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RESEARCH ARTICLE

Neuroprotective effects of *Ginkgo biloba* extract and Ginkgolide B against oxygen–glucose deprivation/reoxygenation and glucose injury in a new *in vitro* multicellular network model

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Abstract **Acute ischemic stroke (AIS), as the third leading cause of death worldwide, is characterized by its high incidence, mortality rate, high incurred disability rate, and frequent reoccurrence. The neuroprotective effects of *Ginkgo biloba* extract (GBE) against several cerebral diseases have been reported in previous studies, but the underlying mechanisms of action are still unclear. Using a novel *in vitro* rat cortical capillary endothelial cell-astrocyte-neuron network model, we investigated the neuroprotective effects of GBE and one of its important constituents, Ginkgolide B (GB), against oxygen–glucose deprivation/reoxygenation and glucose (OGD/R) injury. In this model, rat cortical capillary endothelial cells, astrocytes, and neurons were cocultured so that they could be synchronously observed in the same system. Pretreatment with GBE or GB increased the neuron cell viability, ameliorated cell injury, and inhibited the cell apoptotic rate through Bax and Bcl-2 expression regulation after OGD/R injury. Furthermore, GBE or GB pretreatment enhanced the transendothelial electrical resistance of capillary endothelial monolayers, reduced the endothelial permeability coefficients for sodium fluorescein (Na-F), and increased the expression levels of tight junction proteins, namely, ZO-1 and occludin, in endothelial cells. Results demonstrated the preventive effects of GBE on neuronal cell death and enhancement of the function of brain capillary endothelial monolayers after OGD/R injury *in vitro*; thus, GBE could be used as an effective neuroprotective agent for AIS/reperfusion, with GB as one of its significant constituents.**

Keywords acute ischemic stroke; *Ginkgo biloba* extract; Ginkgolide B; network model; neuroprotection

Introduction

Acute ischemic stroke (AIS) is the primary cause of adult deaths worldwide. It is a devastating condition characterized by its high rates of incidence, mortality, incurred disability, and reoccurrence [1–3]. For the past few years, despite considerable advances in AIS research, the findings did not seem significant because of limited therapeutic options [4]. At present, intravenous alteplase administered 4.5 h within symptom onset is the only reperfusion therapy available in the clinic with proven efficacy in AIS patients. However, its therapeutic effect is limited [5,6].

At a certain time after AIS/reperfusion, the central nervous system (CNS) can incur profound cerebral microcirculatory damage and even neuronal death. Furthermore, damaged or dead neurons can lead to disability and death after AIS [7–9]. Moreover, after

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AIS, the cortical capillary endothelial cells are damaged, which causes a loss of integrity of the blood–brain barrier (BBB). After these phenomena, intravascular monocytes and other inflammatory cells enter the brain tissue, leading to post-stroke inflammation. All those processes are important in the second neuronal hit and death after AIS [10,11]. Therefore, the agents that target both neuronal damage and post-ischemic BBB integrity may possess therapeutic potential in AIS/reperfusion.

Ginkgo biloba extract (GBE) is a classical herbal product extracted from *G. biloba* leaves [12], containing 22%–27% flavonol glycosides, 5.4%–6.6% terpene trilactones, 2.8%–3.4% ginkgolides (A, B, and C), 2.6%–3.2% bilobalide, and less than 5 ppm ginkgolic acid [13]. Substantial laboratory and clinical studies have proven that GBE exerts hemodynamic and neurotransmitter effects, as well as anti-oxidant/free-radical-scavenging effects. GBE has been widely used for treating coronary heart disease and Alzheimer's disease in clinical settings and demonstrated beneficial effects at biochemical and pharmacological levels [14,15]. Ginkgolide B (GB) is one of the major constituents of the terpene trilactones of GBE that has been proven to possess neuroprotective effects against CNS damage secondary to cerebral ischemia [16,17]. However, the cellular and molecular mechanisms of the neuroprotective effects of GBE against AIS remain unclear.

The single-cultured neuron oxygen–glucose deprivation/reoxygenation (OGD/R) model is a common *in vitro* model that mimics AIS/reperfusion; agents are added directly in the Neurobasal Media to observe their neuroprotective effects on neurons [18–20]. However, this research model is too simple compared with the *in vivo* environment and may overlook the effects of astrocytes, BBB, and other factors on neurons. To address this issue, a new *in vitro* AIS/reperfusion model was established. In this model, Sprague–Dawley (SD) rat cortical capillary endothelial cells, astrocytes, and neurons were cocultured according to their relative locations *in vivo* via OGD/R to mimic AIS/reperfusion. The agents were added in the E medium to mimic peripheral blood entering the CNS through BBB *in vivo*.

In this study, we investigated the neuroprotective effects and possible molecular mechanisms of GBE and one of its major constituents, GB, against OGD/R injury using a novel *in vitro* multicellular network model to gain insight into the agents that could be used in treating AIS/reperfusion.

Material and methods

Chemical compounds

GBE constituents, 24% flavonol glycosides, and 6% terpene trilactones were supplied by the Institute for Brain Disorders of Dalian Medical University (Dalian, China). GB was purchased from Sigma (Fig. 1).

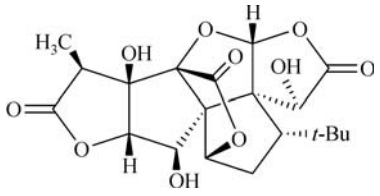

Fig. 1 Chemical structure of GB.

Animals

SD rats (both neonatal and adult) were purchased from Dalian Medical University Animal Center (Dalian, China). All studies were implemented in compliance with institutional guidelines of Dalian Medical University.

Primary culture of rat cortical neurons

The primary cortical neurons were obtained from postnatal day 1 SD rats. Meninges and great vessels were removed from the cortex; then, the cortex was minced into approximately 1 mm^3 pieces in ice cold buffer and digested in 0.125% trypsin–ethylene diamine tetraacetic acid solution at 37 °C for 30 min. The mix was then gently resuspended by adding Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). After filtration, the cortical tissue was centrifuged at 1000 r/min for 5 min and resuspended in DMEM with 10% FBS. The supernatant was discarded and cortical neurons were then plated at 5×10^5/mL into culture plates pre-coated with polylysine (0.1%). The culture plates were stored in a 5% CO_2 humidified incubator at 37 °C for 2 h. The culture medium was then replaced with Neurobasal Media (Gibco 10888022, USA) supplemented with 2% B27 Supplement. Cortical neurons were maintained in primary culture for 6–8 days before being used in experiments.

Primary culture of rat cortical astrocytes

The primary cortical astrocytes were obtained from postnatal day 1 SD rats. Meninges and great vessels were removed and cortical pieces were mechanically dissociated in the astrocyte culture medium (DMEM supplemented with 10% FBS). Dissociated cells were seeded into cell culture flasks and stored in a 5% CO_2 humidified incubator at 37 °C for 7–8 days. To obtain pure astrocytes, flasks with confluent cultures were shaken overnight at 37 °C at 250

r/min. The pure astrocytes prepared in this manner were used in subsequent experiments.

Primary culture of rat cortical capillary endothelial cells

Primary capillary endothelial cells were obtained from adult SD rats (150–180 g body weight). Meninges were removed from forebrains under sterile conditions and the gray matter was minced into small pieces in ice-cold buffer and dissociated by DMEM containing 1% collagenase/dispase for 1 h at 37 °C. The cell pellets were separated by centrifugation in 25% bovine serum albumin (BSA)-DMEM (1000 r/min, 20 min). The precipitate obtained from the pellets was further digested with DMEM containing 1% collagenase/dispase for 2 h at 37 °C. Endothelial clusters were separated on a 33% continuous Percoll gradient, collected, and washed twice in DMEM before plating on a culture bottle coated with collagen type IV. Endothelial cells were maintained in MCDB131 (Gibco M-131-500) supplemented with 10% FBS, 1% microvascular Growth Supplement (Gibco S-005-25), 100 U/mL penicillin, 100 U/mL streptomycin and puromycin (4 mg/mL) (E medium I) and cultured in a humidified atmosphere of 5% CO_2 at 37 °C for 2 days. On the third day, the cells were transferred to a new medium that contained all the components of the above medium except for puromycin (E medium II). When the cultures reached 80% confluency (5–7 days *in vitro*), the purified endothelial cells were considered fit for the subsequent experiments.

Construction of rat cortical capillary endothelial cell–astrocyte–neuron network models *in vitro*

In this study, the SD rat cortical capillary endothelial cells, astrocytes, and neurons were first cocultured according to their relative locations *in vivo*. To construct new models, 5–7 days old primary endothelial cells (2×10^5 cells/cm^2) were seeded on the interior side of the collagen-coated polyester membrane of Transwell inserts (Millipore, 1.0 μm, USA), which were placed in 6- or 24-well culture plates containing no cells. Both luminal and abluminal compartments were maintained in E medium II and the culture plates were cultured in a humidified atmosphere of 5% CO_2 at 37 °C for approximately 4 days. On the 5th day, the insert containing endothelial cells was inverted in a sterile Petri dish, and the astrocyte suspension (5×10^4 cells/cm^2) was seeded on the exterior side of the inserts and placed in a humidified atmosphere of 5% CO_2 at 37 °C for 3 h. After 3 h, the insert containing both endothelial cells and astrocytes was replaced in the well of culture plates, and E–A were cocultured in the humidified atmosphere of 5% CO_2 at 37 °C for approximately 2 days to allow the cells to adhere firmly. Then the E–A cocultured insert was replaced in the well of the 6- or 24-well culture plates with 10 day primary neurons. Under these conditions, the *in vitro* basal network of endothelial cell–astrocyte–neuron (EAN) models was established (Fig. 2).

Construction of the OGD/R EAN model *in vitro*

After 1–2 days of coculturing, the basal EAN model was

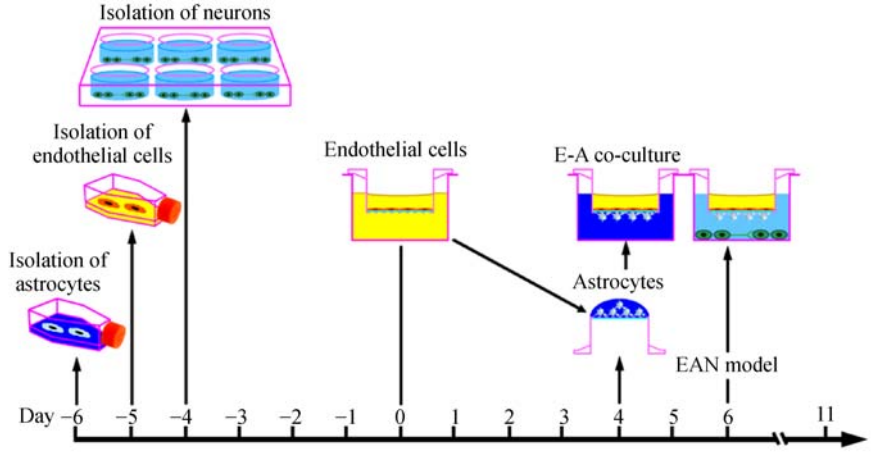

Fig. 2 Schematic illustration of the construction of *in vitro* basal EAN models. The primary SD rat cortical astrocytes, capillary endothelial cells, and neurons were isolated and cultured alone at days 1, 2, and 3, respectively; 5–7 days primary endothelial cells were seeded on the interior side of collagen-coated polyester membrane of the inserts and maintained in E medium II for approximately 4 days. On the 5th day, the insert containing endothelial cells was inverted and the astrocytes suspension was seeded on the exterior side for 3 h. After 3 h, the E–A cocultured insert was maintained in E medium II for approximately 2 days and then replaced in the well containing 10 days primary neurons.

used to construct an OGD/R EAN model to create an AIS model *in vitro*. Four groups were established in this experiment, namely control, OGD/R, GBE (0.1 mg/mL), and GB (100 μmol/L) groups. GBE (0.1 mg/mL) and GB (100 μmol/mL) groups were added to luminal E medium II 24 h before OGD, and the control and OGD/R groups received only 0.05% dimethyl sulfoxide (DMSO). One day later, E medium II, Neurobasal Media, luminal, and abluminal compartment were replaced with glucose-free DMEM (Gibco 11966-025) supplemented with 10% FBS and 1% MVGS and glucose-free Neurobasal Media (Gibco 0050128DJ) supplemented with 2% B_{27} supplement and 2 mmol/L l-glutamine, respectively. The culture plates with E–A cocultured insert were then placed in a hypoxic humidified incubator and flushed with a gas mixture of 95% N_2 and 5% CO_2 (Oligo COY, USA) at 37 °C for 2 h. Two hours later, the cells were cultured in standard medium under normal conditions for reoxygenation and glucose recovery.

MTT assay

After OGD/R, cell viability was assessed using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. The E–A cocultured inserts were replaced from the 24-well culture plates, the medium was drawn from each well, and fresh Neurobasal Media (1 mL/well) was added; then, 100 μL MTT (5 mg/mL diluted by PBS) (Sigma, USA) was added to each culture well and incubated at 37 °C for 4 h. After 4 h, the medium was gently aspirated. Deposited formazan crystals were lysed in 500 μL DMSO by gently shaking the plate. Absorbance at 570 nm was measured using a microplate reader (Thermo, USA). The cell viability (%) was expressed as a percentage relative to the untreated control cells.

Lactate dehydrogenase release assay

Lactate dehydrogenase (LDH) release in the supernatant was determined as an index of cell membrane integrity. After OGD/R, LDH release was measured using a rat LDH assay kit (Jiancheng, Nanjing, China) according to the manufacturer's instructions.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling analysis

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed to determine nucleosomal DNA fragmentation in cells by using the Roche In Situ Cell Death Detection Kit. After OGD/R, E–A cocultured inserts were replaced and neurons were washed twice using Earle's balanced salt solution. The cells were fixed in 4% paraformaldehyde in PBS, pH 7.4, for 1 h at 15 °C to 25 °C. They were then incubated with 3% H_2O_2 in methanol for 10 min at 15 °C to 25 °C before being permeabilized in 1% sodium citrate (tri-sodium salt) containing 0.1% TX-100. The result was visualized using a fluorescent microscope. The cells with green staining within the nucleus were counted as TUNEL-positive cells. The number of TUNEL-positive cells was counted in three nonoverlapping microscopic eyeshots by a person blinded to the group assignment, and amounted to 200 cells in each eyeshot; the apoptotic cells percentage = TUNEL positive cells/total cells.

Transepithelial electrical resistance analysis

Transepithelial electrical resistance (TEER) reflecting paracellular permeability and RBEC layer integrity was measured by the Epithelial-volt-ohm meter and Endohm-12 chamber electrodes (Millicell-ERS2, USA). Each group was measured using TEER values at least thrice after OGD/R injury, and the cell-free filters were subtracted from the measured TEER values.

Transendothelial permeability of the Na-F analysis

The flux of Na-F across the endothelial monolayer was determined as previously described [21]. The E–A cocultured inserts were transferred to 24-well plates containing 1.3 mL Ringer–Hepes buffer (136 mmol/L NaCl, 0.9 mmol/L $CaCl_2$, 0.5 mmol/L $MgCl_2$, 2.7 mmol/L KCl, 1.5 mmol/L KH_2PO_4, 10 mmol/L NaH_2PO_4, 25 mmol/L glucose, 10 mmol/L Hepes, pH 7.4). The culture medium was replaced with 0.5 mL Ringer–Hepes buffer containing 25 mmol/L Na-F in the inserts. The inserts were then transferred at 30, 60, 20, 90, 120, and 150 min to a new well containing Ringer–Hepes buffer. The concentrations of the marker molecules in the samples from luminal and abluminal compartments were determined using a fluorescence microplate reader (Thermo, USA; excitation wavelength, 485 nm; emission wavelength, 535 nm). The flux across cell-free inserts was measured as well. Transendothelial permeability coefficient (Pe) was calculated using the equation: $\mathrm{Pe(cm/s)} = V_{\mathrm{abluminal}}/(A \times 60 \times t) \times C_{\mathrm{abluminal}}/C_{\mathrm{beginning}}$. $V_{\mathrm{abluminal}}$ (mL) is the total volume of the abluminal compartment, A (cm^2) is the surface area of the insert polyester membrane, and t (min) is the time point for detection. $C_{\mathrm{abluminal}}$ (μg/mL) represents the Na-F concentration of abluminal compartments, and $C_{\mathrm{beginning}}$ (μg/mL) represents the initial concentration of Na-F. Transendothelial permeability was calculated according to the equation: $1/P_{\mathrm{endothelial}} = 1/P_{\mathrm{total}} - 1/P_{\mathrm{insert}}$.

Immunofluorescence staining

To characterize different cells, the cultures were washed in PBS and fixed with ethanol for 30 min at 4 °C, then

blocked with 3% BSA for at least 1 h. Endothelial cells were then incubated with anti-von Willebrand factor (vWF) rabbit polyclonal antibody (Abcam, UK), astrocytes with anti-glial fibrillary acidic protein (GFAP) rabbit polyclonal antibody (Abcam, UK), and neurons with anti-microtubule associated protein 2 (MAP-2) mouse monoclonal antibody (Abcam, UK). All primary antibodies were used in a 1:100 dilution following manufacturer's instructions. As secondary antibodies, DyLight 594-labeled anti-rabbit or mouse IgG (both from Abcam, UK) were used in a 1:1000 dilution. The cells were counterstained with 0.1 μg/mL DAPI for 15 min at 15 °C to 25 °C in the dark to visualize the nuclei.

To visualize the brain endothelial tight junction protein zonula occludens 1 (ZO-1), the insert with endothelial cells was washed in PBS and fixed with ethanol (95%) and acetic acid (5%) for 10 min at 20 °C. After blocking with 3% BSA, the cells were incubated with primary antibodies anti-ZO-1 (Abcam, UK) rabbit polyclonal antibody for 1 h at 37 °C. The incubation with secondary antibody DyLight 594-labeled anti-rabbit IgG lasted for 1 h, and the cell nuclei were counterstained by DAPI staining as mentioned above. Finally, the cells were visualized using a Nikon fluorescent microscope.

Western blot analysis

After OGD/R injury, the neurons and endothelial cells from the *in vitro* model were used to detect the protein expression separately. First, the E–A cocultured insert was taken out, astrocytes on the exterior side of the inserts were scraped by a cell scraper, the insert bottom was cut, and then both endothelial cells cultured in the interior side of the inserts and neurons cultured on the bottom of the well were harvested by a cell scraper. The harvested cells were treated with a cell lysis buffer containing phenylmethanesulfonyl fluoride and phosphatase inhibitor (v/v = 99:1) for 40 min on ice. Protein concentration was determined using a BCA protein assay kit. The same amount of protein sample (50 mg) was loaded onto a 12% SDS polyacrylamide gel and transferred onto polyvinylidene difluoride membranes. The blots were blocked with nonfat dry milk powder in PBST for 1 h at room temperature and then incubated with diluted primary antibodies overnight at 4 °C. After washing with PBST, the membranes were incubated with horseradish peroxidase-coupled secondary antibodies and visualized using an enhanced chemiluminescence detection system. The primary antibodies used were: anti-ZO-1 (1:1000) (Abcam, UK), anti-occludin (1:1000) (Abcam, UK), anti-Bcl-2 (1:1000) (Abcam, UK), anti-Bax (1:1000) (Abcam, UK), and anti-β-Actin (1:1000) (Abcam, UK), with β-actin used as the internal standard. The ZO-1, occludin, Bcl-2, and Bax expression levels were normalized to β-actin.

Statistical analysis

All results were presented as mean ± SEM from at least three experiments. One-way analysis of variance was performed for comparisons between more than two groups using SPSS 11.3. Statistical significance was set at $P < 0.05$.

Results

Characterization of cells in the basal EAN model

The morphological characterizations of cells in the basal EAN model were determined using immunofluorescence staining (Fig. 3A). After 1–2 days of co-culturing, endothelial cells, astrocytes, and neurons in the basal EAN model were subjected to immunofluorescence staining. Endothelial cells that were characterized by vWF, a special marker for endothelium immunostaining, presented with non-overlapping continuous monolayers and a tightly apposed, elongated, and fusiform morphology. Astrocytes that were characterized by GFAP immunostaining presented a polygonal morphology with long cell processes that indicated a differentiated phenotype. Neurons that were characterized by MAP-2 immunostaining in the culture spread regularly with irregular dendrites and were interlaced with each other. Calculation of TEER values of endothelial cells were performed from the first day when endothelial cells were seeded on the interior side of the Transwell inserts. The results showed that at the 5th day, TEER values peaked, which means that tight junctions (TJ) were formed between endothelial cells (Fig. 3B). Moreover, the astrocyte processes directly reached the surface of the endothelial cells through the pore of the insert, as observed using an electronic microscope (Fig. 3C).

Effects of GBE and GB pretreatment on paracellular permeability of endothelial cells after OGD/R

The TEER values and the Na-F transendothelial permeability can reflect paracellular permeability of endothelial cells. Compared with the control group, the OGD/R group showed low TEER values and high Na-F permeability. Pretreatment with GBE (0.1 mg/mL) or GB (100 μmol/L) for 3 h increased the tightness of endothelial cell monolayers after OGD/R injury. Moreover, the effect of GBE (0.1 mg/mL) was significantly more pronounced than that of GB (100 μmol/L), as reflected from the TEER values and Na-F permeability (Fig. 4).

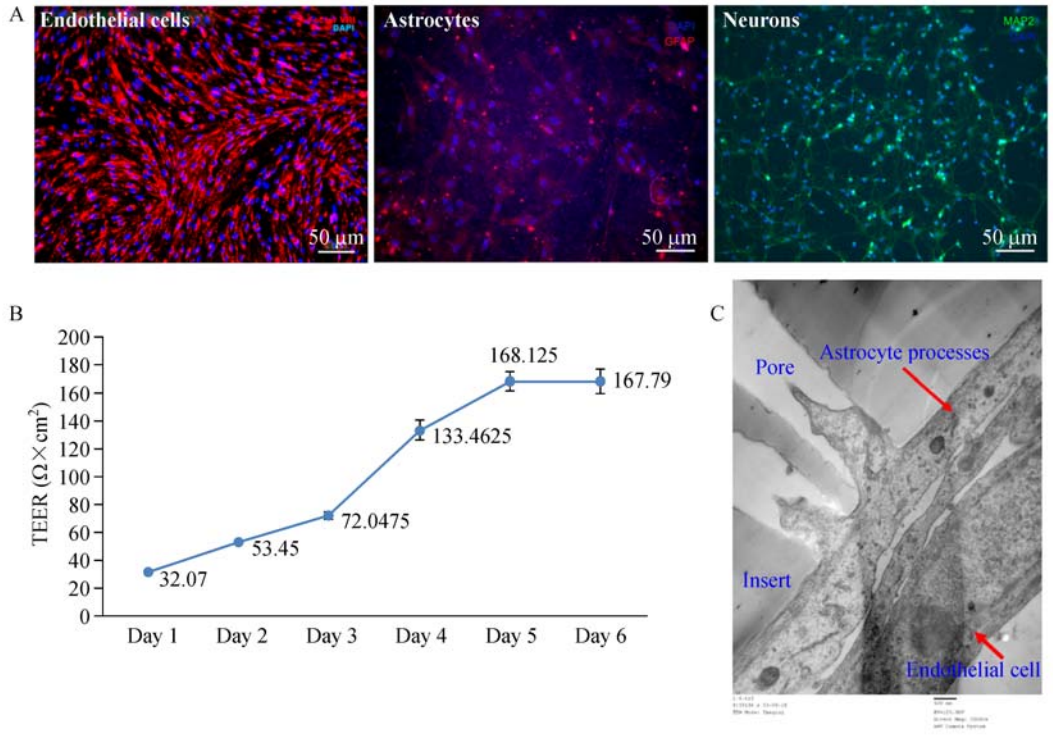

Fig. 3 Characterization of cells in the basal EAN model. (A) Immunofluorescence characterization of endothelial cells, astrocytes, and neurons after 2 days of coculturing in the basal EAN model. Bar = 50 μm. (B) TEER values of endothelial cells from the first day when endothelial cells were seeded on the interior side of the Transwell inserts to the sixth day. (C) Astrocyte processes directly reach the surface of endothelial cells through the pore of the insert, which was observed using an electronic microscope.

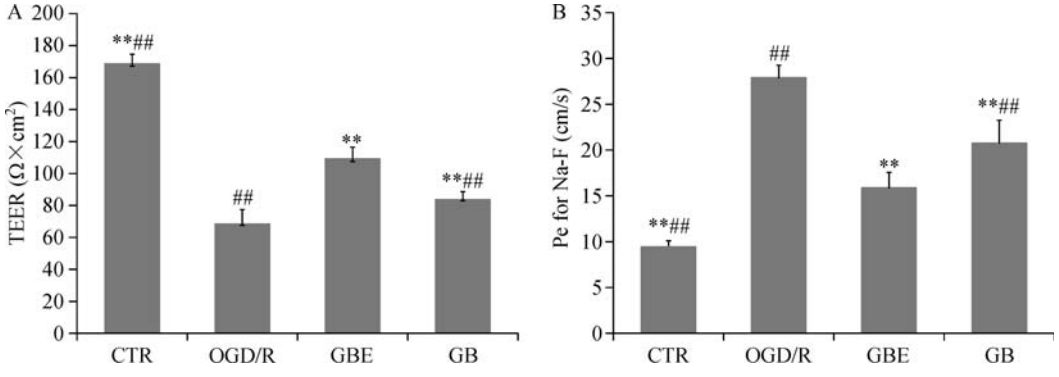

Fig. 4 TEER and Na-F permeability of endothelial cells in different groups after OGD/R injury. (A) Except for the control group, the other groups were subjected to OGD/R injury. The TEER values are presented as mean ± SEM ($n = 4$). $^{**}P < 0.01$ compared with the OGD/R group, ## $P < 0.01$ compared with the GBE group. (B) Except for the control group, the other groups were subjected to OGD/R injury. Pe for Na-F is presented as the mean ± SEM ($n = 4$). $^{**}P < 0.01$ compared with OGD/R group, ## $P < 0.01$ compared with GBE group.

Effects of GBE and GB pretreatment on the expression and localization of interendothelial tight junction proteins after OGD/R

To determine the effects of GBE and GB pretreatment on interendothelial tight junction, the levels of TJ scaffold proteins, ZO-1 and occludin, in RBEC in different groups were determined by Western blot analysis. The results showed that the protein expression levels of ZO-1 and occludin in RBEC were significantly decreased after OGD/R compared with those in the control group. Pretreatment with GBE (0.1 mg/mL) or GB (100

μmol/L) for 3 h increased the levels of those proteins; the protective effects of GBE on ZO-1 and occludin were more pronounced than those of GB (Fig. 5). Moreover, immunofluorescence staining for intercellular junction proteins ZO-1 was used to visualize the cell–cell contacts. In the control group, ZO-1 was localized on the cytomembrane and restricted to intercellular junctions. The cell borders were smooth, continuous, and high density; furthermore, the typical long fusiform endothelial cells could be observed. After OGD/R injury, the distribution of ZO-1 in endothelial cells was uneven, intermittent, low density, and chaotic. This appearance was significantly improved by pretreatment with GBE (0.1 mg/mL) or GB (100 μmol/L) (Fig. 6).

Effects of GBE and GB pretreatment on the MTT assay after OGD/R

To assess the protective effects of GBE and GB pretreatment on cortical neurons, the MTT assay was used to determine the cell survival rate under different conditions. After OGD/R injury, the viability of neurons in the OGD/R group decreased significantly compared with those in the control group. However, the pretreatment with GBE (0.1 mg/mL) or GB (100 μmol/L) for 3 h had a protective effect on cell viability against OGD/R-induced damage to neurons (Fig. 7). Furthermore, the GBE group showed superior protective properties compared with the GB group after OGD/R injury.

Effect of GBE and GB pretreatment on the LDH assay after OGD/R

The LDH assay kit was used to evaluate LDH leakage as a cell injury indicator. The LDH leakage of neurons in the OGD/R group significantly increased compared with those in the control group. Pretreatment with GBE (0.1 mg/mL) or GB (100 μmol/L) for 3 h significantly decreased the LDH release from neurons (Fig. 8). The results of the MTT assay were similar and the GBE group exhibited lower LDH release rates than the GB group, indicating that GBE (0.1 mg/mL) has a better neuroprotective effect on neurons.

Effects of GBE and GB pretreatment on the TUNEL assay after OGD/R

The TUNEL analysis revealed that the apoptosis rate

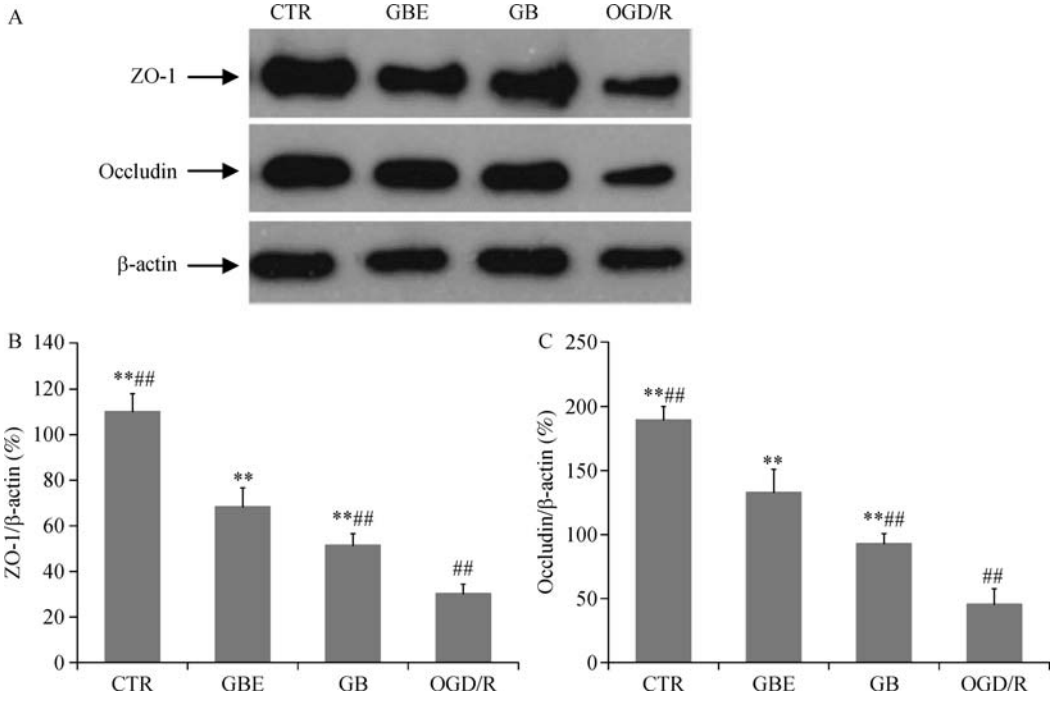

Fig. 5 The ZO-1 and occludin protein expression levels of endothelial cells in different groups were analyzed by Western blot analysis. (A) Except for the control group, the other groups were subjected to the OGD/R injury. The ZO-1 and occludin protein expression of neurons in different groups was analyzed by Western blot analysis. (B) ZO-1 protein expression is presented as the mean ± SEM ($n = 4$). **$P < 0.01$ compared with OGD/R group, ## $P < 0.01$ compared with GBE group. (C) Quantification of occludin protein expression is presented as the mean ± SEM ($n = 4$). **$P < 0.01$ compared with the OGD/R group, ## $P < 0.01$ compared with the GBE group.

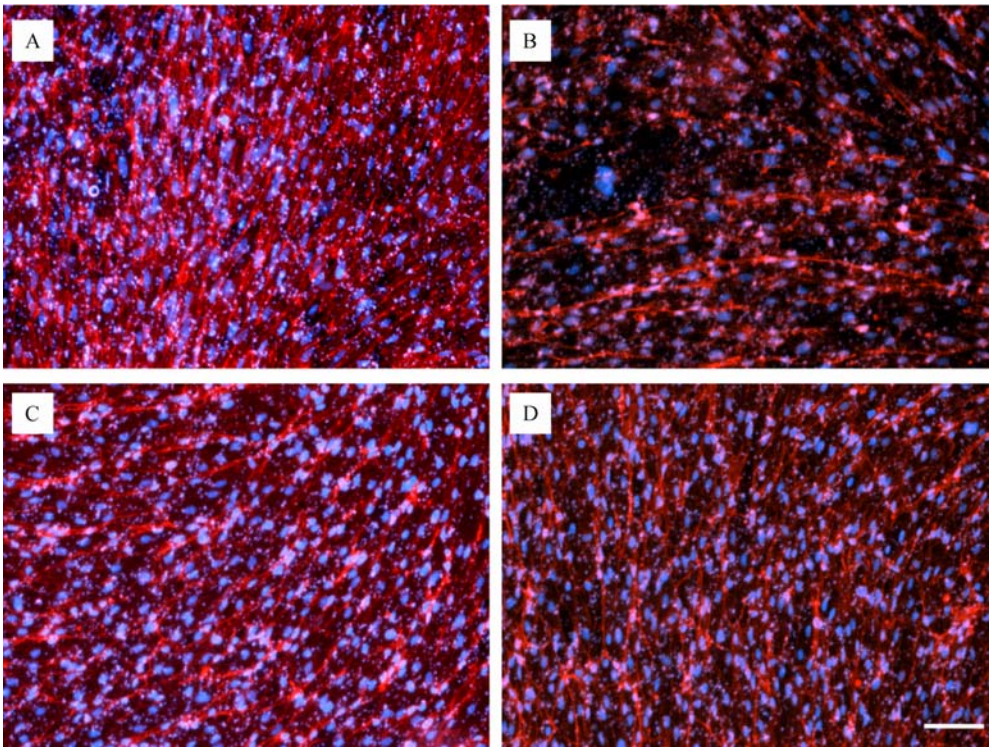

Fig. 6 Immunofluorescence staining for ZO-1 in endothelial cells in different groups. (A) In the control group, ZO-1 was expressed in endothelial cells, (B) in the OGD/R group, ZO-1 was expressed in endothelial cells, (C) in the GBE group, ZO-1 was expressed in endothelial cells, (D) in the GB group, ZO-1 was expressed in endothelial cells. Bar = 50 μm.

(positive cells/total cells × 100%) was relatively higher in the OGD/R group compared with that in control group. Pretreatment with GBE (0.1 mg/mL) or GB (100 μmol/L) for 3 h significantly decreased the neuronal apoptosis rate that was induced by OGD/R. The results indicated that GBE is a highly effective neuroprotectant (Fig. 9).

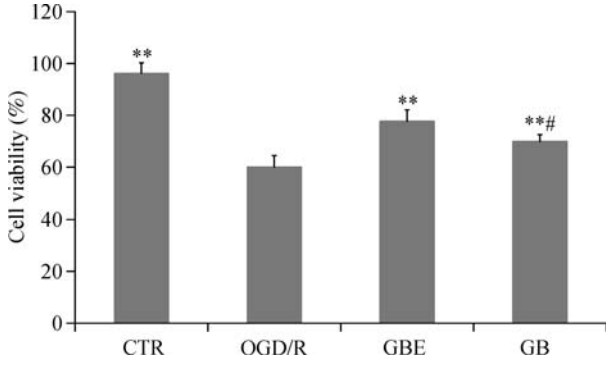

Fig. 7 The neuron cell viability in different groups was analyzed by MTT assay. Except for the control group, the other groups were subjected to the OGD/R injury. The cell viability is presented as mean ± SEM ($n = 4$). $^{**}P < 0.01$ compared with OGD/R group, # $P < 0.05$ compared with GBE group.

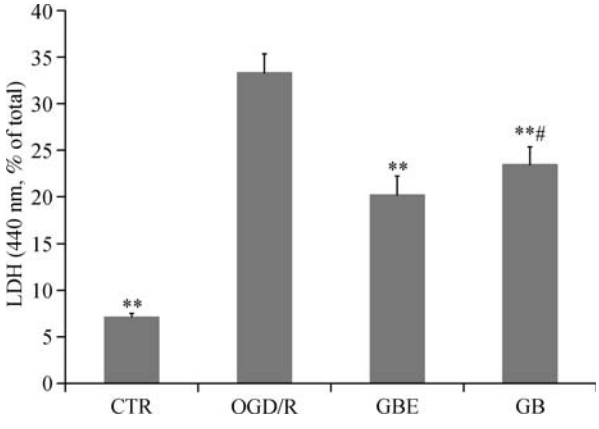

Fig. 8 LDH leakage of neurons in different groups was analyzed by LDH assay. Except for the control group, the other groups were subjected to the OGD/R injury. The LDH leakage of neurons is presented as mean ± SEM ($n = 4$). $^{**}P < 0.01$ compared with OGD/R group, # $P < 0.05$ compared with GBE group.

Effects of GBE and GB pretreatment on Bcl-2 and Bax protein expression levels in neurons

Previous studies have shown that Bcl-2 is an important

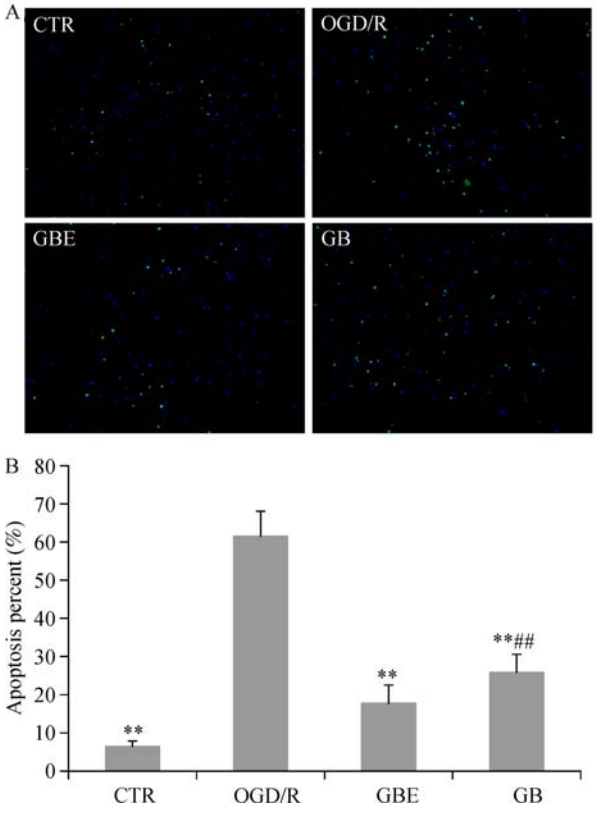

Fig. 9 The apoptosis percent of neurons in different groups was analyzed by the TUNEL assay. (A) Except for the control group, the other groups were subjected to OGD/R injury. The TUNEL assay of apoptosis was conducted in different groups. Green represents apoptotic cells, whereas blue is DAPI. (B) The neuronal apoptosis percent is presented as mean ± SEM ($n = 4$). **$P < 0.01$ compared with OGD/R group, ##$P < 0.01$ compared with GBE group.

anti-apoptotic protein, and that Bax is a pro-apoptotic protein. Given that GBE and GB pretreatment markedly enhance the survival rate and reduce the apoptotic rate of neurons (Fig. 10), the Bcl-2 and Bax protein levels of neurons in different groups were monitored using Western blot analysis. The results showed that the expression of Bcl-2 decreased after OGD/R; moreover, the expression of Bax increased ($P < 0.01$) compared with that in the control group. Pretreatment with GBE (0.1mg/mL) or GB (100 μmol/L) for 3 h significantly increased the expression of Bcl-2 and decreased the expression of Bax in the neurons.

Discussion

In studying AIS, the single-cultured neuron OGD/R model is the commonly used *in vitro* model to mimic AIS/reperfusion. In previous studies, the test substances were directly added to Neurobasal Media to observe their neuroprotective effects [18–20]. This research model is too simple compared with *in vivo* environments and may neglect the effects of astrocytes, BBB, and other factors on neurons. To address this issue, a novel *in vitro* AIS/reperfusion model was established in this study. In the developed model, the SD rat capillary endothelial EAN were cocultured so that they could be synchronously observed in the same system. The test substances were added to the E medium to mimic their entry to the CNS from the peripheral blood through BBB.

In this study, we investigated the neuroprotective effects and possible molecular mechanisms of GBE and one of its major constituents, GB, against OGD/R injury using a novel *in vitro* rat EAN model to gain insight into the agents that could be used in treating AIS/reperfusion.

In this model, the SD rat cerebral capillary EAN were cocultured according to their relative locations in the body. This cocultured system can mimic the CNS environment of AIS/reperfusion through OGD/R. Compared with the previous single-cultured neuron OGD/R model, the new EAN model is instrumental for studying multicellular interactions. In CNS research, multicellular co-culturing has received significant attention

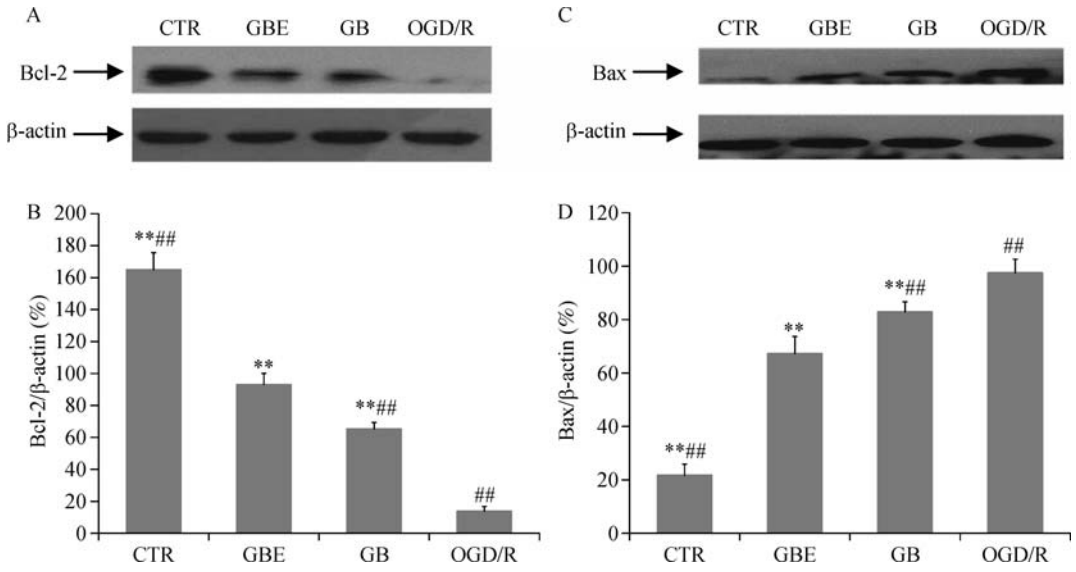

Fig. 10 The Bcl-2 and Bax protein expression of neurons in different groups was analyzed by Western blot analysis. (A) Except for the control group, the other groups were subjected to OGD/R injury and Bcl-2 protein expression of neurons in different groups was analyzed by Western blot analysis. (B) Bcl-2 protein expression is presented as mean ± SEM ($n = 4$). $^{**}P < 0.01$ compared with OGD/R group, ## $P < 0.01$ compared with GBE group. (C) The Bax protein expression in neurons in different groups was analyzed by Western blot analysis. (D) Bax protein expression was quantified and presented as mean ± SEM ($n = 4$). $^{**}P < 0.01$ compared with the OGD/R group, ## $P < 0.01$.

because of the considerable gap between the single-cellular culture model *in vitro* and the complex CNS environment *in vivo* [22]. In these studies, the cells from each coculture model might come from different sources or types, but they all have one thing in common: the cocultured cells were closer to *in vitro* in form and function compared with single-cultured cells [22–24]. Based on previous studies, we selected the SD rat cerebral capillary EAN for the coculture. These three cells are important members comprising the neurovascular unit, which forms the basic CNS structure [25,26]. Moreover, using the single-cultured neuron OGD/R model, the test substances were added to Neurobasal Media directly to observe their neuroprotective effects in pharmacological studies. However, agents could not come in contact with neurons directly *in vitro* because of the existence of BBB [27,28]. This is the reason why some test substances that were effective *in vitro* had no effect *in vivo*. For the above reasons, the test substances in the present research were added to E medium, the luminal compartment of the insert, to mimic their entry to the CNS from the peripheral blood through the BBB.

AIS refers to an intracellular cascade of death of local brain tissue due to a blood circulation disorder [29]. Neuronal damage is an important mechanism of AIS/reperfusion injury [30,31]. Moreover, cerebral capillary endothelial cells are damaged after AIS. TJs are the most prominent features of cerebral capillary endothelial cells and key structures that ensure BBB integrity [32–34]. A damaged BBB allows intravascular monocytes and other inflammatory cells to enter the brain tissue, leading to inflammation after stroke, which is another significant mechanism of AIS/reperfusion injury [10,11]. In this study, the neurons demonstrated decreased cell viability and increased apoptotic rate. The capillary endothelial monolayers showed low TEER values and high Na-F permeability, whereas the expression levels of ZO-1 and occludin in endothelial cells significantly decreased after OGD/R injury. These findings indicated that OGD/R leads to both neuronal and endothelial cell damage.

Previous studies have shown that both GBE and GB exhibit neuroprotective effects against OGD/R injury in single-cultured neurons. The results of the MTT and LDH assays indicated that both GBE and GB could increase cell viability and ameliorate cell injury against OGD/R-induced damage of cortical neurons. In the present study, pretreatment with GBE (0.1 mg/mL) or GB (100 μmol/L) for 24 h in the EAN model significantly increased neuronal cell viability and decreased the cell apoptotic rate against OGD/R injury. Previous studies indicated that neuronal cell death may occur via many different mechanisms after AIS/reperfusion injury [35–37]. After AIS, the expression of apoptosis-associated proteins, such as Bcl-2 and Bax in neurons of the cortex and hippocampus, would be unbalanced [38]. Bcl-2 and Bax are two important members of the Bcl/Bax family. The Bcl-2 protein is an inhibitor of apoptosis and can prevent the release of apoptotic factors, such as cytochrome-c, by inhibiting mitochondrial membrane permeability; whereas Bax can

promote apoptosis [39]. After OGD/R injury, Western blot analysis showed that the expression of Bcl-2 in neurons decreased, whereas that of Bax increased. Pretreatment with GBE or GB could significantly downregulate the expression levels of Bax and upregulate that of Bcl-2. Therefore, the results revealed that both GBE and GB could prevent the cortical neuron death against OGD/R injury due to their anti-apoptotic effects.

We demonstrated that OGD/R impaired the tightness of endothelial cell monolayers and altered the expression and localization of interendothelial TJ proteins (ZO-1 and occludin). However, GBE or GB pretreatment showed high TEER values and low Na-F permeability after OGD/R injury. This result indicated that both GBE and GB were able to ameliorate this damage. Concurrently, the expression and localization of TJs were determined by Western blot analysis and immunofluorescence staining, respectively. The results showed that pretreatment with GBE or GB could normalize the expression and localization of TJs in endothelial cells. This might be the alternative mechanisms of GBE-mediated neuroprotection after AIS/reperfusion.

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Compliance with ethics guidelines

Xiaohan Yang, Tiezheng Zheng, Hao Hong, Nan Cai, Xiaofeng Zhou, Changkai Sun, Liying Wu, Shuhong Liu, Yongqi Zhao, Lingling Zhu, Ming Fan, Xuezhong Zhou, and Fengxie Jin declare no conflict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed.

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