

Protective effects of geniposide and ginsenoside Rg1 combination treatment on rats following cerebral ischemia are mediated via microglial microRNA-155-5p inhibition

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Abstract. Geniposide, an active component of *Gardenia*, has been reported to protect against cerebral ischemia in animals. Ginsenoside Rg1, a component of *Panax notoginseng*, is usually administered in combination with *Gardenia* for the treatment of acute ischemic stroke; however, there are unknown effects of ginsenoside Rg1 that require further investigation. In the present study, the effects of geniposide and ginsenoside Rg1 combination treatment on focal cerebral ischemic stroke were investigated. For *in vivo* analysis, male rats were separated into three groups, including the (control), model and geniposide + ginsenoside Rg1 groups (n=8 per group). A middle cerebral artery occlusion model was established as the model group. The treatment group was treated with geniposide (30 mg/kg, tail vein injection) + ginsenoside Rg1 (6 mg/kg, tail vein injection), and the model group received saline instead. Neurobehavioral deficits, infarct volume, brain edema, and the expression of microRNA (miR)-155-5p and CD11b by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry, were assessed following 24 h of ischemia. For *in vitro* analysis, BV2 mouse microglial cells were cultured and exposed to geniposide (40 µg/ml) + ginsenoside Rg1 (8 µg/ml) during various durations of oxygen-glucose deprivation (OGD). The expression levels of miR-155-5p, pri-miR-155 and pre-miR-155 were detected by RT-qPCR. The results demonstrated that increases in brain infarct volume, edema volume, CD11b-positive cells and miR-155-5p levels were alleviated following geniposide + ginsenoside administration in rats exposed to ischemia. Furthermore, geniposide + ginsenoside

Rg1 treatment suppressed the miR-155-5p, pri-miR-155 and pre-miR-155 expression levels in OGD-injured BV2 microglial cells. The results of the present study demonstrated that tail vein administration of geniposide in combination with ginsenoside Rg1 protected against focal cerebral ischemia in rats through inhibition of microglial miR-155-5p following ischemic injury, which may serve as a novel therapeutic agent for the treatment of strokes.

Introduction

Ischemic stroke caused by blood vessel occlusion is a major cause of morbidity and mortality in humans. Numerous pathological processes are associated with ischemic neuronal damage, including inflammation, energy metabolism disturbance, necrotic and apoptotic cell death, and excitotoxicity (1). Previous studies have aimed to improve the understanding of the underlying mechanisms of ischemic stroke; however, further investigation is required to identify a potential target for the effective prevention and treatment of stroke. Recent clinical and experimental studies have demonstrated that alternative complementary medicine may exhibit a unique efficacy in the treatment of stroke (2,3).

MicroRNA (miRNA/miR), which are non-coding RNAs, are frequently employed in research and are characterized by their regulatory effects on gene expression by inhibiting mRNA translation and/or mediating mRNA degradation (4,5). As numerous pathways are implicated in cerebral ischemic injury, including oxidative stress, excitotoxicity, mitochondrial dysfunction and inflammation, miRNAs have been associated with cerebral ischemic injury due to their roles in ischemic processes, including neuronal cell differentiation (6,7), dendritic plasticity and neuronal outgrowth modulation (8). As a multifunctional miRNA, miR-155 expression is affected by cerebral ischemia (9-11) and has been reported to be involved in the regulation of a variety of physiological and pathological processes, including inflammation and immunity (12,13). Several inflammatory and cytotoxic factors, including tumor necrosis factor- α , interleukin (IL)-1 β and IL-6, secreted by activated microglia may exert an effect on neuronal cell survival (14). miR-155

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may regulate the immune response mediated by microglia in various pathological states (15-17).

Gardenia is the fruit of *Gardenia jasminoides* Ellis (Rubiaceae), which is a commonly used Chinese herb. Gardenia has been reported to exhibit therapeutic effects on hepatic disorders, brain diseases, inflammatory diseases and contusions (18-20). A previous study reported that geniposide, an active component of Gardenia, reduces neuronal cell death and increases cell viability following oxygen and glucose deprivation (OGD) (21). Our previous study also revealed that geniposide exhibits protective effects against infarction volume, microglial activation and inflammatory cytokine release in middle cerebral artery occlusion (MCAO) model rats (22). In clinical and experimental research, Gardenia has previously been used in combination with *Panax notoginseng* to treat brain diseases, including cerebrovascular disease and Alzheimer's disease (23,24). For rats injured by local ischemia, ginsenoside Rg1, a component of *Panax notoginseng*, was reported to alleviate neurological function deficits, as determined by improvements in the learning and memory abilities, in addition to a reduction in the apoptosis of neurons in the hippocampus CA1 zone (25,26). The present study aimed to investigate whether tail vein-administered geniposide in combination with ginsenoside Rg1 may protect against ischemic stroke via miR-155 inhibition. An *in vivo* investigation into miR-155-5p expression, and the effects of geniposide + ginsenoside Rg1 on miR-155-5p expression within the brain and activation of microglia, as determined by immunohistochemistry for CD11b, was performed. In addition, an *in vitro* study was performed to analyze the effects of geniposide + ginsenoside Rg1 on miR-155-5p expression in BV2 microglial cells.

Materials and methods

Animals and grouping. Specific pathogen and virus antibody-free grade male Sprague-Dawley rats (n=56; 253.4±20.3 g; 7-8 weeks-old) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). In accordance with the UK Animals (Scientific Procedures) Act of 1986 (27), the animal experiments were performed with the approval of the ethics committee of the Institute of Basic Theory, China Academy of Chinese Medical Sciences (Beijing, China). The rats were housed in rooms maintained at 23±2°C in a 12 h light/dark cycle and fed a rodent standard diet with free access to water. The animals were randomly divided into three groups (n=8 per group): Sham, model and geniposide (30 mg/kg) + ginsenoside Rg1 (6 mg/kg) groups. For the sham group, the rats underwent similar surgery to the model group, without nylon filament insertion; rats in the model group were administered saline via the tail vein immediately following MCAO. Rats in the geniposide + ginsenoside Rg1 group were administered geniposide (30 mg/kg) + ginsenoside Rg1 (6 mg/kg) via the tail vein immediately following MCAO. Geniposide (purity, >95%; cat. no. 110749-201718) and ginsenoside Rg1 (purity, >95%; cat. no. 110703-201731) were purchased from the National Institutes for Food and Drug Control (Beijing, China).

MCAO model establishment. The MCAO model was established as described by Longa *et al* (28). Rats were anesthetized

with 10% chloral hydrate (350 mg/kg body weight, intraperitoneal injection) (29,30). Subsequently, a midline incision was made to expose the left common carotid artery and the external carotid artery (ECA), including the occipital artery branches. Following this, blood from the occipital artery and superior thyroid artery branches of the ECA was coagulated using a bipolar electrocoagulator. The internal carotid artery (ICA) was isolated and carefully separated from the adjacent vagus nerve. Following ICA exposure, the origin of the left middle cerebral artery was occluded using a 0.25 mm diameter nylon filament coated with poly-L-lysine, which was inserted into the ICA via the ECA. Rectal temperature was maintained at 37°C during and following surgery with a temperature-controlled heating pad (CMA 150 Carnegie Medicin AB, Solna, Sweden). After 2, 6 and 24 h, the ischemic procedure was complete.

Infarct size and brain edema detection. Rats of the 24 h ischemia group were selected for *in vivo* analysis; statistical calculations demonstrated no significant difference when comparing the 2 and 6 h ischemia group rats with the control group. Following the performance of ischemic injury for different time periods, all animals were sacrificed. Brain tissues were removed and prepared into 2 mm coronal sections. The sections were stained with 1.5% 2,3,5-triphenyltetrazolium chloride at 37°C for 10 min and fixed in 4% paraformaldehyde for 24 h at room temperature. The images of the stained slices were analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) to determine the infarct size, which was expressed as the percentage of the whole brain tissue. Brain edema was calculated according to the following equation: [(volume of ipsilateral hemisphere-volume of contralateral hemisphere)/volume of contralateral hemisphere] x100. The experiment was independently repeated three times.

Cell culture and in vitro model. Mouse BV2 microglial cells were purchased from the Institute of Basic Medical Sciences of the China Science Academy (Beijing, China) and cultured in Dulbecco's modified Eagle medium/Ham's F12 (DMEM/F12; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) culture medium containing 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) and 1% penicillin and streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in a humidified 5% CO₂/95% air atmosphere at 37°C. The microglial cells were passaged at 90% confluence, with two to three passages per week. To mimic the OGD pathological process, glucose-free DMEM (Gibco; Thermo Fisher Scientific, Inc.) was used instead of the culture medium and oxygen was restricted in the flasks by keeping the culture flasks in a sealed tank with a persistent low-flow (1.5 l/min) of 95% N₂ and 5% CO₂ mixture. The inlet and outlet ends of the tubes were then clipped and the sealed tank was returned to the incubator. DMEM was used to dissolve geniposide and ginsenoside Rg1 and prepare the storage solution. A total of 40 µg/ml geniposide + 8 µg/ml ginsenoside Rg1 was dissolved within the culture media. After 4, 8 and 16 h of OGD exposure at 37°C, with or without geniposide (40 µg/ml) + ginsenoside Rg1 (8 µg/ml), BV2 cells were collected for the analysis of miR-155-5p expression. The experiment was independently repeated three times.

miR-155-5p, pri-miR-155 and pre-miR-155 quantification by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA of brain tissue and cultured BV2 microglial cells was isolated and analyzed using a Reverse Transcriptase kit (Suzhou GenePharma, Co., Ltd., Suzhou, China). The temperature and duration of reverse transcription was performed at 30°C for 10 min, 42°C for 30 min and 99°C for 5 min. A stem-loop real-time PCR system was used to determine the level of miRNAs using the Maxima SYBR Green qPCR Master Mix (Fermentas; Thermo Fisher Scientific, Inc., Waltham MA, USA) and a StepOne Sequence Detector (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed at 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 60°C for 60 sec. The following primer sequences were used: miR-155 forward, 5'-GCTTCGGTTAATGCTAATCGTG-3'; miR-155 reverse, 5'-AGAGCAGGGTCCGAGGTA-3'; U6 forward, 5'-ATTGGAACGATACAGAGAAGATT-3'; U6 reverse, 5'-GGAACGCTTCACGAATTTG-3'; pre-miR-155 forward, 5'-TGCTAATTGTGATAGGGGTTTT-3'; pre-miR-155 reverse, 5'-TATGGTTGTTTCACGACTCCTTCAC-3'; pri-miR-155 forward, 5'-AGGCTTTTCCTGGGCACC-3'; and pri-miR-155 reverse, 5'-CATGAACAAACCACAACGAGC-3'. RT-qPCR was performed in duplicate and relative expression of miR-155-5p, pri-miR-155 and pre-miR-155 were normalized to U6 expression and determined by the $2^{-\Delta\Delta C_q}$ method (31). The experiment was independently repeated three times.

Immunohistochemistry. Brain tissues from rats were removed for immunohistochemistry analysis at the termination of ischemia. Brain tissues were fixed in 4% paraformaldehyde overnight at room temperature. Sections (5 μ m) embedded in paraffin were deparaffinized in Histo-Clear™ (HS-200; National Diagnostics, Atlanta, GA, USA) rehydrated and washed with 0.01 M PBS; 3% hydrogen peroxide in methanol was used at room temperature for 30 min to inhibit the endogenous peroxidase. Sections were incubated with rabbit anti-CD11b (1:500) antibody (cat. no. ab133357; Abcam, Cambridge, MA) at 4°C overnight following blocking of nonspecific antigens with goat serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C for 20 min. Subsequently, sections were incubated with horseradish peroxidase-conjugated secondary antibodies at 37°C for 2 h. Peroxidase detection was performed using a mixture containing 0.05% 3,3'-diaminobenzidine. Following staining, all sections were coverslipped and analyzed. The immunohistochemistry images were acquired using an Olympus Fluoview FV1000 microscope (LSI3-FV1000-Inverted; Olympus Corporation, Tokyo, Japan) and Image-Pro Plus 6.0 software (Media Cybernetics, Inc.). The experiment was independently repeated three times.

Statistical analysis. Data are presented as the mean + standard deviation. The statistical analysis was conducted with SPSS software (SPSS, Inc. Chicago, IL, USA). Significant differences among the groups were determined by one-way analysis of variance, followed by a Student-Newman-Keuls post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-155-5p expression in rats. In the present study, a rat MCAO model was established. miR-155-5p expression levels increased in a time-dependent manner; miR-155-5p expression levels were significantly increased in the ipsilateral brain tissue of rats in the 24 h ischemia group compared with the sham group ($P < 0.01$; Fig. 1A). The C_q values of 2, 6 and 24 h ischemia groups increased by 1.42-, 1.62- and 2.94-fold, respectively, compared with the sham group (Fig. 1A). To investigate whether the increase of miR-155-5p in the brain was region specific, miR-155-5p expression levels were observed within various brain regions at 24 h post-ischemia. The results demonstrated that miR-155-5p expression was significantly increased in the cortex and basal ganglia of the ipsilateral brain in MCAO models, compared with in the sham group ($P < 0.05$; Fig. 1B and C). However, a significant increase was not observed in the expression levels of miR-155-5p within the hippocampus of MCAO model rats, compared with in the sham group (Fig. 1D). Treatment with geniposide + ginsenoside Rg1 attenuated the miR-155-5p C_q value markedly compared with the model group (1.67 ± 0.52 and 2.93 ± 0.87 , respectively; $P < 0.05$; Fig. 1E). However, geniposide + ginsenoside Rg1 had no effect on the miR-155-5p expression levels in the basal ganglia of MCAO model rats (Fig. 1F).

Geniposide + ginsenoside Rg1 protects brain tissue against ischemic injury. To assess the *in vivo* protective effect of geniposide + ginsenoside Rg1 against ischemic injury, the percentage of cerebral infarction and edema was investigated within an MCAO rat model. Infarction was not observed in the sham group. Cerebral infarction within the model group was observed primarily in the frontal and parietal lobe of the cortex, the caudate nucleus and posterior limbs of the internal capsule (Fig. 2A). The results revealed that geniposide + ginsenoside Rg1 treatment significantly reduced brain infarction size and edema volume compared with the model group ($P < 0.05$). In particular, the percentage of cerebral infarction and brain edema of rats subjected to geniposide + ginsenoside Rg1 was decreased by 34.2 and 42.6%, respectively, compared with the MCAO model group (Fig. 2B and C).

Geniposide + ginsenoside Rg1 inhibits microglia stimulation. Immunohistochemistry demonstrated low levels of CD11b expression within the sham group (Fig. 3A). However, after 24 h of ischemia, CD11b immunoreactivity was visibly increased in the model group (Fig. 3B), which was primarily present in the cortex region, indicating a marked activation of microglial cells within ischemic penumbra. The positive staining of CD11b was primarily located within the cell membranes, while the microglial morphology altered from branch- to amoeba-like, indicating a marked stimulation. Following treatment with geniposide + ginsenoside Rg1, the expression of CD11b was suppressed in the brain parenchyma, compared with the model group (Fig. 3C), as indicated by a reduced number of CD11b-positive cells in the ischemic penumbra region. Semi-quantitative analysis revealed the relative gray values of immunohistochemical staining (Fig. 3D), which indicated that the number of CD11b-positive cells was markedly reduced by geniposide + ginsenoside Rg1 treatment in MCAO model rats ($P < 0.05$).

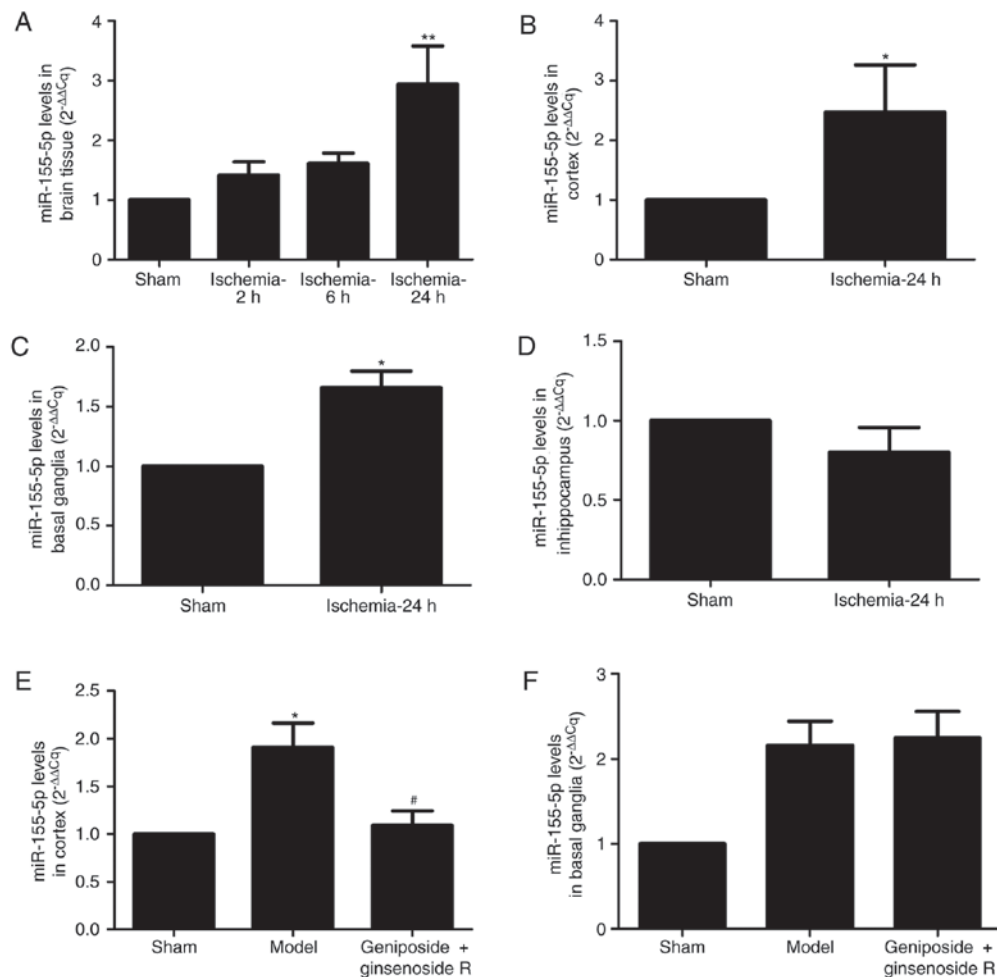


Figure 1. miR-155-5p expression levels are increased in MCAO model rats. (A) miR-155-5p expression levels in the ipsilateral brain tissue of MCAO rats increased in a time-dependent manner; ischemia-2, -6 and -24 h indicates rats subjected to MCAO for 2, 6 and 24 h, respectively. Reverse transcription-quantitative polymerase chain reaction analysis of miR-155-5p expression within the (B) ipsilateral cortex, (C) basal ganglia and (D) hippocampus of sham and 24 h ischemia groups. (E) Geniposide + ginsenoside Rg1 inhibited miR-155-5p expression levels in the cortex of MCAO model rats. (F) Geniposide + ginsenoside Rg1 exhibited no effects on miR-155-5p expression levels in the basal ganglia of MCAO model rats. Values are presented as the mean + standard deviation, n=8 rats per group. *P<0.05 and **P<0.01 vs. sham group, #P<0.05 vs. model group. miR, microRNA; MCAO, middle cerebral artery occlusion; geniposide + ginsenoside R, geniposide + ginsenoside Rg.

miR-155-5p expression within BV2 microglial cells. To further investigate the protective effects of geniposide + ginsenoside Rg1 against ischemic injury *in vitro*, BV2 microglial cells were cultured and the levels of immature forms of miR-155 (pri-miR-155 and pre-miR-155) and mature miR-155-5p were observed by RT-qPCR. As presented in Fig. 4A, miR-155-5p expression levels within the OGD 4 h group were not markedly altered compared with in control group. However, when the duration of ischemic was extended to 8 or 16 h, miR-155-5p expression levels within BV2 microglial cells were significantly increased compared with the control group (P<0.001). Consequently, OGD 8 h was selected as the duration for stimulation for subsequent analysis of BV2 cells. Treatment of cells with geniposide + ginsenoside Rg1 for 8 h significantly decreased the expression levels of the immature and mature forms of miR-155. In particular, miR-155-5p expression within BV2 microglial cells incubated with geniposide + ginsenoside Rg1 was reduced by 93.9% compared with the model group (Fig. 4B; P<0.01). In addition, following treatment with geniposide + ginsenoside Rg1, the expression levels of pri-miR-155 and pre-miR-155, the immature forms of miR-155, were

significantly inhibited by 97.9 and 97%, respectively, compared with the model group (P<0.01; Fig. 4C and D).

Discussion

Ischemic stroke leads to a series of complex, multipart-cascade events that contribute to irreversible brain injury and neuro-vascular network dysfunction within the ischemic core (32). Activation of microglia and astrocytes following stroke leads to enhanced release of proinflammatory factors, which may persist for several weeks (33). Although the immune responses mediated by microglia serve an important role in pathogen clearance and tissue regeneration, without treatment, the inflammatory state may worsen neuronal injury (16). miRNAs are a type of small non-coding RNA that regulate target gene expression and are highly conserved in various eukaryotes (34). Numerous miRNAs have been reported to be involved in the regulation of several inflammation phases and responses to cerebral ischemia (35-37). Of these miRNAs, miR-155 has been demonstrated to be associated with the regulation of various physiological and pathological processes, including inflammation, immunity,

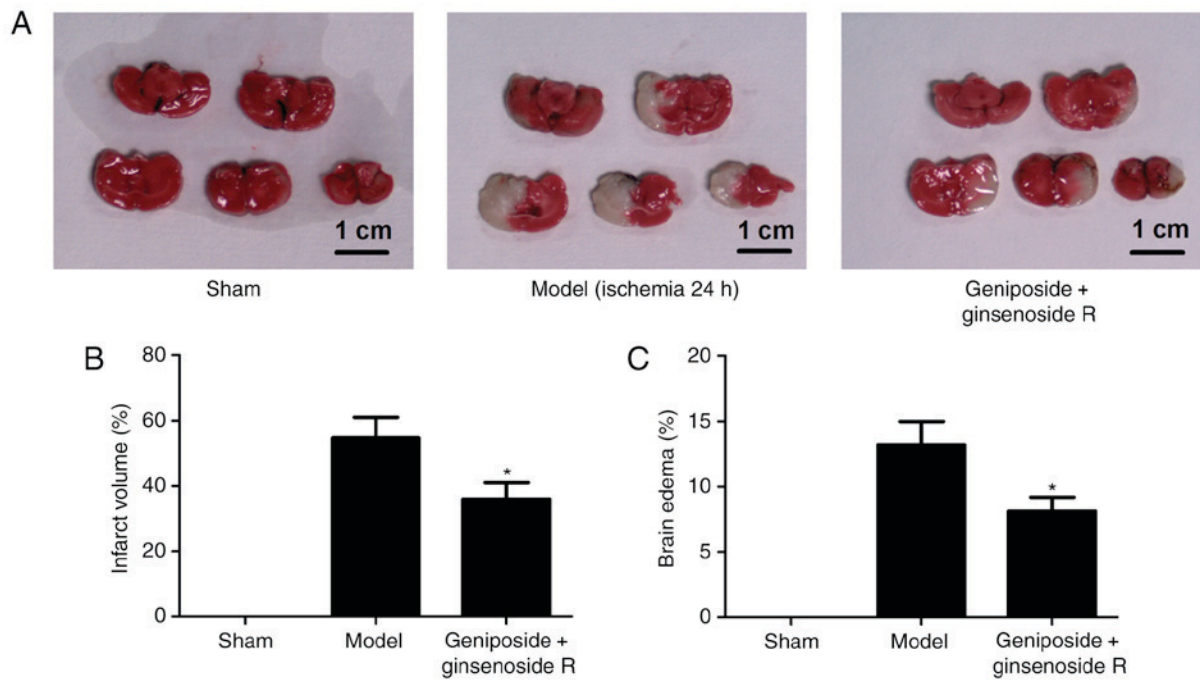


Figure 2. Treatment with geniposide + ginsenoside Rg1 attenuates brain infarction and brain edema following 24 h MCAO in rats. (A) Representative coronal sections stained by 2,3,5-triphenyltetrazolium. Effect of geniposide + ginsenoside Rg1 on (B) brain infarction volume and (C) brain edema in MCAO model rats. Values are presented as the mean + standard deviation, n=8 rats per group. *P<0.05 vs. model group. MCAO, middle cerebral artery occlusion; geniposide + ginsenoside R, geniposide + ginsenoside Rg1.

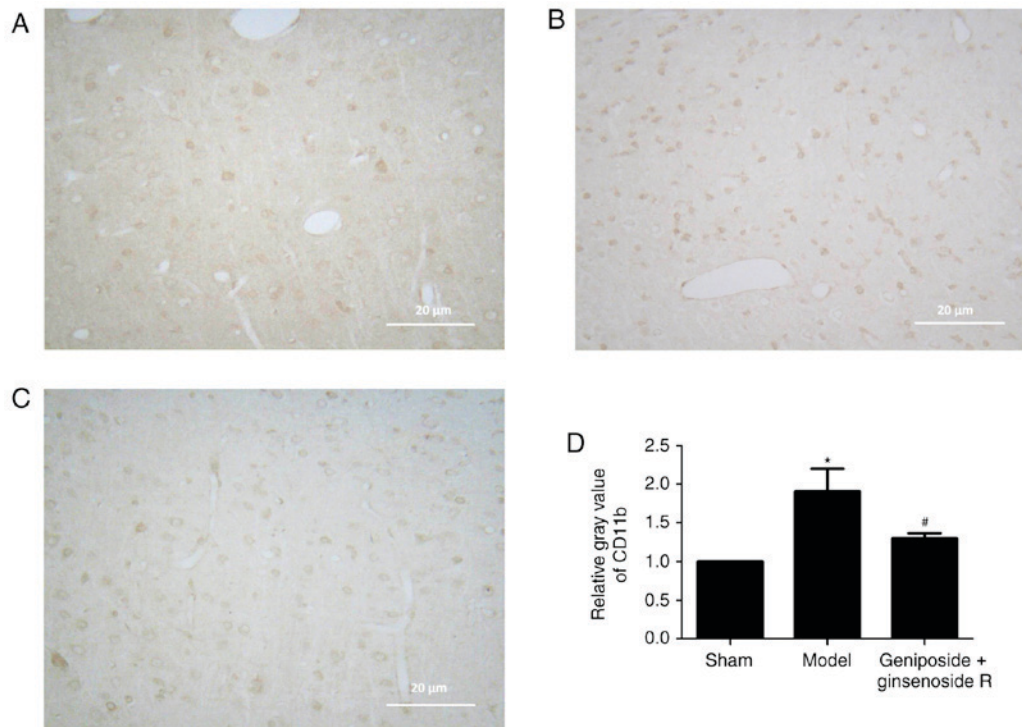


Figure 3. CD11b expression in the brain of middle cerebral artery occlusion model rats with or without geniposide + ginsenoside Rg1 treatment. Sections of brain tissue were incubated with antibody against CD11b for immunohistochemical analysis. (A) A lack of CD11b-antibody labeling was observed in the sham group. (B) Brown staining indicated strong CD11b expression in the model group. (C) Expression of CD11b in the ischemic region of rats was reduced by treatment with geniposide + ginsenoside Rg1, with fewer positively stained cells. Scale bar, 20 μm. (D) Bar graph indicates the results of the relative gray value obtained by semiquantitative analysis of immunohistochemical staining. *P<0.05 vs. sham group, #P<0.05 vs. model group. Geniposide + ginsenoside R, Geniposide + ginsenoside Rg1.

cancer and cardiovascular diseases (12,13). As a critical inflammation-associated miRNA, miR-155 regulates the host immune

response by repressing the expression of target genes (38). The majority of target genes of miR-155 are reported to be associated

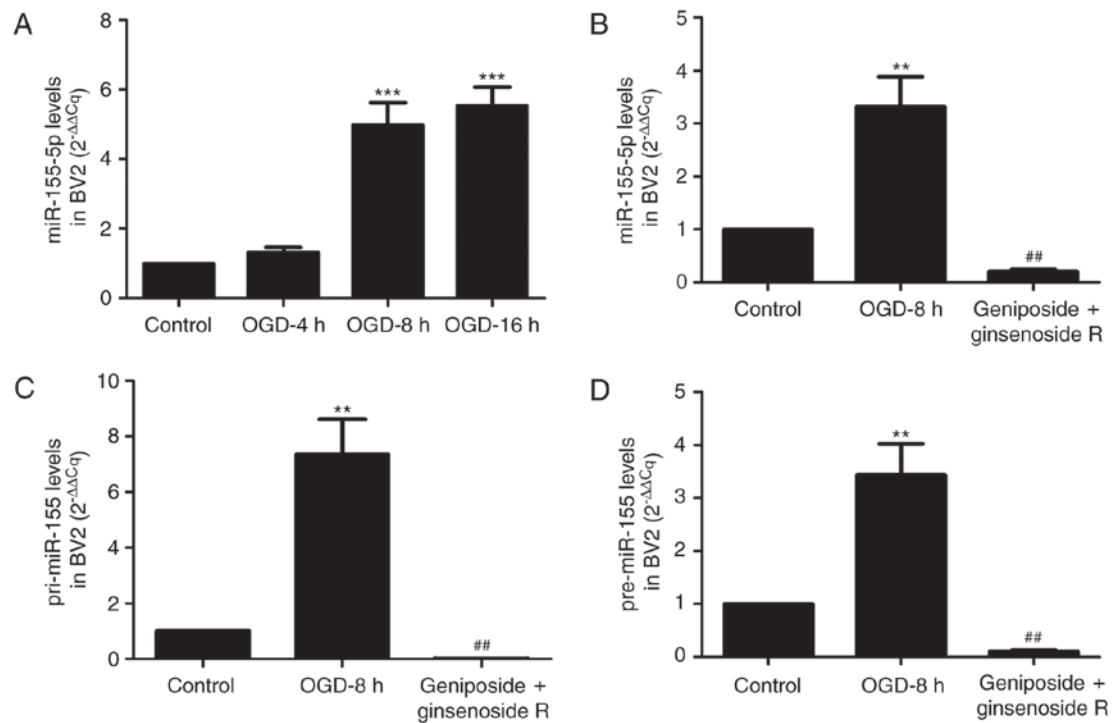


Figure 4. miR-155-5p expression levels in BV2 microglial cells. (A) miR-155-5p expression levels in the microglial cells exposed to OGD injury increased in a time-dependent manner; OGD-4, -8 and -16 h denote BV2 microglial cells subjected to OGD for 4, 8 and 16 h, respectively. (B) Geniposide + ginsenoside Rg1 inhibited the expression of mature miR-155-5p in BV2 microglial cells treated with OGD for 8 h. Geniposide + ginsenoside Rg1 suppressed the expression levels of immature precursor (C) pri-miR-155 and (D) pre-miR-155 in BV2 microglial cells treated with OGD for 8 h. Values are presented as the mean + standard deviation, 5 wells per group. **P<0.01 and ***P<0.001 vs. control group; ##P<0.01 vs. OGD-8 h group. miR, microRNA; OGD, oxygen-glucose deprivation; geniposide + ginsenoside R, geniposide + ginsenoside Rg1.

with the inflammatory regulation process. For example, it has been demonstrated that inflammatory responses mediated by miR-155 in ischemic cerebral tissues were modulated by Toll-like receptor (TLR)4/myeloid differentiation primary response gene 88 and suppressor of cytokine signaling 1 expression, while acetylbrominated lactone may suppress miR-155 expression and inhibit inflammatory actions (39). In addition, an association was reported between the expression of TLRs 3, 7 and 9, and miR-155 transcription, in the olfactory bulbs (40). Based on the reported associations between miR-155 and post-ischemic brain inflammation, therapeutic approaches that target miR-155 may be promising for the treatment of cerebral ischemic damage.

Gardenia, the fruit of *Gardenia jasminoides* Ellis, exhibits varying concentrations of iridoid glycosides. The root of *Panax notoginseng* (Burk.) F. H. Chen is a widely used traditional Chinese medicine that belongs to the genus *Panax* and family Araliaceae. *Panax notoginseng* is also termed Radix notoginseng or Sanchi. In clinical and experimental research, Gardenia is usually used in combination with *Panax notoginseng* to treat central nervous system diseases, including cerebrovascular disease and Alzheimer's disease. Geniposide and ginsenoside Rg1 are the active components of Gardenia and *Panax notoginseng*, respectively. In a carrageenan-induced rat paw edema model, Gardenia and *Panax notoginseng* have been reported to exhibit anti-inflammatory effects via the inhibition of nitric oxide production (41). These compounds were also demonstrated to ameliorate neurological injury, brain infarct volume and the permeability of the blood-brain barrier in MCAO rat models by reducing the expression

of proteinase-activated receptor 1 (42). However, studies concerning the role of geniposide + ginsenoside Rg1 have predominantly been performed in neurons; to the best of our knowledge, investigation regarding their combined effects on microglial cells is limited.

The results of the present study indicated that ischemia caused severe damage in cerebral tissue with a large area of cerebral infarction and brain edema. Geniposide combined with ginsenoside Rg1 reduced the percentage of cerebral infarction size and brain edema in rats subjected to MCAO, which demonstrated a protective effect on ischemia-induced cerebral injury *in vivo*. RT-qPCR revealed that miR-155 expression levels were unaltered in the 2 and 6 h ischemia groups; however, when the duration of ischemia was prolonged to 24 h, miR-155 expression levels increased within the brain tissue, compared with the sham group. Notably, miR-155 expression levels were increased in the cortex and basal ganglia in MCAO rats, compared with the sham group, indicating the potential of miR-155 overexpression as a target for the treatment of cerebral ischemic injury. Rats treated with geniposide combined with ginsenoside Rg1 demonstrated significantly attenuated expression levels of miR-155 expression levels. Based on the results for infarction size and brain edema, the neuroprotective effects of geniposide + ginsenoside Rg1 may be associated with the downregulation of miR-155. In the present study, 10% chloral hydrate was employed for anesthesia during the course of MCAO surgery, according to the previously described method (29,30) in which all mice or rats were treated with 10% chloral hydrate for anesthesia;

however, no symptoms of peritonitis were determined in the present study. In the present study, only the brain tissue was dissected for observation and the abdominal cavity was not observed as there were no obvious symptoms of peritonitis in rats. However, a previous study reported that intraperitoneal administration of 10% chloral hydrate resulted in adynamic ileus and peritonitis in rats, gastric ulcers in rats and peritonitis in swine (43). Therefore, in future experiments, we plan to change to gas anesthesia with isoflurane.

To the best of our knowledge, activated microglia become hypertrophic, rapidly proliferate and migrate to the inflammatory area where they generate excessive amounts of proinflammatory and neurotoxic cytokines that cause neuronal damage (44,45). In the current study, the results of immunohistochemistry demonstrated that microglial cells increased in number and appeared to possess an activated morphology within ischemic-injured regions. Geniposide + ginsenoside Rg1 markedly reversed the CD11b expression, indicating an inhibitory effect of geniposide + ginsenoside Rg1 on microglial cells. As microglial miR-155 expression serves an important role in the pathological process following ischemic stroke, an *in vitro* experiment was performed to observe the effects of geniposide + ginsenoside Rg1 on microglial cells. The results demonstrated that miR-155 expression levels were elevated and microglial cells were activated following OGD injury for 8 h, compared with the control group, indicating microglial activation and high inflammatory potential following OGD. However, geniposide + ginsenoside Rg1 reduced the expression of mature miR-155 and the immature precursors, pri-miR-155 and pre-miR-155, indicating a potential protective role of geniposide + ginsenoside Rg1 in microglial activation. Geniposide + ginsenoside Rg1 treatment may activate microglial cells to release inflammatory factors. miR-155 is an important miRNA that regulates the mRNA expression of inflammatory factors; based on the inhibitory effect on miR-155 in the current study, the anti-inflammatory effect of geniposide + ginsenoside Rg1 may be achieved by inhibiting miR-155 expression.

In conclusion, the *in vivo* and *in vitro* experiments of the present study demonstrated that the administration of geniposide in combination with ginsenoside Rg1 via tail vein injection inhibited ischemia-induced microglial cell activation, which may be associated with the inhibition of microglial miR-155-5p expression. The results of the present study indicated that geniposide in combination with ginsenoside Rg1 may serve as a potential alternative for the prevention of microglial cell injury in ischemic diseases.

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