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Investigating the vasodilatory effects of baicalin and geniposide on cerebral basal arteries via the TRPV4 channel: Insights into the anti-cerebral ischemic mechanism

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Author contributions

Xue-Wei Zhou and An-Dong Zhao wrote the manuscript and data organization. Xue-Wei Zhou, An-Dong Zhao, Jia-Hao Zhang and Tian Hu conducted the animal experiments. Chuan Wang, Ji-Ping Liu, and Bin Wang provided supervision and technical guidance for the entire experimental work.

Competing interests

The authors declare no conflicts of interest.

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Abbreviations

CIS, cerebral ischemic stroke; BC, baicalin; GD, geniposide; TRPV4, Transient receptor potential vanilloid 4; PSS, physiological salt solution; CBA, cerebral basilar artery; VSMCs, vascular smooth muscle cells; OGD, oxygen-glucose deprivation; TRP, Transient receptor potential; MCAO, Middle cerebral artery occlusion.

Citation

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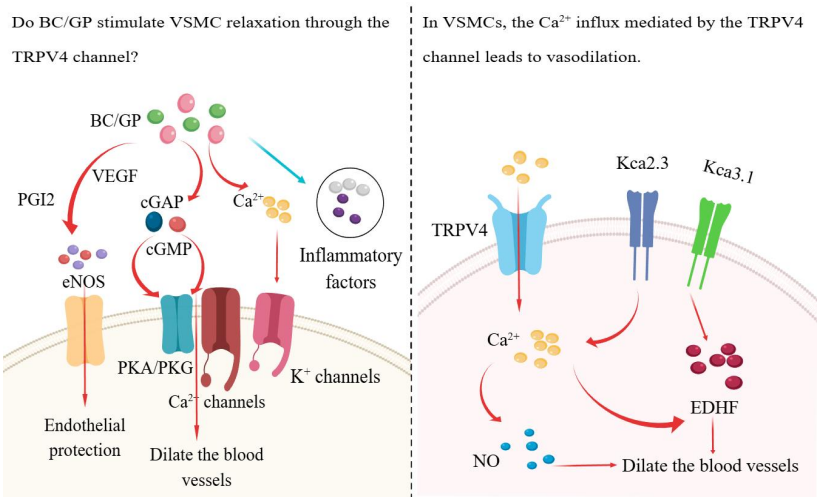
Abstract

Background: Baicalin (BC) and geniposide (GD) are effective components of natural remedies, and studies have shown that they protect against cerebral ischemic stroke (CIS). Transient receptor potential vanilloid 4 (TRPV4) is a calcium-permeable channel that plays important roles in vascular function and vasodilation. However, no studies are available on the effect of BC/GD on the TRPV4 channel and rat cerebral basilar artery (CBA). This study examined the effect of the combination of BC/GD (7:3) on cerebral vascular function after CIS. **Methods:** We used western blotting to determine TRPV4 protein levels and live cell fluorescence Ca^{2+} imaging and patch clamp to determine how BC/GD activates TRPV4 channels. Isolated vessel experiments were used to observe the dilatory effects of BC/GD on CBA under different conditions. Laser Doppler imaging was used to measure cerebral blood flow in rats. Triphenyl tetrazolium chloride and Nissl stainings were used to determine the infarct area in the rat brain and neuronal damage, respectively. **Results:** BC/GD significantly boosted TRPV4 protein levels in vascular smooth muscle cells (VSMCs) during oxygen-glucose deprivation and increased $[Ca^{2+}]_i$ in TRPV4-HEK 293 cells and VSMCs. This effect was not observed in vector-HEK 293 cells. In patch clamp experiments, BC/GD increased Ca^{2+} currents in TRPV4-HEK 293 cells, whereas no significant changes were observed in vector-HEK 293 cells. BC/GD dilated CBA contractions induced by U46619 and KCl, with a concentration-dependent increase of the dilatory effect. In the middle cerebral artery occlusion model, cerebral blood flow in the ischemic side significantly decreased, whereas BC/GD intervention significantly increased cerebral blood perfusion in the ischemic side, reduced the infarct area, and improved neurological function scores and neuronal damage. **Conclusion:** BC/GD activates the TRPV4 channel, leading to Ca^{2+} influx, which in turn activates the intermediate conductance calcium-activated potassium channels channel to regulate vasodilation in vascular smooth muscle.

Keywords: baicalin; geniposide; TRPV4; cerebral basilar artery; cerebral ischemic stroke



Highlights

We examined the effect of the combined compounds baicalin (BC) and geniposide (GD), from the traditional prescription "Huanglian Jiedu Decoction," on cerebral vascular function after cerebral ischemic stroke. The transient receptor potential vanilloid 4 (TRPV4) is a calcium-permeable channel that plays important roles in vascular function and vasodilation. However, no studies were available on the effect of BC/GD on the TRPV4 channel. Our results show that BC/GD activates the TRPV4 channel, ultimately regulating vasodilation in vascular smooth muscle.

Medical history of objective

Scutellaria baicalensis and *Gardenia jasminoides* were first recorded in *Shennong´s Classic of Materia Medica* (unknown author, 25–220 C.E.). *Scutellaria baicalensis* has properties such as clear heat and dry dampness, intense purging heat, and detoxifying and arresting bleeding. It is often used clinically because of its anti-inflammatory and antiviral properties. Baicalin is a flavonoid that is extracted from the dried rhizomes of *Scutellaria baicalensis*. As one of the primary active constituents of *Scutellaria*, it exhibits pharmacological activities including antiviral, anti-inflammatory, antibacterial, antitumor, neuroprotective, antioxidant, hemostatic, and antitumor effects. Studies have shown that baicalin exerts protective effects in tissues such as the brain, myocardium, and liver by reducing cellular inflammatory responses and regulating cell proliferation and apoptosis. *Gardenia jasminoides* clears fire and relieves irritability, clears away heat, promotes diuresis, cools blood to remove apoptogenic heat, and disperses blood stasis. Clinically, it is widely used to treat conditions such as hepatitis, hypertension, and diabetes. geniposide is one of the primary active constituents of *Gardenia jasminoides*, and has demonstrated multiple pharmacological effects both in vivo and in vitro, including anti-inflammatory, neuroprotective, and antitumor effects.

Background

Cerebral ischemic stroke (CIS) occurs when the blood flow to the brain is blocked, leading to a temporary or permanent lack of blood supply, primarily manifesting as local edema, infarction, softening, and loss of neurological function. Major triggers include thrombosis, arteriosclerosis, plaque accumulation, and vascular contraction and relaxation dysfunction [1–4]. According to 2020 estimates, approximately 17.8 million adults in China have experienced a stroke, with a death toll of up to 2.3 million, showing a trend towards younger age, which may be related to the aging population and increased life stress in the country [5]. The brain is the body's largest oxygen- and energy-consuming organ and is extremely sensitive to changes in blood flow and energy supply. Even brief hypoxia can trigger pathological damage, leading to cerebral microvascular dysfunction. If blood flow is not restored in time, permanent infarction may occur if brain tissue ischemia lasts longer than 30 min [6, 7]. Currently, the only approved treatment for ischemic stroke is the tissue plasminogen activator, which carries the risk of inducing reperfusion injury [8]. Therefore, an in-depth exploration of the pathogenesis of ischemic stroke and the search for novel drugs with anti-ischemic effects have become focal points of research.

Changes in the vascular tension are crucial for the occurrence and development of CIS. By regulating vascular tension, cerebral blood vessels maintain stable blood flow to the brain, ensuring normal physiological function of the brain tissue. Vascular smooth muscle cells (VSMCs) are direct executors of vascular tension, adapting to environmental changes by regulating their contraction and relaxation to maintain blood supply within organs and tissues [9, 10]. The contraction and relaxation of VSMCs are complex physiological processes influenced by various factors, including neurotransmitters, hormones, endothelial cell-derived factors, and blood pressure [11–14]. Transient receptor potential (TRP) receptors are a class of nonselective cation channels with homologous amino acid sequences [15]. Studies have shown that the expression and activity of various TRP channel proteins change in neuroinjury diseases, such as CIS [16–18]. It is possible to effectively prevent or alleviate the extent of brain injury by interfering with the regulatory pathways of TRP channels [19]. The pathogenesis of CIS is closely related to the regulation of Ca^{2+}. The transient receptor potential vanilloid 4 (TRPV4) is an important Ca^{2+} channel with selective permeability for Ca^{2+} and is capable of sensing changes in environmental pressure and triggering Ca^{2+} influx, subsequently initiating Ca^{2+}-dependent responses, such as the opening of small conductance calcium-activated potassium channels and intermediate conductance calcium-activated potassium channels (IK_{Ca}) [20–22]. The relaxation of VSMCs occurs through the release of endothelial-derived relaxing factors from endothelial cells, which lowers the Ca^{2+} concentration within the VSMCs, promoting hyperpolarization of smooth muscles, and leading to vasodilation [23]. However, Earley et al. found that in cerebral arterial smooth muscle, TRPV4-mediated Ca^{2+} influx does not induce vasoconstriction, but rather leads to vasodilation [24].

The combination of baicalin (BC) and geniposide (GD) was discovered in the classical prescription "Huanglian Jiedu Decoction" using microdialysis, mass spectrometry, and high-pressure liquid chromatography We found that the combination of BC/GD (7:3) has a significant effect in combating brain ischemic injury. This combination not only acts synergically, improves the penetration rate of the drug in the brain, and alleviates inflammatory damage after cerebral ischemia, but also significantly increases Na^+/K^+-ATPase activity, reduces P-gp content and AQP-4 expression, and mitigates CIS damage through pathways such as reducing excitatory amino acid toxicity and regulating the polarization state of microglia [25–28]. Some studies have indicated that BC exerts concentration-dependent vasodilatory effect. This effect may be related to the promotion of VEGF, eNOS, PGI2, and NO expression, as well as the activation of K^+-ATP channels and Ca^{2+} channels. Therefore, this study focused on the vascular function after CIS to further explore the protective mechanisms of the BC/GD combination [29–31].

Methods

Chemicals and reagents

BC and GD were purchased from Yuanye Biotechnology Co., Ltd., (Shanghai, China). with a purity of ≥ 98%. TRPV4 inhibitor (HC-067047) and IK_{Ca} inhibitor (TRAM-34) were purchased from MCE (Shanghai, China). U46619 with a purity ≥ 98% was purchased from GLPBIO. Dulbecco's modified Eagle medium (DMEM), fluo-4 AM, and Nissl staining solution were purchased from Servicebio (Wuhan, China). TTC solution (2%) was purchased from Solarbio (Beijing, China). The modified Krebs-Henseleit solution (physiological salt solution, PSS) contained the following (in mM): NaCl, 119; KCl, 4.6; $NaHCO_3$, 15; $MgCl_2$, 1.2; $CaCl_2$, 1.5; glucose, 11, and NaH_2PO_4, 1.2. The 60 mM KCl high-potassium salt solution (K-PSS) contained the following (in mM): NaCl 63.6, KCl 60, $NaHCO_3$ 15, $MgCl_2$ 1.2, $CaCl_2$ 1.5, glucose 11, and NaH_2PO_4 1.2. The calcium-free solution with ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), (Ca^{2+}(−) EGTA(+)), contained the following (in mM): NaCl, 119; KCl, 4.6; $NaHCO_3$, 15; $MgCl_2$, 1.2; glucose, 11; NaH_2PO_4, 1.2; and EGTA, 0.5. PSS, K-PSS, and Ca^{2+} (−) EGTA (+) were preheated to 37 °C in a constant temperature water bath, saturated with 95% O_2 and 5% CO_2 gas, and the pH was adjusted to 7.40 using NaOH. Red tetrazole was purchased from Yuanye Biotechnology Co., Ltd., Shanghai, China. The line plug was purchased from Rewod (Shenzhen, China).

Cell culture

VSMCs (kindly provided by Professor Yu Qi of Xi'an Medical University) were cultured in high-glucose complete DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin for 24 h and then switched to glucose-free DMEM. The cells were placed in a tri-gas

incubator (temperature, 37 °C, gas composition, 94% N_2, 5% CO_2, and 1% O_2) and subjected to different durations of hypoxia treatment. By detecting VSMCs viability and morphology at different hypoxia durations (2, 4, 6, 8, 10, and 12 h). The optimal hypoxia treatment duration was selected to establish the VSMCs oxygen-glucose deprivation (OGD) model. Human embryonic kidney (HEK 293) cells (GenEray, Shanghai, China) were cultured in complete DMEM.

Effect of BC/GD on OGD-induced cell viability

Logarithmic growth phase cells were divided into a control group (blank medium) and experimental groups treated with BC/GD at different concentrations (1.56, 3.125, 6.25, 12.5, 25, 50, 100, 200, and 400 μM). The effect of the different concentrations of BC/GD on OGD-induced VSMCs viability was calculated, and the optimal dose was selected for subsequent experiments.

Effect of BC/GD on TRPV4 expression

Based on the results obtained, the cells were divided into the following groups: blank, OGD 8 h, BC/GD 3.125 μM, BC/GD 12.5 μM, BC/GD 50 μM, and BC/GD 50 μM + HC-067047 1 μM. Except for the blank group, the other groups were treated with drugs for 24 h (the blank group and OGD 8 h group were given normal culture medium), followed by 8 h OGD induction.

Construction of a vector and transfection

The TRPV4 mRNA sequence was designed to target the CDS region, and successfully designed primers were synthesized by the Shanghai Jierui Biotechnology Co., Ltd. (Shanghai, China). The following primer sequences were used:

TRPV4-F: CGGGATCCATGGCGGATTCCAGCGAAGGCC BamH1

TRPV4-R: CCGCTCGAGGAGCGGGGCGTCATCAGTCCTC Xho1

The PCR-amplified TRPV4 plasmid was transfected into HEK 293 cells to construct a TRPV4-HEK 293 cell model. After 24 h of culture, processing was performed according to the manufacturer's instructions.

Western blotting

Protein samples were separated using 10% sodium dodecyl sulfate-Tris-glycine polyacrylamide gel electrophoresis. The separated samples were then transferred to a PVDF membrane, which was blocked at room temperature for 2 h with Tris-buffered saline (TBS) containing 0.05% Tween-20 and 5% bovine serum albumin, followed by the addition of the primary antibody TRPV4 (Affinity, Liyang China). The PVDF membrane was incubated overnight at 4 °C with the primary antibody, and then washed with TBST to remove excess primary antibody. The corresponding secondary antibodies were added and incubated for 2 h. The membranes were developed using an ECL substrate (P0018; Beyotime, Shanghai, China), and images were captured using the ChemiDoc XRS+ system (Bio-Rad, Hercules, CA, USA).

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) assay

Cells were incubated in Ca^{2+} detection buffer containing fluo-4AM (10 μL) at 37 °C in the dark for 30 min. Fluorescence signals were recorded using an inverted fluorescence microscope at EX/EM (494 nm/528 nm).

Whole-cell patch clamp assay

Glass recording electrodes were pulled using a Sutter P-1000 microelectrode puller, and a coverslip containing TRPV4-HEK 293 cells was placed in a bath for observation and positioning. Suction was performed when the liquid resistance stabilized at 1/3 to 2/3 of its initial value. The cell membrane was ruptured to establish a whole-cell recording mode and capture cell current signals. Using a gravity accumulation drug administration method, the negative control (0.1% DMSO) and test drugs were introduced sequentially, setting the clamp voltage to −80 mV, applying repolarization stimuli from 0 mV to −120 mV for 1 ms, and then gradually increasing to +120 mV for 200 ms, finally returning to 0 mV, recording the current response of the cell every 10 s.

Animals

We used 100 male SPF-grade Sprague-Dawley rats aged 7–8 weeks, weighing 220 ± 20 g. The animals were provided by Chengdu Dashuo Experimental Animal Co., Ltd. (animal license number: SCXK, Chuan, 2020-0030). Rats were housed in the pharmacology experimental animal room of Shaanxi University of Chinese Medicine, at 23–25 °C, with a relative humidity 45%–55%, with free access to food and water, and were acclimatized for one week to ensure stable physiological conditions. Experiments with rats strictly adhered to the relevant regulations and standards of the Animal Ethics Review of Shaanxi University of Chinese Medicine (Approval No: SUCMDL20240228002).

Isolated vessels experiments

Rats were anesthetized, decapitated, and the brain tissue was removed and immersed in pre-cooled PSS solution at 4 °C. The cerebral basilar artery (CBA) was isolated under a microscope, surrounding tissues were removed, and the artery was cut into rings approximately 2–3 mm long. Two 45 μM tungsten wires were threaded through the vascular ring and fixed in the bath. Starting from 0 mN, the stretching distance slowly increased with a baseline tension of 1 mN, and the PSS was replaced every 30 min. When the contraction force from the two stimuli was greater than 5 mN and showed no significant difference, it was used in the subsequent experiments. The vessels were stabilized in PSS for 1 h, and then K-PSS solution was used to induce vascular contraction. When the contraction response reached a plateau (contraction force of at least 5 mN), the solution was replaced with PSS and K-PSS was used again to induce contraction after the vessels returned to baseline tension. If the difference in contraction values between the two stimuli of the CBA was less than 10%, the sample preparation was considered successful.

Effect of pre-incubation BC/GD on contractile CBA

In different baths, U46619 (0.01–3.0 μM) and KCl (2–80 mM) were cumulatively added to construct concentration-contraction force effect curves, with the maximum contraction force of each constrictor set as 100%. In preliminary experiments, we found that BC/GD at 7×10^{-5} M inhibited vascular contraction; therefore, in formal experiments, 70 μM was chosen as the intermediate concentration, and BC/GD at 7, 70, and 700 μM was added to the bath solution and incubated for 30 min. After pre-incubation, an equal amount of vasoconstrictor was added, and the inhibition rates of BC/GD at 7, 70, and 700 μM on the maximum contraction force of U46619 and KCl were calculated as I_{CHR7}, I_{CHR70}, and I_{CHR700}.

Effect of BC/GD on pre-contraction CBA tension

We reviewed other experiments related to the effective components of natural medicines and vascular function and found that some drug concentrations at 100 μM affected blood vessels [32–35]. Therefore, this experiment started with a final concentration of 10^{-8} M and selected 11 concentration gradients within the range of 10^{-8} to 10^{-3}. Using 0.1 μM U46619 and 60 mM KCl for vascular contraction treatment and after reaching a stable plateau, BC/GD at final concentrations 10^{-8}, 3×10^{-8}, 10^{-7}, 3×10^{-7}, 10^{-6}, 3×10^{-6}, 10^{-5}, 3×10^{-5}, 10^{-4}, 3×10^{-4}, and 10^{-4} M was added sequentially. To eliminate the effect of solvent on the experimental results, another group was treated with the same volume fraction of DMSO after reaching a plateau.

Role of Ca^{2+} in CBA vasodilation

Based on the results of the previous section, BC/GD and 60 mM KCl induced relaxation and were used as controls. When the vascular tension returned to baseline levels, the solution in the bath was replaced with 5 mL Ca^{2+} (−) PSS, and 60 mM KCl was added. When vascular contraction reached a plateau, BC/GD was added sequentially at the same concentration gradient, as described in the previous section.

Role of the TRPV4 channel in BC/GD-induced relaxation

Vessels were contracted with 60 mM KCl, and when contraction reached a plateau, the TRPV4 channel inhibitor HC-067047 (100 nM) and IK_{Ca} channel inhibitor TRAM-34 (1 μM) were used to incubate the vessels. The plateau contraction value at this time was considered as 100%, and the same concentration gradients of BC/GD were added sequentially.

Grouping and administration

After adaptive feeding, the rats were randomly divided into blank, sham operation, middle cerebral artery occlusion (MCAO), BC/GD, BC/GD + HC-067-47 (10 mg/kg), and BC/GD + TRAM-34 (0.5 mg/kg) groups. The optimal effective BC/GD dose from our previous studies was 60 mg/kg [25], therefore, we used this dose. Administration was performed via gavage for seven consecutive days, with the blank, sham operation, and model groups receiving an equal amount of physiological saline. HC-067-47 and TRAM-34 cells were administered via tail vein injection 30 min before modeling.

MCAO model establishment

The rat unilateral ischemic MCAO model was established using the modified Longa suture method [36]. After the last administration, the rats were anesthetized with pentobarbital sodium and the skin was incised using ophthalmic scissors to expose the common, internal, and external carotid arteries. An arterial clamp was used to occlude the internal carotid artery, and the common carotid artery was ligated. The external carotid artery was cut and a suture was inserted to advance to the origin of the anterior cerebral artery. When the suture marker reached the bifurcation of the internal and common carotid arteries, the knot was tightened, and excess sutures were cut off.

Laser doppler for cerebral blood flow

Cerebral blood flow perfusion on the ischemic side of the rats was measured 2 and 24 h after modeling. The rats were anesthetized with pentobarbital sodium. After shaving, the scalp was incised, and the skull was moderately polished using a mini cranial grinder. The treated rat head was placed under a laser doppler imaging detector with a detection distance of 13 cm and a parameter area of 3 cm × 3 cm for a whole-brain scan. Statistical software was used to quantify blood perfusion.

Neurological score and measurement of the cerebral infarct volume

The neurological function score was measured 24 h after modeling using the Zea Longa 5-point scoring system (0, normal neurological function; 1, mild neurological deficit, left forelimb flexion during tail lifting; 2, moderate neurological deficit, turning to the left side while walking; 3, moderate neurological deficit, tilted to the left; 4, non-spontaneous walking, decreased consciousness) [36]. To determine the infarct volume, the whole brain was quickly removed and the cerebellum and brainstem were separated. The brain tissue was frozen at −20 ˚C for 20 min, and cut into six slices of approximately 2 mm along the coronal plane. The slices were incubated in a 2% TTC staining solution in the dark for 20 min and fixed in 4% paraformaldehyde for 24 h. The slices were photographed on a black background and ImageJ software was used to calculate the infarct volume.

Total Infarct Volume = Σ Infarct Area × 2 mm, Infarct Volume Percentage = Total Infarct Volume/Total Brain Tissue Volume × 100%.

Nissl staining

Paraffin sections (10 μm) of rat hippocampal tissue were subjected to dewaxing and dehydration. The sections were stained with Nissl staining solution and excess dye was eluted using an ethanol gradient. After staining, the sections were dried in an oven for 10 min and mounted with neutral resin. The morphology and number of cortical pyramidal cells in the brains of different groups of rats were observed under a microscope.

Statistical analysis

Data were processed using GraphPad Prism 9.0, and results were expressed as mean ± standard deviation. The t-test was used to compare the differences between two groups, and one-way ANOVA was used for comparisons among multiple groups. Statistical significance was set at $P < 0.05$.

Results

Effect of BC/GD concentration and hypoxia duration on VSMCs viability

The CCK-8 assay was used to detect the effects of different OGD durations and BC/GD concentrations on VSMCs viability. The results are shown in Figure 1A, with a 50% inhibitory concentration (IC50) for growth and viability of 8.815 h. VSMCs number significantly decreased after 8 h of OGD treatment. The concentration of BC/GD in the range of 1.56 to 100 μM had no significant effect on VSMCs viability under normal culture conditions, whereas concentrations greater than 100 μM significantly inhibited VSMCs viability. Therefore, in subsequent studies, an OGD duration of 8 h and BC/GD concentrations in the range of 1.56 to 100 μM were selected.

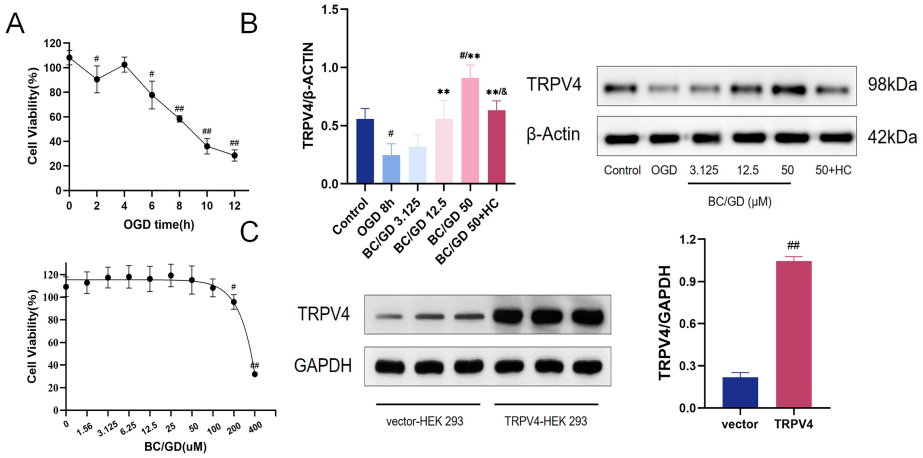


Figure 1 Screening of VSMCs drug concentration and treatment duration. (A) Optimization of Drug Concentration and Treatment Duration for VSMCs Treatment. (B) Effects of BC/GD on TRPV4 expression in OGD-injured VSMCs. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared to control group; $^{**}P < 0.01$, compared to model group; $^{\&}P < 0.05$, compared to BC/GD group. (C) HEK293 cells were transfected with TRPV4 or empty vector, and the levels of TRPV4 were detected by western blot assay. $^{\#\#}P < 0.01$, compared to vector group. n = 3. BC/GD, baicalin/geniposide; OGD, Oxygen-Glucose Deprivation; HC, HC-067047.

BC/GD upregulates the expression of TRPV4 protein

Western blotting results Figure 1B showed that the expression of TRPV4 protein in cells significantly decreased after 8 h of OGD compared to that of the blank group. After treatment with different concentrations of BC/GD, 12.5 μM and 50 μM of BC/GD significantly upregulated TRPV4 protein expression, whereas 3.125 μM showed no significant effect. Compared to the 50 μM BC/GD group, HC-067047 significantly downregulated TRPV4 protein expression.

BC/GD increases $[Ca^{2+}]_i$ in TRPV4-HEK293 cells

Considering that TRPV4 is a member of the Ca^{2+}-permeable ion channels and induces $[Ca^{2+}]_i$ upon activation, we verified the experimental results mentioned above using live cell fluorescence Ca^{2+} imaging. First, the TRPV4 plasmid was transfected into HEK 293 cells (TRPV4-HEK 293), and the transfection efficiency was verified through western blotting. As shown in Figure 1C, TRPV4 protein expression was very low in the empty HEK 293 cells, whereas it significantly increased in the transfected HEK-293 cells. As shown in Figure 2A, compared to the blank group, the BC/GD group showed increased $[Ca^{2+}]_i$ in TRPV4-HEK 293 cells, and HC-067047 inhibited BC/GD-induced $[Ca^{2+}]_i$. In empty vector-HEK 293 cells, owing to low TRPV4 protein expression, BC/GD did not increase $[Ca^{2+}]_i$. These results indicate that BC/GD increased $[Ca^{2+}]_i$ in TRPV4-HEK 293 cells.

BC/GD activates TRPV4 channels

To verify the effect of BC/GD on TRPV4 channels, we performed whole-cell patch-clamp experiments on TRPV4-HEK 293 cells. As shown in Figure 2C, BC/GD significantly increased Ca^{2+} currents in TRPV4-HEK 293 cells, and the current was inhibited when cells were treated with HC-067047. In vector-HEK 293 cells, BC/GD failed to increase cell current. These results indicate that BC/GD activates TRPV4 channels.

BC/GD increases $[Ca^{2+}]_i$ in VSMCs

As TRPV4 is widely present in VSMCs, we preliminarily explored the effect of BC/GD on VSMCs by conducting Ca^{2+} influx experiments. As shown in Figure 2B, 50 μM of BC/GD significantly induced Ca^{2+} influx in VSMCs compared to that in the blank group. After HC-067047 treatment, the effect of BC/GD on Ca^{2+} influx significantly decreased. The results of Ca^{2+} influx experiments conducted in VSMCs indicate that BC/GD activates TRPV4 channels in VSMCs, leading to Ca^{2+} influx.

BC/GD inhibits vasoconstriction

As shown in Figure 3A, 3B, U46619 and KCl induced concentration-dependent contractions in isolated CBA, and pre-incubation with BC/GD (7, 70, and 700 μM) caused the dose-response curves of both vasoconstrictors to shift downwards to the right. At the highest concentration of the constrictor, the inhibition rates of pre-incubated BC/GD at 7, 70, and 700 μM on contraction were ICHR7, ICHR70, and ICHR700, respectively: 3.62 ±

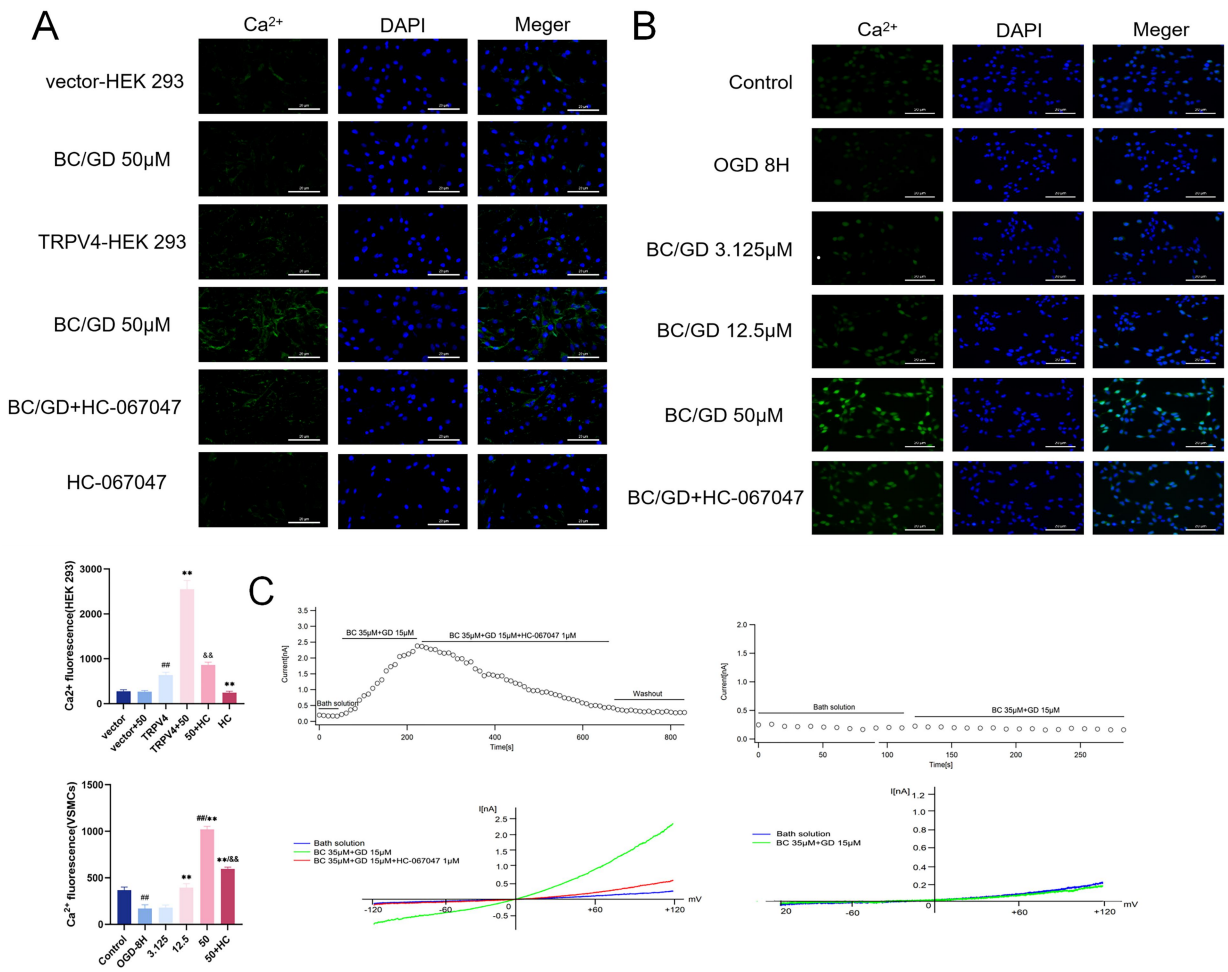


Figure 2 BC/GD increases $[Ca^{2+}]_i$ in TRPV4–HEK293 cells. (A) Representative images of BC/GD (50 μM)-induced $[Ca^{2+}]_i$ changes in cells with or without HC067047 (1 μM). $^{\#\#}P < 0.01$, compared to vector group; $^{**}P < 0.01$, compared to TRPV4 group; $^{\&\&}P < 0.01$, compared to TRPV4 + BC/GD 50 μM group. (B) Representative images of BC/GD (3.125, 12.5, 50 μM)-induced $[Ca^{2+}]_i$ changes in cells with or without HC067047 (1 μM). $^{\#\#}P < 0.01$, compared to contrl group; $^{**}P < 0.01$, compared to OGD-8H group; $^{\&\&}P < 0.01$, compared to BC/GD 50 μM group. (C) Representative I–V curve for BC/GD (50 μM)-stimulated cation current in TRPV4– and vector–HEK293 cells. 400×, Scale bar = 20 μm, n = 3. BC/GD, baicalin/geniposide; OGD, Oxygen-Glucose Deprivation; HC, HC-067047.

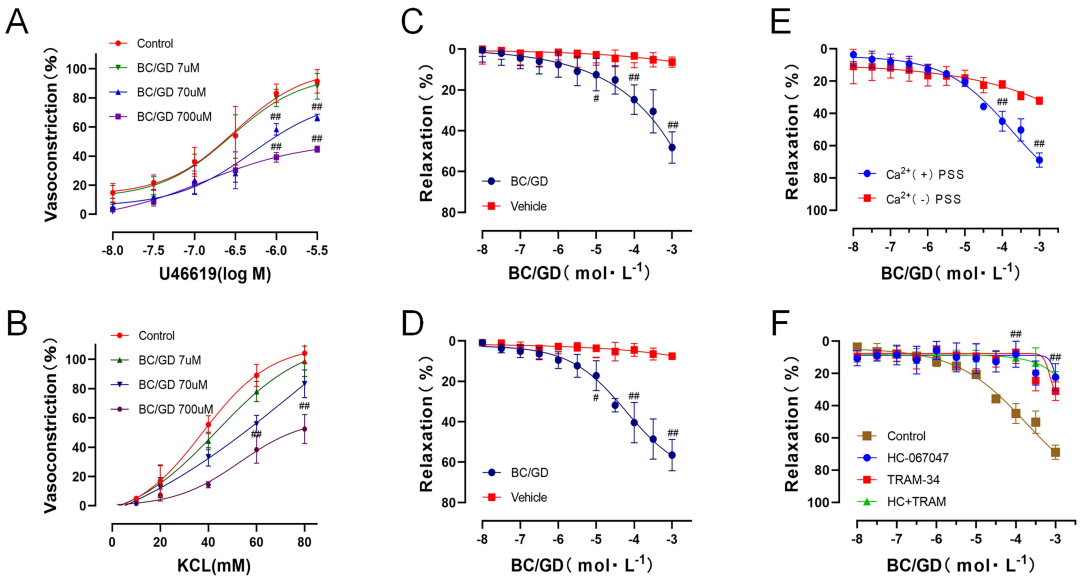


Figure 3 BC/GD improves vasorelaxation in isolated CBA involving the TRPV4-IK$_{Ca}$ axis. (A, B) Inhibitory Effects of Pre-incubated BC/GD (7, 70, 700 μM) on U46619 (0.01, 3.00 μM) and KCl (2.00, 80.00 mM)-Induced Vasoconstriction. $^{\#\#}P < 0.01$, compared to control group. (C, D) BC/GD Induces Vasodilation in Pre-contracted CBA with U46619 (0.1 μM) and KCl (60 μM). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared to Vehicle group. (E) BC/GD Exhibits Weakened Vasodilation in Ca^{2+} (−) PSS Solution. $^{\#\#}P < 0.01$, compared to Ca^{2+} (+) group. (F) Vasodilation Induced by BC/GD in the Presence of HC-067047 (100 nM) and TRAM-34 (1 μM). $^{\#\#}P < 0.01$, compared to control group. n = 3. BC/GD, baicalin/geniposide.

8.22% and 5.25 ± 8.70%, 26.9 ± 4.28% and 20.12 ± 6.49%, 50.84 ± 4.13%, and 49.91 ± 6.22%, indicating that BC/GD can inhibit the contraction induced by vasoconstrictors.

BC/GD induces vasodilation

As shown in Figure 3C, 3D, BC/GD induced the concentration-dependent relaxation of CBA pre-contracted with U46619 and KCl, with no significant effect observed in the solvent control group (vehicle) on pre-contracted CBA.

Role of Ca^{2+} in the vasodilation effect induced by BC/GD

As shown in Figure 3E, BC/GD exhibited concentration-dependent vasodilatory effects in Ca^{2+}(+) PSS culture solution. When Ca^{2+}(−) PSS was used, the maximum dilation rate significantly decreased.

Role of TRPV4 channel in BC/GD-induced vasodilation of CBA

The results showed Figure 3F that 10^{-3}M of BC/GD produced a dilation rate of 68.83 ± 4.33% on 60 mM KCl contracted CBA, which decreased to 22.33 ± 8.34% and 30.93 ± 5.61% after treatment with HC-067047 (100 nM) and TRAM-34 (1 μM), respectively. Combining the experimental results from the previous three sections, we found that BC/GD inhibited vasoconstriction and induced vasodilation, and that this effect was related to Ca^{2+} influx. The TRPV4 channel mediates Ca^{2+} influx, and Ca^{2+} is associated with vasodilation mediated by IK$_{Ca}$ channels. Therefore, we speculate that BC/GD induces the relaxation of CBA by activating TRPV4 channels, leading to Ca^{2+} influx, with the involvement of IK$_{Ca}$ channels.

BC/GD increases cerebral blood flow perfusion

Laser Doppler blood flow imaging was used to monitor cerebral blood flow on the ischemic side of the rats before modeling, 30 min after injection of the inhibitors, and 2 and 24 h after modeling. As shown in Figure 4A, 30 min after the injection of inhibitors, cerebral blood flow in the HC-067047 and TRAM-34 groups significantly decreased compared to the blood flow before modeling, suggesting that HC-067047 and TRAM-34 may reduce cerebral blood flow in rats by inhibiting vascular dilation. Two hours after modeling, the cerebral blood flow on the ischemic side of all groups significantly decreased; however, no statistical differences were observed among the groups. Twenty-four hours after modeling, the cerebral blood flow in the ischemic side of the MCAO group significantly decreased compared to that in the blank group. The cerebral blood flow in the ischemic side of the BC/GD, HC-067047, and TRAM-34 groups significantly increased compared to that in the MCAO group. The cerebral blood flow in the ischemic side of the HC-067047 and TRAM-34 groups significantly decreased compared to that in the BC/GD group. This suggests that BC/GD improves blood perfusion in the ischemic area, whereas HC-067047 and TRAM-34 inhibit the improvement in cerebral blood flow caused by BC/GD.

BC/GD attenuates neurological deficit and infarct volume

The neurological deficit scores of the rats in the MCAO group significantly increased, indicating the successful establishment of the MCAO model. The neurological deficit scores in the BC/GD group significantly decreased compared to those in the MCAO group. The neurological deficit scores in the HC-067047 and TRAM-34 groups significantly increased compared to those in the BC/GD group Table 1. Figure 4C shows TTC-stained brain slices, where MCAO injury led to a significant increase in cerebral infarct volume, with no infarct areas observed in the blank and sham groups. The BC/GD group showed a significantly reduced infarct volume compared to that in the MCAO group. The infarct volumes in the HC-067047 and TRAM-34 groups significantly increased compared to those in the BC/GD group. These results indicate that BC/GD effectively alleviates CIS damage and that this effect is inhibited by HC-067047 and TRAM-34, which is consistent with the results of the cerebral blood flow.

Comparison of Nissl staining results in rats from each group

As shown in Figure 4B, the number of pyramidal cells in the hippocampal region of rats in the MCAO group significantly decreased, with irregular cell morphology, extensive vacuolation, and pale-stained cells. The BC/GD group showed a significant reduction in vacuolated cells with compact and orderly arranged pyramidal cells, compared to that in the MCAO group. The HC-067047 and TRAM-34 groups exhibited some degree of improvement in neuronal pathological changes; however, the effect was not as pronounced as in the BC/GD group. These experiments indicate that BC/GD may mediate the vasodilation of cerebral vessels in MCAO rats through the TRPV4-IK$_{Ca}$ axis.

Discussion

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The brain uses most of the oxygen and energy in the body and is extremely sensitive to changes in energy supply. It is estimated that, under normal physiological conditions, approximately 750–1,000 mL of blood flows through the brain per minute, with the consumption of O_2 and glucose accounting for approximately 20% of the body's total consumption. Therefore, insufficient O_2 and glucose levels can lead to CIS. The CBA is formed by the convergence of the left and right vertebral arteries near the medulla and pons, supplying one-third of the blood flow to the posterior brain. The posterior inferior cerebellar artery, which connects below the CBA, is the largest branch of the vertebral artery and is a common site for embolism due to its multiple curves.

In cellular experiments, TRPV4 protein expression significantly decreased after OGD treatment, and treatment with different concentrations of BC/GD significantly upregulated TRPV4 protein expression, which was inhibited by HC-067047, suggesting that BC/GD may enhance VSMCs function by regulating TRPV4 expression. We generated TRPV4-HEK 293 cells to test for Ca^{2+} influx and verified that BC/GD induced Ca^{2+} influx in TRPV4-HEK 293 cells. In vector-HEK 293 cells with very low TRPV4 protein expression, BC/GD did not induce Ca^{2+} influx. Furthermore, using whole-cell patch-clamp experiments, we confirmed that BC/GD induced Ca^{2+} currents in TRPV4-HEK 293 cells, and no current changes were observed in vector-HEK 293 cells, showing that BC/GD can activate TRPV4 channels. TRPV4 channels on the surface of VSMCs play an important role in the regulation of vascular tension. Therefore, we conducted Ca^{2+} influx experiments in VSMCs and found that BC/GD activated TRPV4 channels in VSMCs, increasing Ca^{2+} influx. These results indicated that BC/GD activates TRPV4 channels and regulates vascular smooth muscle function.

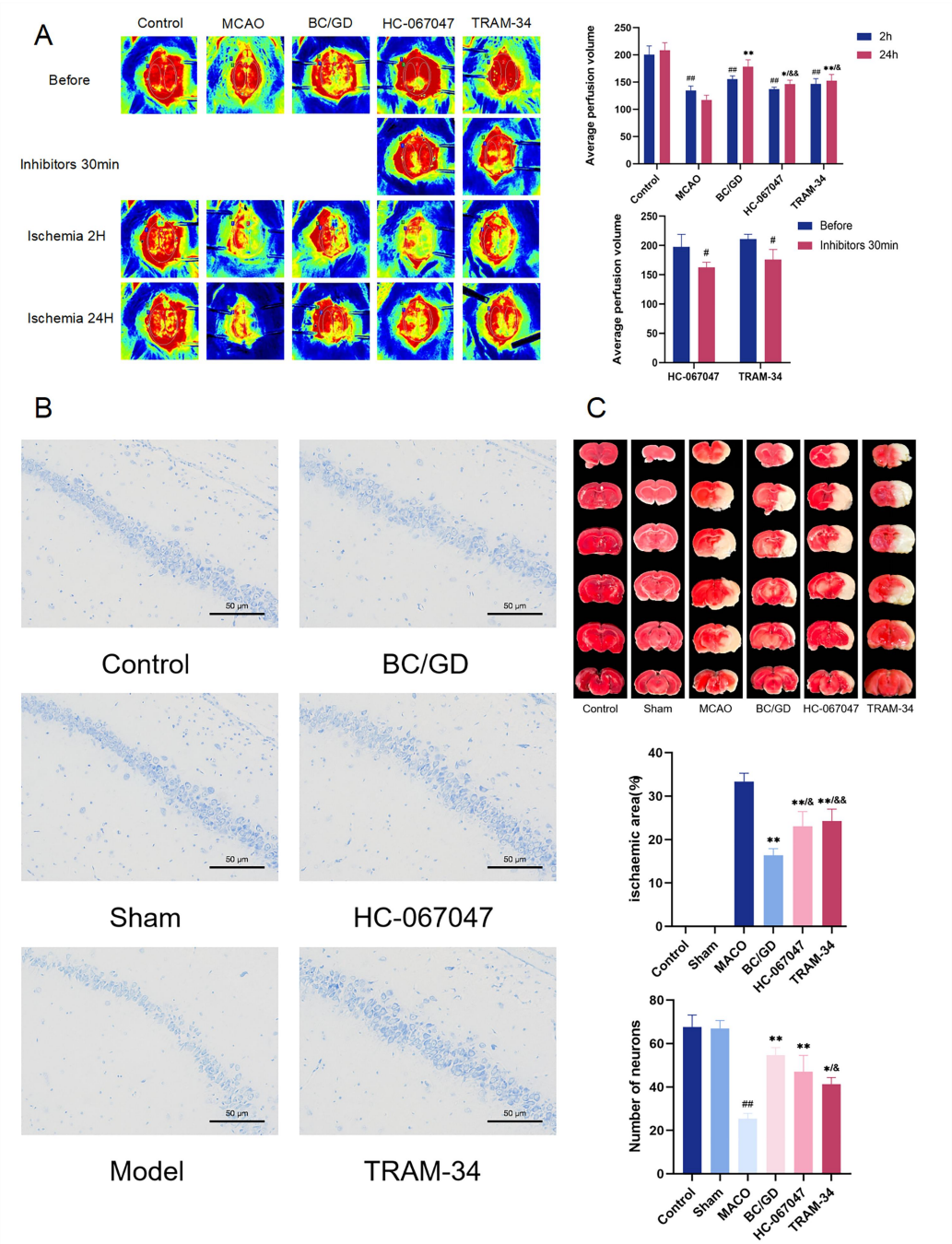

Figure 4 BC/GD has ameliorative effects on CIS. BC/GD intervention increased the cerebral blood flow in the ischemic hemisphere of MCAO rats (A), improved neurological function (B), and reduced the infarct area (C). 200×, Scale bar= 50 μm, n = 3. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared to control group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared to model group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ compared to BC/GD group. BC/GD, baicalin/geniposide.

Table 1 Neurological scores of rats in each group (n = 9)

Groups	Neurological score
Control	0 ± 0.00
Sham	0 ± 0.00
MACO	3.00 ± 0.74$^{\#\#}$
BC/GD	1.67 ± 0.65**
HC-067047	2.67 ± 0.78$^{\&\&}$
TRAM-34	2.75 ± 0.75$^{\&\&}$

$^{\#\#}P < 0.01$, compared to control group; $^{**}P < 0.01$, compared to model group; $^{\&\&}P < 0.01$, compared to BC/GD group. BC/GD, baicalin/geniposide.

To explore the preliminary mechanism of BC/GD-induced vasodilation, isolated vascular ring techniques were used to observe its effects on CBA contraction. The experimental results showed that BC/GD effectively relaxed CBA contractions induced by U46619 and KCl in a concentration-dependent manner. When the vessels were treated with the $Ca^{2+}(-)$ solution, the relaxation effect of BC/GD was significantly weakened, indicating the critical role of Ca^{2+} in BC/GD-induced vasodilation. HC-067047 and TRAM-34 inhibited the relaxation of BC/GD The results of the laser Doppler imaging indicated that BC/GD effectively improved cerebral blood perfusion on the ischemic side. The TTC staining showed that BC/GD significantly reduced the cerebral infarct volume, which was consistent with the improvement in cerebral blood flow. Nissl staining and neurological function score results simultaneously demonstrated

that BC/GD reduced neurological deficits in rats of the MCAO group and significantly decreased neuronal damage; these improvements were inhibited by treatment with HC-067047 and TRAM-34. Therefore, we speculate that BC/GD activates the TRPV4 channel, leading to Ca^{2+} influx, which in turn activates the IK_{Ca} channel to regulate the vasodilation function of the vascular smooth muscle.

Although this study preliminarily elucidated the vascular protective effects of BC/GD in CIS, some limitations must be acknowledged. First, the experiment did not deeply explore the detailed mechanisms of action of the BC/GD and TRPV4 channels. We speculate that the activation of TRPV4 may involve a series of complex signaling pathways, such as the secretion of NO, PGI2, and other vasodilatory factors, as well as intercellular signaling between endothelial cells and VSMCs. This study only explored the possible roles of IK_{Ca} and TRPV4 channels, without considering other K_{Ca} channels. Second, the animal models used in this study mainly focused on the MCAO model, which, although highly clinically relevant, may have different mechanisms because of the complexity of human CIS. Further validation using clinical samples is required.

Conclusions

In summary, this study demonstrates that BC/GD induces Ca^{2+} influx by activating TRPV4 channels, further activating IK_{Ca} channels to regulate VSMCs function, improving cerebral blood perfusion, and alleviating ischemic brain injury. This mechanism provides new insights into how BC/GD can treat CIS, demonstrating its significant potential for improving cerebrovascular function.

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