Protective Effects of Guizhi-Fuling-Capsules on Rat Brain Ischemia/Reperfusion Injury

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Abstract. Previous studies revealed that Guizhi-Fuling-Capsules (GZFLC), a traditional Chinese medical (Kampo) formulation composed of five kinds of medicinal plants, Cinnamomum cassia BLUME (Cinnamomi Cortex), Paeonia lactiflora PALL. (Peonies Radix), Paeonia suffruticosa ANDREWS (Moutan Cortex), Prunus persica BATSCH (Persicae Semen), and Poria cocos WOLF (Hoelen), exerts a protective effect against vascular injury and has a protective effect against glutamate- or nitro oxide-mediated neuronal damage. In the present study, the effect of GZFLC in a rat in vivo model of focal cerebral ischemia and reperfusion was investigated. Administration of GZFLC (0.3 and 0.9 g/kg, p.o.) after focal cerebral ischemia significantly decreased brain infarction and water contents in rats subjected to 2-h ischemia followed by 24-h reperfusion from 31.72 ± 2.49%, 84.76 ± 1.63% in the model group to 17.31 ± 3.66%, 82.51 ± 1.36% and 8.30 ± 3.73%, 81.35 ± 1.73%, respectively. Furthermore, analysis of inflammatory cytokines in ischemic brain showed that GZFLC treatment significantly down-regulated expressions of pro-inflammatory cytokines including interleukin (IL)-1β and tissue necrosis factor-α and markedly up-regulated expressions of anti-inflammatory cytokines IL-10 and IL-10R both in mRNA and protein levels. The serum levels of these inflammatory cytokines were also regulated the same way. These results suggested that GZFLC may be beneficial for the treatment of brain ischemia-reperfusion injury partly due to its anti-inflammatory properties.

Keywords: cerebral ischemia, cytokine, inflammation, traditional Chinese medicine

Introduction

Ischemic stroke, characterized by the disruption of cerebral blood flow, remains one of the leading causes of death and of severe disability and places a large burden on healthcare and social service resources in most countries (1). There is evidence that the inflammatory immune reaction is one of the most relevant processes in the pathogenesis of cerebral ischemia, especially in the period of reperfusion (2–4). Major players in the initiation and modulation of the post-ischemic inflammatory response are inflammatory cytokines. Pro-inflammatory cytokines such as interleukin (IL)-1β and tissue necrosis factor-α (TNF-α) contribute to the pathogenesis, causing exacerbation of ischemic-reperfusion brain tissue damage. Anti-inflammatory cytokines, such as IL-10, possess anti-inflammatory properties and can provide neuroprotection and tissue repair in ischemic stroke. A better understanding of cytokines’ role in cerebral ischemia may result in the design of improved therapy.

However, no specific and effective therapy that limits neuronal damage and neurological dysfunction for stroke is currently available. In China, Korea, and Japan, extensive experience and abundant clinical data on the treatment of stroke have been documented with traditional Chinese medicine, which has been developed over a period of thousands of years (5, 6).

Guizhi-Fuling-Capsules (GZFLC) (Keishi-bukuryo-gan in Japanese) is a traditional Chinese medical
A cerebral infarction (11). GZFLC improves erythrocyte and subjective symptoms of patients with asymptomatic ischemia and reperfusion in rats. Meanwhile, the effect of GZFLC treatment on the post-ischemic inflammatory response was also examined. Specifically, we assessed the mechanisms of GZFLC-treated brain ischemia should be further studied.

Previous studies revealed that GZFLC exerts a protective effect against vascular injury (7), inhibits the progression of atherosclerosis (8, 9), and has protective effects against glutamate- or nitro oxide-mediated neuronal damage in cultured cerebellar granule cells (10). Clinically, Guizhi Fuling Wan (Keishi-bukuryogan in Japanese) has been used to treat stroke in Japan, which improved intellectual ability, emotional disorder, and subjective symptoms of patients with asymptomatic cerebral infarction (11). GZFLC improves erythrocyte deformability, erythrocyte membrane viscoelasticity (12), and erythrocyte aggregability (13) in patients with old cerebral infarction. However, the efficacy and mechanism of GZFLC-treated brain ischemia should be further studied.

The objective of the present study was to evaluate the effects of GZFLC administration on focal cerebral ischemia and reperfusion in rats. Meanwhile, the effect of GZFLC treatment on the post-ischemic inflammatory response was also examined. Specifically, we assessed the expressions of the pro-inflammatory cytokines IL-1β and TNF-α, as well as that of anti-inflammatory IL-10 and its receptor (IL-10R) in the brain.

Materials and Methods

Animals and reagents

Male Sprague-Dawley (SD) rats weighing 230–250 g provided by Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China) were housed in controlled conditions and received a standard rat chow and tap water ad libitum. All the animals used in this work received humane care in compliance with institutional animal care guidelines, and the research protocol was approved by the Local Institutional Committee.

Rat IL-1β, TNF-α, and IL-10 ELISA kit were purchased from Biosource (Camarillo, CA, USA); Trizol reagent was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA); TaKaRa RNA PCR kit (AMV) ver2.1 was purchased from Takara Biotechnologies (Carlsbad, CA, USA); EnVision kit were purchased from DaKo (Glostrup, Denmark). All other reagents were from local commercial sources.

GZFLC (Lianzhong Brand) was manufactured by Jiangsu Kanion Pharmaceutical Co., Ltd. (Lianyungang, China) and the lot number was 030209. The storage location of the specimen used in the study is the Department of Pharmacology, School of Pharmacy, Second Military Medical University, China. GZFLC is Traditional Chinese Patent Medicine, which is recorded on page 453 and 454 in volume I of Pharmacopoeia of the People’s Republic of China 2005 (English version). Identification and assay of GZFLC is carried out by the TLC and gas chromatography method. The gas chromatography system and system suitability are as follows: a column packed with 5% SE-30 as the stationary phase, 1.6-m-long; and column temperature maintained at 130°C. The number of theoretical plates of the column is not less than 600, calculated with the reference to the peak of paeonol (C_{15}H_{20}O_{3}), and the resolution between the peak of paeonol and internal standard substance is not less than 2. The details of the procedure are as follows: Weigh accurately 0.5 g of the content, obtained under the test of weight variation; distill with steam and collect about 400 ml of distillate; extract with successive quantities of 60, 40, 40, and 30 ml chloroform and combine the chloroform extracts; dehydrate with 2 g of anhydrous sodium sulfate; filter; wash with sodium sulfate and container with 10 ml of chloroform in portions; and filter. Combine all of the chloroform filtrates, recover chloroform at 70°C on a water bath to a quantity, transfer to a 5-ml volumetric flask, dilute with chloroform to volume, and shake well. Measure accurately 2 ml of the solution to a 5 ml volumetric flask, add 2 ml of internal standard solution, dilute with chloroform to volume, and shake well. Inject 1 µl into the column, and calculate the content. Each capsule contains not less than 1.2 mg of paeonol, referred to Cortex Moutan.

We did not use any positive control drug in our experiments since no drugs are used clinically for prevention of brain ischemic damage through an anti-inflammation mechanism regulating cytokines levels.

Cerebral ischemia model

Transient focal cerebral ischemia in the SD rats was conducted according to procedures described previously (14). Briefly, each animal was anesthetized with 400 mg/kg chloral hydrate intraperitoneally, a 30-mm segment of 4-0 ethylon monofilament, whose tip was rounded by heating, was gently introduced from the external carotid artery into the internal carotid artery lumen approximately 17–18-mm distal to the carotid...
bifurcation until a mild resistance was felt. Reperfusion was accomplished by with drawing the filament 120 min after induction of ischemia. In sham-operated rats, the external carotid artery was surgically prepared for insertion of the filament, but the filament was not inserted. Rectal temperature was monitored continuously and maintained between 36.5°C and 37.5°C by surface heating and cooling. Animals were then returned to their cages and closely monitored until they recovered from anesthesia.

Experimental groups
A total of 80 rats were randomly and equally divided into four groups: Group 1 served as the control (sham-operated group); Group 2 was the ischemia and reperfusion without medication group (model group); Group 3 and Group 4 animals were treated with GZFLC intra-gastric administration at dose of 0.3 and 0.9 g/kg, respectively. GZFLC was diluted in normal saline (pH 7.4) and was given at the time of ischemia for 30 min and again 9.5-h later. The analyses were performed blindly in each experimental group.

Evaluation of ischemia area and water contents
Rats were sacrificed after 24 h of reperfusion and the brains were removed quickly. Six rats of each group were cooled on ice and were cut into five coronal sections (2 mm). Sections were stained with a 2% solution of 2,3,5-triphenyltetrazolium (TTC) dye (Sigma, St. Louis, MO, USA) at 37°C for 20 min. Stained sections were then recorded using a digital camera and the volume of total brain and infarct area were measured by an image analysis software (MedBrain 4.0; Medease Science and Technology, Nanjing, China). The water content in the brain of 6 other rats in each group was determined by the dry to wet weight ratio [(wet weight − dry weight) / wet weight × 100%].

Determination of the cytokine protein levels
After 24 h of reperfusion, a serum sample was obtained, and the brain tissue was homogenized in normal saline (1:2 ratio) and then centrifuged for IL-10 level determinations, which were performed by enzyme-linked immunosorbent assay (ELISA) as specified in manufacturer’s kit. There are 5 rats in each group for determination of the cytokine protein levels.

Reverse-transcriptase polymerase chain reaction (RT-PCR)
There were 3 rats in each group for the RT-PCR and Western blotting test. Total RNA from brain regions was isolated with Trizol according to the manufacturer’s protocol. For each reaction, 1 µg of total RNA served as a template. For amplification, specific primer pairs for β-actin, IL-1β, TNF-α, IL-10, and IL-10R were as follows and the size of the products were 587, 578, 315, 174, and 319 bp after 30 amplification cycles, respectively: β-actin, forward 5'-AAGGCCAACCGTGAAA AGATGAC-3' and reverse 5'-GGTGATCATGTGGTGG CCACCGAC-G-3'; IL-1β, forward 5'-GACCTGTCTCTT TGAAGCTGAC-G-3' and reverse 5'-TCCATCTCCTTC TTTGGGTATTGT-3'; TNF-α, forward 5'-CATGATC CGAGATGTGGAACTGGC-3' and reverse 5'-CTGG CTCAGCCACTCCAGC-G-3'; IL-10, forward 5'-TAAG GGTTACTTGGTTGCAAAGCC-3' and reverse 5'-AG GGGAGAAATCGATGACAGCGC-3'; and IL-10R, forward 5'-CCAACTGGACCATCAGCAACTGAAAACCTC-3' and reverse 5'-GCCTTGTTAATTGCGATTCCAC-3'. In all experiments, control reactions were performed by substituting sterile nuclease-free water for the RNA template in the reaction. β-Actin was amplified as a reference. The RT-PCR products were visualized on a 1% agarose gel with ethidium bromide.

Western blotting
Rat brain tissues were cleaned and homogenized in ice-cold RIPA buffer. Equal amounts of protein preparations (10 µg in 25 µl buffer) were then separated on SDS-polyacrylamide gels, electrotransferred to nitrocellulose membranes, and blotted with primary antibodies. Blots were then developed by using enhanced chemiluminescence reagent and visualized on the Kodak X-Omat film.

Statistical analysis
All data are expressed as the mean ± S.D. Data analyses were performed with SPSS10.0. The data were analyzed by one-way ANOVA. Differences between groups were considered statistically significant at P<0.05.

Results
The protective effects of GZFLC on rat cerebral ischemia/reperfusion injury
Infarct area and oedema occurred in ischemic brain tissue in the rats subjected to 2-h ischemia and 24 h of reperfusion. Treatment with GZFLC significantly decreased infarct volume percent and water contents (Fig. 1: A and B).

Effects of GZFLC on serum levels of TNF-α, IL-1β, IL-10
As shown in Fig. 2, serum levels of IL-1β, TNF-α, and IL-10 increased after 2-h cerebral ischemia followed by 24-h reperfusion compared to sham animals (P<0.01).
Treatment with GZFLC significantly decreased expression of TNF-\(\alpha\) and IL-1\(\beta\) in serum and significantly increased the expression of IL-10.
Effects of GZFLC on brain tissue expression of TNF-α, IL-1β, and IL-10

Compared to the sham operation group, IL-1β, TNF-α, and IL-10 in ischemic brain tissue homogenate all increased after 2-h cerebral ischemia followed by 24-h reperfusion as shown in Fig. 3. Treatment with GZFLC significantly decreased expression of IL-1β and TNF-α in tissue homogenate and significantly increased the expression of IL-10. RT-PCR analysis showed that the expressions of IL-1β, TNF-α, IL-10, and IL-10R mRNA were seldom in the brain of sham groups and all increased in the brain of cerebral ischemia/reperfusion rats (Fig. 4); treatment with GZFLC significantly decreased the mRNA expressions of IL-1β and TNF-α, while the expressions of IL-10 and IL-10R mRNA significantly elevated. These results from RT-PCR were next confirmed by western blotting at protein levels (Fig. 5).
Discussion

According to preliminary test and clinical doses, GZFLC intragastric administration at dose of 0.3 and 0.9 g/kg was used in this study. The data presented in this work demonstrated that administration of GZFLC after focal cerebral ischemia decreased brain infarction and water contents, and the effect of treatment with GZFLC on decreasing brain infarction at dose of 0.9 g/kg was better than that of 0.3 g/kg (P<0.01). From this, it is suggested that GZFLC has protective effect against cerebral ischemia, which is in accordance with Zhang’s report that Guizhi Fuling Pills help alleviate ischemia-induced cerebral injuries (15). Furthermore, analysis of inflammatory cytokines in ischemic brain showed that this protection was associated with significant down-regulated expressions of pro-inflammatory cytokines including IL-1β and TNF-α and up-regulated expressions of anti-inflammatory cytokines IL-10 and IL-10R, both in mRNA and protein levels. The current study also showed serum levels of these inflammatory cytokines were regulated in the same way.

Indirect evidence and direct experimental data exist to suggest a critical role for cytokines in the pathogenesis of ischemic cerebral damage. Impairment of the balance of pro- and anti-inflammatory cytokines may lead to disease progression and a more severe outcome after cerebral ischemia. It has been reported that the expressions of both pro- and anti-inflammatory cytokines are induced rapidly by focal or global brain ischemia (3, 16, 17). Over expression of IL-1β or TNF-α, the two major pro-inflammatory cytokines, exacerbates the brain infarction after cerebral ischemia, whereas their inhibition has been reported to attenuate the infarct volume (18–20). In our study, the expressions of IL-1β and TNF-α were significantly up-regulated by ischemia, and both were markedly reduced by GZFLC. This finding indicates that the protective effect of GZFLC against cerebral damage after ischemia is likely attributable to its anti-inflammatory properties.

Inflammatory cytokines that contribute to the pathogenesis, causing exacerbation of brain injury, and other cytokines that possess anti-inflammatory properties and may mediate neuroprotection and tissue repair. A neuroprotective role for anti-inflammatory cytokines in brain damage after cerebral ischemia has been highlighted in previous studies. In the recent years, it was found that IL-10, predominantly secreted by activated monocytes, macrophages, and smooth cells under certain conditions, is just an important one of these molecules. Intracerebroventricular and systemic injection of IL-10 (21) and posts ischemic gene transfer of IL-10 into the lateral ventricle (22) both attenuated brain infarction and hippocampal damages. Moreover, a C1-inhibitor protects against brain ischemia-reperfusion injury via inhibition of inflammation partly due to increasing protective cytokine (IL-6, IL-10) production (23). Up-regulating production of anti-inflammatory cytokines such as IL-10 may be a promising method for treatment of brain ischemia. The results in our study demonstrate that IL-10, IL-1β, and TNF-α are induced following brain ischemia-reperfusion. The temporal profile of these cytokines is similar to that seen in MCAO (16), where TNF-α induces IL-10; subsequently, IL-10 inhibits TNF-α expression. IL-10 acts as negative feedback regulator of TNF-α release (24), exerting its beneficial effects on brain 1/R injury. Furthermore, IL-10 limits the role of glutamate cytotoxicity by inactivation of NF-kappaB (25), a transcription factor that modulates inflammation and key regulatory proteins in cerebral ischemia (26). IL-10 clearly has a protective effect against brain ischemia. Our present results also showed that production of IL-10 and its receptor were increased significantly and cerebral ischemia injury was ameliorated markedly in GZFLC-treated rats, suggesting a potential involvement of the anti-inflammatory effect of IL-10 in GZFLC treatment. The elevation of the protective cytokine IL-10 in 1/R rats was further accelerated by GZFLC, which appears to contribute to the neuroprotective effect of GZFLC. Moreover, the action of GZFLC on brain ischemia injury and cytokines showed dose-effect dependency. In addition, GZFLC was reported to possess a hypotensive effect in patients with asymptomatic cerebral infarction (10). Clinically, gastric discomfort and dull pain are seen in some patients. So, the dose and adverse effects of GZFLC should be thoughtful considered in clinical application.

In summary, GZFLC has protective effects on rat brain ischemia-reperfusion injury. Part of the mechanism is that GZFLC can enhance the expression of anti-inflammatory cytokines and down-regulate the expression of inflammatory cytokines. These results also suggested that interventions aimed at down-regulating pro-inflammatory cytokines, particularly up-regulating anti-inflammatory cytokines, would eventually become an effective therapeutic strategy for the treatment and prevention of brain ischemia damage.

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References


