The American Journal of Chinese Medicine, Vol. 38, No. 1, 51–64

Institute for Advanced Research in Asian Science and Medicine

The Anti-Inflammatory Effect of Paeoniflorin on Cerebral Infarction Induced by Ischemia-Reperfusion Injury in Sprague-Dawley Rats

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Abstract: *Paeoniflorin*, a component in *Paeonia lactiflora* Pall, inhibits nuclear factor-κB expression in chronic hypoperfusion rat and has anti-inflammatory properties. Therefore, the aim of the present study was to investigate the effect of paeoniflorin on cerebral infarct, and the involvement of anti-inflammation. We established an animal model of cerebral infarct by occluding both the common carotid arteries and the right middle cerebral artery for 90 min, followed by reperfusion of 24 hours. The ratios of cerebral infarction area to total brain area, and neuro-deficit score were used as an index to observe the effects of *paeoniflorin* on cerebral infarct. ED1 (mouse anti rat CD68), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), intercellular adhesion molecular-1 (ICAM-1), myeloperoxidase (MPO) immunostaining and apoptotic cells in the cerebral infarction region also were studied. The results indicated that both pre-treatment and post-treatment with *paeoniflorin* reduced the ratio of cerebral infarction area; pre-treatment with *paeoniflorin* also reduced the neurological deficit score. The counts of ED1, IL-1β, TNF-α, ICAM-1 of microvessels and MPO immunoreactive cells and apoptotic cells were increased in the cerebral infarction region; however, these increases were reduced by *Paeoniflorin* pre-treatment.

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In conclusion, *Paeoniflorin* reduced cerebral infarct and neurological deficit in ischemia-reperfusion injured rats, suggesting that *paeoniflorin may have a* similar effect in humans and might be a suitable treatment for stroke. *Paeoniflorin* reduced cerebral infarct, at least in part, involves the anti-inflammatory properties.

Keywords: *Paeoniflorin*; Cerebral Infarct; Neurological Deficit; Anti-Inflammation.

Introduction

Stroke is the third leading cause of death and long-term disability in Taiwan; therefore, the study of effective treatments of stroke is an important task. Recombinant tissue plasminogen activator (t-PA) given within 3 hours after onset of stroke symptoms is the only approved stroke treatment at present. However, t-PA is known to increase the risk of cerebral hemorrhage (Marler *et al.*, 1995; Hacke *et al.*, 1998). *Paeoniflorin* is a component in *paeonia lactiflora* Pall and has been reported to reduce cerebral infarction volume and improve learning and memory impairment in rat with chronic stage middle cerebral artery occlusion (MCAo) (Xiao *et al.*, 2005).

Microglia activation may indicate neuronal damage and the severity of neuronal injury in rats with MCAo (Morioka *et al.*, 1993). Interleukin-1β (IL-1β) plays an important role in the reperfusion period of ischemic brain damage (Yamasaki *et al.*, 1995); furthermore, it has been shown that tumor necrosis factor-α (TNF-α) concentration in plasma is higher in lacunar stroke patients who exhibit early neurological deterioration, indicating that TNF-α may contribute to brain damage (Castellanos *et al.*, 2002). Apoptosis may represent a model of ischemia cell death in cerebral hypoxic ischemia (Pulera *et al.*, 1998). Intercellular adhesion molecule-1 (ICAM-1) expression was increased and mediating leukocyte-endothelial adhesion to facilitate leucocytes migration into brain damage region (Zhang *et al.*, 1995), whereas myeloperoxidase (MPO) activity is a quantitative index of polymorphonuclear neurtrophils (PMN) which may contribute to the delay of progressive brain damage in stroke patients (Barone *et al.*, 1991). Therefore, the purpose of the present study was to investigate the effect of *paeoniflorin* on cerebral infarct and its anti-inflammatory action. We established an animal model of cerebral infarct by blocking the blood flow in both common carotid arteries and right middle cerebral artery of Sprague-Dawley (SD) rats for 90 min followed by reperfusion for 24 hours (Hsieh *et al.*, 2006; Cheng *et al.*, 2008). We used 2% 2, 3, 5-triphenyl-tetrazolium chloride (TTC) staining to differentiate between the white cerebral infarction area and the reddish-purple normal brain tissue (Yang *et al.*, 1998). The ED1 (mouse anti rat CD68), proinflammatory cytokines IL-1β and TNF-α, and ICAM-1 and MPO immunostaining and apoptotic cells were also studied.

Methods and Materials

The Preparation of Paeoniflorin

Paeoniflorin (30 mg) (Nacalai Tesgue, Kyoto, Japan) was dissolved in glycofurol solution (500 μl; Sigma, USA) to generate a 0.18 M solution. The solution was then added to 29.5 ml of phosphated buffer saline, thus, the final concentration of *paeoniflorin* was 1 mg/ml.

Animals

Male SD rats, weighing 300–350 g, were purchased 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 levels were between 55 ± 5%, and the rats were maintained on a 12 hours light-dark cycle at 22 ± 2°C. All animal experiments were performed in accordance with the Guidelines of the Chinese Society for the Laboratory Animals Science, Taiwan.

Animal Model Establishment of Cerebral Infarction

The SD rats were anesthetized with an intraperitoneal injection (i.p.) of chloral hydrate (400 mg/kg). The hair on the right inguinal region was shaved and the skin was incised followed by exposure of the right femoral artery and vein. A PE-50 tube was placed into the artery and the other end of the PE- tube was connected to a heart rate-blood pressure monitoring apparatus (LE 5001 pressure meter, Panlab. S. L. L., Barcelona, Spain). Another PE-50 tube was placed into the vein for the administration of *paeoniflorin*. First, both common carotid arteries of the rat were exposed through a middle neck incision in a supine position; the arteries were then wrapped by a loop of plastic line (0.1 mm in diameter) and a PE-50 tube (0.2 mm in diameter), respectively. Second, the head of the rat was placed in a stereotactic apparatus in the prone position; the scalp was incised from the midpoint of the binaural line to expose the skull. A bone widow (3.5 mm in diameter) was created, through which the olfactory tract and the right middle cerebral artery were clearly visible. An 8-0 nylon line was placed under the right middle cerebral artery just at the immediate upper margin of the olfactory tract, and tied loosely by a surgical needle. The cerebral blood perfusion of the rat brain was monitored by a laser Doppler perfusion monitor (DRT4, Moor Instruments Inc. Wilmington, USA). The marker from 900 changed to 200 when the blood flow of both common carotid arteries was blocked. The monitor marker decreased from 200 to 50 following the occlusion of blood flow through the right middle cerebral artery. The blood flow was re-established 90 min after cerebral ischemia, i.e. reperfusion. The blood pressure and heart rate of the rat were monitored throughout the experimental procedure. The rectal temperature was maintained at 37 ± 0.5°C by using a heated pad.

A total of 42 SD rats were divided into 7 groups of 6 rats as follows: (A) Sham group, the bilateral common carotid arteries and the right middle cerebral artery were exposed, but blood flow was not blocked; (B) Control group, the blood flow of bilateral common carotid arteries and the right middle cerebral artery was blocked for 90 min followed by reperfusion for 24 hours; (C) P-10 group, the methods of blood flow occlusion were identical to that in the control group, except that *paeoniflorin* 10 mg/kg intravenously (i.v.) was administered 10 min prior to blocking the blood flow; (D) P-15 group, the methods were identical to those in the P-10 group, except that *paeoniflorin* 15 mg/kg (i.v.) was administered; (E) P-20 group, the methods were identical to those in the P-10 group, except that *paeoniflorin* 20 mg/kg; (F) D-20 group, the methods were identical to those in the control group, except that *Paeoniflorin* 20 mg/kg (i.v.) was administered 30 min after blocking the blood flow; (G)

PBS group, the methods were identical to those in the P-10 group, except that PBS 1 ml/kg (i.v.) was administered. The neurological status of each rat was evaluated blindly after reperfusion for 24 hours; the rats were then anesthetized with chlorate hydrate (400 mg/kg, i.p.). Whole blood (3 ml) was obtained from the heart chamber to measure counts of erythrocytes, leucocytes, platelets and lymphocytes, and to measure the amount of hematocrit and hemoglobin. Finally, the rat was sacrificed and the brain was removed for the measurement of cerebral infarction area.

The other 18 SD rats were randomly divided into 3 groups of 6 rats each as follows: (A) Sham group, both common carotid arteries and the right middle cerebral artery were exposed, but blood flow was not blocked; (B) Control group, the blood flow of both common carotid arteries and the right middle cerebral artery was blocked for 90 min followed by reperfusion for 24 hours; (C) P-20 group, the methods of blood flow blocking occlusion were identical to those in the control group, except that *paeoniflorin* 20 mg/kg was administered intravenously 10 min prior to blocking the blood flow. The rat was anesthetized with chloral hydrate (400 mg/kg, i.p.) followed by perfusion from the left intra-cardiac ventricle with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain was removed and post fixed in 4% paraformaldehyde for 3 days, and then cryoprotected in 30% w/v sucrose in PBS for 1–2 days. Finally, the brain was embedded in optimal cutting temperature (OCT) medium and cut into 20 μm sections for immunostaining. The number of ED1, IL-1β, TNF-α, ICAM-1 and MPO immunoreactive cells and apoptotic cells in the cerebral infarction zone of the third section from the frontal lobe was calculated under a light microscope. The data are represented as number/1 mm^2 (Fig. 1).

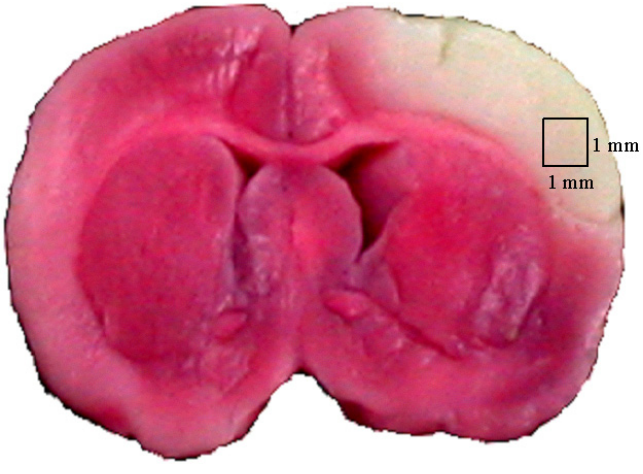

Figure 1. View of the third total brain coronal section from the frontal lobe. The square line within the cerebral infarction region indicates the area for calculating ED1 (mouse anti rat CD68), interleukin-1β, tumor necrosis factor-α, intercellular adhesion molecule-1, and myeloperoxidase immunoreactive cells and apoptotic cells. The length and width of the square line 1 mm. $n = 6$ in each group.

Measurement of Cerebral Infarction Size

The rat brain was placed into a plastic model of a rat brain, 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. Tissue which stained white indicated cerebral infarction area and the reddish-purple indicated normal brain tissue. 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 slice was calculated, and the data were expressed as a percentage (%).

Evaluation of Neurological Status

The neurological status of the rat was evaluated by an evaluator, who was blinded to the treatment groups, according to the neurological examination grading scale system (Bederson *et al.*, 1986) as follows: Grade 0, no neurological deficit; Grade 1, left forelimb flexion; Grade 2, decreased resistance to a lateral push; Grade 3, circling behavior.

Measurement of Peripheral Blood Cells

White blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT) and lymphocyte (LYM) were measured by a blood cell analysis apparatus (System KX-21, System Ltd., Kobe, Japan).

Immunostaining

The primary antibodies used in this study were mouse antibodies to ED1 (1: 500; Serotec Ltd.), IL-1β (1: 400; Endogen Ltd.), TNF-α (1:200; Bender MedSystems, Austria), ICAM-1 (1:100 dilution, Santa Cruz, USA) and MPO (1:500 dilution, Cell Science Inc.). Brain sections were washed briefly with PBS followed by incubation with 3% H_2O_2 in methanol for 15 min. After washing with PBS, tissues were incubated with 10% normal animal serum (LsAB kit, Zymed, San Francisco, CA, USA) for 20 min. Primary antibodies were applied to tissues for 30 min in a humid chamber, and then incubated with biotinylated secondary antibodies for 10 min. Tissues were subsequently incubated with labeled-(strept) avidin-biotin (Ls-AB)-peroxidase complex for 10 min followed by diaminobenzidine tetrahydrochloride (DAB) solution (Liquid DAB substrate kit, Zymed, San Francisco, CA, USA) for 2–10 min, and counterstained with hematoxylin.

In situ Analysis of Apoptosis

A DNA fragmentation detection kit purchased from Oncogene (TdT-FragEL^{TM} kit; Oncogene, Boston, MA, USA). 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. Terminal deoxynucleotidyl transferase (TdT) was then added to the tissues at 37°C for 1.5 hours, followed by the addition of the stop buffer solution was added to terminate the reaction. Apoptotic cells were detected by incubating tissues with DAB, followed by counterstaining with methyl green.

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

Effect of Paeoniflorin on Cerebral Infarct Induced by Ischemia-Reperfusion Injured Rats

The ratio of cerebral infarction area in the control group (12.1 ± 1.9%; Figs. 2 and 3) was greater than that in the P-10 group (8.1 ± 0.8%; $p < 0.05$; Figs. 2 and 3), P-15 group (3.9 ± 1.6%; $p < 0.001$; Figs. 2 and 3), P-20 group (2.7 ± 1.7%; $p < 0.001$; Figs. 2 and 3) and D-20 group (7.9 ± 1.4%; $p < 0.01$; Figs. 2 and 3). Similarly, the ratio of cerebral infarction area in the PBS group (12.6 ± 2.5%; Figs. 2 and 3) was greater than that in the

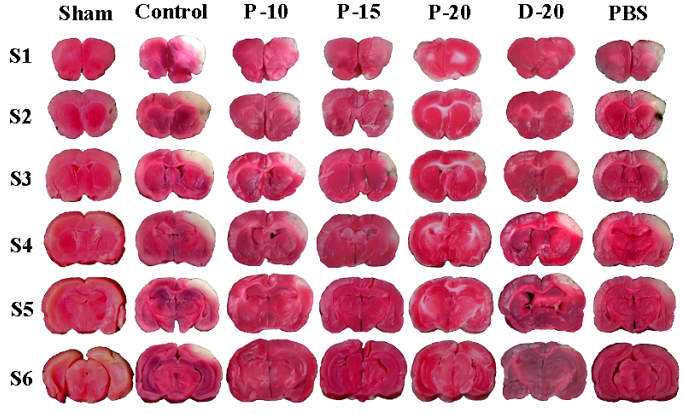

Figure 2. Focal cerebral infarction development after blocking both common carotid arteries and right middle cerebral artery for 90 min followed by reperfusion for 24 hours. After staining with 2, 3, 5-triphenyl-tetrazolium chloride, the infarction areas were white and the non-infarction areas were red-purple in color. In this and subsequent figures, the graphs are defined as follows: Sham: sham group; Control: control group, no *paeoniflorin* treatment; P-10: P-10 group, pre-treatment with *paeoniflorin* 10 mg/kg; P-15: P-15 group, pre-treatment with *paeoniflorin* 15 mg/kg; P-20: P-20 group, pre-treatment with *paeoniflorin* 20 mg/kg; D-20: D-20 group, post-treatment with *paeoniflorin* 20 mg/kg; PBS: PBS group, pre-treatment with phosphate buffer saline 1 ml/kg; S1: first slice from frontal lobe; S2: second slice from frontal lobe; S3: third slice from frontal lobe; S4: fourth slice from frontal lobe; S5: fifth slice from frontal lobe; S6: sixth slice from frontal lobe. $n = 6$ in each group.

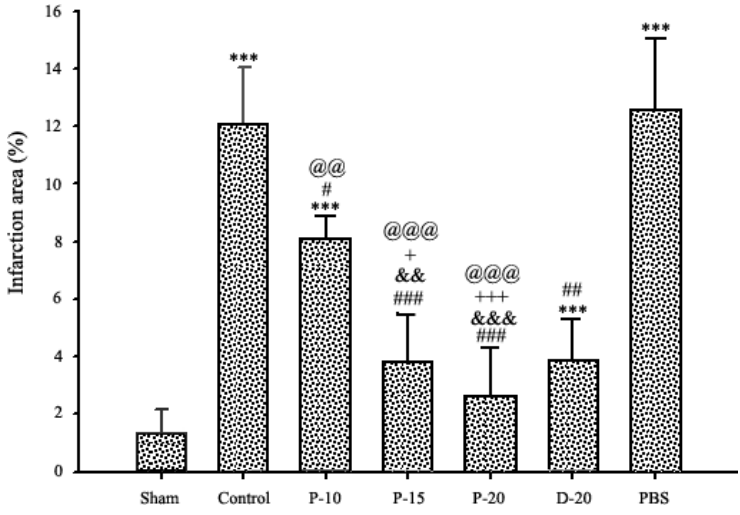

Figure 3. Effect of *paeoniflorin* on cerebral infarct in ischemia-reperfusion injured rats. Pre-treatment with *paeoniflorin* 10, 15 and 20 mg/kg, and post-treatment with *paeoniflorin* 20 mg/kg reduced cerebral infarction area. $^{***}p < 0.001$ compared to the sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to the control group. $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ compared to P-10 group; $^{+}p < 0.05$, $^{+++}p < 0.001$ compared to D-20 group; $^{@@}p < 0.01$, $^{@@@}p < 0.001$ compared to the PBS group. $n = 6$ in each group.

P-10 group ($p < 0.01$; Figs. 2 and 3), P-15 group ($p < 0.001$; Figs. 2 and 3) and P-20 group ($p < 0.001$; Figs. 2 and 3). The ratio of cerebral infarction area was similar between the control and PBS groups ($p > 0.05$; Figs. 2 and 3). The ratio of cerebral infarction area in the sham group ($1.4 \pm 0.9\%$; Figs. 2 and 3) was lower than that in the control group ($p < 0.001$; Fig. 2 and 3), P-10 group ($p < 0.001$; Fig. 2 and 3), D-20 group ($p < 0.001$; Figs. 2 and 3) and PBS group ($p < 0.001$; Figs. 2 and 3). The ratio of cerebral infarction area was similar among the sham, P-15 and P-20 groups (all $p > 0.05$; Figs. 2 and 3). The ratio of cerebral infarction area was greater in the P-10 group than in the P-15 group ($p < 0.01$; Figs. 2 and 3) and P-20 group ($p < 0.001$; Figs. 2 and 3). The ratio of cerebral infarction area was greater in the D-20 group than in the P-15 group ($p < 0.05$; Figs. 2 and 3) and P-20 group ($p < 0.001$; Figs. 2 and 3).

Effect of Paeoniflorin on Neurological Deficit in Ischemia-Reperfusion Injured Rats

The neurological deficit score in the sham group (0.7 ± 0.5; Fig. 4) was lower than that in the control group (2.2 ± 0.4; $p < 0.001$; Fig. 4), the P-10 group (2.3 ± 0.5; $p < 0.001$; Fig. 4), the D-20 (2.2 ± 0.4; $p < 0.001$; Fig. 4) and the PBS group (2.2 ± 0.4; $p < 0.001$; Fig. 4). The neurological deficit score in the sham group was similar to that in the P-15 (1.2 ± 0.4; $p > 0.05$; Fig. 4) and P-20 groups (0.8 ± 0.4; $p > 0.05$; Fig. 4). The neurological deficit scores in the P-15 and P-20 groups were lower than that in the control group (both $p < 0.05$; Fig. 4). The neurological deficit score in the P-10 group was greater than that in the P-15 group ($p < 0.001$; Fig. 4) and P-20 group ($p < 0.01$; Fig. 4). The neurological

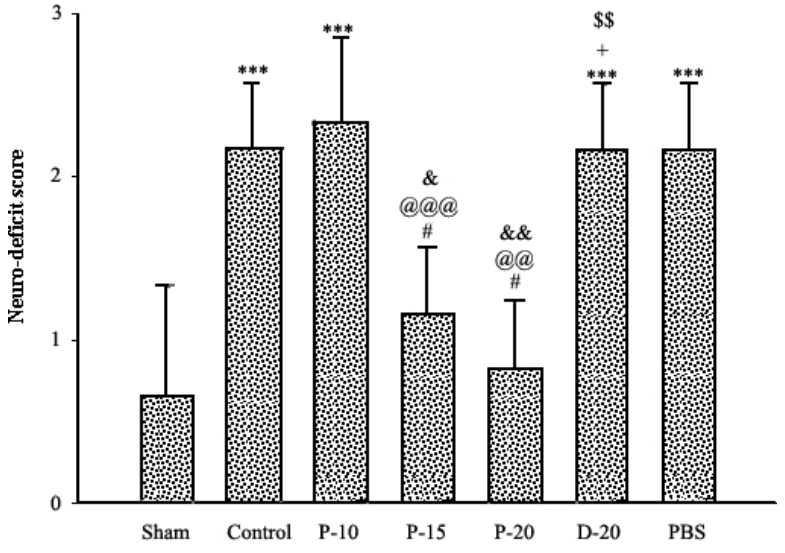

Figure 4. Effect of *paeoniflorin* on neurological deficit induced by ischemia-reperfusion. Pre-treatment with *paeoniflorin* 15 and 20 mg/kg significantly reduced neurological deficit. $^{***}p < 0.001$ compared to the sham; $^{\#}p < 0.05$ compared to the control; $^{@@}p < 0.01$, $^{@@@}p < 0.001$ compared to P-10 group; $^{+}p < 0.01$ compared to P-15; $^{\$\$}p < 0.01$ compared to P-20 group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ compared to the PBS group. $n = 6$ in each group.

score in the PBS group was greater than that in the P-15 group ($p < 0.05$; Fig. 4) and the P-20 group ($p < 0.01$; Fig. 4). The neurological score was greater in the D-20 group than in the P-15 group ($p < 0.05$; Fig. 4) and P-20 group ($p < 0.01$; Fig. 4).

Effect of Paeoniflorin on Peripheral Blood Cells in Ischemia-Reperfusion Injured Rats

The levels of WBC, RBC, HGB, HCT, MCV, MCH, MCHC, PLT and LYM in peripheral blood were similar among the sham, control, P-10, P-15, P-20, D-20 and PBS groups (all $p > 0.005$; Table 1).

Effect of Paeoniflorin on ED1, TNF-1α, IL-1β, ICAM-1 and MPO Immunoreactive Cells, and Apoptosis in Ischemia-Reperfusion Injured Rats

The counts of ED1 immunoreactive cells in the control group were greater than those in the sham group ($p < 0.001$; Table 2, Fig. 5) and P-20 group ($p < 0.001$; Table 2, Fig. 5). The counts of ED1 immunoreactive cells were greater in the P-20 group than in the sham group ($p < 0.001$; Table 2, Fig. 5).

The counts of IL-1β immunoreactive cells in the control group were greater than those in the sham group ($p < 0.001$; Table 2, Fig. 5) and P-20 group ($p < 0.001$; Table 2, Fig. 5). The counts of IL-1β immunoreactive cells were greater in the P-20 group than in the sham group ($p < 0.01$; Table 2, Fig. 5).

Table 1. The Effect of *Paeoniflorin* on Peripheral Blood Cells in Ischemia-Reperfusion Injured Rats

Groups	WBCX (10^3/ul)	RBC ($X10^6$/ul)	HGB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	PLT ($X10^3$/ul)	LYM (%)	LYM ($X10^3$/ul)
Sham	8.6 ± 4.7	6.2 ± 1.6	12.1 ± 2.7	36.9 ± 8.4	60.0 ± 3.5	19.8 ± 1.1	32.9 ± 0.6	505.5 ± 262.1	53.0 ± 24.6	3.9 ± 2.3
Control	7.5 ± 2.1	6.2 ± 0.7	12.7 ± 1.4	37.6 ± 3.5	61.1 ± 1.2	20.7 ± 0.6	33.8 ± 0.7	571.7 ± 384.6	43.9 ± 16.0	7.8 ± 11.5
P-10	10.3 ± 4.3	6.1 ± 1.8	11.9 ± 3.3	35.7 ± 10.1	59.3 ± 4.4	19.7 ± 1.0	31.4 ± 5.0	397.7 ± 214.3	47.0 ± 23.8	4.5 ± 2.51
P-15	9.2 ± 3.3	6.4 ± 0.8	12.0 ± 2.0	36.8 ± 6.2	57.7 ± 4.7	18.8 ± 1.5	32.6 ± 0.5	607.2 ± 195.0	48.3 ± 17.0	4.7 ± 1.9
P-20	12.4 ± 4.7	7.7 ± 1.0	15.0 ± 1.7	44.4 ± 5.3	58.1 ± 1.6	19.8 ± 1.0	34.3 ± 1.6	715.0 ± 434.0	29.3 ± 13.4	3.6 ± 1.8
D-20	9.6 ± 6.8	6.4 ± 1.1	12.8 ± 2.5	33.3 ± 13.4	60.2 ± 4.1	20.0 ± 1.6	33.3 ± 0.8	541.2 ± 421.8	60.6 ± 21.5	3.3 ± 1.6
PBS	7.0 ± 4.4	5.3 ± 1.8	10.5 ± 3.5	35.7 ± 20.4	59.2 ± 3.1	20.0 ± 1.0	32.0 ± 5.1	434.8 ± 194.4	57.6 ± 12.7	3.0 ± 2.2

Mean ± SD. WBC: White blood cell; RBC: red blood cell; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet; LYM: lymphocyte; Sham: sham group; Control: control group; P-10: P-10 group; P-15: P-15 group; P-20: P-20 group; D-20: D-20 group; PBS: PBS group. $n = 6$ in each group.

Table 2. The Effect of *Paeoniflorin* on ED1, IL-1β, TNF-α, ICAM-1 and MPO Immunoreactive Cells, and Apoptotic Cells in Ischemia-Reperfusion Injured Rats

	Sham	Control	P-20
ED1	24.0 ± 15.3	$451.2 \pm 73.1^{***}$	$191.7 \pm 47.6^{***\#}$
IL-1β	18.3 ± 12.6	$508.3 \pm 103.1^{***}$	$173.8 \pm 55.2^{**\#}$
TNF-α	23.0 ± 13.8	$441.3 \pm 77.5^{***}$	$193.2 \pm 23.0^{***\#}$
ICAM-1	2.8 ± 2.8	$67.3 \pm 17.3^{***}$	$21.0 \pm 8.8^{*\#}$
MPO	20.2 ± 15.1	$482.5 \pm 58.8^{***}$	$172.0 \pm 45.9^{***\#}$
Apoptosis	97.0 ± 33.2	$931.5 \pm 176.3^{***}$	$355.3 \pm 78.9^{**\#}$

Mean $\pm$ SD. ED1: ED1 (mouse anti-rat CD68) immunoreactive cells; IL-1β: interleukin 1β immunoreactive cells; TNF-α: tumor necrosis factor-α immunoreactive cells; ICAM-1: intercellular adhesion molecule-1 immunoreactive cells of microvessels; MPO: myeloperoxidase immunoreactive cells; Apoptosis: apoptotic cells; *p $<$ 0.05, **p $<$ 0.01, ***p $<$ 0.001 compared to the sham group; $^{\#}$p $<$ 0.001 compared to the control group. $n = 6$ in each group.

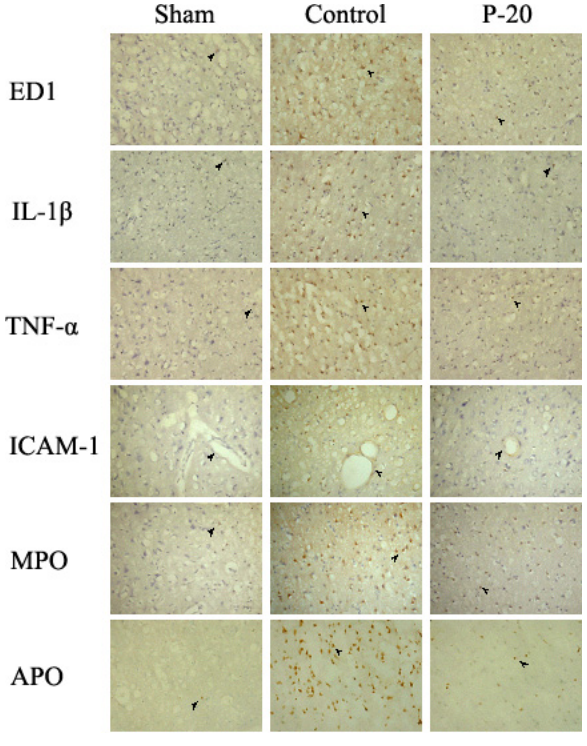

Figure 5. Effect of *paeoniflorin* on ED1(mouse anti rat CD68)-, interleukin-1β (IL-1β)-, tumor necrosis factor-α (TNF-α)-, intercellular adhesion molecule-1 (ICAM-1)-, myeloperoxidase (MPO)- immunoreactive cells and apoptosis in ischemia-reperfusion injured rats. ED1-, interleukin-1β-, tumor necrosis factor-α-, intercellular adhesion molecule-1-, myeloperoxidase- immunoreactive cells, and apoptosis cells (APO) (arrow) increased in the control group compared to the sham group. These increases were not seen in the P-20 group. $n = 6$ in each group.

The counts of TNF-α immunoreactive cell in the control group were greater than those in the sham group ($p < 0.001$; Table 2, Fig. 5) and P-20 group ($p < 0.001$; Table 2). The counts of TNF-α immunoreactive cell were greater in the P-20 group than in the sham group ($p < 0.001$; Table 2, Fig. 5).

The counts of ICAM-1 immunoreactive cells of microvessels in the control group were greater than those in the sham group ($p < 0.001$; Table 2, Fig. 5) and P-20 group ($p < 0.001$; Table 2, Fig. 5). The counts of ICAM-1 immunoreactive cells of microvessels were greater in the P-20 group than in the sham group ($p < 0.05$; Table 2, Fig. 5).

The counts of MPO immunoreactive cells in the control group were greater than those in the sham group ($p < 0.001$; Table 2, Fig. 5) and P-20 group ($p < 0.001$; Table 2, Fig. 5). The counts of MPO immunoreactive cells were greater in the P-20 group than in the sham group ($p < 0.001$; Table 2, Fig. 5).

The counts of apoptotic cells in the control group were greater than those in the sham group ($p < 0.001$; Table 2, Fig. 5) and P-20 group ($p < 0.001$; Table 2, Fig. 5). The counts of apoptotic cells were greater in the P-20 group than in the sham group ($p < 0.01$; Table 2, Fig. 5).

Discussion

The results of the present study indicated that *paeoniflorin* pre-treatment and post-treatment can reduce cerebral infarction size; furthermore *paeoniflorin* pretreatment also can reduce neurological-deficit score in rats with cerebral infarct induced by ischemia-reperfusion. *Paeoniflorin* did not change the levels of WBC, RBC, HGB, HCT, MCV, MCH, MCHC, PLT and LYM in peripheral blood suggesting that *paeoniflorin* may have a similar effect in stroke patients. These results are consistent with those reported in an earlier study which found that *paeoniflorin* reduces cerebral infarct volume in MCAo rats in the chronic stage (Xiao *et al.*, 2005). It has been reported that *paeoniflorin* may inhibit nuclear factor-κB expression in chronic hypoperfusion rat, indicating that *paeoniflorin* may suppress neuro-inflammatory reaction (Liu *et al.*, 2006). *Paeoniflorin* can inhibit I_{Na} in the hippocampus neuron of mice (Zhang *et al.*, 2003). The influx of Na^+ and accumulation of intracellular Na^+ leading to Ca^{2+} overload contributes to brain damage in the ischemic brain (Zhang *et al.*, 2003). *Paeoniflorin* has an anti-thrombotic effect, and this effect may relate to the inhibition of arachidonic acid metabolism, leading to increase in the activity of t-PA (Ye *et al.*, 2001).

Microglia is activated rapidly in response to damage to the central nervous system, such as cerebral ischemia or inflammation (Kreutzberg, 1996), and activated microglia can be identified by ED1 immunostaining (Tzeng and Wu, 1999; Lao *et al.*, 2005). Activated microglia releases neurotoxic substances, such as nitric oxide (NO) and free oxygen radical (Chao *et al.*, 1992; Boje and Arora, 1992; Chuang *et al.*, 2008). Microglia has been shown to be a sensitive indicator of neuronal damage in the early stage of transient cerebral ischemia in rats (Morioka *et al.*, 1991). In a model of cerebral ischemia, IL-1β generated from endothelial cells and microglia; Zhang *et al.* (1998) reported that activated microglia may mediate via IL-1β to exacerbate brain damage. TNF-α has been shown to increase blood-brain-barrier

permeability (Barone *et al.*, 1997) and facilitate inflammatory cells infiltration (Liu *et al.*, 1994) in MCAo rats, indicating that TNF-α exacerbates cerebral ischemic damage. ICAM-1 is expressed after cerebral ischemia and has been reported to play a role in mediating leucocyte-endothelial adhesion to facilitate leucocytes migration into ischemic zone (Zhang *et al.*, 1995). PMN has been shown to cause oxidative stress injury and therefore may contributes to post-cerebral ischemic brain damage (Beray-Berthat *et al.*, 2003), MPO is also a marker of PMN infiltration (Beray-Berthat *et al.*, 2003; Miljkovic-Lolic *et al.*, 2003). Apoptosis may contribute to neuron loss and provide evidence of neuron preservation after cerebral ischemia (Du *et al.*, 1996; Linnik *et al.*, 1993). Our results indicated the presence of ED1, IL-1β, TNF-α, ICAM-1 in microvessel and MPO immunoreactive cells and apoptotic cells in the cerebral infarction region; however, these increase were inhibited by*paeoniflorin* pretreatment. *Paeoniflorin* reduced cerebral infarction size and neurological deficit in ischemia-reperfusion injured rats, indicating that *paeoniflorin* has an anti-inflammatory effect.

In conclusion, *paeoniflorin* reduced cerebral infarct and neurogical deficit in the ischemia-reperfusion injured rats, suggesting *paeoniflorin* may have similar effect in stroke patients. *Paeoniflorin* reduced cerebral infarct, due to in part to its anti-inflammatory properties.

Acknowledgments

This study was supported by grant CMU93-CM-03 and CMU94-013 from the China Medical University, Taichung, Taiwan.

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