Gualou Guizhi Granule Suppresses LPS-induced Inflammatory Response of Microglia and Protects Against Microglial-Mediated Neurotoxicity in HT22 via Akt/NF-κB Signaling Pathways

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Research

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Abstract

**Background** Neuroinflammation plays a crucial part in the commence and advancement of ischemic stroke. Gualou Guizhi Granule (GLGZG) is known to exhibit well neuroprotective effect, but it is not known whether GLGZG can regulate inflammatory process at the cellular level in BV2 microglia cells and protect against microglial-mediated neurotoxicity in neurons. Herein, we aimed to investigate the anti-inflammatory effects of GLGZG on BV2 microglia cells and protection against microglial-mediated neurotoxicity in neurons.

**Methods** Cell model of neuroinflammation was constructed by lipopolysaccharide (LPS) to observe the effect of GLGZG in the presence or absence of GLGZG. The production of nitric oxide (NO), inflammatory mediators were detected. Moreover, potential mechanisms associated with anti-inflammatory effect, such as inhibition of microglial activation, the nuclear factor kappa B (NF-κB) were also investigated. In addition, to prove whether GLGZG protects against microglial-mediated neurotoxicity, neuronal HT22 cells were cultured in conditioned medium. And cell survivability, neuronal apoptosis of HT22 were evaluated.

**Results** It was found that a main regulator of inflammation, NO, is suppressed by GLGZG in BV2 microglial cells. Moreover, GLGZG dose-dependently decreased the mRNA and protein levels of inducible NO synthase (iNOS) in LPS-stimulated BV2 cells. Additionally, GLGZG inhibited the expression and secretion of proinflammatory cytokines in BV2 microglial cells. And, GLGZG inhibited LPS-activated nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in BV2 microglial cells at the intracellular level. GLGZG negatively affected Akt phosphorylation: phosphorylated forms of Akt decreased. To check whether GLGZG protects against microglial-mediated neurotoxicity, neuronal HT22 cells were incubated in the conditioned medium. GLGZG showed a neuroprotective effect by promoting cell survivability, suppressing neuronal apoptosis.

**Conclusions** GLGZG exerted its potential effects on suppressing inflammatory responses in LPS-induced BV2 cells by attenuating NF-κB and Akt pathways. In addition, GLGZG could protect against microglial-mediated neurotoxicity in HT22.

**Background**

Stroke, especially ischemic stroke, is a life-threatening disease which is considered the most common cause of disability and mortality around the world [1, 2]. Although ischemic stroke involve the interaction of numerous pathophysiological processes, accumulating evidence proves that neuroinflammation plays an important role in the pathological process involved in ischemic stroke [3–6]. Recent studies proved have proved that microglia is critically involved in regulating neuroinflammation [7].

Microglia as the main immune cells in the brain, act as the first major safeguard of the central nervous system (CNS). In physiological state, microglia are at the resting state, they survey the CNS microenvironment via continuously extending and retracting ramified processes; while they were
activated in a number of neurodegenerative diseases and different types of brain injury \([8–10]\). Ischemic stroke is a powerful stimulus triggers microglial activation. Once activated, microglia develop macrophage-like capabilities, and polarize into M1 or M2 phenotype. They are considered as a double-edged sword \([11, 12]\). M2 microglia are regarded as “healing cells”, they clear debris, release anti-inflammatory mediators and function to help repair injury. On the other hand, M1 microglia are considered as proinflammatory, releasing pro-inflammatory mediators, and function to exacerbate ischemic injury, at the same time, peripheral leukocytes infiltrate into the brain and the normally immune-privileged brain environment is exposed to systemic responses that further exacerbate inflammation and brain damage, resulting in a vicious cycle. Thus, regulating microglial phenotype might be a potential treatment for neuroinflammation of ischemic stroke.

It is proved that the activated microglia-mediated neuroinflammation is mainly regulated by transcription factor nuclear factor kappa B (NF-\(\kappa B\)), a crucial modulator of various kinds of inflammation \([13]\). As we know, NF-\(\kappa B\) is bound to the inhibitor of kappaB (\(\kappa B\)) proteins in the cytosol which is inactive. And when the \(\kappa B\) kinase (IKK) firstly phosphorylates and degrades \(\kappa B\), NF-\(\kappa B\) begin to be activated, NF-\(\kappa B\) is dissociated from complex and transfers to the nucleus. Then, triggering the generation of pro-inflammatory molecules. What’s more, the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) signal pathway is also involved in NF-\(\kappa B\) activation and pro-inflammatory responses \([14]\).

Chinese classical prescription are important resources to develop safe and effective candidates for neuroinflammation of ischemic stroke. Gualou Guizhi granule (GLGZG, Min drug system approval No.S20130001), is a standard hospital prescription at Fujian University of TCM Affiliated Second People's Hospital (Fuzhou, China), which is first reported by Zhang Zhongjing in Jin Kui Yao Lue \([15]\). It consists of 6 kinds of Chinese herbs: *Trichosanthes kirilowii* Maxim., *Cinnamomum cassia* Presl., *Paeoniae lactiflora* Pall., *Zingiber officinale* Rosc., *Ziziphus jujuba* Mill. and *Glycyrrhiza uralensis* Fisch. It has long been used in clinic to treat muscular spasticity and dyskinesia following stroke, epilepsy or spinal cord injury in China with a weight ratio of 10:3:3:3:2:3 \([16–21]\).

Moreover, in recent years, studies have also documented that GLGZG exerts well effects of anti-inflammation, anti cerebral ischemia reperfusion injury and neuroprotection \([22–25]\). Our group have shown that GLGZG exhibited significant anti-inflammatory effects to reduce inflammatory cytokines, chemokines and mediators, such as tumor necrosis factor (TNF\(\alpha\)), interleukin (IL)1\(\beta\), IL6, monocyte chemotactic protein 1 (MCP1) and nitric oxide (NO) in vivo\([26]\). Although preliminary researches suggested a potential relationship between GLGZG and neuroinflammation, whether GLGZG can regulate neuroinflammation, and the underlying mechanisms, have not been well investigated. In the current study, we aimed to investigate the anti-neuroinflammatory effects of GLGZG in LPS-stimulated BV-2 microglial cells. Furthermore, the roles of GLGZG in Akt/NF-\(\kappa B\) related signaling pathway were investigated.

## Methods

### Reagents and Chemicals
GLGZG was presented by Pharmaceutical Department of Fujian University of Traditional Chinese Medicine Affiliated Second People's Hospital (Fuzhou, China). It was certified and standardized on the basis of labeled compounds (The Food and Drug Administration in Fujian Province 2013). Our phytochemical studies also illustrated that 104 compounds in GLGZG were identified or tentatively characterized and several bioactive components, such as citrulline, luteolin, puerarin, liquiritin, taxifolin, naringin, formononetin, isoliquiritigenin, 6-gingerol, curcumin, caffeic acid, ferulic acid, jujuboside A, protocatechuic acid, cinnamic acid, catechin, paoniflorin [22,29–31]. Lipopolysaccharide (LPS) was purchased from Sigma (St. Louis, USA). RPMI Medium 1640 basic and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Carlsbad, CA, USA). CellTiter 96® AQ aqueous one solution cell proliferation assay was bought from Promega (Madison, USA). IL-1β, IL-6, IL-10 and TNF-α mouse ELISA kits were obtained from (ABclonal (Wuhan) biotechnology co. LTD, Wuhan, China). Rabbit antibodies against Akt, p-Akt(Ser473), PI3K(p85), PI3K(p110α), IKKβ, p-1kBα, IkBα, NF-κBp65 and β-actin were purchased from Cell Signaling Technology (Boston, MA, USA). The secondary antibodies conjugated with horseradish peroxidase (HRP) were all bought from Xiamen LuLong Biotech Co., Ltd. (Xiamen, China).

**Cell Culture And Treatment**

BV-2 microglial cells and neuronal HT22 were obtained from Beijing Beina Chuanglian Biotechnology Research Institute (Beijing, China). In brief, cells were cultured in RPMI 1640 medium with 10% FBS and 1% penicillin-streptomycin mixed solution (Gibco Invitrogen Corporation, Carlsbad, CA, USA) at 37 °C in a 5% CO₂ incubator.

In these studies, the cells were seeded on 96-well plates or 6-well plates and were divided into three groups randomly: (1) pure BV2 cell group as control group (BV2 group); (2) LPS group, where BV2 cells were incubated with LPS (1 µg/mL) for 24 h; and (3) LPS plus GLGZG (50, 100, 200 µg/mL) group, where cells were co-incubated with LPS (1 µg/mL) and GLGZG (50, 100, 200 µg/mL) for 24 h. Culture supernatants were harvested for ELISA experiments and nitric oxide assay. Additionally, the cells were then harvested for RNA or protein isolation.

To determine protective effect of GLGZG against microglial-mediated neurotoxicity, neuronal HT22 were incubated in conditioned medium from the control, LPS, and LPS plus GLGZG groups of BV2 cells. Conditioned media were then tested for cell viability using CellTiter 96® AQueous one solution cell proliferation and LDH assays, and cell lysates were used to test for apoptosis from conditioned media-treated HT-22 cells.

**Cell Viability Assay**

After treatment, the cell viability was assessed by CellTiter 96® AQueous one solution cell proliferation assay. CellTiter 96® AQueous one solution was added and incubated for another 4 h at 37 °C. Then the
absorbance at 570 nm was taken by a microplate reader (Infinite M200 Pro, TECAN). The cell viability was calculated according to the OD value. Survival rate (%) = OD_{experimental group}/OD_{control group} ×100%.

**Elisa Experiments**

After treatment, culture supernatants were collected and then centrifuged prior to the determination of IL-1β, IL-6, IL-10 and TNF-α production. Detailed manipulation process was performed by manufacturer protocols of mouse ELISA kits.

**Nitric Oxide Assay**

After treatment, the culture supernatants were collected, and NO production was measured by assessing the nitrite level in the culture media. It was executed by mixing the medium with griess reagent. Optical concentration was analyzed at 540 nm after 10 minutes incubation.

**Quantitative Real-time Pcr Analysis**

After treatment, cells were washed with PBS, and the total RNA was extracted by RNeasy® Mini Kit, and the RNA concentration were determined. Then Revert Aid First strand cDNA Synthesis Kit was used to reverse transcription to cDNA. The cDNA product was used as a template for quantitative PCR amplification, and it was then carried out on ABI 7900HT real-time PCR system (Applied Biosystems, Inc., Foster City, CA, USA). The data were analyzed by $2^{-\Delta\Delta CT}$ relative quantification method. The relative transcriptional level of target genes normalized to GAPDH was calculated. The primer sequences for amplification of the target genes are shown in Table 1. The relative transcriptional level of target genes normalized to GAPDH was calculated.
Table 1
Primers used for quantitative real-time PCR analysis

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<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>CD32</td>
<td>5′-AATCCTGCGTTCCTACTGATC-3′</td>
<td>5′-GTGTCACCGTGCTTTCTTGAG-3′</td>
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<tr>
<td>CD86</td>
<td>5′-GACCGTTGTGTGTTCTGG-3′</td>
<td>5′-GATGAGCAGCATCAACAGGA - 3′</td>
</tr>
<tr>
<td>CD206</td>
<td>5′-CAAGGAAGTGTGCCATTTG-3′</td>
<td>5′-CCTTTCAGTCTTGGCAACG-3′</td>
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<td>5′-CTGAAGGAGCCTGTCTGTG-3′</td>
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<tr>
<td>Ym1</td>
<td>5′-CAGGGAATGAGTGTTGG-3′</td>
<td>5′-CACGGACCTCCTAATTTG-3′</td>
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<tr>
<td>IL-1β</td>
<td>5′-ATG ACC TGT TCT TTG AGG CTG AC-3′</td>
<td>5′-CGA GAT GCT GCT GTG AGA TTT G-3′</td>
</tr>
<tr>
<td>IL-6</td>
<td>5′-GAC CAA GAC CAT CCA ACT CAT C-3′</td>
<td>5′-ACA TTC ATA TTG CCA GTT CT GT A-3′</td>
</tr>
<tr>
<td>IL-10</td>
<td>5′-CCAAGCCTTTATCGGAAATGA-3′</td>
<td>5′-TTTCAGGGGAGAAATCG-3′</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5′-ATG AGC ACG GAA AGC ATG-3′</td>
<td>5′-TAC GGG CTT GTC ACT CGA GTT-3′</td>
</tr>
<tr>
<td>iNOS</td>
<td>5′-CAA GCA CCT TGG AAG AGG AG-3′</td>
<td>5′-AAG GCC AAA CAC AGC ATA CC-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-AGC CCA GAA CAT CAT CCC TG-3′</td>
<td>5′-AGC CCA GAA CAT CAT CCC TG-3′</td>
</tr>
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</table>

Western Blot Analysis

Western Blot Analysis
The cells were inoculated into 6-well plate with a density of 3 × 10^5/well and cultured for 24 h, then treated with indicated concentrations of GLGZG and LPS (1 µg/mL) for 24 h. Cells were collected and lysed by lysis buffer, then they were centrifuged at 12,000 g for 15 min. The supernatant was collected and the protein concentration was determined by the BCA method. Then protein mixed with loading buffer and incubated in 100 °C for 6 min. Ultimately, samples were analyzed for western blot analysis with primary antibodies to iNOS(1:1000), CD206(1:1000), p-AKT(Ser473)(1:2000), AKT(1:1000), PI3K(p85)(1:1000), PI3K(p110α)(1:1000), IKKβ(1:1000), p-IκBα(1:1000), IκBα(1:1000), NF-κBp65(1:1000), Bax(1:1000), Bcl-2(1:1000), NeuN(1:1000) and β-actin(1:1000) overnight at 4 °C followed by incubating with the horseradish peroxidase-conjugated secondary antibody IgG (HRP Goat Anti-Rabbit IgG secondary antibody (1:5000), Goat Anti-Mous IgG secondary antibody (1:5000), LuLong Biotech Co., Xiamen, China) at room temperature. Finally they were evaluated using the ECL western detection reagents and the relative expression level of target genes normalized to β-actin was analyzed.

Immunofluorescence Assay
Immunofluorescence assay was performed to detect the nuclear translocation of NF-kBp65 subunit. In brief, cells were fixed using 4% paraformaldehyde for 15 min at room temperature, followed by washing with PBS for 5 min 3 times. The cells were blocked with 5% BSA prepared in 0.1% Tween 20 (PBST) and 0.3% Triton X-100 for 1 h at room temperature. Afterward, cells were incubated with NF-κBp65 specific primary antibody (1:100, Cell Signaling Technology, MA, USA) overnight at 4 °C. After washing, cells were incubated with FITC-labeled IgG secondary antibody (Beijing Zhongshan Jinqiao Biotechnology Co. LTD, Beijing, China, 1:200 dilution in 5% BSA solution) for 1 h in dark. After that, cells were stained with 25 µg/mL of 4′-6-diamidino-2-phenylindole (DAPI) in PBST. Finally, samples were documented using Olympus IX73 fluorescence microscope (Tokyo, Japan).

**Statistical analysis**

All the experiments were repeated three times. The data were statistically analyzed by SPSS 19.0 software and expressed as mean ± standard deviation (x ± s). The difference between groups was analyzed by one-way ANOVA followed by the Student-Newman-Keuls q test with post-hoc correction was employed for the multiple comparisons, and P<0.05 was considered to indicate statistical significance.

**Results**

**GLGZG modulate M1/M2 microglial polarization in the LPS-induced activation of microglia**

According to the past research result, 50, 100 and 200 µg/mL of GLGZG were employed in these experiments [26]. As illustrated in Fig. 1, GLGZG in the presence and absence of LPS did not have a significant effect on microglial viability.

Morphological examination result is presented in representative photomicrographs in Fig. 2. As shown in Fig. 2, the LPS-activated cells were characterized by round and an absence of thin processes, which was considered as an “active stage” phenotype. While, as compared to LPS-activated cells, the control and GLGZG-treated LPS-activated BV2 cells showed less rounded and more elongated cells with thinner processes, which is considered as “resting stage” phenotype.

To further confirm these findings, expression of M1 and M2 markers of BV2 in culture were determinated by real-time PCR. As shown in Fig. 3A-3C, LPS treatment caused a significantly increase in mRNA levels of three M1 markers: iNOS (P<0.01), CD32 (P<0.01) and CD86 (P<0.05), and GLGZG treatment obviously attenuated the effect for iNOS (P<0.05, P<0.01), CD32 (P<0.05, P<0.01) and CD86 (P<0.05, P<0.01). Western blot analysis of the M1 marker-iNOS (Fig. 3D-3E), it was indicates that the protein express of iNOS is significantly up-regulated after LPS activation of BV2 microglia cells (P<0.01, Fig. 3D-3E), while this effect is obviously attenuated by GLGZG treatment (P<0.05). Likewise, mRNA expression of three M2 markers and the protein express of the M2 marker-CD206 were examinated. As shown in Fig. 4, LPS treatment have no significant effect on CD206, Arginase1 and Ym1, three M2 markers,
however GLGZG treatment significantly elevated the expression for CD206, Arginase1 and Ym1 ($P < 0.05$, $P < 0.01$). Protein result of CD206 revealed that LPS had no significant effect on CD206 protein, while GLGZG treatment significantly up-regulated CD206 protein.

**Glgzg Suppressed Lps-induced Inflammatory Response In Lps-activated Bv-2 Microglial**

To further investigate the effect of GLGZG on neuroinflammation in LPS-activated BV-2 microglial, we determined the effect of GLGZG on neuro-inflammatory responses, such as cytokines including IL-1β, IL-6, IL-10, MCP-1 and TNF-α, inflammatory mediators including NO. The effect of GLGZG on LPS induced microglial release IL-1β, IL-6, IL-10, MCP-1, TNF-α and NO were shown in Fig. 5. Compared with the control group, the content of IL-1β, IL-6, MCP-1, TNF-α and NO in the LPS group increased significantly, while compared with the LPS group, IL-1β, IL-6, MCP-1, TNF-α and NO were decreased significantly in the cells of the 50, 100 and 200 µg/mL dose group of GLGZG. In addition, GLGZG also markedly increased the production of IL-10.

Moreover, the mRNA expression of IL-1β, IL-6, TNF-α and iNOS were measured, all of which were inhibited by treatment with GLGZG (Fig. 6). These results suggest that GLGZG not only inhibits LPS induced NO production, iNOS, IL-1β, IL-6, IL-10, TNF-α, and also significantly reduces mRNA expression of them.

3.3 GLGZG inhibites activation of the NF-κB pathway in LPS-activated BV-2 microglial

As shown in Fig. 7A-E, GLGZG dose-dependently suppressed NF-κBp65 expression in the nuclear compared with the average levels in LPS group ($P < 0.01$). And to confirm the reduced nuclear translocation, regulators of NF-κB in the cytosol, such as IκB, p-IκB and IκKβ, were investigated. As expected, IκBa expression decreased and increased with GLGZG treatment, while p-IκBa and IκKβ expression respectively increased and decreased with GLGZG treatment compared to LPS group ($P < 0.01$).

Furthermore, in order to check the decrease in translocation of the nuclear transcription factor, we directly assayed the intracellular translocation of the factor by immunofluorescence, and NF-κB nuclear translocation in LPS-treated cells reduced with GLGZG treatment, as observed by immunofluorescence microscopic analysis, indicating specific suppression of NF-κB nuclear translocation (Fig. 8).

**Glgzg Suppresses Akt Phosphorylation In Lps-activated Bv-2 Microglial**

As shown in Fig. 9, GLGZG could significantly increase the expression of p-AKT(Ser473) and PI3K(p85) compared with LPS group. While, the expression of AKT and PI3K(p110α) had no significant change among groups.
Glgzg Protect Against Microglial-mediated Neurotoxicity

As shown in Fig. 10A, the results show that conditioned medium from LPS plus GLGZG-treated BV2 microglia cells significantly enhanced cell viability (69.56%, 72.13%, 86.34%) on HT-22 cells, as compared to the LPS-treated BV2 microglia cell conditioned medium (P < 0.01). Furthermore, as shown in Fig. 10B-E, the levels of Bcl-2 notably declined, while cleaved caspase-3 and Bax increased in LPS-treated BV2 microglia cell conditioned medium. While GLGZG exhibited a more pronounced effect on Bcl-2, cleaved caspase-3 and Bax expression.

Discussion

Neuroinflammatory responses is inevitable and vital pathological processes in ischemic stroke, and microglial activation-mediated inflammatory responses play an important role in brain neuroinflammation and subsequent neuronal injuries. Activated microglia can be characterized by M1 (proinflammatory) and M2 (neuroprotective/anti-inflammatory) phenotype [32]. BV2 cells stimulated by LPS could provide a very reproducible and robust model for induction of M1 phenotype and an inflammatory activation profile of BV2 microglia cells, and it is highly similar to activation of primary microglia [33, 34]. Since GLGZG was found to be anti-inflammatory and neuroprotective in several reports, in this study, whether GLGZG could act directly upon microglia cells to regulate microglial polarization, pro- and anti-inflammatory cytokine gene expression, and the neurotoxic ability of activated microglia, were evaluated in vitro.

Indeed, the results revealed that GLGZG decreased production of the M1 markers, iNOS, CD32, TNF-α and IL-1β, while increased production of the M2 marker, CD206, arginase1 and Ym1. Correspondingly, GLGZG also obviously inhibited the production of the pro-inflammatory mediators IL-1β, IL-4, IL-6, TNF-α and IFNγ and increased the level of IL-10 in LPS-activated BV2 microglia cells. As we know that, pro-inflammatory cytokines, e.g. TNF-α, IL-6, and IL-1β, can promote neuronal damage, and also induce more microglia activation as feedback [35]. In addition, some studies indicated that enhanced M2 polarization may be beneficial due, in large part, to a switch of production from M1 “proinflammatory cytokines” to M2 “anti-inflammatory cytokines” thus decreasing inflammation and facilitating tissue and cellular repair [36, 37]. The findings suggested that GLGZG exerted anti-neuroinflammatory effects by inhibiting BV2 activation.

Moreover, at the intracellular level, GLGZG was also shown to inhibit LPS-activated nuclear translocation of NF-κB in BV2 cells. To our knowledge, NF-κB signaling pathway is the most common involved pathway in the inflammatory response [38, 39]. NF-κBp65, one of the NF-κB subunits, is inactivated at normal physiological conditions and is bound to the inhibitor of kappaB (IκB) proteins in the cytosol. While when it is stimulated by inflammatory stimuli, such as viruses, bacterial toxins LPS, IKK firstly phosphorylates and degrades IκB, NF-κB begin to be activated, NF-κB is dissociated from complex and transfers to the nucleus. In this study, we found that GLGZG markedly inhibited LPS-induced NF-κB activation in BV2 cells by decreasing the phosphorylation level of IκBα, NF-κB p65 nuclear level and IKK induced by LPS stimulation. Furthermore, it is proved that the classical NF-κB pathway mediates not only microglial
activation, but also neuron death [36]. Our previous experiment found that GLGZG produces its protective effect by inhibiting the apoptosis of neurons. And in this report, our data support that GLGZG suppressed microglial activation via inhibiting NF-κB signal pathways.

PI3K/Akt pathway, a classical signaling pathway, is involved in NF-κB-mediated neuroinflammation. Activation of Akt take part in regulating pro-inflammatory responses in microglia by modulating NF-κB signal pathways [40]. As expected, herein, we found that GLGZG activates the phosphorylation of Akt induced by LPS in BV-2 microglia. Therefore, these results suggested that GLGZG might specifically suppress neuroinflammatory responses and the activation of NF-κB signaling pathways in activated BV-2 cells via activation of the Akt signal pathways.

Activated microglia release NO and proinflammatory cytokines, induce neuroinflammation, which are known to damage neurons [41]. Next, to examine whether GLGZG treatment protected against activated microglia-mediated neuroinflammation. As shown in results (Fig. 9A), GLGZG significantly improved cell viability in LPS treatment-induced HT22 cells and reduced neuron apoptosis. Thus, these results suggest that GLGZG inhibition of microglial activation could prevent injury to neurons.

Conclusions

To conclude, findings from this study illuminated that GLGZG could attenuate the inflammatory response of LPS-activated microglia via NF-κB and Akt signaling pathway, and protect neurons against activated microglia-mediated neuroinflammation. The study provides evidence that GLGZG may be a potential treatment for neuroinflammation of ischemic stroke.

Abbreviations

GLGZG, Gualou Guizhi Granule; LPS, lipopolysaccharide; iNOS, inducible NO synthase; NF-κB, nuclear factor kappa B; IκB, inhibitor of kappaB; IKK, IκB kinase; TNFα, tumor necrosis factor, IL, interleukin; MCP1, monocyte chemotactic protein 1; NO, nitric oxide.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no conflict of interest.

**Funding Statement**

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**Author Contributions**

ZYQ conceived the study, designed the experiment, carried out the real-time PCR, ELISA and Western blotting measurement; and drafted the manuscript. LYJ and NLH mainly cultured cells and performed the Western blot analysis, the ELISA test and analyzed the data. FLM did the measurement of real-time PCR. CKD and XW contributed to the statistical analysis and manuscript editing. YGH and LY contributed to the research design and coordination, secured funding for the project, helped to draft the manuscript, and took responsibility for the work. All authors read, discussed, and approved the final manuscript.

**Acknowledgements**

Not applicable

**References**


**Figures**

![Bar chart showing effects of Gualou Guizhi Granule (GLGZG) on cell viability of BV-2 microglial cells with or without LPS stimulation.](image)

**Figure 1**

Effects of Gualou Guizhi Granule (GLGZG) on the cell viability of BV-2 microglial cells with or without LPS stimulation. Cells were treated with 50, 100 and 200 µg/mL of GLGZG and incubated in the presence and
absence of 1 µg/mL LPS for 24 h. Cell viability was determined by CellTiter 96® AQ aqueous one solution. Results were expressed as percentages relative to the control group. Data were shown as means ± SD of six independent experiments in triplicate.

Figure 1

Effects of Gualou Guizhi Granule (GLGZG) on the cell viability of BV-2 microglial cells with or without LPS stimulation. Cells were treated with 50, 100 and 200 µg/mL of GLGZG and incubated in the presence and absence of 1 µg/mL LPS for 24 h. Cell viability was determined by CellTiter 96® AQ aqueous one solution. Results were expressed as percentages relative to the control group. Data were shown as means ± SD of six independent experiments in triplicate.
Figure 2

Effects of Gualou Guizhi Granule (GLGZG) on the morphological characteristics with or without LPS stimulation. Representative bright field microscopy images show the morphological differences among nonactivated control, LPS-activated and activated + GLGZG (50, 100 and 200 μg/mL)-treated BV2 microglia cells.
Figure 2

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Suppression of M1 phenotype markers by Gualou Guizhi Granule (GLGZG) in the LPS-activated BV2 microglia cells. (A-C) mRNA was collected from control, LPS-activated and LPS activated + GLGZG (50, 100 and 200 μg/mL)-treated BV2 cells at 24 hours after activation and treatment. (D, E) Western blot analysis and quantification of the M1 marker, iNOS. Data were shown as means ± SD. ##P<0.01 versus the control group. *P<0.05 and **P<0.01 versus the LPS-treated group.
Figure 4

Upregulation of M2 phenotype markers by Gualou Guizhi Granule (GLGZG) in the LPS-activated BV2 microglia cells. (A-C) mRNA was collected from control, LPS-activated and LPS activated + GLGZG (50, 100 and 200 μg/mL)-treated BV2 cells at 24 hours after activation and treatment. (D, E) Western blot analysis and quantification of the M2 marker, CD206. Data were shown as means ± SD. *P<0.05 and **P<0.01 versus the LPS-treated group.
Upregulation of M2 phenotype markers by Gualou Guizhi Granule (GLGZG) in the LPS-activated BV2 microglia cells. (A-C) mRNA was collected from control, LPS-activated and LPS activated + GLGZG (50, 100 and 200 μg/mL)-treated BV2 cells at 24 hours after activation and treatment. (D, E) Western blot analysis and quantification of the M2 marker, CD206. Data were shown as means ± SD. *P<0.05 and **P<0.01 versus the LPS-treated group.

Figure 5

Effect of Gualou Guizhi Granule (GLGZG) on the secretion of IL-1β, IL-6, IL-10, TNF-α, NO and MCP-1 in LPS-stimulated BV2. Cells were treated with 50, 100 and 200 μg/mL of GLGZG and incubated in the presence of 1 μg/mL LPS for 24 h. Data were shown as means ± SD. ##P<0.01 versus the control group. *P<0.05 and **P<0.01 versus the LPS-treated group.
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Effect of Gualou Guizhi Granule (GLGZG) on the secretion of IL-1β, IL-6, IL-10, TNF-α, NO and MCP-1 in LPS-stimulated BV2. Cells were treated with 50, 100 and 200 μg/mL of GLGZG and incubated in the presence of 1 μg/mL LPS for 24 h. Data were shown as means ± SD. ##P<0.01 versus the control group. *P<0.05 and **P<0.01 versus the LPS-treated group.

Figure 6
Effect of Gualou Guizhi Granule (GLGZG) on mRNA expression of IL-1β, IL-6, IL-10, TNF-α and iNOS in LPS-stimulated BV2. Cells were treated with 50, 100 and 200 μg/mL of GLGZG and incubated in the presence of 1 μg/mL LPS for 24 h. The mRNA levels were expressed as relative fold to the control group. Data were shown as means ± SD. **P<0.01 versus the LPS-treated group.

Figure 6

Effect of Gualou Guizhi Granule (GLGZG) on mRNA expression of IL-1β, IL-6, IL-10, TNF-α and iNOS in LPS-stimulated BV2. Cells were treated with 50, 100 and 200 μg/mL of GLGZG and incubated in the presence of 1 μg/mL LPS for 24 h. The mRNA levels were expressed as relative fold to the control group. Data were shown as means ± SD. **P<0.01 versus the LPS-treated group.
Figure 7

Effect of Gualou Guizhi Granule (GLGZG) on NF-κB signaling in LPS-stimulated BV2. Cells were treated with 50, 100 and 200 μg/mL of GLGZG and incubated in the presence of 1 μg/mL LPS for 24 h. After incubation, the protein was extracted from cells, and the levels of NF-κBp65 (A, B) in the nucleus, IkKβ (C, D), p-κB and IκB (C, E) were measured by Western blot. Data were shown as means ± SD. ##P<0.01 versus the control group. *P<0.05 and **P<0.01 versus the LPS-treated group.
Figure 8

Effect of Gualou Guizhi Granule (GLGZG) on the NF-κB p65 protein localization in LPS-stimulated BV2. Cells were treated with 50, 100 and 200 μg/mL of GLGZG and incubated in the presence of 1 μg/mL LPS for 24 h. After incubation, the NF-κB p65 protein localization was detected via immunofluorescence staining. The NF-κB p65 was labelled by FITC-conjugated secondary antibody, and nuclei were counterstained with DAPI. Scale bar, 50 μm.
Figure 8

Effect of Gualou Guizhi Granule (GLGZG) on the NF-κB p65 protein localization in LPS-stimulated BV2. Cells were treated with 50, 100 and 200 μg/mL of GLGZG and incubated in the presence of 1 μg/mL LPS for 24 h. After incubation, the NF-κB p65 protein localization was detected via immunofluorescence staining. The NF-κB p65 was labelled by FITC-conjugated secondary antibody, and nuclei were counterstained with DAPI. Scale bar, 50 μm.
Figure 9

Effect of Gualou Guizhi Granule (GLGZG) on Akt signaling in LPS-stimulated BV2. Cells were treated with 50, 100 and 200 μg/mL of GLGZG and incubated in the presence of 1 μg/mL LPS for 24 h. After incubation, the protein was extracted from cells, and the levels of PI3K(p85) (A, B), PI3K(p110α) (A, C), p-Akt(Ser473) and Akt (A, D) were measured by Western blot. Data were shown as means ± SD. ##P<0.01 versus the control group. **P<0.01 versus the LPS-treated group.
Figure 9

Effect of Guizhi Granule (GLGZG) on Akt signaling in LPS-stimulated BV2. Cells were treated with 50, 100 and 200 μg/mL of GLGZG and incubated in the presence of 1 μg/mL LPS for 24 h. After incubation, the protein was extracted from cells, and the levels of PI3K(p85) (A, B), PI3K(p110α) (A, C), p-Akt(Ser473) and Akt (A, D) were measured by Western blot. Data were shown as means ± SD. ##P<0.01 versus the control group. **P<0.01 versus the LPS-treated group.
Figure 10

Effect of Gualou Guizhi Granule (GLGZG) on the neurotoxicity of LPS-activated BV2 microglia cells in HT-22 cells. BV2 cells were activated by LPS. Conditioned media from control, LPS-activated, and LPS + GLGZG (50, 100 and 200 μg/mL)-treated cells were transferred to neuronal cell line, HT-22 cells. HT-22 cells were treated with these conditioned media, supernatant was harvested for cell viability, and cells were harvested for cleaved-caspase3, Bax and Bcl-2 protein analysis. (A) Cell viability was determined by CellTiter 96® AQ ueous one solution. Results were expressed as percentages relative to the control group. Data were shown as means ± SD of six independent experiments in triplicate. (B-E) the protein was extracted from cells, and the levels of cleaved-caspase3, Bax and Bcl-2 were measured by Western blot. Data were shown as means ± SD. #P<0.05 and ##P<0.01 versus the control group. *P<0.05 and **P<0.01 versus the LPS-treated group.
Figure 10

Effect of Gualou Guizhi Granule (GLGZG) on the neurotoxicity of LPS-activated BV2 microglia cells in HT-22 cells. BV2 cells were activated by LPS. Conditioned media from control, LPS-activated, and LPS + GLGZG (50, 100 and 200 μg/mL)-treated cells were transferred to neuronal cell line, HT-22 cells. HT-22 cells were treated with these conditioned media, supernatant was harvested for cell viability, and cells were harvested for cleaved-caspase3, Bax and Bcl-2 protein analysis. (A) Cell viability was determined by CellTiter 96® AQ ueous one solution. Results were expressed as percentages relative to the control group. Data were shown as means ± SD of six independent experiments in triplicate. (B-E) the protein was extracted from cells, and the levels of cleaved-caspase3, Bax and Bcl-2 were measured by Western blot. Data were shown as means ± SD. #P<0.05 and ##P<0.01 versus the control group. *P<0.05 and **P<0.01 versus the LPS-treated group.

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