Gui-Zhi-Fu-Ling-Wan Reduces Cerebral Infarction Area via the Anti-inflammatory Effects in Ischemia-reperfusion Injured Rats

Ching-Liang Hsieh[1,2], Tin-Yun Ho[3], Shan-Yu Su[1,4], Chin-Yi Cheng[3], Wei-Tsung Hsu[1], Ching-Tou Hsieh[5], Hsiao-Yun Pu[2], Nou-Ying Tang[4]

[1]Department of Chinese Medicine, China Medical University Hospital; [2]Graduate Institute of Integrated Medicine, [3]Graduate Institute of Chinese Medical Science, [4]School of Chinese Medicine, China Medical University; [5]Deparment of Internal Medicine, Jen-Ai Hospital, Taichung, Taiwan.

Background/Purpose. Blood stasis, a pathological concept of blood circulation disturbance in Traditional Chinese Medicine, has been demonstrated to be associated with the process of stroke. Gui-Zhi-Fu-Ling-Wan (GZFLW) is known to improve the status of severe blood stasis. The purpose of this study is to investigate the effects of GZFLW on cerebral infarct.

Methods. A rat model of cerebral infarct was established by blocking both common carotid arteries and the right middle cerebral artery for 90 min, followed by reperfusion for 24 h. Neuro-deficit score and the percentage of cerebral infarction area to total brain area were used as a therapeutic effect index of GZFLW, (a Chinece Medicine formula), and MK801(a non-competitive N-methyl-D-asparate receptor antagonist) treatment. The numbers of ED1 (mouse anti rat CD68)-, interleukin-1β (IL-1β)-, tumor necrosis factor-α (TNF-α)-, intercellular adhesion molecule-1 (ICAM-1)-, myeloperoxidase (MPO)-immunoreactive cells, and apoptotic cells in the cerebral infarction region were counted under light microscope. In addition, the phosphorylation of mitogen-activated protein (MAP) kinases was detected by Western blotting 90 min after blocking the blood flow.

Results. Pre-treatment with GZFLW 0.6 g/kg, GZFLW 0.8 g/kg, MK801 1.0 mg/kg and post-treatment with GZFLW 0.6 g/kg reduced the cerebral infarction area; pre-treatment with GZFLW 0.6 g/kg and MK801 1.0 mg/kg reduced the neuro-deficit score and also reduced the numbers of ED1-, IL-1β-, TNF-α-, ICAM-1-, MPO-immunoreactive and apoptotic cells in the cerebral infarction region. GZFLW 0.6 g/kg reduced the cerebral ischemia-induced phosphorylation of c-Jun N-terminal kinases (JNKs).

Conclusion. GZFLW reduced the cerebral infarction area and neuro-deficit in rats, suggesting that GZFLW has the potential to reduce cerebral infarction size in humans. GZFLW also inhibited microglia activation, neutrophil infiltration, and TNF-α, IL-1β, ICAM-1 expression, suggesting that GZFLW has an anti-inflammatory effect. Moreover, our data indicate that the anti-inflammatory effect of GZFLW might be associated with the JNK signaling pathway in cerebral ischemia. **(Mid Taiwan J Med 2008;13:1-11)**

Key words

cerebral infarct, c-Jun N-terminal kinases, ED1, Gui-Zhi-Fu-Ling-Wan, intercellular adhesion molecule-1, neurologic deficit.

Received : 2 July 2007. Revised : 7 September 2007.
Accepted : 13 September 2007.
Address reprint requests to : Nou-Ying Tang, School of Chinese Medicine, China Medical University, 91 Hsueh-Shih Road, Taichung 404, Taiwan.

INTRODUCTION

Several studies have shown that stroke patients with increased red blood cell (RBC) deformity, RBC aggregation, blood viscosity and

platelet aggregation exhibit the phenomenon of severe blood stasis. However, these symptoms can be improved by treatment with Gui-Zhi-Fu-Ling-Wan (GZFLW) [1,2].

Activation of microglias is characteristic of neuronal damage after middle cerebral artery occlusion (MCAo) [3]. Activated microglias secrete interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) , cytokines which play a critical role in ischemic brain damage [4,5]. Neuronal apoptosis occurs after severe cerebral ischemia [6], and could represent a mode of ischemic cell death [7]. Intercellular adhesion molecule-1 (ICAM-1) participates in leukocyte-endothelial adhesion and in the migration of leukocytes into the damaged region of the brain [8]. Myeloperoxidase (MPO) activity, a quantitative index of polymorphonuclear neurtrophils (PMN) [9], increases in the cerebral infarction region after cerebral ischemia in rats. Neutrophils are the main source of oxidative free radicals in myocardial ischemia-reperfusion [10,11]. Mitogen-activated protein (MAP) kinases, including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase (JNKs) and p38, are activated by extracellular stimulation and participate in cell growth and stress signaling [12,13]. The activation of the p38 pathway during sustained ischemia can be inhibited by inhibitors of IL-1β and TNF-α [14], suggesting an association between ischemia, inflammatory cytokines and the activation of MAP kinases.

The purpose of this study was to investigate the effects of GZFLW on cerebral infarct in a rat model of cerebral infarction.

SUBJECTS AND METHODS

Extraction procedures of GZFLW

The plant materials used to concoct GZFLW were authenticated and extracted by Koda Pharmaceutical Co. Ltd. (Taoyuan, Taiwan). The 240 g sample of dried GZFLW comprised *Cinnamon twig* 44 g, *Prunus persica* (L) Batsch 74 g, *Poria cocos* (Schw.) Wolf 22 g, *Paeonia lactiflora* Pall 45 g, and *Paeonia suffruticosa* Andr 55 g. Crude GZFLW was extracted twice with 1.68 and 1.2 liters of boiling water for 40 min and 50 min, respectively. The aqueous extracts were filtered and freeze-dried. The total yield was 35.6 g (14.83%). The freeze-dried GZFLW extracts were qualified by high-performance liquid chromatography; paeoniflorin and cinnamic acid were used as standards.

Animals

Male Sprague-Dawley (SD) rats, weighing 300 to 400 g, were obtained from the National Laboratory Animal Breeding and Research Center, National Science Council, Taiwan. The rats were housed in standard iron cages in the animal center of the China Medical University. The humidity level was controlled at 55 ±5%, and the rats were maintained on a 12-h light-dark cycle at 22 ±2°C. All animal experiments were performed in accordance with the Guidelines of the Chinese Society for Laboratory Animal Science, Taiwan, R.O.C.

Establishment of the cerebral infarction animal model

The cerebral infarction animal model was established as described by Hsieh et al. (2001) [15]. Each rat was placed supine and both common carotid arteries were exposed through a midline incision in the neck. Then, both common carotid arteries were tied off with plastic line (0.1 mm in diameter). Rats were then placed prone and the head of each rat was fixed in a stereotactic apparatus. The scalp was incised to create an anterior-posterior direction wound (1.5 cm in length), exposing the skull from the midpoint of the binaural line. The olfactory tract and right middle cerebral artery were clearly visible through the bone window measuring 3.5 mm in diameter. An 8-0 nylon line mediated via a surgical needle was placed under the right middle cerebral artery just at the immediate upper margin of the olfactory tract, and tied loosely. The markers for laser Doppler perfusion were monitored (DRT4, Moor Instruments Inc. Wilmington, USA). The markers changed from 900 units to 200 units when the plastic line loops were drawn to block the blood flow of both common carotid arteries. The markers changed from 200 units to 50 units when the blood flow of

the right middle cerebral artery was blocked. Cerebral blood flow was reestablished after blocking the blood flow of both common carotid arteries and the right middle cerebral artery for 90 min. The blood gas was measured from the right femoral artery before and 90 min after blocking the blood flow.

GZFLW treatment and measurement of infarction in ischemia-reperfusion injured SD rats

A total of 42 SD rats were randomly divided into 7 groups of 6 rats: 1) the sham group comprised rats in which both common carotid arteries and the right middle cerebral artery were exposed, but the blood flow was not blocked; 2) the control group was composed of rats in which the blood flow of both common carotid arteries and the right middle cerebral artery was blocked for 90 min followed by reperfusion for 24 h; 3) the $G_{0.4}$ group of rats received intraperitoneal injection (i.p.) of GZFLW 0.4 g/kg 10 min before the blood flow was blocked; 4) the $G_{0.6}$ group of rats was administered GZFLW 0.6 g/kg (i.p.) 10 min before the blood flow was occluded; 5) the $G_{0.8}$ group received GZFLW (i.p.) 0.8 g/kg 10 min before the blood flow was blocked; 6) the MK group was administered MK801 (Sigma, Steinheim, Germany) 1.0 mg/kg (i.p.) 10 min before the blood flow was occluded; 7) the $DG_{0.6}$ group was administered GZFLW 0.6 g/kg (i.p.) 30 min after the blood flow was blocked.

Evaluation of neurological status

After reperfusion for 24 h, the neurological status of the rats was estimated by an evaluator who was blinded to the grouping [16]. The neurological examination grading scale system ranged from 0-3, where grade 0 indicates no neurological deficit; grade 1, forelimb flexion; grade 2, decreased resistance to a lateral push; grade 3, circling behavior.

Measurement of cerebral infarction area

After evaluation of neurological status, 3 mL of whole blood was collected from the right femoral artery to measure blood biochemistry and blood cells. Then, the brain of each rat was removed after transcardiac perfusion of 0.9% NaCl and 4% paraformaldehyde. Each brain was then placed into a plastic rat brain model, and the coronals were sectioned into 2 mm slices. The samples were then placed in 2% 2, 3, 5-triphenyl-tetrazolium chloride (TTC) solution in a 37°C room for 15 min to allow for differentiation between the white cerebral infarction area and the reddish-purple normal brain tissues [17]. Finally, the samples were fixed with 10% formalin solution.

The cerebral infarction areas of the first six sections from the frontal lobe were measured using a microscopic image-analysis system (Image-Pro Lito Version 3.0, Media Cybernetics, USA). The ratio of infarction area to total brain area in each section of the rat brain was calculated, and the data were expressed as a percentage (%).

The measurement of blood cells, blood biochemistry and blood sugar levels

Whole blood (0.5 mL) was analyzed for red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), lymphocytes (LYM) and platelets (PLT) by a blood cell analysis apparatus (System KX-21, System Ltd., Kobe, Japan). The other 2.5 mL of whole blood was centrifuged at 3400 rpm for 10 min; aminotransferase aspirate (GOT), aminotrans-ferase alanine (GPT), blood urea nitrogen (BUN), creatinine, and blood sugar levels of supernatants were determined by a biochemistry analysis apparatus (Roche, COBUS, MIRA).

Immunostaining and apoptotic cell detection of brain tissues in GZFLW treated ischemia-reperfusion injured SD rats

The 24 SD rats that had been randomly divided into the sham, control, $G_{0.6}$ and MK groups were scarified. The brains were removed, embedded in optimal cutting temperature (OCT) medium and cut into 20 μm sections for immunostaining as described by Hsieh et al. (2006) [15]. The numbers of ED1-, IL-1β-, TNF-α-, ICAM-1-, MPO-immunoreactive cells and apoptotic cells in the cerebral infarction zone of the 3rd section from the frontal lobe were

counted under a light microscope. The data represent the number of cells within 1 mm^2.

In situ analysis of apoptosis

A DNA fragmentation detection kit (TdT-FragEL™ kit; Oncogene, Boston, MA) was used to study apoptotic cell death. Tissues were rinsed briefly with TBS, and then incubated with proteinase K for 20 min followed by 3% H_2O_2 in methanol for 5 min to inactivate endogenous peroxidases. Terminal deoxynucleotidyl transferase (TdT) was then added to the tissues at 37°C for 1.5 h. The addition of stop buffer solution terminated the reaction. Apoptotic cells were detected by incubating tissues with 3,3'-Diaminobenzidine tetrahydrochloride substrate (DAB), followed by counterstaining with methyl green.

Western blotting of the brain tissues in GZFLW treated ischemic SD rats

A total of 9 SD rats were randomly divided into 3 groups of 3 rats as follows: 1) sham group; 2) control group; 3) $G_{0.6}$ group. The rats were sacrificed and the brain tissues were removed for Western blotting as described by Hsieh et al. (2007) [18].

Statistical analysis

The data are represented as mean ± SD. Groups were compared by one-way analysis of variance (ANOVA) followed by Scheffe's test. A p-value < 0.05 was considered statistically significant.

RESULTS

Effects of GZFLW on infarction areas in ischemia-reperfusion injured rats

Blood gas analysis indicated that the pH values and the percentages of pCO_2 and pO_2 were similar among the sham, control, $G_{0.4}$, $G_{0.6}$, $G_{0.8}$, $DG_{0.6}$ and MK groups before and 90 min after blocking the blood flow (all $p > 0.05$, Table 1).

After blocking the blood flow of both common carotid arteries and the right cerebral artery for 90 min, all rats developed cerebral infarction after 24 h of reperfusion. The cerebral infarction areas were visibly white, and the non-infarction areas were redish-purple in color after TTC staining (Fig. 1A). The percentage of cerebral infarction area in the $G_{0.6}$ group, $G_{0.8}$ group, $DG_{0.6}$ group and MK group were 5.1 ± 0.9% ($p < 0.001$), 6.2 ± 0.5% ($p < 0.001$), 10.1 ± 1.5% ($p < 0.05$) and 3.7 ± 1.4% ($p < 0.001$), respectively. The percentages of infarction areas in the above groups were smaller than the percentage in the control group (12.7 ± 1.0%; Fig. 1B). The percentage of cerebral infarction area in the $G_{0.4}$ group was 11.3 ± 1.1% greater than that in the $G_{0.6}$, $G_{0.8}$ and MK groups (all $p < 0.001$). The percentage of cerebral infarction area was similar in the control group and $G_{0.4}$ group ($p > 0.05$). The percentage of cerebral infarction area was greater in the $DG_{0.6}$ group than that in the $G_{0.6}$ group ($p < 0.001$). The percentage of cerebral infarction area in the $G_{0.8}$ group was larger than that in the MK group ($p < 0.05$, Fig. 1B).

Table 1. Blood gas analysis in the Gui-Zhi-Fu-Ling-Wan-treated ischemia-reperfusion injured rats

	Before*			0-90*		
Group	pH	pCO_2	pO_2	pH	pCO_2	pO_2
S	7.30 ± 0.02	43.7 ± 2.3	99.7 ± 15.9	7.36 ± 0.06	35.4 ± 7.8	122.8 ± 22.7
C	7.31 ± 0.02	43.3 ± 5.1	106.5 ± 12.0	7.36 ± 0.07	35.0 ± 2.7	117.7 ± 10.8
$G_{0.4}$	7.32 ± 0.03	46.0 ± 5.1	101.3 ± 11.8	7.32 ± 0.03	45.7 ± 4.0	105.5 ± 7.5
$G_{0.6}$	7.31 ± 0.03	42.9 ± 4.8	98.2 ± 8.6	7.35 ± 0.04	34.0 ± 4.1	116.8 ± 13.6
$G_{0.8}$	7.32 ± 0.02	46.9 ± 6.1	103.7 ± 16.0	7.33 ± 0.03	41.6 ± 4.1	115.8 ± 17.7
$DG_{0.6}$	7.34 ± 0.04	40.9 ± 7.9	117.5 ± 19.1	7.39 ± 0.08	35.1 ± 9.2	124.3 ± 14.3
MK	7.32 ± 0.04	44.1 ± 4.4	111.2 ± 10.7	7.31 ± 0.02	43.0 ± 2.7	109.8 ± 11.9

*Mean ± SD. S = sham group; C = control group; $G_{0.4}$ = $G_{0.4}$ group; $G_{0.6}$ = $G_{0.6}$ group; $G_{0.8}$ = $G_{0.8}$ group; $DG_{0.6}$ = $DG_{0.6}$ group; MK = MK group. Before: blood gas analysis before blocking the blood flow; 0-90: blood gas analysis 90 min after blocking the blood flow.

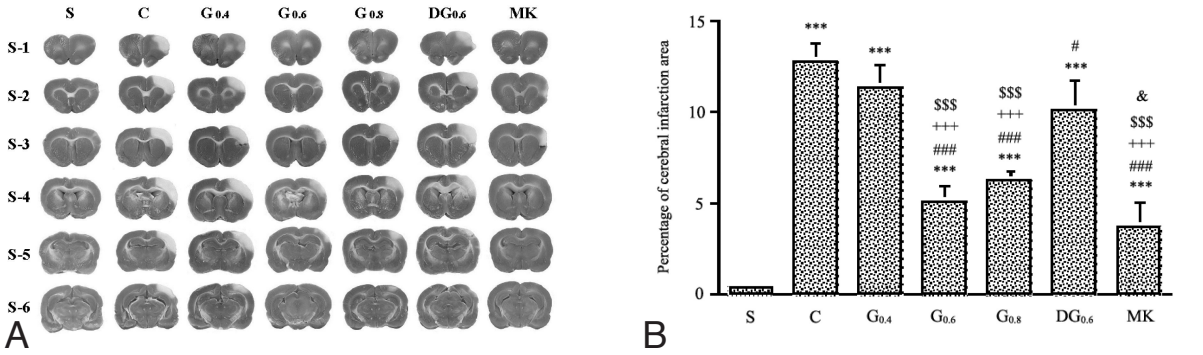

Fig. 1. Effects of GZFLW on cerebral infarction induced by ischemic-reperfusion injury. A: Coronal sections of brains after ischemia for 90 min followed by reperfusion for 24 h. After staining with 2,3,5-triphenyl-tetrazolium chloride, the infarction areas were white and the non-infarction areas were red-purple in color. In this and subsequent figures, S = sham group; C = control group; $G_{0.4}$ = $G_{0.4}$ group; $G_{0.6}$ = $G_{0.6}$ group; $G_{0.8}$ = $G_{0.8}$ group; $DG_{0.6}$ = $DG_{0.6}$ group; MK = MK group; S-1 = first slice from frontal lobe; S-2 = second slice from frontal lobe; S-3 = third slice from frontal lobe; S-4 = 4th slice from frontal lobe; S-5 = 5th slice from frontal lobe; S-6 = 6th slice from frontal lobe. B: The percentage of cerebral infarct area in ischemia-reperfusion injured rats. Pre-treatment with Gui-Zhi-Fu-Ling-Wan (GZFLW) 0.6 g/kg, 0.8 g/kg, MK801 1 mg/kg and post-treatment with GZFLW 0.6 g/kg reduced cerebral infarction area. *$p < 0.05$, ***$p < 0.001$ compared with of S; #$p < 0.05$, ###$p < 0.001$ compared with C; +++$p < 0.001$ compared with $G_{0.4}$; \$\$\$$p < 0.001$ compared with $DG_{0.6}$; &$P < 0.05$ compared with $G_{0.8}$.

Effects of GZFLW on neurological deficit in ischemia-reperfusion injured SD rats

The average neurological deficit score in the $G_{0.6}$ group was 1.3 ± 0.5 and that in the MK group was 1.2 ± 0.4. Both were lower than the average score in the control group (2.8 ± 0.4) (all $p < 0.001$). The neurological deficit score in the $G_{0.4}$ group (2.7 ± 0.5) was larger than that in the $G_{0.6}$ group ($p < 0.01$) and in the MK group ($p < 0.001$).

The neurological deficit score in the $DG_{0.6}$ group (2.5 ± 0.5) was larger than that in the $G_{0.6}$ group ($p < 0.05$, Fig. 2).

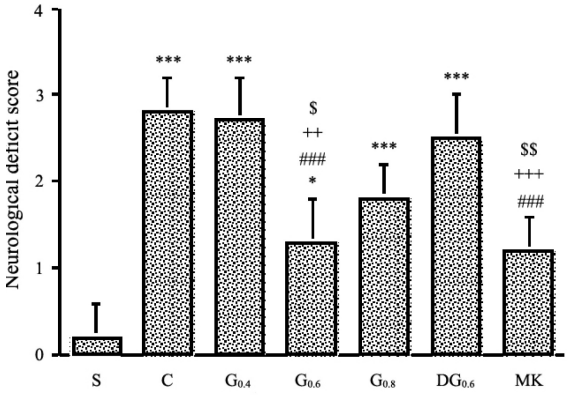

Fig. 2. Effects of GZFLW on neurological deficit induced by ischemia-reperfusion injury. Pre-treatment with GZFLW 0.6 g/kg and MK801 1 mg/kg significantly reduced neurological deficit. ***$p < 0.001$ compared with the value of S; ###$p < 0.001$ compared with the value of C; ++ $p < 0.01$, +++$p < 0.001$ compared with the value of $G_{0.4}$; \$ $p < 0.05$, \$\$$p < 0.01$ compared with the value of $DG_{0.6}$.

Effects of GZFLW on peripheral blood cells and blood biochemistry in ischemia-reperfusion injured SD rats

There were no significant differences in numbers of RBC, WBC, LYM and PLT, the concentration of HGB, or percentage of HCT between the sham, control, $G_{0.4}$, $G_{0.6}$, $G_{0.8}$, $DG_{0.6}$ and MK groups (all $p > 0.05$, Table 2).

There were no significant differences in levels of GOT, GPT, BUN, creatinine or blood sugar between the sham, control, $G_{0.4}$, $G_{0.6}$, $G_{0.8}$, $DG_{0.6}$ and MK groups (all $p > 0.05$; Table 3).

Effects of GZFLW on ED1-, IL-1β-, TNF-α-, ICAM1-, MPO-immunoreactive cells and apoptotic cells in ischemia-perfusion injured SD rats

SD rats were pre-treated with GZFLW 0.6 g/kg 10 min before ischemia-reperfusion procedures took place. The brain tissues were removed for immunostaining and the immunoreactive cells were counted within a 1 mm^2 zone (Fig. 3A). The number of ED1-immunoreactive cells in the control group was greater than that in the sham group ($p < 0.001$); however, the number of ED1-immunoreactive cells in the $G_{0.6}$ and MK groups was lower than that in the control group (both $p < 0.001$; Fig. 3B, Table 4).

Table 2. Effects of Gui-Zhi-Fu-Ling-Wan on peripheral blood cells in ischemia-reperfusion injured rats

Group	RBC* (106/μL)	HGB* (g/dL)	HCT* (%)	WBC* (103/μL)	LYM* (103/μL)	PLT* (103/μL)
S	7.4 ± 0.7	14.1 ± 1.6	42.4 ± 4.7	6.0 ± 1.3	2.6 ± 0.8	739.3 ± 92.8
C	7.5 ± 1.3	12.5 ± 2.5	40.7 ± 7.8	7.3 ± 0.9	2.7 ± 0.7	796.5 ± 187.9
$G_{0.4}$	7.6 ± 0.5	13.8 ± 1.0	44.2 ± 2.3	6.9 ± 2.5	3.0 ± 0.7	799.5 ± 166.2
$G_{0.6}$	7.7 ± 0.8	13.8 ± 1.9	44.2 ± 5.2	6.4 ± 1.6	2.3 ± 0.8	818.5 ± 181.4
$G_{0.8}$	7.1 ± 0.9	12.4 ± 1.4	37.9 ± 2.6	6.7 ± 2.2	3.5 ± 0.6	786.8 ± 122.4
$DG_{0.6}$	7.3 ± 0.9	12.5 ± 1.8	38.3 ± 6.3	7.2 ± 0.7	2.3 ± 0.9	885.2 ± 95.9
MK	7.2 ± 0.8	13.6 ± 1.0	41.7 ± 3.2	7.5 ± 1.0	2.2 ± 0.9	786.5 ± 81.6

*Mean ± SD. RBC = red blood cell; HGB = hemoglobin; HCT = hematocrit; WBC = white blood cell; LYM = lymphocyte; PLT = platelet.

Table 3. Effects of Gui-Zhi-Fu-Ling-Wan on biochemistry in ischemia-reperfusion injured rats

Group	GOT* (U/L)	GPT* (U/L)	BUN* (mg/dL)	Creatinine* (mg/dL)	Blood Sugar* (mg/dL)
S	359.7 ± 107.9	40.0 ± 18.6	19.4 ± 6.9	0.6 ± 0.08	163.0 ± 18.1
C	472.0 ± 124.4	66.0 ± 43.2	37.7 ± 20.9	0.7 ± 0.1	148.7 ± 24.9
$G_{0.4}$	353.8 ± 143.6	43.8 ± 20.1	43.8 ± 20.1	0.7 ± 0.1	158.0 ± 31.1
$G_{0.6}$	382.8 ± 120.3	51.5 ± 15.2	33.8 ± 9.8	0.7 ± 0.2	177.5 ± 31.6
$G_{0.8}$	446.5 ± 122.1	58.3 ± 17.5	23.2 ± 7.4	0.6 ± 0.1	141.5 ± 22.3
$DG_{0.6}$	384.2 ± 137.2	52.8 ± 14.6	33.0 ± 14.0	0.7 ± 0.1	138.8 ± 22.9
MK	352.0 ± 170.5	48.8 ± 16.2	33.6 ± 15.2	0.7 ± 0.2	152.2 ± 26.5

*Mean ± SD. GOT = aminotransferase asparate, GPT = aminotransferase alanine; BUN = blood urea nitrogen.

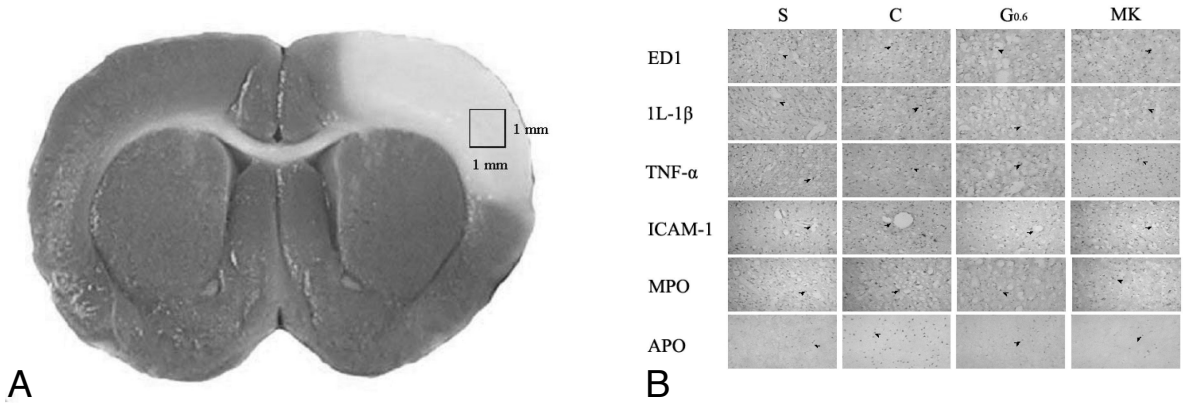

Fig. 3. Effects of GZFLW on ED1-, interleukin-1β-, tumor necrosis factor-α-, intercellular adhesion molecule-1-, myeloperoxidase-immunoreactive cells and apoptosis in ischemia-reperfusion injured rats. A: View of the third section from the frontal lobe. The ED1-, tumor necrosis-α-, interleukin-1β-, intercellular adhesion molecule-1-, myeloperoxidase- immunoreactive cell numbers within the square in the cerebral infarction zone were counted. Square box = 1 mm^2. B: ED1-, interleukin-1β-, tumor necrosis factor-α-, intercelular adhesion molecule-1-, myeloperoxidase-immunoreactive cells, and apoptosis cells (arrow) increased in the control group compared with the sham group. These increases were not seen in the $G_{0.6}$ and MK groups. ED1 = ED1 immunoreactive cell; IL-1β = interleukin-1β immunoreactive cell; TNF-α = tumor necrosis factor-αimmunoreactive cell; ICAM-1 = intercellular adhesion molecule-1 immunoreactive cell; MPO = myeloperoxidase immunoreactive cell; APO = apoptotic cell.

The number of IL-1β-immunoreactive cells in the control group was greater than that in the sham group ($p < 0.001$); however, the number of IL-1β-immunoreactive cells in the $G_{0.6}$ and MK groups was lower than that in the control group (both $p < 0.001$). The number of IL-1β immunoreactive cells was smaller in the MK group than in the $G_{0.6}$ group ($p < 0.05$; Fig. 3B, Table 4).

The number of TNF-α-immunoreactive

Table 4. Effects of Gui-Zhi-Fu-Ling-Wan on the number of ED1-, IL-1β-, TNF-α-, MPO- and ICAM-1-immunoreactive cells and apoptotic cells in ischemia-reperfusion injured rats

	S*	C*	G06*	MK*
ED1	24.7 ±9.2	384.5 ±35.6†††	103.3 ±28.1†††‡‡‡	86.7 ±30.1†††‡‡‡
IL-1β	24.2 ±11.6	474.5 ±28.0†††	135.2 ±37.3†††‡‡‡	77.7 ±24.3†††‡‡‡
TNF-α	19.8 ±8.1	357.7 ±46.0†††	103.7 ±29.2†††‡‡‡	76.7 ±28.8†††‡‡‡
ICAM-1	4.2 ±1.7	80.0 ±12.6†††	29.8 ±11.2†††‡‡‡	14.0 ±5.1‡‡‡
MPO	18.2 ±12.5	400.3 ±60.1†††	155.7 ±59.3†††‡‡‡	107.5 ±18.2†††‡‡‡
Apoptotic cells	99.7 ±19.6	807.8 ±76.1†††	331.2 ±38.0†††‡‡‡	158.8 ±41.1‡‡‡

*Mean ±SD. ED1= ED1-immunoreactive cell; IL-1β = interleukin-1β-immunoreactive cells; TNF-α = tumor necrosis factor-α-immunoreactive cells; ICAM-1= intercellular adhesion molecule-1-immunoreactive cells; MPO = myeloperoxidase-immunoreactive cells; Apoptosis= apoptotic cells. $^{\dagger\dagger\dagger}p < 0.001$ compared with sham group; $^{\ddagger\ddagger\ddagger}p < 0.001$ compared with C group.; $^{\S}p < 0.05$, $^{\S\S\S}p < 0.001$ compared with $PG_{0.6}$ group.

cells in the control group was greater than that in the sham group ($p < 0.001$); however, the number of TNF-α-immunoreactive cells in the $G_{0.6}$ and MK groups was lower than that in the control group (both $p < 0.001$; Fig. 3B, Table 4).

The number of ICAM-1-immunoreactive cells in the control group was greater than that in the sham group ($p < 0.001$); however, the number of ICAM-1-immunoreactive cells in the $G_{0.6}$ and MK groups was lower than that in the control group (both $p < 0.001$). The number of ICAM-1 immunoreactive cells in the $G_{0.6}$ group was lower than that in the MK group ($p < 0.05$; Fig. 3B, Table 4).

The number of MPO immunoreactive cells in the control group was greater than that in the sham group ($p < 0.001$); however, the number of MPO-immunoreactive cells in the $G_{0.6}$ and MK groups was lower than that in the control group (both $p < 0.001$; Fig. 3B, Table 4).

The number of apoptotic cells in the control group was greater than that in the sham group ($p < 0.001$); however, the number of apoptotic cells in the $G_{0.6}$ and MK groups was lower than that in the control group (both $p < 0.001$). The number of apoptotic cells in the $G_{0.6}$ group was lower than that in the MK group ($p < 0.01$; Fig. 3B, Table 4).

Effects of GZFLW on the MAP kinases pathways in cerebral ischemic SD rats

After cerebral ischemia for 90 min, the levels of phosphorylated JNKs in the cerebral cortex and hippocampus region increased in the control group (Fig. 4); however, this increase was reduced in $G_{0.6}$ group. Cerebral ischemia for 90 min and pre-treatment with GZFLW did not change the levels of JNKs, ERKs, p38 protein, phosphorylated ERKs or phosphorylated p38 (Fig. 4).

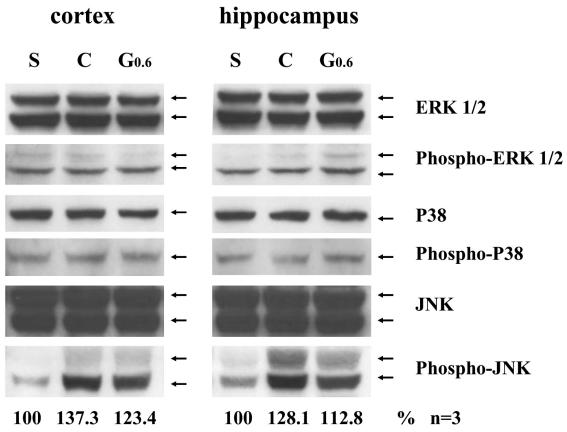

Fig. 4. Effects of GZFLW on MAP kinase activation in ischemic brain injury. After ischemia for 90 min, brain tissues in the cerebral cortex and hippocampus were analyzed for the protein levels and phosphorylation of JNK, p38 and ERK by Western blotting. The percentage on the bottom represents the amount of phospho-JNK protein relative to the S. S: sham group; C = control group; $G_{0.6}$ = pre-treatment with GZFLW 0.6 g/kg.

DISCUSSION

The results of this study demonstrated that cerebral infarction size was reduced in rats pre-treated with GZFLW and MK801 and post-treated with GZFLW; pre-treatment of GZFLW and MK 801 also reduced the neuro-deficit in ischemia-reperfusion injured rats. MK801 is a neuroprotective drug that has been shown to reduce cerebral infarction size induced by MCAo in rats [19] and in rabbits [20]. The consistency of

the neuro-deficit and infarction areas in our study suggests that there is an association between the symptoms and the size of brain tissue damage.

When the CNS encounters pathological changes, including inflammation and ischemia, microglias are rapidly activated and transform into phagocytes [21]. ED1 immunoreactivity is a marker of microglia/macrophage activity [22,23]. Microglia activation provides a sensitive indicator when there is no apparent neuronal damage during the early stage of cerebral ischemia [24]. In addition, microglias participate in neurotoxicity via nitric oxide and reactive nitrogen oxides [25,26] and they secrete cytokines including TNF-α and IL-1β. The expression of TNF-α increases in the ischemic cortex, which facilitates neurtophil infiltration, thereby exacerbating brain tissue damage in MCAo rats [27]. The increase in cerebral infarction size by TNF-α is dose-dependent in spontaneous hypertensive MCAo rats. Therefore, the inhibition of TNF-α may be a strategy for treating ischemic stroke [28]. In addition, the level of TNF-α in blood is an indicator of early neurological deterioration in lacunar stroke patients [29]. IL-1β also facilitates the infiltration of neurophils into the cerebral infarction region, and is an important factor in the formation of brain edema [5]. IL-1β, produced by endothelial cells and microglias, regulates the expression of adhesion molecules after cerebral ischemia in mice [30,31]. Adhesion molecule ICAM-1 plays a role in mediating leucocyte-endothelial adhesion to facilitate leucocyte infiltration into the ischemic brain region [8,32]. ICAM-1 mRNA and protein expressed in endothelial cells [8] have been shown to increase during the second hour of reperfusion, and to persist for one week in MCAo rats. ICAM-1 has been shown to be an inflammatory marker [32,33]; ischemic brain cell death is associated with inflammation [33]. A quantitative index of PMN infiltration into the damaged brain tissues in MCAo rats is MPO [9,34]. PMN increases in cerebral infarction and is associated with inflammation, which contributes to delayed progressive brain tissue damage [9]. Anti-beta-z integrin inhibits neutrophils and, therefore, acts as a neurprotector in MCAo-induced cerebral infarct [35]. In addition, reactive oxygen species [36], especially superoxide [37], are produced by neurtrophils in cerebral ischemia-reperfusion injured rats. Several reports have found that oxygen free radicals play a critical role in triggering ischemia neuronal damage [38]. Neutrophils prompt the release of oxygen free radicals, proinflammatory mediators and cytokines, including TNF-α in myocardial ischemia-reperfusion injury [10,11]. After severe cerebral ischemia, apoptotic cells are present, and contribute to the delayed death of nonneuronal brain cells [6]. Apoptosis occurs after cerebral ischemia both in spontaneous hypertensive and non-hypertensive rats; therefore, targeting apoptotic cells might represent a therapeutic strategy for stroke [7,39]. In our study, we demonstrated that GZFLW reduced the cerebral infarction size, neuro-deficit, the ED1-, IL-1β-, TNF-α-, ICAM-1-, MPO-immunoreactive cells and apoptotic cells in the cerebral infarction region, suggesting that GZFLW inhibits microglia activation and neutrophil infiltration in the infarction region.

In our study, the phosphorylation of JNK increased in the cerebral cortex and hippocampus region 90 min after blocking the blood flow of both common carotid arteries and the right middle cerebral artery; however, this increase was reduced by pre-treating rats with GZFLW. Several studies have reported that the dynamic balance between the activation of ERK and the activation of the JNK-p38 pathway plays a critical role in determining whether a cell will survive or undergo apoptosis. JNK is a stress-activated protein and the sustained over-stimulation of JNK signaling pathway has been shown to induce cell death in PC-12 cells [14]. Ischemia induces the production of cellular inflammatory cytokines, such as TNF-α and IL-1β, and induces apoptosis [14]. In addition, Yamasaki et al reported that anti-IL-1β decreased infarction size and neutrophil infiltration in MCAo rats [5]. Inhibitors targeting the JNK pathway also exhibit

anti-inflammatory activity [40]. Therefore, our results suggest that GZFLW exhibits anti-inflammatory activity by inhibiting the JNK signaling pathway in cerebral ischemia.

In our study, GZFLW did not change the numbers of RBC, WBC, LYM or PLT, the concentration of HGB, the percentage of HCT, GOT, GPT, BUN, creatinine or the level of blood sugar in peripheral blood flow of ischemia-reperfusion injured rats, suggesting that short-term administration of GZFLW does not influence blood cells, blood sugar, liver function or kidney function.

In conclusion, GZFLW reduced the cerebral infarction area and neuro-deficit in ischemia-reperfusion injured rats, suggesting that it has the potential to induce similar effects in humans. GZFLW simultaneously inhibited microglia activation, TNF-α, IL-1β, ICAM-1, and neutrophil infiltration, suggesting that the reduction of infarction areas, at least in part, is associated with its anti-inflammatory activity. Finally, our data indicate that the anti-inflammatory effects of GZFLW might be associated with the JNK signaling pathway in cerebral ischemia.

ACKNOWLEDGMENT

This study was supported by grant NSC 93-2320-B-039-036 from the National Science Council, Taiwan, ROC.

REFERENCES

1. Kohta K, Hikiami H, Shimada Y, et al. Effects of keishi-bukuryo-gan on erythrocyte aggregability in patients with multiple old lacunar infarction. *Journal of Medical and Pharmaceutical Society for WAKAN-YAKU* 1993;10:251-9.
2. Hikiami H, Goto H, Sekiya N, et al. Comparative efficacy of keishi-bukuryo-gan and pentoxifylline on RBC deformability in patients with “oketsu” syndrome. *Phytomedicine* 2003;10:459-66.
3. Morioka T, Kalehua AN, Streit WJ. Characterization of microglial reaction after middle cerebral artery occlusion in rat brain. *J Comp Neurol*. 1993;327: 123-32.
4. Liu T, McDonnell PC, Young PR, et al. Interleukin-1£] mRNA expression in ischemic rat cortex. *Stroke* 1993; 24:1746-51.
5. Yamasaki Y, Matsuura N, Shozuhara H, et al. Intereukin-1 as a pathogenetic mediator of ischemic brain damage in rats. *Stroke* 1995;26:676-81.
6. Du C, Hu R, Csernansky CA, et al. Very delayed infarction after mild focal cerebral ischemia: a role for apoptosis? *J. Cereb. Blood Flow Metab*. 1996;16:195-201.
7. Pulera MR, Adams LM, Liu H, et al. Apoptosis in a neonatal rat model of cerebral hypoxia-ischemia. *Stroke* 1998;29:2622-30.
8. Zhang RL, Chopp M, Zaloga C, et al. The temporal profiles of ICAM-1 protein and mRNA expression after transient MCA occlusion in the rat. Brain Res. 1995;682:182-8.
9. Barone FC, Hillegass LM, Price WJ, et al. Polymorphonuclear leukocyte infiltration into cerebral focal ischemic tissue: myeloperoxidase activity assay and histologic verification. *J Neurosci Res*. 1991; 29:336-45.
10. Hansen PR. Role of neutrophils in myocardial ischemia and reperfusion. *Circulation* 1995;91:1872-85.
11. Jordan JE, Zhao ZQ, Vinten-Johansen J. The role of neutrophils in myocardial ischemia-reperfusion injury. *Cardiovasc. Res*. 1999;43:860-78.
12. Kim IJ, Lee KW, Park BY, et al. Molecular cloning of multiple splicing variants of JIP-1 preferentially expressed in brain. *J Neurochem*. 1999;72:1335-43.
13. Seger R, Krebs EG. The MAPK signaling cascade. *FASEB* 1995;9:726-35.
14. Barone FC, Feuerstein GZ. Inflammatory mediators and stroke: new opportunities for novel therapeutics. *J Cereb Blood Flow Metab*. 1999;19:819-34.
15. Hsieh CL, Cheng CY, Tsai TH, et al. Paeonol reduced cerebral infarction involving the superoxide anion and micoglia activation ischemia-reperfusion injured rats. *J. Ethnopharmacol* 2006;106:208-15.
16. Bederson JB, Pitts LH, Tsuji M, et al. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. *Stroke* 1986;17:472-6.
17. Yang Y, Shuaib A, Li Q. Quantification of infarct size on focal cerebral ischemia model of rats using a simple and economical method. *J. Neurosci. Methods* 1998;84:9-16.

18. Hsieh CL, Lin JJ, Chiang SY, et al. Gastrodia elata modulated activator protein 1 via-Jun N-terminal kinase signaling pathway in kainic acidinduced epilepsy in rats. *J Ethnopharmacol* 2007; 109:241-7.
19. Relton JK, Martin D, Thompson RC, et al. Peripheral administration of interleukin-1 receptor antagonist inhibits brain damage after focal cerebral ischemia in the rat. *Exp. Neurol*. 1996; 138:206-13.
20. Pan Y, Lo EH, Matsumotor K, et al. Quantitative and dynamic MRI of neuroproction in experimental stroke. *J Neurosurg Sci* 1995;131:128-34.
21. Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. *Trends Neurosc* 1996;19:312-8.
22. Lao CJ, Lin JG, Kuo JS, et al. Microglia, apoptosis and interleukin-1β expression in the effect of Sophora Japonica L. on cerebral infarct induced by ischemia-reperfusion in rats. *Am. J. Chin. Med*. 2005;33:425-38.
23. Wang GJ, Deng HY, Maier CM, et al. Mild hypothermia reduces ICAM-1 expression, neutrophil infiltration and microglia/monocyte accumulation following experimental stroke. *Neuroscience* 2002; 114:1081-90.
24. Morioka T, Kalehua AN, Streit WJ. The microglial reaction in the rat dorsal hippocampus following transient forebrain ischemia. *J Cereb Blood Flow Metab.* 1991;11:966-73.
25. Boje KM, Arora PK.. Microglial-produced nitric oxide and reactive nitrogen oxides mediate neuronal cell death. *Brain Res*. 1992;587:250-6.
26. Chao CC, Hu S, Molitor TW, et al. Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. *J Immunol*. 1992;149:2736-41.
27. Liu T, Clark RK, McDonnell PC, et al. Tumor necrosis factor-α expression in ischemic neurons. *Stroke* 1994; 25:1481-8.
28. Barone FC, Arvin B, White RF, et al. Tumor necrosis factor-α a mediator of focal ischemic brain injury. *Stroke* 1997;28:1233-44.
29. Castellanos M, Castillo J, García MM, et al. Inflammation-mediated damage in progressing lacunar infarctions. *Stroke* 2002;33:982-7.
30. Sairanen TR, Lindsberg PJ, Brenner M, et al. Global forebrain ischemia results in differential cellular expression of interleukin-1β (IL-1β) and its receptor at mRNA and protein level. J. Cereb. *Blood Flow Metab*. 1997;17:1107-20.
31. Zhang Z, Chopp M, Goussev A, et al. Cerebral vessels express interleukin 1£] after focal cerebral ischemia. *Brain Res*. 1998;784:210-7.
32. Deng H, Han HS, Cheng D, et al. Mild hypothermia inhibits inflammation after experimental stroke and brain inflammation. *Stroke* 2003;34:2495-501.
33. Koh SH, Park Y, Song CW, et al. The effect of PARP inhibitor on ischaemic cell death, its related inflammation and survival signals. *Eur J Neurosci.* 2004;20:1461-72.
34. Beray-Berthat V, Croci N, MPlotkine M, et al. Polymorphonuclear neutrophils contribute to infarction and oxidative stress in the cortex but not in the striatum after ischema-reperfusion in rats. *Brain Res.* 2003;987:32-8.
35. Miljkovic-Lolic M, Silbergleit R, Fiskum G, et al. Neuroprotective effects of hyperbaric oxygen treatment in experimental focal cerebral ischemia are associated with reduced brain leukocyte myeloperoxidase activity. *Brain Res*. 2003;971:90-4.
36. Takahashi R, Edashige K, Sato EF, et al. Luminol chemiluminescence and active oxygen generation by activated neutrophils. *Arch Biochem Biophysics* 1991;285:325-30.
37. Fabian RH, Kent TA. Superoxide anion production during reperfusion is reduced by an antineutrophil antibody after prolonged cerebral ischemia. *Free Radic Biol Med.* 1999;26:355-61.
38. Kitagawa K, Matsumoto M, Oda T, et al. Free radical generation during brief period of cerebral ischemia may trigger delayed neuronal death. *Neuroscience* 1990;35:551-8.
39. Linnik MD, Zobrist RH, Hatfield MD. Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. *Stroke* 1993;24:2002-9.
40. Kaminska B. MAPK signalling pathways as molecular targets for anti-inflammatory therapy-from molecular mechanisms to therapeutic benefits. *Biochim Biophys Acta* 2005;1754:253-62.

桂枝茯苓丸經由抗發炎作用減少缺血—再灌流損傷大鼠的腦梗塞

謝慶良[1,2] 侯庭鏞[3] 蘇珊玉[1,4] 程錦宜[3] 徐維聰[1] 謝慶竇[5] 蒲曉韻[2] 唐娜櫻[4]

中國醫藥大學附設醫院 中醫部[1] 中國醫藥大學 中西醫結合研究所[2]

中國醫學研究所[3] 中醫學系[4] 財團法人仁愛綜合醫院 內科部[5]

背景/目的 血瘀是傳統中醫的病理概念，許多研究已顯示血瘀伴隨著中風的發展，而桂枝茯苓丸能改善嚴重血瘀狀態，因此本研究的目的是觀察桂枝茯苓丸對腦梗塞的效用。

方法 將Sprague-Dawley大鼠兩側總頸動脈和右側中大腦動脈的血流阻斷90分鐘，隨後再灌流24小時建立一個腦梗塞的大鼠動物模型。以大鼠的神經缺損程度和腦梗塞面積與該片腦切片總腦面積的百分率做爲桂枝茯苓丸和MK801的療效指標。同時也計算梗塞區域的ED1(mouse anti rat CD68)、interleukin-1β (IL-1β)、tumor necrosis factor-α (TNF-α)、intercellular adhesion molecule-1 (ICAM-1)、myeloperoxidase(MPO)免疫反應細胞和凋亡細胞(apoptotic cell)。另外，也利用西方點墨(western blotting)法偵察mitogen activated protein(MAP)kinase的磷酸化(phosphorylation)。

結果 桂枝茯苓丸0.6 g/kg、0.8 g/kg、MK801(非競爭性N-methyl-D-asparate受體拮抗劑)1.0 mg/kg前治療，以及桂枝茯苓丸0.6 g/kg後治療減少腦梗塞面積；桂枝茯苓丸0.6 g/kg和MK801 1.0 mg/kg前治療減少神經神經缺損程度，以及減少ED1，Il-1β、TNF-α、ICAM-1、MPO免疫反應細胞和凋亡細胞的數目。桂枝茯苓丸0.6 g/kg減少腦缺血所誘發的c-jun N-terminal kinase(JNK)的磷酸化路徑。

結論 桂枝茯苓丸減少大鼠的腦梗塞面積和神經缺損，推測桂枝茯苓丸可能減少人類的腦梗塞。桂枝茯苓丸也能抑制microglia的活化、TNF-α、IL-1β、ICAM-1的表現，以及嗜中性白血球的浸潤(neurotrophil infiltration)，推測桂枝茯苓丸有抗發炎作用。進而，我們的資料顯示桂枝茯苓丸的抗發炎作用是經由腦缺血的JNK信息傳導路徑。（**中台灣醫誌2008;13:1-11**）

關鍵詞

腦梗塞、c-Jun N-terminal kinase、ED1、桂枝茯苓丸、intercellular adhesion molecule-1、神經缺損

聯絡作者：唐娜櫻

地 址：404台中市北區學士路91號

中國醫藥大學 中醫學系

收文日期：2007年7月2日 修改日期：2007年9月7日

接受日期：2007年9月13日