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*Original Article*

# Berberine attenuates cerebral ischemia-reperfusion injury via activating PI3K-Akt signaling in a rat model of type 2 diabetes

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**Abstract:** Berberine (BBR) is an isoquinoline alkaloid, which originally isolated from Chinese Herb Coptischinensis. Accumulating evidences have demonstrated that BBR executes a wide range of pharmacological effects. The present study aims to identify the effects and molecular mechanisms of BBR on cerebral ischemia/reperfusion (I/R) injury of a rat model of type 2 diabetes. Ninety male Sprague-Dawley (SD) rats with diabetes were randomized equally into three groups (n=30): sham group, I/R group, and I/R+BBR group. Rats were treated with saline or BBR for 7 days then subjected to cerebral ischemia reperfusion by middle cerebral artery occlusion for 2 h followed 12 h reperfusion. Cerebral infarct volume was observed and evaluated by hematoxylin-eosin (HE) staining and transmission electron microscopy (TEM). The levels of SOD, MDA and NO in infarct district were examined by enzyme-linked immunosorbent assay (ELISA). Cerebral cell apoptosis was detected by terminal dexynucleotidyltransferase (TdT)-mediated dUTP nick end labeling (TUNEL). Besides, the expression levels of PI3K, Akt and phosphorylation of Akt (p-Akt) were detected by Western blot analysis. Our results showed that treatment with BBR markedly decreased cerebral infarct volume in the I/R+BBR group compared to that in the I/R group, and significantly down-regulated expression of SOD, MDA and NO. Furthermore, BBR reduced cell apoptosis in cerebral infarct district of the I/R+BBR group compared to that in the I/R group, increased Bcl-2 and decreased Caspase-3 and Bax expression. Moreover, we found that BBR promoted PI3K and p-Akt expression to alleviate cerebral I/R injury via activating PI3K-Akt signaling. In conclusion, our data first demonstrate that BBR exerts anti-apoptotic effect and attenuates cerebral I/R injury via activating PI3K-Akt signaling in a rat model of type 2 diabetes.

**Keywords:** I/R, diabetes, BBR, apoptosis, PI3K-Akt

## Introduction

Ischemic brain disease remains the leading cause of mortality and disability all over the world [1]. Although timely reperfusion treatment is the primary method, cerebral ischemia/reperfusion (I/R) still generates serious damages in cerebral infarct district [2-4]. Diabetes mellitus (DM) has reached epidemic proportions in the general adult population in most developed countries [5]. Patients with diabetes have a higher risk of developing ischemic cerebral disease, and more severe cerebral infarctions than non-diabetic people [6, 7]. Therefore, novel therapeutic strategies are required to prevent and/or protect the cerebral tissues against cerebral I/R in patients with type 2 diabetes.

Berberine (BBR) is an isoquinoline-derived alkaloid extracted from Rhizoma Coptidis, which has been widely used in clinical owing to its multiple biochemical and pharmacological effects [8, 9]. It has been well documented that BBR has beneficial effects on decreasing hyperglycemia, alleviating insulin resistance and inhibiting lipid synthesis [10-13]. Growing evidences from both animals model and patients have demonstrated that BBR attenuates myocardial I/R injury by inhibiting autophagy, apoptosis and inflammation in type 2 diabetes [14, 15]. In recent years, studies have shown that BBR alleviates cerebral I/R injury via down regulation of adenosine-5' monophosphate kinase activity [16]. The potential protective effect of BBR on cerebral ischemia prompted us to investigate whether it is capable of exerting fa-

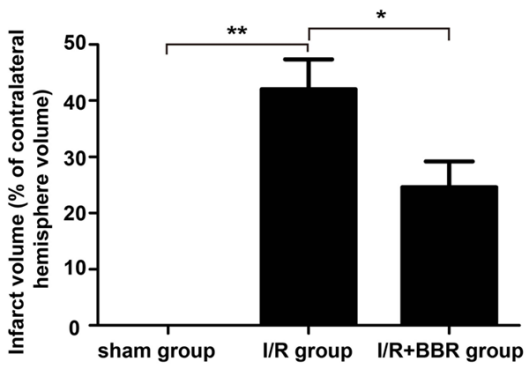


**Figure 1.** Effect of BBR on the cerebral in infarct volume. BBR reduced cerebral I/R injury volume in a rat model of type 2 diabetes. Data were expressed as mean ± SD (n=30). Ischemia-reperfusion: I/R. **p<0.01 and *p<0.05.

vorable effect during cerebral I/R injury in a rat model of type 2 diabetes and the underlying mechanism responsible for this action.

In this study, we performed a model of middle cerebral artery occlusion for 2 h followed by 12 h reperfusion in a rat model of type 2 diabetes, to evaluate the potential effect and molecular mechanism of BBR on cerebral I/R injury.

## Materials and methods

### *Animals*

Ninety adult male Sprague-Dawley (SD) rats (250-300 g) were purchased from the Southern Medical University Animal Center (Guangzhou, China). All rats were treated under a pathogen-free condition at about 22~24°C with a 12 h light-dark cycle. Rats were treated with low-dose STZ (30 mg/kg) (Sigma, St. Louis, MO, USA) every other day for two times. One week after STZ injection, the fasting blood glucose level of each rat was measured by using a glucose meter (Accu-Chek; Roche, Nutley, NJ, USA). The rats with fasting blood glucose levels ≥11.1 mmol/L at week 8 were considered to have successful developed diabetes. The research was in accord with the National Institutes of Health Guidelines for the Use of Laboratory Animals and approved by the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital.

### *Reagents*

BBR was purchased from Beyotime Biotechnology (Shanghai, China). A terminal dexynucleotidyltransferase (TdT)-mediated dUTP nick end labeling (TUNEL) kit was obtained from Invitrogen (Invitrogen, Carlsbad, CA, USA). DAPI was purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA). The superoxide dismutase (SOD), malondialdehyde (MDA) and superoxide generation ELISA assay kits were purchased from Maixin Biotechnology (Fuzhou, Fujian, China). The primary antibodies against PI3K (ab86714), Akt (ab8805), p-Akt (ab38449), Caspase-3 (ab2171), Bcl-2 (ab32124), Bax (ab-32503), and Gapdh (ab8245) were purchased from Abcam (Abcam, Cambridge, UK). Goat anti-rabbit (sc-2774) and goat anti-mouse (sc-2060) secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### *In vivo animal experiments*

Ninety adult male SD rats of diabetes were randomized equally into three groups (n=30): sham group, I/R group, and I/R+BBR group. The rats in sham and I/R group were gavaged with saline at doses of 200 mg/kg body weight every day for 4 weeks, while rats in I/R+BBR group were treated with BBR. Middle cerebral artery occlusion (MCAO) was performed to establish focal ischemia as previous reported [17, 18]. The rats in I/R and I/R+BBR group were subjected to 2 h ischemia by MCAO followed by reperfusion for 12 h though suture removal. Briefly, the rats were firstly anesthetized with 10% chloral hydrate (300 mg/kg). Then, the left common carotid artery (CCA), the internal carotid artery (ICA), and the external carotid artery (ECA) were fully ligated near bifurcation. After careful isolated the surrounding vessel and nerve tissues, a small incision was made on the CCA and a 0.3 mm monofilament surgical nylon suture (Ethicon, Somerville, NJ, USA) was introduced via the CCA into the ICA. After ischemic for 2 h, blood flow was restored carefully by removing the nylon suture followed by reperfusion for 12 h. The rats in the sham group were received the same surgical procedures without inserting the monofilament surgical nylon suture.

### *Determination of brain infarction volume*

Thirty rats brain tissues from each group were quickly collected and stained with 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, St. Louis, MO, USA). Briefly, each brain of rat was sliced into 5 coronal sections, 4 mm thick per slide, fixed with 4% paraformaldehyde (Sigma, St. Louis, MO, USA) and then stained with 1% TTC phosphate buffer (pH 7.4) at room temperature for 15 min. Normal brain tissue exhibits a deep

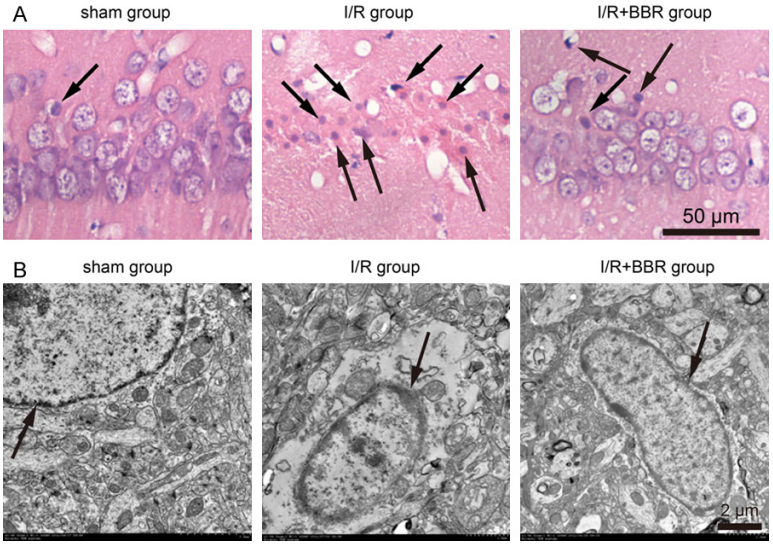


**Figure 2.** H&E staining and TEM study of infarct district. A. HE staining showed that the death nerve cells in infarct area of the I/R group was increased compared to that in the I/R+BBR group. Black arrow: death nerve cells. Bars: 50 μm. B. TEM revealed that nuclear membrane of neuron in infarct area of the I/R group was dissolved compared to that in the I/R+BBR group. Bars: 2 μm. Black arrow: karyolemma. TEM: transmission electron microscopy.

red staining, while the infarct brain district shows a pale gray staining. Stained tissues were photographed and the digital images were analyzed by using Image-Pro Plus 6.0 system (Media Cybernetics Inc., Bethesda, MD, USA), in order to calculate the cerebral infarct volume.

*Histology*

Cerebral tissues were fixed with 4% paraformaldehyde (Sigma, St. Louis, MO, USA). Then, followed by dehydrated, paraffin embedded and sectioned 3~5 μm thickness with a Ultra-Thin Semiautomatic Microtome (KD-3358, Leica, Bensheim, Germany). After that, deparaffinized with xylene solution (Sigma, St. Louis, MO, USA), and stained with hematoxylin and eosin (HE, Sigma, St. Louis, MO, USA) according to the standard protocols. The results were visualized using a Leica microscope with Dialux 20 Model (Leica, Bensheim, Germany) and analyzed with Leica FireCam software (Leica, Bensheim, Germany).

*Transmission electron microscopy (TEM)*

TEM was performed as previously reported [19]. Briefly, cerebral tissues were fixed with 1.5~2% glutaraldehyde (Sigma, St. Louis, MO, USA). Then, the tissues were cut into small pieces, dehydrated, and embedded in spurr's resin (Electron Microscopy Science, Hatfield, PA, USA). After that, the spurr sections were directly post-stained with aqueous uranyl acetate/lead citrate (Electron Microscopy Science, Hatfield, PA, USA). The results were observed *with a Hitachi AMT XR-40 CCD camera (Hitachi, Japan).*

*Assay of cerebral apoptosis*

Cerebral cell apoptosis was analyzed by terminal deoxy-nucleotidyltransferase UTP nick end labeling (TUNEL) assay. Terminal deoxynucleotidyl transferase UTP nick end labeling was performed by using an in situ cell death detection kit (Invitrogen, Carlsbad, CA, USA). Briefly, 100 μl TUNEL reaction mixtures were added on each sample, and the slides were incubated at 37°C for 60 min, then rinsed with PBS three times with 5 min for each time, and observed with a Axio Observer Z1 fluorescence microscopy (Zeiss, Germany).

*Western blot assay*

Cerebral samples were isolated from RIPA buffer (Invitrogen, Carlsbad, CA, USA) on ice for 30 min, and the lysates were purified by centrifugation at 4°C for 30 min at 13,000 rpm. After quantitation of protein concentration, approximately 40 μg of total protein was separated by 8~12% SDS-PAGE and then transferred to PVDF (Polyvinylidene Fluoride) membranes (Millipore, CA, USA). The membranes were blocked for 60 min at 37°C with 5% non-fat dry milk, then incubated with primary antibody including PI3K (1:1000), Akt (1:2000), p-Akt (1:1000), Caspase-3 (1:2000), Bcl-2 (1:2000), Bax (1:1000), and Gapdh (1:5000) over night at 4°C. After three washings with TBST (Tris Buffered Saline and Tween-20, pH 8.0) with 10 min for three times, the membranes were incubated with corresponding secondary antibody (1:2000) in TBST solution for 60 min at 37°C. The positive protein bands were detected by using a chemiluminescent system (Amersham Biosciences, Little Chalfont, UK), and then the bands were scanned and quantified by densitometric analysis using an image analyzer

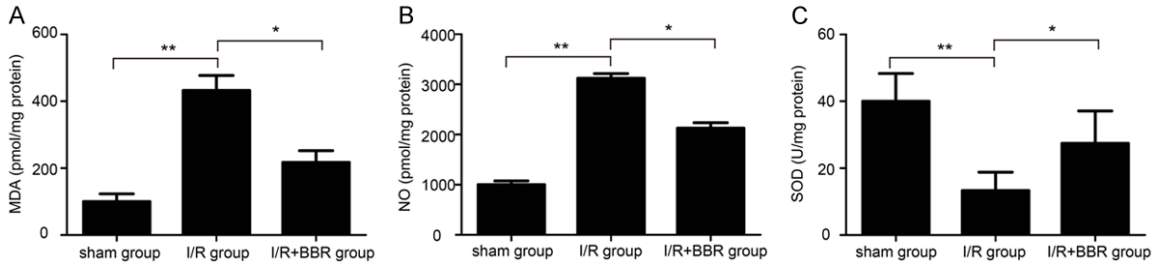


**Figure 3.** BBR reduces MDA and NO and increases SOD activity in infarct area of diabetes rats subjected to cerebral I/R injury. MDA (A), NO (B) and SOD (C) activities were detected in infarct district of cerebral tissues. **p<0.01 and *p<0.05.

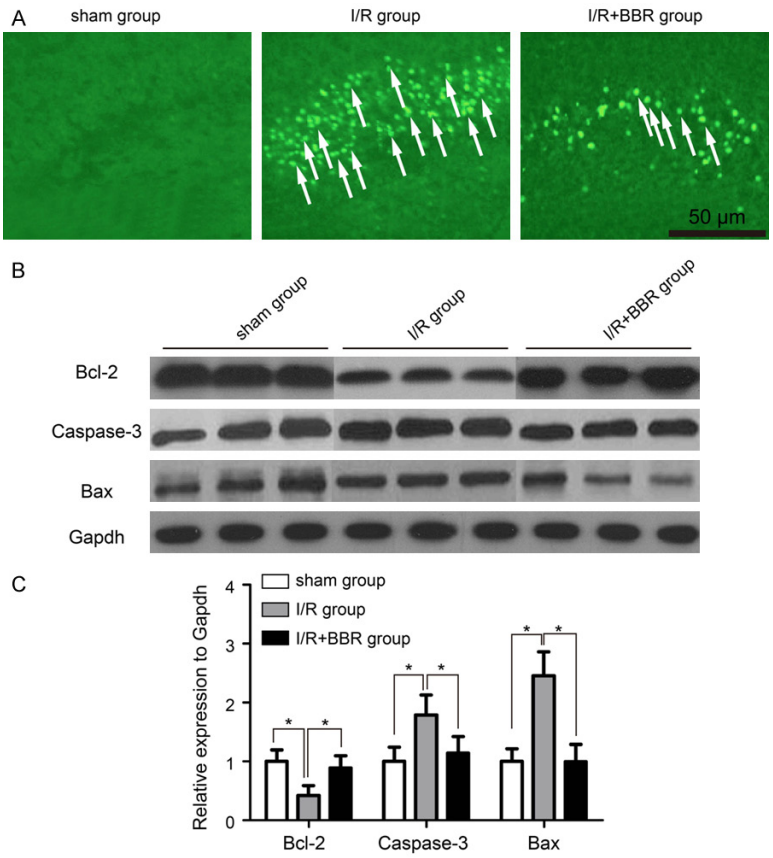


**Figure 4.** BBR attenuates cerebral cell apoptosis following I/R injury in a rat model of type 2 diabetes. A. Representative photomicrographs of in situ detection of cell apoptosis in infarct district by TUNEL staining. Black arrow: apoptosis cells. B. The Bcl-2, Caspase-3 and Bax expression in infarct area was detected by using Western blot analysis. TUNEL: terminal deoxy-nucleotidyltransferase UTP nick end labeling. C. Comparison of the relative protein expression of Bcl-2, Caspase-3 and Bax in the three groups. *p<0.05.

Quantity One System (Bio-Rad, Richmond, CA, USA).

*Enzyme-linked immunosorbent assay (ELISA)*

Cerebral tissues were homogenized in ice-cold saline. Then, the homogenate was centrifuged at 13,000 g at 4°C for 60 min. The activities of MDA, SOD and NO was determined in the supernatants using a ELISA kit following the manufacturer's protocols. Briefly, SOD activity was measured by using the nitroblue tetrazolium (NBT) method. MDA was assayed as thiobarbituric acid-reactive substances after precipitating the proteins with trichloroacetic acid. NO concentration was detected by using the nitrate reductase method.

*Statistical analysis*

Statistical analysis was detected by using SPSS17.0. All assays were repeated at least three times. The data are expressed as mean ± SD (Standard Deviation). Quantitative data were evaluated by using analysis of variance (ANOVA), and followed by Dunnett's test. Values of $p<0.05$ was considered to be statistically significant.

## Results

*BBR alleviates cerebral I/R injury in a rat model of type 2 diabetes*

In vivo experiment, we firstly evaluated the effect of exogenous BBR treatment on cerebral I/R injury of diabetes rats. Compared with the I/R group, the cerebral infarct volume in the I/R+BBR group was markedly reduced (**Figure 1**,

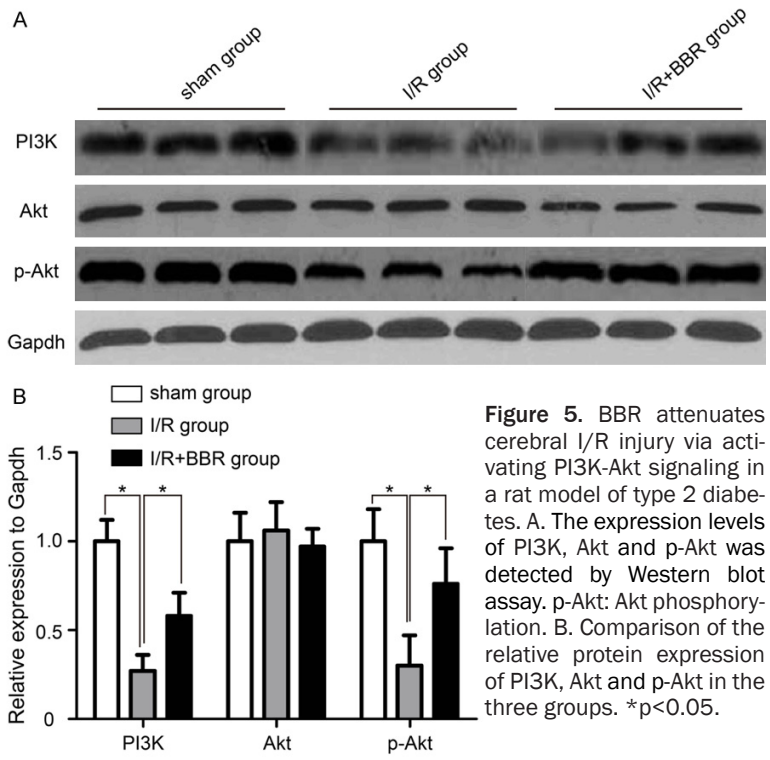


**Figure 5.** BBR attenuates cerebral I/R injury via activating PI3K-Akt signaling in a rat model of type 2 diabetes. A. The expression levels of PI3K, Akt and p-Akt was detected by Western blot assay. p-Akt: Akt phosphorylation. B. Comparison of the relative protein expression of PI3K, Akt and p-Akt in the three groups. *p<0.05.

p<0.05). HE staining shown that the death cells in infarct district of the I/R group were significantly increased compared to that in the I/R+BBR group (**Figure 2A**). Meanwhile, TEM revealed that cell karyolemma in infarct area of the I/R group was significantly degraded compared to that in the I/R+BBR group (**Figure 2B**).

*BBR reduces MDA and NO and increases SOD activity in infarct district of diabetes rats subjected to cerebral I/R injury*

As shown in **Figure 3**, the activity of MDA and NO was significantly increased in the I/R group compared with the sham group (p<0.01), while SOD activity was markedly decreased (p<0.01). MDA and NO activity was down-regulated in the I/R+BBR group compared to that in the I/R group (p<0.05), while the SOD activity was up-regulated (p<0.05).

*BBR attenuates cerebral cell apoptosis following I/R injury in a rat model of type 2 diabetes*

An increased cell apoptosis of TUNEL staining was observed in the infarct district of I/R group than Sham group (**Figure 4A**). Pretreatment of rats with BBR significantly reduced cell apoptosis in the infarct district of the I/R+BBR group compared to that in the I/R group (**Figure 4A**). Meanwhile, Western blot analysis showed that the protein expression levels of Bax and Caspase-3 in infarct area of the I/R group were significantly increased than that in the Sham group, while Bcl-2 protein level was markedly decreased (**Figure 4B** and **4C**, all p<0.05). Pretreatment of diabetes rats with BBR resulted in attenuation of Bax and Caspase-3 expression, and promotion Bcl-2 expression in the I/R+BBR group compared to the I/R group (**Figure 4B** and **4C**, all p<0.05). These data indicated that BBR exerted neuroprotective effect for cerebral I/R injury by inhibiting nerve cell apoptosis in a rat model of type 2 diabetes.

*BBR treatment attenuates cerebral I/R injury via PI3K-Akt signaling in a rat model of type 2 diabetes*

Our previous data demonstrated that BBR significantly reduced the nerve cell apoptosis caused by cerebral I/R injury as indicated by markedly decreased expression levels of Caspase-3 and Bax and increased expression of Bcl-2. To further understand the molecular mechanisms of BBR's neuroprotective action, we investigated the changes of PI3K-Akt signaling pathway in cerebral I/R injury. As shown in **Figure 5**, the PI3K and p-Akt expression in the I/R group was down-regulated compared to that in the sham group (p<0.05). Furthermore, BBR markedly up-regulated the PI3K and p-Akt expression levels in the I/R+BBR group compared to that in the I/R group (p<0.05). These results strong suggested that BBR attenuates cerebral I/R injury via activating PI3-Akt signaling in a rat model of type 2 diabetes.

## Discussion

In this study, our results provided directly in vivo evidences that BBR protected the brain against I/R injury in a rat model of type 2 diabetes. The results demonstrated that pretreatment of rats with BBR significantly improved

brain function and reduced cerebral apoptosis. Our findings also identified that BBR attenuated cerebral I/R injury in diabetic rats through activating PI3-Akt signaling.

BBR is an alkaloid extracted from the coptischinensis species (Huanglian), and it has a long history for treating diarrhea in Chinese traditional medicine. Accumulating studies have suggested that berberine has a wide variety of biological effects, including cardiovascular-protective action, anti-tumor, decrease insulin resistance, etc [13, 20-22]. Zeng, et al. [23] found that BBR improved cardiac function in patients with severe congestive heart failure. Lv, et al. [24] identified that BBR suppressed doxorubicin-activiated cardiomyocyte apoptosis. Here, our data showed that BBR treatment for four weeks improved the cerebral I/R injury, as indicated by the cerebral infarct volume and the activity of MDA, NO and SOD in infarct area.

The pathogenesis of cerebral I/R injury is apparently multifactorial, and cerebral cell apoptosis is one of the major pathogenic mechanism underlying cerebral I/R injury [25]. Blocking the apoptosis process of nerve cell could minimize I/R-induced cerebral injury. Here, we found an increase cerebral apoptosis was observed by TUNEL staining in infarct area of the I/R group than that in the sham group. More importantly, treatment with BBR reduced cerebral cell apoptosis after I/R injury in diabetic rats.

The PI3K-Akt signaling pathway is central to physical and pharmacological pre- and post-conditioning and salvaging the I/R condition [25, 26]. Previous studies have suggested that PI3K-Akt-dependent signaling pathway plays crucial roles in anti-apoptotic action from I/R injury [27, 28]. Data from the present study also revealed that pretreatment with BBR not only promoted PI3K and Akt phosphorylation expression, but also markedly increased Bcl-2 expression and decreased active Caspase-3 and Bax expression as compared to that the I/R group, suggesting that BBR attenuates cerebral injury following I/R via activating PI3-Akt signaling in a rat model of type 2 diabetes.

Taken together, our results first show that BBR exerts anti-apoptotic action against cerebral I/R injury via activating PI3K-Akt signaling in diabetic rats. The findings suggest a potential therapeutic value of BBR in the prevention and rescue for ischemic brain disease with diabetes.

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## Disclosure of conflict of interest

None.

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