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Orthogonal design to sift the optimal parameter of *Neiguan* acupuncture for cerebral infarction

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Research Highlights

(1) This study performed acupuncture at *Neiguan* (PC6) using a custom lifting- and thrusting-controlled machine. An orthogonal design was used to sift the optimal parameter of *Neiguan* acupuncture for stroke.
(2) Main parameters included: lifting and thrusting frequency 1 Hz, duration 180 seconds or lifting and thrusting frequency 2–3 Hz, duration 5/60 seconds. The effects of acupuncture significantly elevated cerebral blood flow and reduced infarct volume. The interaction between frequency and duration played a critical role in quantified acupuncture therapy for cerebral infarction.

Abstract

The individual difference and non-repeatability in acupuncture have not only restricted the development of acupuncture, but have also affected the specificity of acupoints. The present study used instruments to control needle depth, lifting and thrusting frequency, and the duration of acupuncture. Effects of the quantified acupuncture were observed at *Neiguan* (PC6) with different stimulation parameters. A frequency of 1, 2, or 3 Hz and duration of 5, 60, or 180 seconds were used to observe cerebral blood flow and ratio of infarct volume recovery. Results showed that stimulation at *Neiguan* with a frequency of 1 Hz and long duration of 180 seconds or 2/3 Hz and long duration of 5/60 seconds significantly increased cerebral blood flow and decreased the ratio of infarct volume. Interactions between frequency and duration play a critical role in quantified acupuncture therapy.

Key Words

neural regeneration; acupoint; quantified acupuncture; *Neiguan*; middle artery occlusion injury; lifting and thrusting method; orthogonal design; lifting and thrusting frequency; acupuncture duration; grants-supported paper; neuroregeneration

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Conflicts of interest: None declared.

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INTRODUCTION

Currently, there is controversy over the specific effects of acupuncture meridians, with some denying that meridians have specific effects[1]. Germany, the Netherlands, the United Kingdom, and other research institutions have found no positive effects of acupuncture for the treatment of chronic asthma, back pain, and smoking cessation[2-4]. American Acupuncture Council Expert Group performed a retrospective analysis for acupuncture literature from January 1970 to October 1997. They found that, although many studies have shown acupuncture's beneficial effects for post-surgery- or chemotherapy-induced nausea and vomiting, toothache, stroke rehabilitation, headache, dysmenorrhea, and tennis elbow, there are varying degrees of defects in the experimental design, sample size, or control group design in these studies. Studies with more stringent design involving more diseases are needed to show the benefits and effectiveness of acupuncture[5].

It has been reported by the World Health Organization in the year 2003 that acupuncture is effective for 28 kinds of diseases, including stroke[6]. However, the systematic review of acupuncture for acute and chronic stroke rehabilitation by Wu *et al* [7] has shown that there is no evidence to support the acupuncture as a routine treatment for stroke rehabilitation. Recent studies on evidence-based medicine have also shown that acupuncture does not improve motor function in stroke patients[8-9]. Acupuncture factors included in the evaluation were acupuncture method (electroacupuncture, hand acupuncture), acupuncture points, needling frequency, and number of treatments. However, factors such as uniformity of manipulation and choice of acupoints were not listed in the systematic review[8-9].

Neiguan (PC6) is the *Luo*-connecting point of the Pericardium meridian. It has been used to treat stroke for decades[10-13]. Electroacupuncture at *Neiguan* can significantly improve cerebral pathology in the damaged zones of rat brain, and promote recovery of neurological function[14]. Acupuncture at *Neiguan* can also adjust the excitability of the cardiovascular system possibly *via* reduction of sympathetic response. The rostral ventrolateral medulla of thalamus, arcuate nucleus, and ventrolateral periaqueductal gray matter are also involved in the process[15]. *Neiguan* is the traditional point for sports-related nausea and vomiting, and is also used to treat morning sickness and chemotherapy side effects[16]. Mind, in Chinese medicine, is governed by the heart. In modern times, mind is associated with the brain, including consciousness and mental and emotional aspects. In recent years, *Neiguan* was mostly used to reduce smokers' psychological dependence[17]. Acupuncture at *Neiguan* can activate several brain areas, as confirmed by MRI. However, other points with analgesic effects have no such effect on brain activity[18]. Therefore, *Neiguan* is strongly associated with the brain, and can be used to treat brain-related diseases. Wang *et al* [19] stimulated middle cerebral artery occlusion rats with a high frequency and short duration and found that neuronal ultrastructure and cerebral blood flow were significantly improved. This resulted in the improvement of brain energy metabolism, reduction in free radical production, protection of brain cells from mitochondrial membrane attack, and reduction in mitochondrial DNA damage and mutations. Wu *et al* [20-21] performed acupuncture at *Neiguan*, *Renzhong* (DU26), and *Sanyinjiao* (SP6) of rabbits with global cerebral ischemia/reperfusion injury. They found that the ultrastructural damage of neurons was significantly reduced, with the presence of larger quantities of intact mitochondrial structure, and increased bulk density. This was probably to ensure structural integrity of capillaries and good and effective blood flow. Moreover, acupuncture has a positive regulatory role in high coagulation in rabbits with cerebral ischemia. Liu *et al* [22] have shown that acupuncture significantly reduced damage to endothelial cells and basement membranes, and stabilized the ultrastructure of the blood-brain barrier, resulting in improved blood circulation of the cerebral cortex.

Differences in rotation angle and stimulation frequency of the twirling technique will cause changes in effect indicators and alter the effectiveness of acupuncture[23]. Therefore, we propose to evaluate three issues: Is acupuncture effective for treating stroke? What is the most important factor of the lifting and thrusting method in acupuncture that determines its effect? What is the optimal parameter for *Neiguan* to treat stroke? Each fundamental question remains unanswered because of limitations in clinical studies. The present study uses an instrument called a lifting- and thrusting-controlled machine to provide standard and quantified research of acupuncture. This instrument lays a foundation for acupuncture experimental repeatability. The present study used an optimal analysis to sift optimal parameters of *Neiguan*.

RESULTS

Quantitative analysis of experimental animals

A total of 198 male adult Wistar rats were randomly assigned to normal (normal feeding without intervention, n = 18), model (middle cerebral artery occlusion, n = 18), and quantified acupuncture (middle cerebral artery occlusion + quantified acupuncture, n = 162) groups. The quantified acupuncture group was subdivided into nine different parameter groups in accordance with the $L_9(3^4)$ (Table 1) orthogonal experimental design (Group I–IX), with 18 rats in each parameter group (Table 2). Sixty rats from model and various quantified acupuncture parameter groups died within 3 days after the middle cerebral artery occlusion surgery. In addition, eight rats were excluded from quantified acupuncture treatment, cerebral blood flow, ratio of infarct volume testing because of unqualified neurological scores. An additional 68 rats were supplemented to ensure 18 qualified animals for each parameter group. A total of 268 rats were included in the final analysis.

Table 1 Factors and levels of acupuncture frequency and duration analyzed in the orthogonal design

Level	Frequency (Hz)	Duration (second)
1	1	5
2	2	60
3	3	180

Effects of acupuncture frequency and duration on cerebral blood flow and infarct volume in middle cerebral artery occlusion rats

The results of cerebral blood flow and cerebral infarct volume ratio after nine frequencies and durations of acupuncture treatment for stroke are shown in Table 3. The orthogonal experimental visual analysis (Table 4) and analysis of variance (Table 5) shows the effects and the major or minor roles of quantified acupuncture parameters on the cerebral blood flow and cerebral infarct volume ratio.

Table 2 Nine quantified acupuncture subgroups and quantified acupuncture parameters in orthogonal design

Group	Frequency (Hz)	Duration (second)
I	1	5
II	1	60
III	1	180
IV	2	5
V	2	60
VI	2	180
VII	3	5
VIII	3	60
IX	3	180

Eighteen rats in each group.

Table 3 Effect of different frequencies and durations of acupuncture on cerebral blood flow and infarct volume ratio in middle cerebral artery occlusion rats

Group	Cerebral blood flow	Infarct volume ratio
I	378.97±213.02	0.108±0.065
II	402.78±194.98	0.123±0.146
III	495.29±240.74	0.074±0.039
IV	520.20±245.84	0.086±0.058
V	426.41±172.53	0.118±0.068
VI	471.41±234.68	0.140±0.083
VII	445.90±179.23	0.062±0.043
VIII	464.48±213.53	0.093±0.068
IX	360.73±155.65	0.129±0.092

Infarct volume ratio = (the volume of the contralateral hemisphere – the volume of non-infarct tissue in the infarcted ipsilateral hemisphere)/the volume of the contralateral hemisphere. The data are expressed as mean ± SD of 18 rats in each group.

Rat cerebral blood flow

As shown in Table 4, interaction exerted the most significant effect on decreasing the ratio of infarct volume, and the order of importance that influenced the acupuncture effect was as follows: interaction (A × B) > frequency (A) > duration (B). The R(A × B), R(A), and R(B) were similar, and the three factors should be considered synthetically. According to Table 5, interaction factor is statistically significant (P < 0.05). As interaction factor was the primary influencing factor, the frequency and duration are the primary influencing factors in improving the cerebral blood flow.

In accordance with this principle, the optimal level of interaction of the two factors depends on the optimal level of the interaction column in the orthogonal experiment. The larger the F value, the greater the influence it has on the result.

Table 4 Direct-viewing of cerebral blood flow and infarction volume in middle cerebral artery occlusion rats treated with two-factor and three-level quantified acupuncture parameters

Item	1 Frequency (A)	2 Duration (B)	3 A×B (1)	4 A×B (2)	5 Cerebral blood flow	6 Infarction volume
1	1	1	1	1	378.97	0.108
2	1	2	2	2	402.78	0.123
3	1	3	3	3	495.29	0.074
4	2	1	2	3	520.20	0.086
5	2	2	3	1	426.41	0.118
6	2	3	1	2	471.41	0.140
7	3	1	3	2	445.90	0.062
8	3	2	1	3	464.48	0.093
9	3	3	2	1	360.73	0.129
Cerebral blood flow	K1	425.680	448.357	438.287	388.703	
	K2	472.673	431.223	427.903	440.030	
	K3	423.703	442.477	455.867	493.323	
	R	48.970	17.134	27.964	104.620	
Infarction volume	K1	0.102	0.085	0.114	0.118	
	K2	0.114	0.111	0.113	0.108	
	K3	0.094	0.114	0.084	0.084	
	R	0.020	0.029	0.030	0.034	

A × B: Interaction between frequency and duration R(A × B) = R(A × B) (1) + R (A × B)(2)/2. The K-value: the sum of test results of corresponding factors under a certain level. K1–3: the sum of each result of a factor under 1–3 levels. R-value reflected the indicator range with corresponding factor changes, which suggested an order of importance in the influence of various factors, *i.e.*, the minimum of K subtracted from a maximum.

Table 5 Variance analysis of cerebral blood flow in middle cerebral artery occlusion rats treated with two-factor and three-level quantified acupuncture parameters on *Neiguan* acupoint

Factor	Degree of freedom	Square of deviance	*F*	*P*
A	2	41 492.615	0.961	> 0.05
B	2	4 095.155	0.095	> 0.05
A×B(I)	2	10 790.577	0.250	> 0.05
A×B(II)	2	147 785.897	3.423	< 0.05

A: Frequency; B: duration; A × B: interaction between frequency and duration.

Table 6 Sum of *K*-values of different levels of frequency and duration factor

Cerebral blood flow				Infarct volume			
K	A1	A2	A3	K	A1	A2	A3
B1	827	921	896	B1	0.23	0.2	0.19
B2	868	845	932	B2	0.22	0.2	0.2
B3	949	878	817	B3	0.16	0.22	0.23

A1, 2 and 3: Frequency 1, 2, and 3 Hz. B1, 2 and 3: Duration 5, 60, and 180 seconds, respectively.

According to the sum of the K-values of different frequencies and durations in Table 6, it was concluded that *Neiguan* quantified acupuncture at 1 Hz; 180 seconds, 2 Hz; 5 seconds and 3 Hz; 60 seconds significantly increased cerebral blood flow in middle cerebral artery occlusion rats.

Variance analysis ratio of infarct volume in middle cerebral artery occlusion rats

According to Table 4, interaction (A × B) > duration (B) > frequency (A). The R(A×B), R(A), and R(B) were similar, and the three factors should be considered synthetically. A smaller K-value was preferred for analyzing the ratio of infarct volume. Table 6 shows that A1B3 (lifting and thrusting 1 Hz for 180 seconds) and A3B1 (lifting and thrusting 3 Hz for 5 seconds) resulted in an optimal therapeutic effect for decreasing the ratio of infarct volume.

Needling at *Neiguan* increased cerebral blood flow and decreased infarct volume in middle cerebral artery occlusion rats

Cerebral blood flow in rats of the model group was significantly decreased when compared with the normal group ($P < 0.01$). Quantified acupuncture at *Neiguan* (1 Hz; 180 seconds, 2 Hz; 5 seconds, 3 Hz; 5 seconds and 3 Hz; 60 seconds) significantly increased cerebral blood flow in middle cerebral artery occlusion rats ($P < 0.01$; Figure 1). The ratio of infarct volume in the model group rats was significantly increased compared with the normal group ($P < 0.01$). Quantified acupuncture at *Neiguan* 3 Hz for 5 seconds significantly decreased the ratio of infarct volume in middle cerebral artery occlusion rats ($P < 0.05$; Figures 2, 3).

Neurological function

There were no significant differences in neurological function scores between the model and quantified acupuncture groups before treatment ($P > 0.05$; Figure 4).

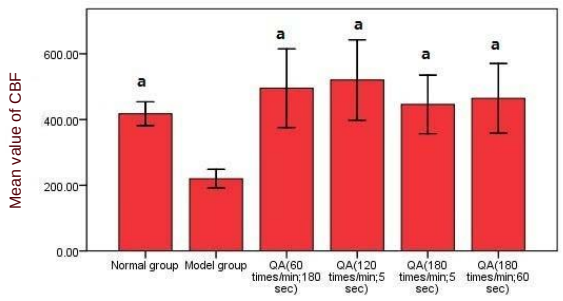

Figure 1 Comparison of cerebral blood flow (CBF) between groups.

[a]*P* < 0.01, *vs*. model group. Quantified acupuncture (QA) Group III (1 Hz; 180 seconds); QA Group IV (2 Hz; 5 seconds); QA Group VII (3 Hz; 5 seconds); QA Group VIII (3 Hz; 60 seconds).

DISCUSSION

In this study, cerebral blood flow and ratio of infarct volume in rats of the model group were significantly decreased when compared with the normal rats after stroke, indicating that this modeling approach can simulate the stroke pathology. Acupuncture at *Neiguan* can improve motor function in rats, although the result was not statistically significant. However, even a weak limb motor function improvement in clinic will improve the quality of life of patients in the future. Acupuncture at *Neiguan* increases cerebral blood flow and decreases the ratio of infarct volume in middle cerebral artery occlusion rats, which further proves that acupuncture at *Neiguan* adjusted vascular function and increased blood flow[24]. Therefore, it is essential to select the correct acupuncture techniques in treatment of diseases. Moreover, as acupuncture techniques are diverse, even a single acupuncture manipulation by the same person is not always the same. This is obviously not conducive to rigorous research on acupuncture techniques. The present study used instruments to control needle depth, lifting and thrusting frequency, and the duration of acupuncture, which reduces uncontrollable possibilities caused by the instability of acupuncture manipulation.

Nonetheless, there are still limitations to this study: (1) The cycle of experimental observation was 72 hours; it failed to observe the long-term efficacy of acupuncture for stroke. (2) Experimental conclusions and clinical practices are varied, possibly because in clinic, twisting techniques at *Neiguan* are combined with lifting and thrusting. Lifting and thrusting techniques are not always used alone.

MATERIALS AND METHODS

Design

A traditional Chinese medicine, orthogonal, clinical study.

Time and setting

Experiments were performed at the Acupuncture Institute of First Affiliated Hospital, Tianjin University of Traditional Chinese Medicine in China from July 2007 to November 2008.

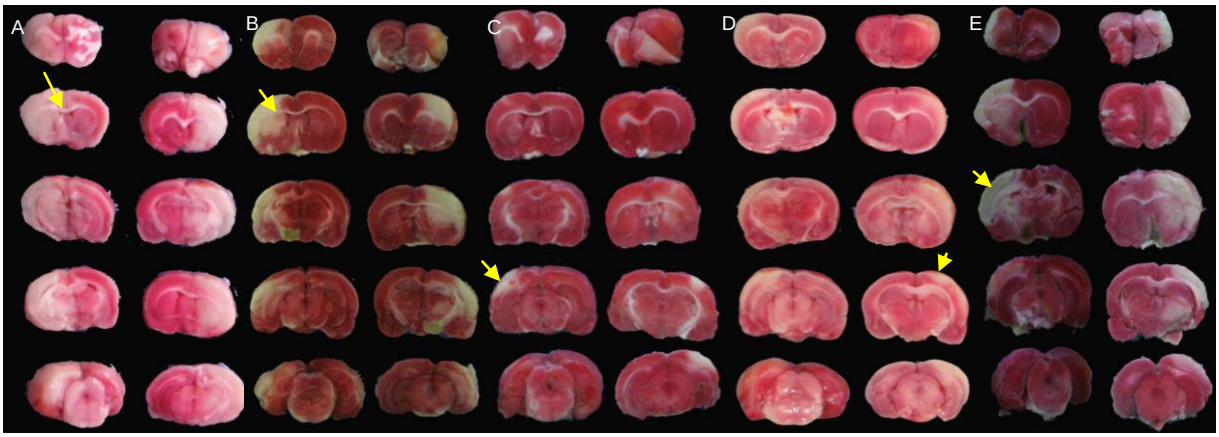

Figure 2 Comparison of ratio of infarction volume between groups (2,3,5-triphenyltetrazolium chloride staining).

(A) Model group; (B) quantified acupuncture Group III (1 Hz; 180 seconds); (C) quantified acupuncture Group IV (2 Hz; 5 seconds); (D) quantified acupuncture Group VII (3 Hz; 5 seconds); (E) quantified acupuncture Group VIII (3 Hz; 60 seconds). Arrows show infarct region. Quantified acupuncture at *Neiguan* at 3 Hz, 5 seconds significantly decreased ratio of infarction volume in middle cerebral artery occlusion rats.

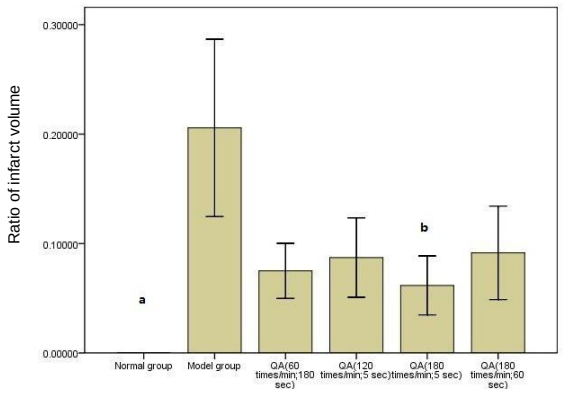

Figure 3 Comparison of infarct volume between groups.

Infarct volume ratio = (the volume of the contralateral hemisphere – the volume of non-infarct tissue in the infarcted ipsilateral hemisphere)/the volume of the contralateral hemisphere.

The data are expressed as mean ± SD of 18 rats in each group. The differences among groups were compared using analysis of variance, followed by least significant difference test. [a]*P* < 0.01, *vs*. model group; [b]*P* < 0.05, *vs*. model group. Quantified acupuncture (QA) Group III (1 Hz; 180 seconds); QA Group IV (2 Hz; 5 seconds); QA Group VII (3 Hz; 5 seconds); QA Group VIII (3 Hz; 60 seconds).

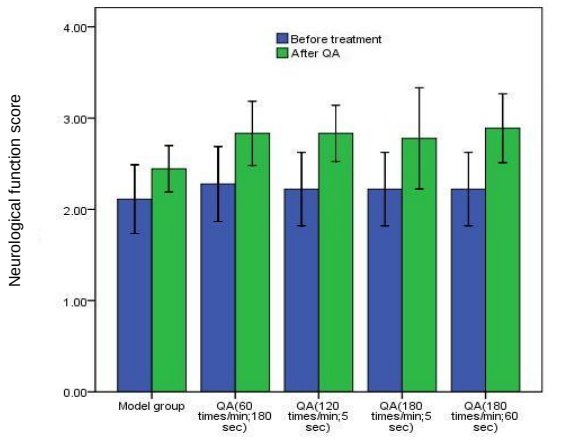

Figure 4 Neurological function scores before and after quantified acupuncture (QA) treatment.

Neurological score ranged from 0 to 5; high score indicates neurological function closer to normal. Data are expressed as mean ± SD of 18 rats in each group. The differences among groups were compared using analysis of variance, followed by least significant difference test. QA Group III (1 Hz; 180 seconds); QA Group IV (2 Hz; 5 seconds); QA Group VII (3 Hz; 5 seconds); QA Group VIII (3 Hz; 60 seconds).

Materials

Adult specific pathogen free male Wistar rats aged 2–3 months and weighing 250–300 g were provided by Vital River, Beijing, China, certificate No. SCXK (Jing) 2007-0001. All rats were allowed free access to food and water and maintained at a 12-hour light/dark cycle. All experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[25].

Methods

Model preparation

The animal models in middle cerebral artery occlusion were established according to the Zea-Longa method[26-27]. The main steps were as follows: after fasting and water deprivation for 12 hours, 10% chloral hydrate was injected intraperitoneally to anesthetize the animal until the response to pain stimulus disappeared. The rat was fixed supinely to the operating table, and the skin was partly sterilized. A cut of 2–2.5 cm was made to the left of the median of the neck. After segregating the thyroid gland, the left triangular area between the sternomastoid muscle and sternohyoid muscle was exposed. The left common carotid artery and external carotid artery were bluntly separated. The external carotid artery was ligated with number 0 operation thread. An occlusion was made on the bifurcation and the proximal end with two small artery clips. A small hole was pricked on the proximal end with a 1 mL injector. A 0.26-mm-diameter fishing line was inserted slowly. The artery clip on the bifurcation was loosened. The fishing line was inserted into the internal carotid artery until resistance was felt. The depth of the fishing line was about 18–20 mm. The fishing line exactly closed the middle cerebral artery, and blocked the blood flow of the middle cerebral artery. After the fishing line was successfully inserted, the common carotid artery was ligated, and then another artery clamp was loosened. The skin was sutured.

Neurological evaluation

Neurological deficits were assessed by professional staff blinded to the middle cerebral artery occlusion surgery and quantified acupuncture treatment. Neurological function of all awakened animals was evaluated following middle cerebral artery occlusion and after the 3-day quantified acupuncture treatment using the Zausinger 6-point neurological function score. Rats with neurological function scoring grades 1–3 qualified as an established model and entered the next phase of the experiment.

Quantified acupuncture treatment

Neiguan is located at the flexor aspect of the forearm between the tendons of palmaris longus and flexor carpi radialis. Acupuncture needles were purchased from Suzhou Huatuo Medical Instruments, Suzhou, Jiangsu

Province, China, and were 1.5 cun (cun is a unit of length, which refers to the width of the interphalangeal joint of the patient's thumb) in length and 0.3 mm in diameter. The lifting- and thrusting-controlled machine was used for quantified acupuncture treatment (Haifu Technology Co., Ltd., Chongqing, China). No anesthesia was administered before acupuncture treatment, which was completed with one person holding the rats while another person operated the machine. Rats in the quantified acupuncture groups underwent quantified acupuncture treatment after the qualified models were established. Quantified acupuncture frequency and duration were selected in accordance with the $L_9(3^4)$ orthogonal design scheme. Stimulation took place twice a day, six times in total (Figure 5).

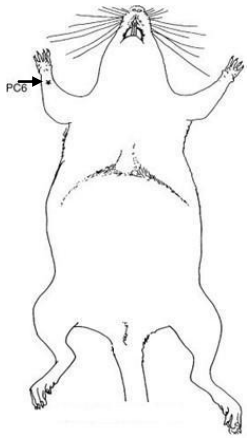

Figure 5 Schematic localization of acupuncture points (*Neiguan*, PC6) stimulated in the rats.

Cerebral blood flow detection with a laser-Doppler flow meter

Cerebral blood flow was monitored in the cerebral cortex ipsilateral to the occluded middle cerebral artery with a laser-Doppler flow meter (DRT4, Moor, Wilmington, DE, USA). The left dorsolateral portion of the calvaria was carefully abraded with a mini-drill until only a paper-thin layer of bone remained to prevent cerebral spinal fluid leakage. A hole 2.0 mm in diameter at 3.0 mm lateral and 1.0 mm caudal to the bregma was drilled. Each animal was placed in a supine position, where the head was firmly immobilized in a stereotaxic frame. A laser-Doppler probe was held in a micromanipulator and gently touched the intact dura mater to measure the regional cerebral blood flow and the flow values were recorded during 1-minute intervals every 10 minutes until the laser-Doppler flow meter baseline was stable and the data were calculated as averaged values.

Detection of the ratio of infarct volume using 2,3,5-triphenyltetrazolium chloride staining

Following the evaluation of neurological deficits at 72 hours after ischemia, the rats were killed rapidly under anesthesia and their brains were sliced into 2.0-mm sections. The brain slices were incubated in a solution of triphenyl-tetrazolium chloride (0.2%) for 30 minutes at 37°C and then fixed in paraformaldehyde (0.4%). The infarct region presented as white in color while "normal" tissues showed up in red. The images of the brain slices were taken with a digital camera attached to a computer system. The infarct volume was analyzed by a computer-assisted image system with Image-Pro Plus (Rockville, MD, USA). Relative infarct ratio was calculated using the following equation: (VC – VL)/ VC, where VC is the volume of the contralateral hemisphere and VL is the volume of non-infarct tissue in the infarcted ipsilateral hemisphere. The analysis was carried out under blinded conditions.

Statistical analysis

All data were expressed as mean ± SD. SPSS 13.0 software (SPSS, Chicago, IL, USA) was applied for the data analysis. The orthogonal experimental design adopted direct-viewing analysis and orthogonal analysis of variance. One-way analysis of variance was used to compare between groups, and the least significant difference test was used to compare between experimental groups. A value of $P < 0.05$ was considered statistically significant.

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