expressed as mean ± SD, *P < 0.01 vs normal control; *P < 0.05, **P < 0.01 vs DM control; $$$P < 0.01 vs BBR + CAN
inhibited the increase in serum Cre levels in the diabetic mice, compared with the vehicle control, whereas berberine or canagliflozin alone did not show significant effects. Compared with canagliflozin, BC significantly decreased urine excretion and total urine glucose, which is a key issue frequently seen in canagliflozin-treated patients.

Effects of BC on kidney MDA and SOD levels, pAMPK and AMPK, and TNFα expression levels

Kidneys from the diabetic mice showed a significant increase in MDA levels and decreased SOD levels, compared with normal controls (Figs. 5A and 5B). However, kidneys of the animals treated with all drugs showed a significant decrease in MDA levels and increase in SOD activities, compared with the untreated diabetic controls. No significant differences in kidney MDA levels and SOD activities between any drug-treated groups were observed.

Western blot analysis showed significantly decreased kidney pAMPK and AMPK levels in the diabetic mice, compared with normal controls (Figs. 5C and 5D). However, all the drugs significantly increased the kidney pAMPK and AMPK expression levels in diabetic mice, and BC showed a stronger effect on pAMPK expression than berberine or canagliflozin alone. Particularly, BC showed a significant increase in pAMPK/AMPK ratios, compared with canagliflozin alone.

Furthermore, IHC assays showed that the diabetic mice exhibited a significant increase in TNFα in the glomeruli but not in other parts of kidney tissues, compared with normal controls (Fig. 6). This increase was significantly attenuated by berberine or BC, suggesting that the effect of BC was stronger than that of berberine alone. However, canagliflozin alone did not show any significant effects. Compared with canagliflozin, BC significantly decreased the TNFα in the glomeruli of diabetic mice.

Discussion

In the present study, STZ successfully induced the classic syndromes of diabetes mellitus in mice, such as significant increases in blood glucose, dietary and water intake, and urine excretion, as well as reduction of body weight and insulin levels, which were consistent with our previous study [13]. In the current research, canagliflozin alone could significantly control blood glucose at a stable level and seemed to be involved in the inhibition of glucose reabsorption in the
Fig. 6 Changes in TNFα levels in kidneys of mice after oral administration of drugs. Normal, normal mice; DM, diabetic mice; BBR, berberine-treated diabetic mice; CAN, canagliflozin-treated diabetic mice; BBR+CAN, diabetic mice treated with the combination of berberine and canagliflozin. The data were expressed as mean ± SD, ##P < 0.01 vs normal control; **P < 0.01 vs DM control; $$$P < 0.01 vs BBR + CAN

kidneys by targeting SGLT2 [14]. Interestingly, the total urine glucose excretion of canagliflozin alone did not exceed that of the untreated diabetic control in the late weeks, although canagliflozin inhibited the renal glucose reabsorption. Therefore, if the use of canagliflozin presumably deteriorates the increasing urine glucose levels in diabetic individuals is not a concern. However, canagliflozin did not significantly inhibit water intake and excessive urine excretion, as exhibited by diabetic patients [5]. Berberine alone did not maintain the glucose-reducing effect stable for a long time since this disease was a chronic process. Berberine alone significantly reduced the body weight, which may be mistaken for weight loss usually observed in type 1 diabetes mellitus. Nevertheless, we found that BC alleviated diabetic syndromes better than berberine or canagliflozin alone in diabetic mice.

In the present study, the canagliflozin-treated mice showed a declining trend in diet intake (P = 0.061), compared with the untreated diabetic controls, which might be attributed to the reduced blood glucose levels. The decrease in diet intake caused by canagliflozin might improve insulin sensitivity or promote glucose utilization as berberine did because the serum insulin levels did not increase. This finding was further confirmed by the glucose tolerance test, and the results seemed to be consistent with previous reports [9]. In previous studies, berberine activates AMPK and enhances glucose metabolism and utilization [15]. In our preliminary study, berberine and canagliflozin did not cause any significant change on PDX-1 and insulin gene expression levels in pancreatic islet tissues of STZ-treated diabetic mice (data not shown). Therefore, canagliflozin or berberine obviously exhibited a glucose-reducing effect in an insulin-independent manner.

Diabetes is associated with the increase of nephropathy [16]. In the present study, the diabetic mice showed a significant increase in serum BUN and Cre levels, suggesting that this diabetic model demonstrated kidney dysfunctions. BC significantly improved this impairment; thus, we determined the underlying mechanism. Diabetic nephropathy is associated with increased oxidative stress and inflammation [17]. We assayed the oxidative stress and inflammatory levels in kidneys of diabetic mice. We found that diabetic mice exhibited a significant increase in kidney MDA levels and TNFα expression levels in the glomeruli, which might contribute to kidney dysfunction. However, compared with berberine or canagliflozin alone, BC showed increased inhibition effects on the key inflammatory factor (TNFα) but not on oxidative stress, which might explain why BC showed more improvement in kidney function. In addition, the inhibition of AMPK activity plays an important role in oxidative stress, inflammatory response, and kidney dysfunction; conversely, the activation of AMPK enhances these dysfunctions [12, 18]. In the present study, we found that the diabetic mice showed reduced pAMPK levels, compared with normal controls, but this decrease was normalized by all the drugs tested. However, BC showed a more preferable
effect than berberine or canagliflozin alone. Therefore, BC may prevent diabetic nephropathy caused by the promotion of pAMPK activity and the decrease of TNFα expression in the kidneys.

Although canagliflozin showed an ideal hypoglycemic effect, BC seemed to have more improvement in the increased fasting and postprandial blood glucose than canagliflozin alone. On the other hand, canagliflozin might cause potential urinary tract infections and kidney dysfunction; however, berberine has been demonstrated to have some antibacterial properties [10] and protective effects on kidneys [12, 19]. Canagliflozin results in the increased low-density lipoprotein cholesterol levels [20], whereas berberine demonstrates cholesterol-reducing activity [10]. In the present study, BC significantly reduced LDL-c, compared with canagliflozin alone. Besides of these results, BC significantly decreased urine excretion and total urine glucose compared with canagliflozin alone. In addition, BC seemed to have more improvement in kidney functions than canagliflozin alone. Overall, BC seems to be a promising combination for the treatment of diabetes mellitus and its complications compared with canagliflozin alone.

In summary, the results from the present study indicated that BC enhanced the antidiabetic or complementary effects, including enhanced insulin sensitivity, improved serum lipid levels, and protection of kidney functions. These synergic antidiabetic mechanisms might be mediated by the renal-SGLT2-inhibiting effect of canagliflozin and the AMPK-activating effect of berberine. In particular, the improvement of kidney functions in diabetes might be associated with the synergic effect of increased pAMPK activity and reduced inflammation response in the kidneys. The present results first suggested that the combination of berberine and canagliflozin might offer a promising application in reducing blood glucose and alleviating adverse complications in diabetic patients who cannot tolerate insulin administration. However, the exact underlying mechanisms should be investigated in future studies.

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Cite this article as: TIAN Cai-Ming, JIANG Xin, OUYANG Xiao-Xi, ZHANG Ya-Ou, XIE Wei-Dong. Berberine enhances antidiabetic effects and attenuates untoward effects of canagliflozin in streptozotocin-induced diabetic mice [J]. *Chin J Nat Med*, 2016, 14(7): 518-526