DGMI alleviates OGD/R-induced cell injury by regulating inflammatory and apoptosis signaling pathways

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Abstract: *Diterpene ginkgolides meglumine injection* (DGMI), a kind of *Ginkgo biloba* special extract injection, is now used for the treatment of ischemic stroke in convalescence. In the present study, we aimed to confirm whether DGMI could suppress inflammatory responses and apoptosis and explore the potential mechanisms underlying these effects. Cell viability and lactate dehydrogenase (LDH) release were measured by MTS and LDH assays after the cells were exposed to oxygen-glucose deprivation/reoxygenation (OGD/R). The extent of anti-apoptotic effect of DGMI was detected by flow cytometry using Annexin V-FITC/PI double staining assay kit. Pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6 and IL-10, were quantified by a specific Bio-Plex Pro^{TM} Reagent Kit. Additionally, activities of TLR2/4, NF-κB p65, MAPK pathway and apoptosis-related proteins as well as cellular localization of NF-κB p65 were determined by Western blotting analysis and immunofluorescence staining, respectively. DGMI at 50 μg/mL significantly increased the cell viability and decreased the secretion of IL-1β, IL-6, IL-10 and TNF-α in OGD/R-induced BV2 microglia cells. These effects were also confirmed by LDH assay and Annexin V-FITC/PI staining. Meanwhile, DGMI not only inhibited the protein expressions of TLR2, TLR4, MyD88, p-TAK1, p-IkBα, p-IKKβ and Bak, but also decreased the cleaved caspase-3/caspase-3, Bax/Bcl-2 and cleaved PARP-1/PARP-1 ratio in OGD/R-induced BV2 microglia cells. Furthermore, OGD/R-enhanced p-JNK1/2 and p-p38 MAPK expressions and nuclear translocation of NF-κB p65 were also partially inhibited by DGMI. The present study showed that inflammatory responses were triggered in BV2 microglia cells activated by OGD/R, leading to the release of pro-inflammatory cytokines and apoptosis. DGMI suppressed the inflammatory response and apoptosis by regulating the TLR/MyD88/NF-κB signaling pathways and down-regulation of p-JNK1/2 and p-p38 MAPK activation.

Keywords: Ginkgolides; BV2 microglia cells; TLR; Cerebral ischemia; NF-κB; MAPK; Inflammation

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1. Introduction

Ischemic stroke, a pathological condition characterized by a transient or permanent reduction in cerebral blood flow in a major brain artery, is a leading cause of death and adult disability worldwide. In terms of the mechanism of ischemic stroke, rapid reperfusion as soon as possible is considered to be critical in the therapy of cerebral ischemic disease. However, restoration of blood flow usually causes exacerbation of brain injury and a profound inflammatory response. Although multiple mechanisms are involved in cerebral ischemia/reperfusion, accumulating evidence demonstrates that inflammation contributes to stroke progression[1,2]. Microglia, the main type of mononuclear macrophages in the brain, have been proposed to be involved in immune-inflammatory reaction in the central nervous system (CNS), and reportedly, is an important contributor of homeostasis and neuro-inflammation after cerebral ischemic[3,4]. In addition, numerous studies, including ours, have shown that the pro-inflammatory mediators, such as Tumor necrosis factor α (TNF-α), IL-6 and IL-1β, secreted from activated microglial are harmful to the survival of the neuron cells[5,6]. Therefore, it is possible that inhibition of

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inflammatory signaling can attenuate microglial hypoxic injury through reducing inflammatory cytokine production, thus promoting the survival of neurons.

Pharmacological research and clinical evidence indicates that concentrated and partially purified extracts of Ginkgo biloba leaves have many beneficial effects against some kinds of neural and vascular damage, such as membrane stabilization, anticoagulant, vasodilator, antioxidant, free radical scavenger and anti-inflammatory effects[7–11]. Diterpene ginkgolides meglumine injection (DGMI) is a kind of Ginkgo biloba special extract injection, and it has been approved by China Food and Drug Administration (CFDA) in 2012 for the treatment of ischemic stroke in convalescence stage after phases II–III of clinical trials. Evidence base on previous studies has shown that DGMI exerts multiple pharmacological activities to resist the cerebral ischemia-reperfusion injury in rats, and attenuates oxygen-glucose deprivation (OGD)-induced apoptosis of nerve cells *via* regulation of PI3K/Akt/Bad/caspase-3/7, cAMP/PKA/Bad/caspase-3/7 and calpain signaling pathway *in vitro*[12]. It can also obviously reduce the infract area, increase the activities of SOD, and decrease the content of MDA, CK-BB in blood serum and caspase-3 in MACO/R rat[13]. Phase III clinical trials also show that it is safe and effective in the treatment of the syndrome of stagnant phlegm blocking collaterals in convalescence of ATCI and acute cerebral infarction[14]. However, studies on the role of DGMI have usually been conducted in neurons, and the work regarding its effect on microglial cells is limited to our knowledge.

In the present study, we demonstrated the protective effect of DGMI on BV2 microglia cells after oxygen-glucose deprivation/reoxygenation (OGD/R). Furthermore, we investigated the neuroprotective effects as well as anti-inflammatory and apoptotic action of DGMI *in vitro* and explored its underlying mechanisms.

2. Methods

2.1. Ethical approval

This was a preclinical research, the experimental system was a cell line, no animal and human samples were used in the present study, and the drug has also been approved for marketing by the Chinese CFDA.

2.2. Materials

BV2 microglia cells were purchased from Kunming Institute of Botany, Chinese Acasdemy of scuences (Kunming, China). DGMI was supplied by Kanion Pharmaceutical (JiangSu, China). Ginaton injection solution (GIs) was purchased from Chi Sheng Chemical Corporation (Taiwan, China).

2.3. Cell culture

Murine BV2 microglial cells with phenotypic and functional properties of microglial cells were purchased from Chinese Academy of Science (Shanghai, China) and cultured in DMEM containing 10% FBS and 1% penicillin–streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Cells between passages 3 and 15 were used in this study.

2.4. OGD/R and drug administration

BV2 cells (>93% viability) were seeded into 96-well plates and 100-mm cell culture dish at a density of 3.0×10^4 cells/100 μL/well (MTS and LDH assays) or 8.0×10^3 cells/100 μL/well or 1.0×10^6/mL (protein and protein kinase assays), respectively. After a 24-h incubation to allow cell attachment, cells were washed with glucose-free 1× PBS twice, and then placed into glucose-free DMEM. BV2 cells were then placed into a sealed chamber with persistent low-flow (20 L/min) of 95% N_2 and 5% CO_2 mixture to expel oxygen for 20 min.

Subsequently, the air inlet and the outlet of the tubes were clamped, and the chamber was placed into CO_2 incubator. After 4 h of OGD exposure, the culture medium was removed, and the drugs (DGMI and GIs) at various concentrations were added to the fresh medium. Next, BV2 cells were reoxygenated by exposing cells at 37 °C, 95% air, 5% CO_2 for different durations based on the requirement of the experimental object, such as 1 h for protein kinase assays and immunofluorescence detection, 3 h for MTS and LDH assays and 6 h for total protein assays.

2.5. Cell viability assay

BV2 cell viability in response to OGD/R and DGMI treatment was assessed by MTS assay. Briefly, 3.0×10^4 cells/100 μL/well BV2 cells were seeded into 96-well plates and strictly exposed to OGD for 4 h according to the above procedure. Subsequently, DGMI at various concentrations (6.25, 12.5, 25, 50 and 100 μg/mL) was added to cells and then reoxygenated for additional 3 h. Cellular MTS assay (Promega, Madison, WI, USA) was performed by addition of 20 mL MTS stock solution to each well. After incubation for 3–4 h, the absorbance was measured using a microplate spectrophotometer (Molecular Devices Flex Station 3, America) at 490 nm.

2.6. Cell death detection and apoptosis assay

The amount of LDH released in culture medium was determined by LDH assay kit according to the manufacture's protocol (Beyotime Institute of Biotechnology, Haimen, China). Briefly, following a 3-h reoxygenation and treatment with 50 μg/mL DGMI, the cells were centrifuged at 400× g for 5 min, and 120 μL of the supernatant was then transferred to a 96-well plate for LDH activity determination. Absorbance values after the colorimetric reaction were measured at 490 nm with a reference wavelength of 655 nm using a Flex Station 3 microplate spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA, USA). Apoptosis was determined by flow cytometry using Annexin V-FITC/PI double staining detection kit (Beyotime, Nantong, China) as described in the manufacturer's instruction. Briefly, 2.0×10^5 cells/500 μL/well BV2 cells were plated into 24-well palte and strictly exposed to OGD for 4 h, followed by 50 μg/mL DGMI and GIs treatment for additional 3 h at 37 ºC, 5% CO_2, respectively. Cell collection and apoptosis detection were conducted according to a previous study[5]. The stained cells were analyzed by NovoCyte D2040R flow cytometry (ACEA Biosciences, California San Diego, USA) using the NovoExpress 1.1.0 software. The experiment was repeated for three times, and six wells were used for each group.

2.7. Detection of inflammatory cytokines

The treatments of BV2 cells under each condition were the same as those described in 2.6. Cell death detection and apoptosis assay. Similarly, after the cells were exposed to OGD/R or OGD/R plus drugs (50 μg/mL DGMI and GIs), 100 μL of the supernatant was then collected and stored at –80 ºC. The cells without any treatment were defined as the control group. Interleukin-1β, 6, 10 (IL-1β, IL-6, IL-10) and TNF-α levels in the culture supernatants of each group were determined according to the protocol provided by the Bio-Plex ProTM Reagent Kit (Bio-Rad Laboratories, Hercules, CA, USA). Six wells of each sample were analyzed in each assay.

2.8. Western blotting analysis

Toll-like receptors 2/4(TLR2/4), myeloid differentiation protein 88 (MyD88) (Abcam, Cambridge, UK), p-TAK1 (Thermo Fisher, USA), p-IKKβ, Bak, PARP-1, p-ERK1/2, p-JNK, and p-p38 MAPK (Cell Signaling Technology, CST, Co., USA), p-IkBα, Bcl-2, Bax, Bak, PARP-1 and Caspases-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) levels in BV2 cells were analyzed by Western blotting analysis. Briefly, the cells were washed with

ice-cold 1× PBS and harvested under non-denaturing conditions by incubation at 4 °C with lysis buffer. The insoluble material was removed by centrifugation at 14 000× g for 10 min at 4 °C. The protein content was measured by the Bradford assay, and then 50 μg proteins were denatured, subjected to SDS-PAGE in 10% polyacrylamide gels and blotted onto PVDF membranes. Standard procedures for Western blotting were carried out as previously described[15].

2.9. Immunofluorescence detection of NF-κB

Double labeled immunocytochemistry was performed to demonstrate the expression and localization of Nuclear transcription factor κB (NF-κB) p65 in BV2 cells. Briefly, the cells were fixed with 4% (v/v) paraformaldehyde for 15 min at room temperature and washed with 1× PBS for three times, followed by incubation with 0.3% Triton X-100 for 10 min at room temperature. Non-specific binding was then blocked by incubation with 1% (w/v) BSA in 1× PBS for additional 30 min. Cells were subsequently incubated with NF-κB p65 primary antibody (1:100 dilution, Cell signaling technology, CST, Co., USA) at 37 ºC for 2 h. After washing with 1× PBS for three times, cells were then incubated with goat anti-rabbit IgG-CFL 488 (1:250 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 37 ºC. Nuclei were stained with 2 μg/mL Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA) for additional 10 min at 37 ºC. Fluorescent images were visualized under by a fluorescence reverse microscope (Leica DMI 4000B, Leica Co., Germany).

2.10. Statistical analysis

All data were presented as mean±standard deviation (SD). SPSS Statistics V17.0 software was used for further statistical analyses. One-way analysis of variance (ANOVA) was used to determine statistically significant differences among the groups. Differences were considered to be significant when $P<0.05$.

3. Results

3.1. DGMI protects BV2 microglia against OGD/R-induced cell injury

According to our previous study (data not shown), OGD for 4 h and reoxgenation for 3 h are the best time points to evaluate the drug efficacy[5]. In order to assess the effect of DGMI on OGD/R-induced cell injury, we examined the occurrence of ischemic damage using the optimized OGD and reoxygenation time above by comparing the viability between the OGD/R group and control group, and the efficacy of different doses of DGMI (6.25–100 μg/mL) was evaluated. The results were shown in Figure 1 that decreased cell viability was observed in the OGD/R group (61.1%±8.9%, $P<0.001$ *vs.* control group). However, DGMI at 50 and 100 μg/mL significantly improved the cell viability of BV2 cells exposed to OGD/R (74.6%±3.9%; 70.9%±2.3%, $P<0.01$ *vs.* OGD/R group). This finding suggested that DGMI could protect BV2 cells form OGD/R-induced injury. We used 50 μg/mL in the following studies since the cell viability peaked at this dosage.

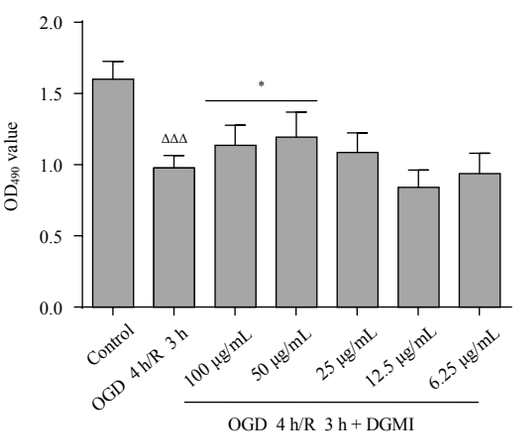

Figure 1. Cell viability of BV2 microglia cells exposed to OGD/R. Cell viabilities were tested using MTS assay by absorbance at 490 nm. All the results were expressed as OD_{490} values. Each value indicates the mean±SD and is representative of results obtained from six wells in all experiments. $^{\Delta\Delta\Delta}P<0.0001$ as compared with control group; $^{*}P<0.05$ as compared with model group.

To further verify the protective effect of DGMI, LDH assay and Annexin V-FITC/PI staining were also performed to confirm the above-mentioned results. As shown in Figures 2A and 2B, the percentage of apoptotic cells was measured by flow cytometry at the indicated time points after staining with Annexin V-FITC and PI. The results indicated that the percentage of apoptotic cells was increased after the cells were subjected to OGD 4 h/R 3 h (14.13%±1.5% in OGD/R group *vs*. 2.04%±0.08% in OGD group), while the cell death percentage was significantly declined in drug treatment (50 μg/mL DGMI and GIs) groups (6.14%±0.69% in DGMI group, 8.41%±0.35% in GIs group *vs*. 14.13%±1.5% in OGD/R group, respectively). The inhibition of OGD/R-induced cell death in the case of DGMI intervention was also verified by cell death detection (LDH assay) (Fig. 2C).

3.2. Measurement of proinflammatory cytokines in microglial cells

In order to confirm whether activation of BV2 microglia triggered the release of proinflammatory cytokines IL-1β, IL-6, IL-10 and TNF-α, the supernatants from OGD/R-induced BV2 microglia cells were collected and tested by Bio-Plex 200 System using Bio-Plex ProTM Reagent Kit. As shown in Figure 3, OGD/R could promote IL-1β, IL-6, IL-10, and TNF-α secretion above the basal levels (IL-1β, 1.63-fold, P<0.01; IL-6, 2.43-fold, P<0.01; IL-10, 1.57-fold, P<0.01; TNF-α, 7.12-fold, P<0.05 *vs*. control group, respectively). Compared with the OGD/R group, DGMI at 50 μg/mL significantly decreased IL-1β, IL-6, IL-10 and TNF-α levels (P<0.05 or P<0.01). The results indicated that DGMI had a good anti-neuroinflammatory activity.

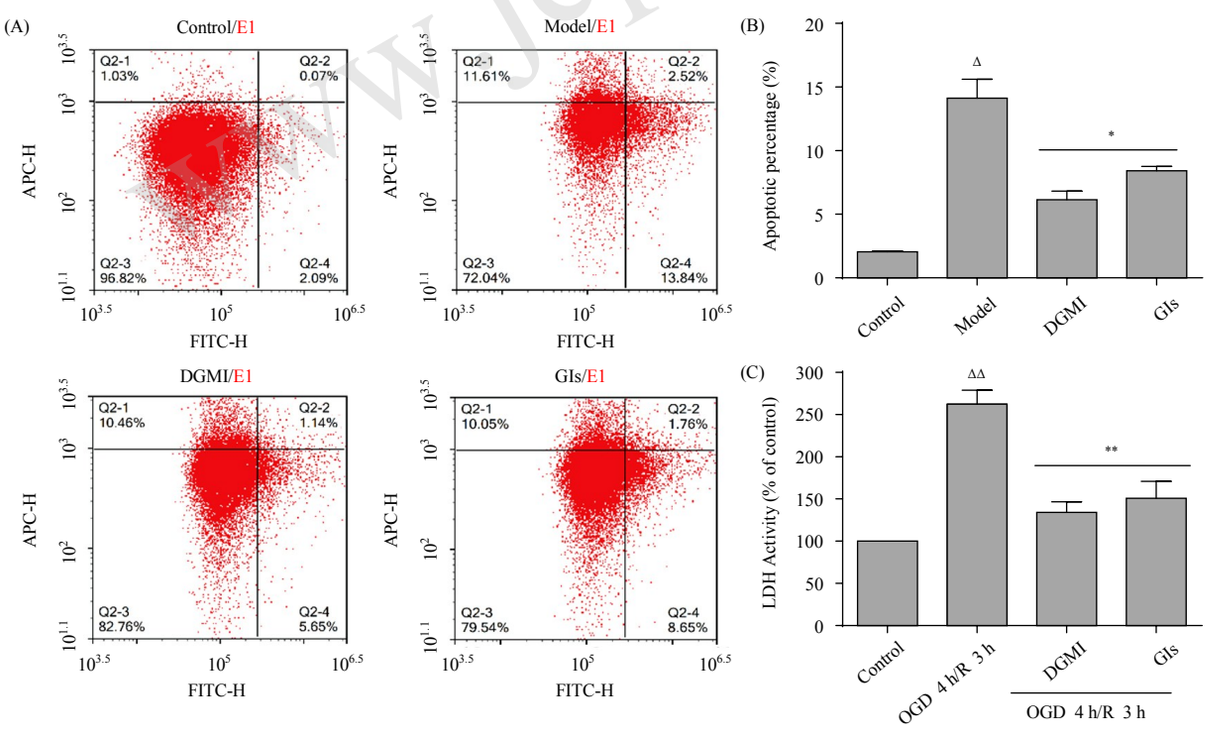

Figure 2. DGMI protects BV2 microglia cells against OGD-induced cell injury. (A) and (B) Relative percentage of the Annexin V-positive and PI-negative cells analyzed by ACEA NovoCyte D2040R. (C) DGMI (50 μg/mL) decreased LDH release from BV2 microglia cells at 3 h after OGD/R. Each data point is a mean±SD (n = 3). $^{\Delta}P$<0.05, $^{\Delta\Delta}P$<0.01 as compared with control group; $^{*}P$<0.05, $^{**}P$<0.01 as compared with model group.

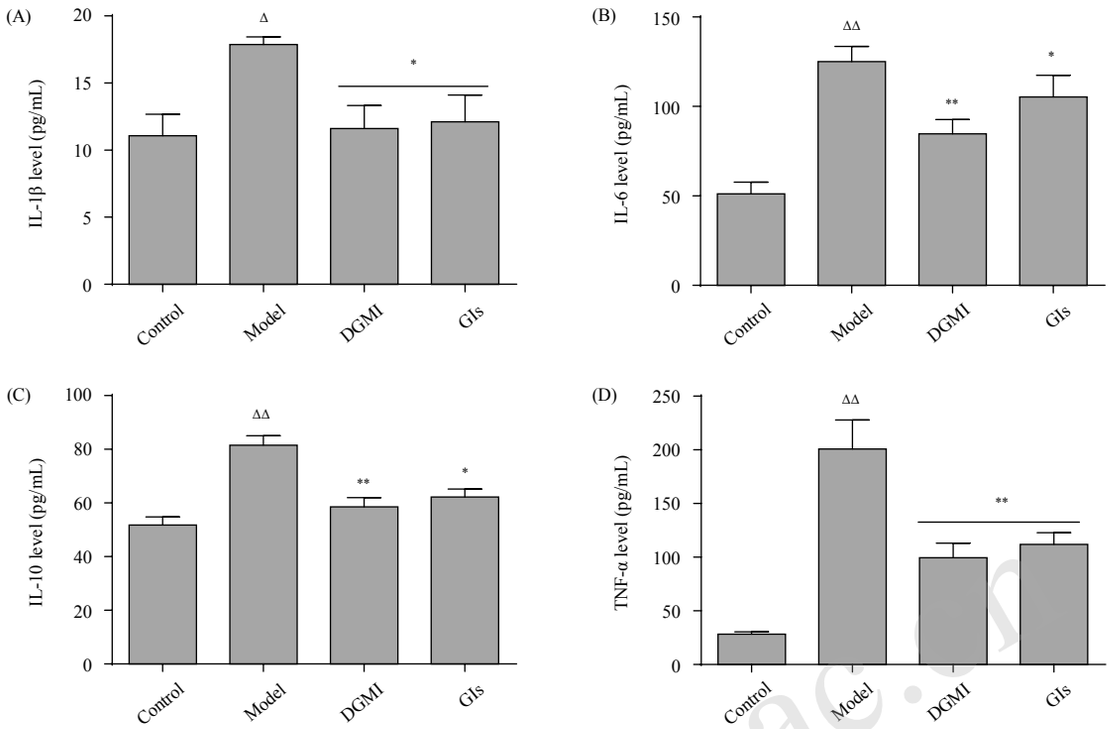

Figure 3. Secretion of pro-inflammatory factors from BV2 microglial cells after OGD/R. Inflammatory cytokines IL-1β (A), IL-6 (B), IL-10 (C) and TNF-α (D) levels in cell supernatant were detected by Bio-Plex Pro™ Reagent kit according to manufacturer's instructions. $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.001$, as compared with control group; $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$ as compared with control group; $^{*}P<0.05$, $^{**}P<0.01$ as compared with model group. Each value indicates the mean±SD and is representative of results obtained from six wells in all experiments.

3.3. Effects of DGMI on TLR signaling pathways in BV2 microglia cells exposed to OGD/R

The TLR signal pathway plays myriad pivotal roles in promoting and modulating immune and inflammatory responses[5,16]. Therefore, to further explore the anti-neuroinflammatory mechanism of DGMI, we examined the effects of DGMI on TLR2/4 and MyD88 in OGD/R-induced BV2 microglia cells. It was observed that BV2 cells subjected to 4-h OGD followed by 6-h reoxygenation had significantly enhanced TLR2, TLR4 and MyD88 protein expressions compared with the control group (1.63-fold, $P<0.05$; 1.36-fold, $P<0.05$; 1.64-fold, $P<0.05$ *vs.* control group, respectively). However, this increase was significantly suppressed by DGMI at 50 μg/mL. Meantime, OGD/R increased the expression of MyD88 protein in BV2, which was also inhibited by treatment with 50 μg/mL DGMI (Fig. 4C). Next, we analyzed the effects of DGMI on the expressions of key kinases in downstream TLR signaling pathways. Decreased levels of IkappaB-α, IKKβ (IkBα), IkappaB kinase-α/β (IKKβ) and Transforming growth factor-beta-activated kinase 1 (TAK1) were detected after 4-h OGD and 1-h reoxygenation, which were accompanied by the enhancement of their corresponding phosphorylation levels (2.69-fold, $P<0.01$; 1.24-fold, $P<0.05$; 1.57-fold, $P<0.05$ relative to control respectively). DGMI at 50 μg/mL significantly reduced the protein levels of p-TAK1, p-IKKβ and p-IkBα levels in OGD/R-induced BV2 microglia cells (Fig. 5). Overall, all the results suggested that OGD/R activated the TLR signaling pathway and enhanced the inflammatory reaction, and the anti-inflammatory mechanism of DGMI might be, at least in part, related to its inhibitory effect on TLR signaling.

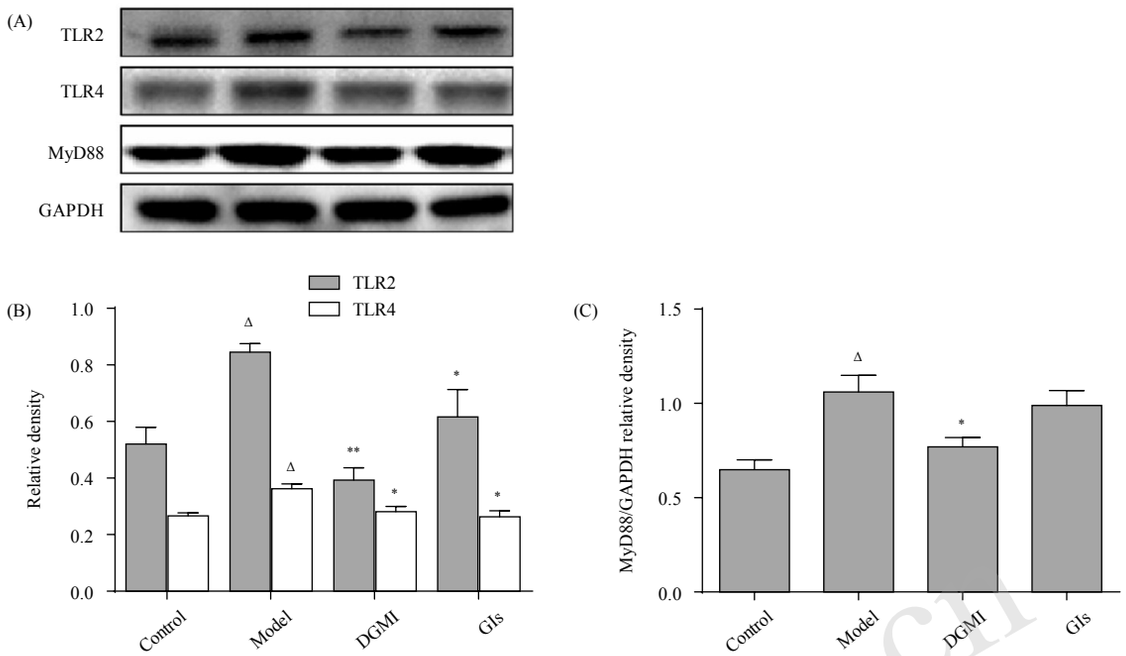

Figure 4. Expressions of TLR2, TLR4 and MyD88 in BV2 microglia cells after OGD/R. Representative graph (A) and quantitative data TLR2/4 (B), MyD88 (C) levels in BV2 microglia cells. GAPDH was used as the loading control. Western blotting images are representatives of three independent experiments. Each data point is a mean±SD (n = 3). $^{\Delta}P$<0.05 as compared with corresponding control group; $^{*}P$<0.05, $^{**}P$<0.01 as compared with corresponding model group.

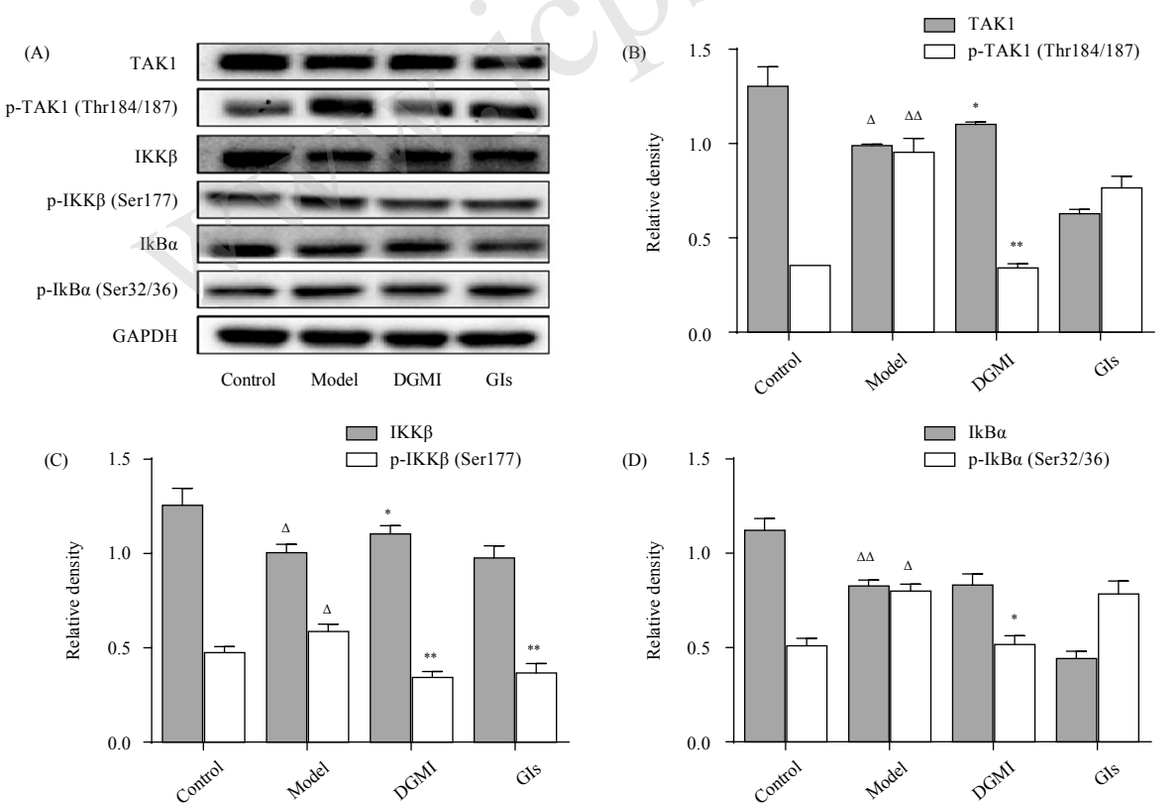

Figure 5. Expressions of p-TAK1, p-IKKβ and p-IkBα in BV2 microglia cells after OGD/R. Representative graph (A) and quantitative data p-TAK1 (B), p-IKKβ (C) and p-IkBα (D) levels in BV2 microglia cells. GAPDH was used as the loading control. Western blotting images are representatives of three independent experiments. Each data point is a mean±SD (n = 3). $^{\Delta}P$<0.05, $^{\Delta\Delta}P$<0.01, as compared with corresponding control group; $^{*}P$<0.05, $^{**}P$<0.01, as compared with corresponding model group.

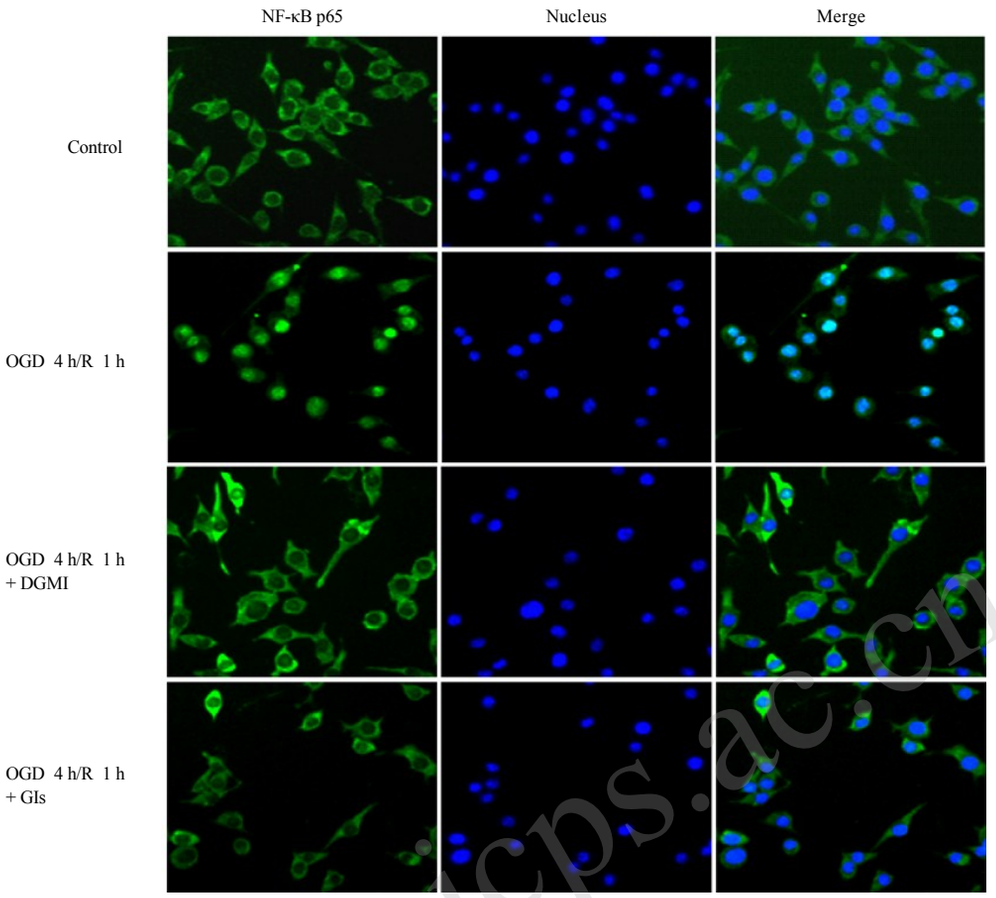

Figure 6. Cellular location of NF-κB p65 in BV2 microglia cells exposed to OGD/R. After exposure to OGD for 4 h, BV2 cells were reoxygenated for either 1 h or 1 h followed by treatment with 50 μg/mL DGMI. Cells were subjected to immunocytochemical analysis with an antibody directed against NF-κB p65 (green). To reveal nuclear morphology, the nuclei were stained with Hoechst 33258 (blue). The fluorescence was imaged by a fluorescence microscopy.

3.4. DGMI reduces the transfer of NF-κB p65 from cytoplasm to nucleus in BV2 microglia cells

As a central mediator, NF-κB is involved in microglia related inflammatory processes. Activation of NF-κB leads to up-regulation of downstream genes, such as IL-1β, IL-6, TNF-α and inducible nitric oxide synthase (iNOS)[17]. For this reason, it's very necessary to detect whether DGMI has inhibitory effect on the activation of NF-κB. In this study, localization of NF-κB p65 in BV2 cells subjected to OGD/R was observed by double labeled immunofluorescence. Figure 6 shows that NF-κB p65 was clearly translocated into the nucleus (merged images, blue-green) compared with untreated cells (shown as control, green). The level of p65 in the nucleus was dramatically reduced by treatment with DGMI at the concentration of 50 μg/mL, indicating that the activation of NF-κB induced by OGD/R was likely to be blocked by DGMI.

3.5. DGMI inhibits OGD/R-induced JNK, p38-phosphorylation in BV2 microglia cells

Mitogen-activated protein kinase (MAPK) pathway has been previously implicated in inflammatory processes. MAPK pathway-associated molecules, including ERK, JNK and p38, are involved in middle cerebral artery occlusion and reperfusion (MCAO/R) and OGD/R-induced injury in rats and microglia[18]. To find out the underlying mechanisms elucidating the altered

inflammatory factors caused by DGMI, we detected the activity of MAPK pathways in DGMI-treated and untreated groups. Our results showed that following 4 h of OGD stimulation and 6 h of reoxygenation, the phosphorylation levels of extracellular signal-regulated Kinase (ERK), c-Jun NH_2-terminal kinase (JNK) and p38 MAPK in BV2 microglial cells were significantly increased in the model group by 1.19-fold (42 KD, P<0.05), 1.71/3.37-fold (54 KD/46 KD, P<0.01) and 3.14-fold (P<0.01), respectively. Quantitative analysis revealed statistically significant downregulation of JNK and p38 phosphorylation, but not JNK in DGMI group compared with the model group (Fig. 7).

3.6. DGMI attenuates OGD/R-induced apoptosis in BV2 microglia cells

Previous studies have demonstrated that apoptosis is an important pathophysiological mode of cell death in ischemic brain injury[19,20]. For this reason, the protein levels of B-cell lymphoma-(Bcl-2) family members, including pro-apoptotic proteins Bcl-2 Associated X protein, Bcl-2 homologous antagonist/killer, poly (ADP-ribose) polymerase-1, cleaved Cysteinyl aspartate specific proteinase-3 (Bax, Bak, cleaved PARP-1, cleaved Caspase-3) and anti-apoptotic protein 2 (Bcl-2), two important regulators triggering mitochondrial depolarization in the process of apoptosis were investigated. Western blotting analysis suggested that the levels of Bak, Bax/Bcl-2, cleaved caspase-3/ caspase-3 and cleaved PARP-1/PARP-1 ratio were all significantly increased after the cells were exposed to OGD/R (2.03-fold, P<0.05; 5.42-fold, P<0.01; 6.41-fold, P<0.01 and 2.16-fold, P<0.01 relative to control). However, All of the above up-regulations were significantly reversed by 50 μg/mL DGMI (Fig. 8B−E), implying that DGMI inhibited apoptosis by regulating both pro- and anti-apoptotic proteins in OGD/R-exposed BV2 microglia cells.

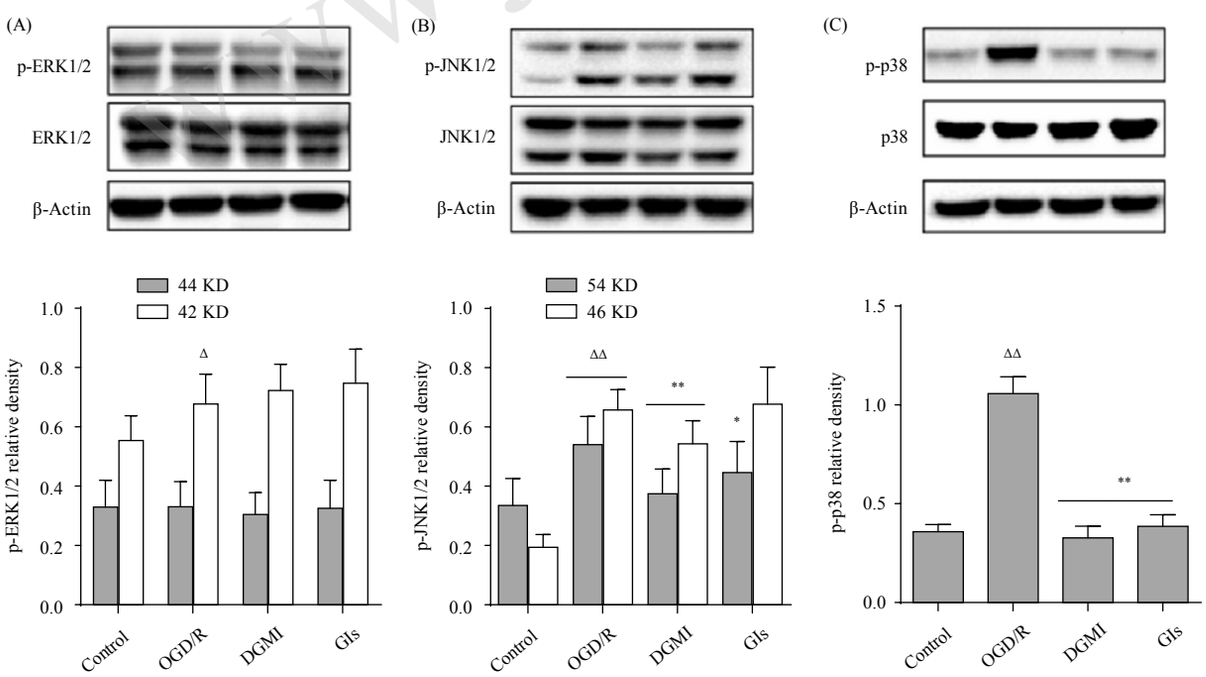

Figure 7. Expressions of ERK1/2, JNK and p38 phosphorylation in BV2 microglia cells after OGD/R. Representative graph and quantitative data of ERK1/2 (A), JNK1/2 (B) and p38 (C) phosphorylation levels in BV2 microglia cells. GAPDH was used as the loading control. Western blotting images are representatives of three independent experiments. Each data point is a mean±SD (n = 3). $^{\Delta}P$<0.05, $^{\Delta\Delta}P$<0.01 as compared with control group; $^{*}P$<0.05, $^{**}P$<0.01, as compared with model group.

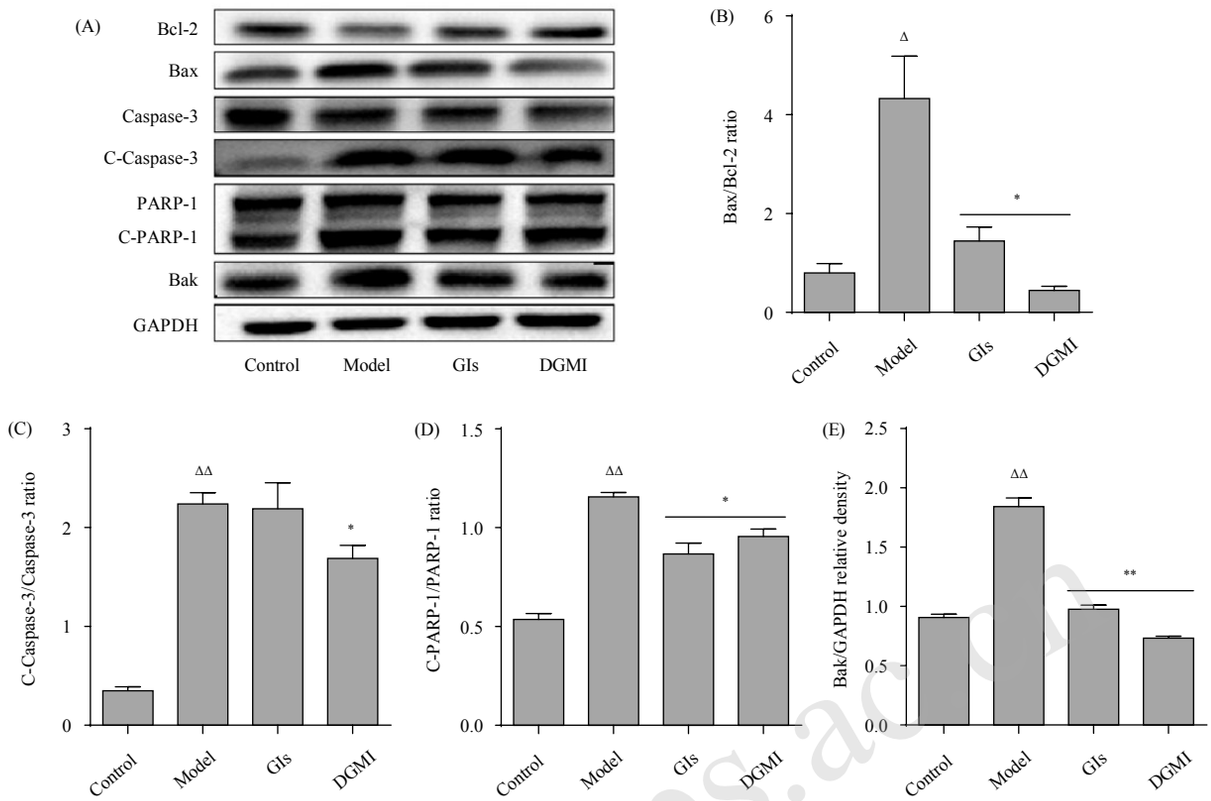

Figure 8. Expressions of Bcl-2, Bax, Bak, Caspase-3 and PARP-1 levels in BV2 microglia cells after OGD/R. Representative graph (A) and quantitative data on the ratio of Bax/Bcl-2 (B), C-Caspase-3/Caspase-3 (C), C-PARP-1/ PARP-1 (D) and expression of Bak (E) in BV2 microglia cells. GAPDH was used as the loading control. Western blotting images are representative of three independent experiments. Each data point is a mean±SD (n = 3). $^{\Delta}P$<0.05, $^{\Delta\Delta}P$<0.01 as compared with control group; $^{*}P$<0.05, $^{**}P$<0.01, as compared with model group.

4. Discussion

Nowdays, more and more attention has been paid to pharmacodynamic constituents from natural medicines in ischemic stroke therapy[18,21,22]. DGMI is a type of Ginkgo biloba special extract injection, which contains ginkgolid A (35%), ginkgolid B (60%), ginkgolid C (2%) and ginkgolid K (2%). Previous studies have shown that GA and GB possess excellent neuroprotective capability against ischemia-induced damage in rat permanent MCAO models and *in vitro* studies, which are related to inhibition of inflammatory signaling[23–25]. GK, a derivative compound of GB and isolated from the leaves of the Ginkgo biloba, can significantly inhibit the release of beta-glucuronidase from neutrophils induced by PAF[26]. Other study has also demonstrated that GA, GB, GK and BB can exert antioxidant effects against cerebral ischemia injury by activating Akt/Nrf2 pathway *in vitro* and *in vivo*[27]. However, whether the neuroprotective effects of DGMI are associated with anti-inflammatory pathways needs further investigation. In this study, the anti-inflammatory and apoptosis effects of DGMI were extensively investigated in OGD/R-induced cell injury *in vitro*. The major finding of the present work was that treatment with DGMI significantly increased the cell viability and decreased the LDH, IL-1β, IL-6, IL-10 and TNF-α production after the BV2 cells were exposed to OGD/R by regulating the TLR/MyD88/NF-κB signaling pathways and down-regulation of p-JNK1/2 and p-p38 MAPK activation. Further study demonstrated that DGMI appeared to ameliorate some protein expressions and regulate multiple signaling pathways associated with

inflammatory response and apoptosis. The current study provided the experimental evidence that DGMI could suppress inflammation progression and apoptosis, which might contribute to the treatment of ischemic stroke.

TLR-mediated signaling plays a key role in the induction of host innate immunity and inflammatory responses during cerebral ischemia/reperfusion. Once TLRs are activated, MyD88 is recruited to TLR domains, leading to subsequent downstream intracellular signaling activation, such as NF-κB and MAPK signaling pathways, which are involved in induction of proinflammatory cytokines and chemokines in ischemic stroke[28–30]. In the resting cells, NF-κB is normally sequestered in an inactive form in the cytoplasm as a heterotrimer complex consisting of p50, p65 and IκB subunits. Once NF-κB signaling is activated, IκB is phosphorylated by IκB kinase (IKK) leading to ubiquitination and degradation, and the NF-κB p65-p50 dimer s released, which then NF-κB is translocated to the nucleus, resulting in gene up-regulation. In addition, TAK1 is responsible for activating IKK and the canonical NF-κB signaling pathway[31,32]. Acute inhibition of TAK1 protects against neuronal death, indicating that TAK1 is a key target in neuroprotection of cerebral ischemia[33]. Therefore, we firstly investigated the changes of upstream TLR signaling-related proteins and found that treatment with DGMI significantly suppressed the upregulated expressions of TLR2/4 and MyD88 induced by OGD/R in BV2 cells (Fig. 4). Meanwhile, DGMI significantly increased TAK1, IKKβ and IκBα expressions and inhibited TAK1, IKKβ and IκBα phosphorylation in the injured microglial cells, indicating that DGMI prevented the nuclear translocation of p65 probably through increasing the level of IκBα (Fig. 5). NF-κB p65 immunofluorescence experiments also strongly supported the above-mentioned results from another aspect (Fig. 6). These observations might at least partially elucidate the anti-inflammatory mechanisms of DGMI.

A wealth of evidence suggests that activated microglia become hypertrophic, undergo rapid proliferation and migrate to inflammatory sites where they release excess amounts of various cytotoxic molecules, such as NO, oxygen radicals, and cytokines that mediate neuronal damage. A certain amount of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-10 and TNF-α, released by microglia are related to bad stroke event[34,35]. Targeting such molecular helps reduce neuron death in ischemic penumbra[36]. Our study proved that DGMI protected BV2 microglia and decreased the expressions of downstream pro-inflammatory cytokines (Fig. 3). Therefore, inhibition of TLR signaling pathway up and downstream caused by DGMI might protect neurons survival *in vivo* and finally alleviate ischemic injury. In addition, the inflammatory response is a highly regulated process, in which the balance between cell survival and apoptosis is orchestrated to ultimately drive and resolve inflammation[37].

NF-κB regulates a wide range of genes involved in apoptosis, and NF-κB activation may be proapoptotic[38,39]. This study demonstrated that OGD/R-induced cell apoptosis might be dependent on functional activation of TLRs. DGMI significantly reduced the Bax/Bcl-2 ratio and the levels of Bak, as well as a resulted in a decrease in cleaved caspase-3 and cleaved PARP-1 (Fig. 8). The flow cytometric analysis results also demonstrated that DGMI could inhibit the apoptosis of BV2 microglial cells induced by OGD/R. Our results suggested that DGMI inhibited the intrinsic pathway of apoptosis and increased their antiapoptotic capacity to prevent OGD/R injury. Taken together, we found that DGMI could increase the cell survival and reduce the LDH levels and cell apoptosis, indicating that DGMI might have anti-apoptosis function by regulating the balance of cell survival and death/apoptosis.

Mitogen-activated protein kinases (MAPKs) are activated after focal cerebral I/R and play crucial roles in regulating neuronal survival or damage. Several components of the MAPK pathway, including extracellular signal-regulated kinase 1/2 (ERK1/2), c-jun *N*-terminal kinase (JNK) and p38 kinase (p38), have been implicated in the signaling pathways relevant to inflammation[18,40]. Increasing evidence shows that p38 and JNK MAPK are induced in the neuron astrocytes and microglia after focal ischemia, Inhibition of p38 and JNK MAPK or depletion of JNK in mice reveals a pivotal role of p38 and JNK in neuronal apoptosis and inflammation, showing a beneficial effect of the inhibitors in focal stroke models[18,41,42]. Our results demonstrated that the phosphorylation of ERK1/2, JNK1/2, and p38 MAPK protein was significantly increased after OGD/R. DGMI inhibited JNK1/2 and p38 MAPK activation, but had no effect on the increased p-ERK1/2 expression, suggesting that JNK1/2 and p38 MAPK pathways could be involved in the neuroprotective effects of DGMI (Fig. 7).

In summary, the present study revealed that DGMI displayed excellent anti-inflammatory and apoptotic effects *in vitro* by decreasing the production of pro-inflammatory mediators in OGD/R-induced BV2 microglia cells *via* regulating multiple signaling pathways, including TLR/MyD88/NF-κB and p38/JNK MAPK, which was considered to be an additional function of DGMI in the treatment of ischemic injury. In conclusion, this study provided the evidence that DGMI might control the functional activation of microglia, suggesting that DGMI could alleviate neuro-inflammatory and apopotosis-related diseases, such as stroke and AD. Further studies need to focus on co-culturing of neuron, microglia and astrocyte and utilize suitable animal model to investigate the cell interactions and anti-neuroinflammatory effect *in vivo*.

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银杏二萜内酯葡胺注射液通过调控炎症和凋亡信号通路减轻OGD/R诱导的细胞损伤

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摘要: 银杏二萜内酯葡胺注射液(DGMI)是一种银杏叶提取物注射液, 目前用于缺血性中风恢复期的治疗。在本研究中, 我们旨在证实DGMI是否可以抑制炎症反应和细胞凋亡, 并探讨这些作用的潜在机制。将细胞暴露于氧-糖剥夺/复氧(OGD/R)后, 通过MTS和LDH测定法测量细胞活力和乳酸脱氢酶(LDH)释放量。使用Annexin V-FITC/PI双重染色分析试剂盒通过流式细胞仪检测了DGMI的抗凋亡作用。通过特定的Bio-Plex Pro™剂盒对包括TNF-α、IL-1β、IL-6和IL-10在内的促炎性细胞因子进行定量检测。此外, 分别通过蛋白质印迹分析和免疫荧光染色测定TLR2/4, NF-κB p65, MAPK通路激活和凋亡相关蛋白的活性以及NF-κB p65的细胞定位。结果显示, 50 μg/mL的DGMI在OGD/R诱导的BV2小胶质细胞中显著提高了细胞活力并降低了IL-1β, IL-6, IL-10和TNF-α的分泌。通过LDH测定和Annexin V-FITC/PI染色也证实了这些作用。同时, DGMI不仅抑制TLR2, TLR4, MyD88, p-TAK1, p-IkBk, p-IKKK和Bak的蛋白表达, 且降低了OGD/R诱导的BV2小胶质细胞中C-caspase-3/caspase-3, Bax/Bcl-2和C-PARP-1/PARP-1的比率。此外, DGMI还部分抑制了OGD/R增强的p-JNK1/2和p-p38 MAPK表达以及NF-κB p65的核移位。本研究表明, OGD/R激活的BV2小胶质细胞中触发了炎症反应, 导致促炎细胞因子的释放和细胞凋亡。DGMI通过调节TLR/MyD88/NF-κB信号通路以及下调p-JNK1/2和p-p38 MAPK激活来抑制炎症反应和细胞凋亡。

关键词: 银杏内酯; BV2神经小胶质细胞; TLR; 脑缺血; NF-κB; MAPK; 炎症