Lin YP, Liu Q, Chen CT, *et al*. Effect of acupuncture plus mild hypothermia on MAPK/ERK pathway of brain tissues in rats with cerebral ischemia-reperfusion injury. J Acupunct Tuina Sci, 2016, 14(5): 311-319
DOI: 10.1007/s11726-016-0942-2

Basic Study

Effect of acupuncture plus mild hypothermia on MAPK/ERK pathway of brain tissues in rats with cerebral ischemia-reperfusion injury

针刺联合亚低温对脑缺血再灌注大鼠脑组织 MAPK/ERK 通路的影响

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Abstract

Objective: To observe the protective effect of acupuncture plus mild hypothermia on brain tissues in rats with cerebral ischemia-reperfusion injury (CIRI), and the influence on protein expression levels of phosphorylated Raf-1, MEK-2 and ERK1/2 in the mitogen-activated protein kinase (MAPK)/extracellular regulated protein kinases (ERK) pathway, and to explore the mechanism of acupuncture plus mild hypothermia therapy for the ischemic stroke.

Methods: Ninety Sprague-Dawley (SD) rats were randomly divided into a blank control group, a sham operation group, a model group, an acupuncture group, a mild hypothermia group and an acupuncture plus mild hypothermia group, 15 rats in each group. Except the rats in the blank control group, the remaining rats were used to prepare the middle cerebral artery occlusion (MCAO) models according to the modified occlusion method using lines, while only the occlusion lines were inserted without blocking the brain arteries of rats in the sham operation group. When the vital signs of rats were stable, rats in the blank control group did not receive any intervention; rats in the sham operation group and the model group received fastening without treatment; rats in the acupuncture group, the mild hypothermia group, and the acupuncture plus mild hypothermia group were treated with the corresponding therapeutic methods. 72 h later, observed neurologic injury score, evaluated infarction area ratio by 2,3,5-tripheyl tetrazolium chloride (TTC) staining, determined apoptosis by TUNEL assay, and measured the phosphorylated Raf-1, MEK-2 and ERK1/2 protein expression levels in rat ischemic hippocampal tissues by Western blot assay.

Results: Compared with the blank control group and the sham operation group, after modeling, the neurologic injury score, infarction area ratio and apoptotic cells were increased, and phosphorylated Raf-1, MEK-2 and ERK1/2 protein expression levels were significantly increased in the model group; the differences were statistically significant ($P<0.05$ or $P<0.01$). Compared with the model group, after acupuncture or mild hypothermia therapy, neurologic injury score and infarction area ratio were decreased; apoptotic cells and phosphorylated Raf-1, MEK-2 and ERK1/2 protein expression levels were significantly decreased; the differences were statistically significant ($P<0.05$ or $P<0.01$). Compared with the acupuncture group, neurologic injury score and phosphorylated Raf-1, MEK-2 and ERK1/2 protein expression levels were decreased in the acupuncture plus mild hypothermia group; differences between the groups were statistically significant ($P<0.05$ or $P<0.01$). Compared with the mild hypothermia group, phosphorylated Raf-1, MEK-2 and ERK1/2 protein expression levels decreased in the acupuncture plus mild hypothermia group, and differences were statistically significant ($P<0.01$).

Conclusion: Acupuncture or mild hypothermia therapy can improve neurologic injury, reduce infarction area and apoptosis, which brought about protective effect on the brain tissues, in the MCAO model. The protective effect of acupuncture plus mild hypothermia group is the strongest. The mechanism may involve the MAPK/ERK pathway, by reducing the phosphorylated Raf-1, MEK-2 and ERK1/2 protein expression levels.

Keywords: Acupuncture Therapy; Reperfusion Injury; Hypothermia Induced; Brain Ischemia; Apoptosis; Mitogen-activated Protein Kinases; Rats

【摘要】目的：观察针刺联合亚低温疗法对脑缺血再灌注大鼠脑组织的保护作用，以及对丝裂原激活的蛋白激酶(mitogen-activated protein kinase, MAPK)/细胞外调节蛋白激酶(extracellular regulated protein kinases, ERK)通路上磷

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酸化 Raf-1、MEK-2、ERK1/2 蛋白表达水平的影响，探讨针刺联合亚低温疗法干预缺血性中风的机制。**方法**：将 90 只 Sprague-Dawley (SD)大鼠随机分为空白组、假手术组、模型组、针刺组、亚低温组和针刺联合亚低温组，每组 15 只。随机挑选 15 只为空白组，其余大鼠根据线拴法并改良以复制大脑中动脉缺血(middle cerebral artery occlusion, MCAO)模型，假手术组只插入线栓不阻断大脑中动脉。待大鼠生命体征稳定后，空白组不做任何处理，假手术组和模型组只捆绑不治疗，针刺组、亚低温组、针刺联合亚低温组进行相应的处理。72 h 后，观察神经功能缺损评分，氯化三苯基四氮唑(2,3,5-tripheyl tetrazolium chloride, TTC)染色检测梗死面积比，TUNEL 法检测凋亡细胞，Western blot 法检测大鼠缺血侧海马组织磷酸化 Raf-1、MEK-2、ERK1/2 蛋白的表达水平。 **结果**：造模后，模型组大鼠神经功能缺损评分、梗死面积比值及凋亡细胞增多，磷酸化 Raf-1、MEK-2、ERK1/2 蛋白的表达水平明显增高，与空白组及假手术组差异有统计学意义($P<0.05$ 或 $P<0.01$)。经针刺及亚低温治疗后，各治疗组神经功能缺损评分、梗死面积比值减少，凋亡细胞及磷酸化 Raf-1、MEK-2、ERK1/2 蛋白的表达水平均明显降低，与模型组差异均有统计学意义($P<0.05$ 或 $P<0.01$)。与针刺组比较，针刺联合亚低温组神经功能缺损评分，磷酸化 Raf-1 蛋白激酶(Raf-1)、丝裂原活化蛋白激酶-2 (mitogen-activated protein kinase-2, MEK-2)、细胞外信号调节激酶 1/2 (extracellular signal regulated kinase1/2, ERK1/2)蛋白的表达水平下降，组间差异有统计学意义($P<0.05$ 或 $P<0.01$)。与亚低温组比较，针刺联合亚低温组磷酸化 Raf-1、MEK-2、ERK1/2 蛋白的表达水平下降，组间差异有显著统计学意义($P<0.01$)。**结论**：针刺及亚低温疗法均可改善 MCAO 模型大鼠的神经功能缺损，减少脑梗死面积及细胞凋亡，对脑组织起保护作用，并且针刺联合亚低温组的保护作用较强。其机制可能是通过 MAPK/ERK 通路，降低磷酸化 Raf-1、MEK-2、ERK1/2 蛋白的表达水平。

【关键词】针刺疗法；再灌注损伤；亚低温；脑缺血；细胞凋亡；丝裂原激活的蛋白激酶；大鼠
【中图分类号】R2-03　　**【文献标志码】**A

Cerebrovascular diseases seriously affect human health, with the clinical characteristics of high incidence, high mutilation rate, high morbidity, high mortality, high recurrence rate and low cure rate. Ischemic cerebral vascular disease (ICVD) is the most common cerebrovascular disease, accounting for 70% of all cerebral vascular diseases. Thrombolytic therapy is a preferred clinical treatment method in ultra-early acute cerebral infarction patients, however, appearance of the cerebral ischemia-reperfusion injury (CIRI), after the recanalization of the occluded vessels and recovery of ischemic brain region perfusion, will lead to further exacerbation of clinical symptoms of patients and severe nervous system dysfunction.

With the stimulation, Ras, Raf-1 protein kinase (Raf-1), mitogen-activated protein kinase (MEK) and extracellular signal regulated kinase (ERK) can be sequentially phosphorylated (P) in vivo. Then the extracellular signal is transmitted into the cells to regulate cell growth, differentiation, proliferation and apoptosis[1]. In recent years, as important therapies for CIRI, acupuncture and mild hypothermia have been widely used in clinic. However, it is rarely reported the clinical treatment of stroke with combination of these two therapies. This study was designed to explore whether acupuncture together with mild hypothermia can more effectively improve the pathological condition of rat's focal CIRI. The mitogen activated protein kinases/extracellular signal regulated kinase (MAPK/ERK) pathway was investigated to compare the therapeutic effects between acupuncture treatment and acupuncture plus mild hypothermia therapy, in the treatment of stroke, and to further explore their protective mechanism in brain tissues.

1 Materials and Methods

1.1 Materials

1.1.1 Experimental animals

Ninety SPF grade and male Sprague-Dawley (SD) rats, weighing 250-280 g, were provided by the Experimental Animal Center of Hunan University of Traditional Chinese Medicine, certificate number: scxk (Xiang) 2011-0003.

1.1.2 Main instruments

Shandon325 Wax slicer (Shandon, UK), MIAS medical image analysis system (Beijing BUAA Tianhua Technology Co., Ltd., China), LEICA DM LB2 binocular microscope (LEICA, Germany), bipolar coagulation device (Shanghai Medical Laser Instrument Factory, China), TES 1310 TYPE-K digital thermometer (Taiwan TES Electrical Electronic Corp., China), rat rectal temperature thermometer (Beijing Jinuotai Technology Development Co., Ltd., China), Huatuo brand cosmetic acupuncture needles (Suzhou Medical Supplies Co., Ltd., China), fish lines.

1.1.3 Main reagents

Anti-Raf1 (phospho S259) and anti-MEK2 (phospho T394) antibodies (Abcam, UK), phospho-p44/42 MAPK ERK1/2 antibody (Cell Signaling Technology, USA), β-actin antibody (Proteintech, USA), TUNEL apoptosis detection kit (KeyGENE BioTECH, China), 10% chloral hydrate (Tianjin Kermel Chemical Reagent Co., Ltd., China), 1.5% 2,3,5-tripheyl tetrazolium chloride (TTC) solution (Well-Biology Co., Ltd., China).

1.2 Methods

1.2.1 Animal grouping

Ninety healthy SD rats were adaptively fed for 7 d and randomly divided into 3 groups, a blank control group (n=15), a sham operation group (n=15) and a middle cerebral artery occlusion (MCAO) model group (n=60). MCAO rat models were prepared according to the literature[2]. After modeling, rats in the MCAO model group were then randomly divided into a model group, an acupuncture group, a mild hypothermia group and an acupuncture plus mild hypothermia group, 15 rats in each group.

1.2.2 Model preparation

The method developed by Longa EZ, *et al*[2] was referenced. In brief, rats were anesthetized with 10% chloral hydrate [3 mL/(kg·bw)] after fasting for 12 h, and then fixed on the rat board in a supine position. Median incision of the neck was conducted to expose the right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA). The communicating branches and ECA were electrically coagulated after ligation. CCA and ICA were occluded by artery clamp. An oblique incision was performed on the ECA. The end (5 mm in the length) of a monofilament nylon fishing line (3 #) was paraffin embedded, and marked at the site of 18 mm in length. The occlusion line was inserted from the incision for about 18-20 mm (based on the animal body weight), from CCA bifurcation, to embolize the right middle cerebral artery (MCA). Sutured the skin and fixed the trailing end of the occlusion line to the rat skin. Two hours after the ischemia, carefully pulled out the occlusion line for about 8 mm, the reperfusion model was then established. In the sham operation group, only nylon fishing line was inserted for about 10 mm without blocking, and other procedures were same as that in the model group. When the vital signs of the modeling animals were stable (1 h after the CIRI) and the neurologic function was scored 1-3 points, the model was a success and could be used. Rats with successful modeling were fed at 20 ℃, one in each cage, with free access to food and water, and were watered with drip tube if necessary.

1.2.3 Acupoints locations and methods

Acupoints: Baihui (GV 20), Dazhui (GV 14) and Shuigou (GV 26).

Methods: Acupoint was located according to the *Experimental Acupuncture Science*[3], *Laboratory Animal Acupoint Atlas*[4], and simulation of human bone-length measurement method for acupoints. Philtrum is at the middle of 1 mm below the rat cleft and nose tip. Oblique needling was conducted for 2 mm with the needle tip toward the nasal septum. Baihui (GV 20) is in the middle of the parietal bone, and the needle was inserted horizontally for 2 mm. Dazhui (GV 14) is between the 7th cervical vertebra and the 1st thoracic vertebra, at the middle of the back, and perpendicular needling was conducted for 3 mm. Acupuncture treatment was performed once every 12 h, with once needling manipulation during the needle retaining (30 min). A total of 7 acupuncture treatments were conducted.

1.2.4 Mild hypothermia therapy

Mild hypothermia method used in this study was the modification of the one developed by Shu X[5] and Yin YH, *et al*[6]. Temperature measuring: rectal temperature (measured by inserting approximately 4 cm of the thermometer into the rat anus) represented the deep body temperature; eardrum temperature (measured with digital thermometer) represented the brain tissue temperature. When the vital signs of model rats became stable, rats in the mild hypothermia group and the acupuncture plus mild hypothermia group were placed in the metabolic cages with ice packs and processed ice. Rat's rectal temperature should be reduced to (33±1) ℃, and rat eardrum temperature should be reduced to (31±1) ℃ within the first 30 min, measured once every 10 min during this period. Rectal temperature and eardrum temperature were detected once every 1 h, when the temperatures were stable. Mild hypothermia therapy was continued for 72 h. Adjusted the ice pack numbers in the metabolic cages according to the rat's body temperature. The rats were warmed with a stove if rat's body temperature was too low. After 72 h, rats were taken out for natural rewarming, with normal water and food ingestion.

The rats were fastened and fixed for 30 min each time without any treatment, when all the rats, in addition to those in the blank control group, came around; the breathing, heart rate and other vital signs of rats were stable. Rats in the acupuncture group only received acupuncture treatment; rats in the mild hypothermia group only received mild hypothermia therapy; rats in the acupuncture plus mild hypothermia group received both therapies.

1.2.5 Detection indicators and methods

Neurologic injury score: The method for neurologic injury score developed by Longa EZ, *et al*[2] was referenced, and performed immediately in the rats, after reperfusion and consciousness recovery from anesthesia, and repeated once 72 h later. No neurological damage symptom was recorded as 0 point; contralateral forelimb of the embolized artery bending, when lifting rat tail, was recorded as 1 point; rotation toward the contralateral side of the embolized artery when walking was recorded as 2 points; toppling

toward the contralateral side of the embolized artery when walking was recorded as 3 points; loss of spontaneous walking and consciousness was recorded as 4 points.

TTC staining for infarct area ratio: 72 h after the treatment, 5 rats in each group were randomly selected. Rats were anesthetized with 10% chloral hydrate by intraperitoneal injection. The brains were removed quickly by decollation and kept in 20 ℃ refrigerator for 15 min after rapidly rinsed with iced saline. Cut the brain into five slices via 4 coronal incises after the olfactory bulb, and cerebellum and lower brain stem were removed. The first cut was at the midpoint of the line between anterior pole and optic chiasma; the second cut was at the optic chiasma; the third cut was at the infundibular stalk; the fourth cut was between the infundibular stalk and the end pole of posterior lobe. The sections were then quickly placed in 1.5% TTC solution and incubated for 15-30 min at 37 ℃ water bath in dark, fliped once every 5min. Fixed in 10% formalin and photographed after staining. The biggest ischemic cross-section 'A slice' was selected. Scanned and calculated the total area of the uninjured side of the 'A slice' and non-infarcted area of the affected side using MIAS medical image analysis system. In order to eliminate errors due to edema of cerebral infarction hemisphere, the infarct area percentage (IS%) of the 'A slice' was calculated after correction with Swanson method[7].

$IS\% = (S_1 - S_r) \div 2S_1 \times 100\%$ (S_1 was the total area of the uninjured side of the 'A slice'; S_r was the non-infarcted area of the affected side).

TUNEL assay for apoptotic cell number of the ischemic brain tissue: 5 rats in each group were randomly selected and anesthetized. The right side brain was removed quickly on ice by decollation, and fixed in 10% formalin solution, paraffin embedded. Serial sections with a thickness of 2 μm were prepared. The protocol of TUNEL apoptosis detection kit was strictly followed. Positive cells were those with brown stained nuclei. Rat brain tissue immunohistochemistry images were collected by SONY camera, under 400 times amplification optical microscope, and analyzed using MIAS medical image analysis system. Two slices were randomly selected for each rat; five non-overlapping high-power (HP) fields were randomly selected in the hippocampus area for each slice. Apoptotic cell numbers of different fields were calculated and averaged for statistical analysis.

Western blot was used to detect the expression levels of phosphorylated Raf-1, MEK-2 and ERK1/2 proteins in the ischemic hippocampus tissue of each group: three rats were randomly selected from the five of each group. Total proteins were extracted from the hippocampus tissues. The protein concentrations were determined, and then the proteins were transferred to a membrane after the electrophoresis. The membrane was blocked with 5% nonfat milk in 1 × TBST and incubated with the primary antibodies against Raf1 (phospho S259), MEK2 (phospho T394) (ab30622), phospho-p44/42 MAPK ERK1/2 and β-actin respectively, at 4 ℃ overnight, and followed by incubation with the second antibodies of HRP goat anti-mouse IgG or HRP goat anti-rabbit IgG for 45-60 min. The film was scanned after ECL chromogenic exposure, and analyzed by quantity one, the professional grayscale analysis software. Measured the grayscale values of the target bands, and calculated the ratio between the target band and β-actin.

1.3 Statistical analysis

All data were analyzed using SPSS 17.0 statistical software. The normality test was analyzed first. The normal distribution data were statistically described by mean ± standard deviation ($\bar{x} \pm s$). Multiple groups of measurement data were compared using One-way ANOVA. Data with homogeneity of variance were analyzed using least significant difference (LSD). Data with heterogeneity of variance were analyzed using Tamhane's T2 method. Data unfit the normal distributions were statistically described by the median and quartile [M (Q)] and analyzed using the non-parametric tests. $P<0.05$ was used as the test standard.

2 Results

2.1 Influences of acupuncture plus mild hypothermia on rat's nerve function score after CIRI

Compared with the blank control group and the sham operation group, the neurological function score increased in the model, the acupuncture, the mild hypothermia, and the acupuncture plus mild hypothermia groups before treatment. The differences were statistically significant ($P<0.01$), indicating the successful modeling. Differences of neurologic injury scores among all modeling groups were not statistically significant ($P>0.05$); compared with the model group, neurologic injury scores in the acupuncture, the mild hypothermia, the acupuncture plus mild hypothermia groups were all decreased, after the treatments, and the differences between groups were statistically significant ($P<0.05$ or $P<0.01$); compared with the acupuncture group, neurologic injury score was significantly decreased in the acupuncture plus mild hypothermia group, the difference was statistically significant ($P < 0.01$); and differences among the treatment groups were not significant ($P > 0.05$), (Table 1).

Table 1. Comparing neurological function scores of rats before and after the treatment [M (Q), point]

Group	*n*	After reperfusion and stable in rats	After 72 h
Blank control	10	0 (0)	0 (0)
Sham operation	10	0 (0)	0 (0)
Model	10	2 (1)[1)2)]	1.5 (1)[1)2)]
Acupuncture	10	1 (1)[1)2)]	1 (0)[1)2)3)]
Mild hypothermia	10	1.5 (1)[1)2)]	1 (1)[1)2)3)]
Acupuncture plus mild hypothermia	10	1 (1)[1)2)]	0 (1)[1)2)4)5)]

Note: Compared with the blank control group, 1) $P<0.01$; compared with the sham operation group, 2) $P<0.01$; compared with the model group, 3) $P<0.05$, 4) $P<0.01$; compared with the acupuncture group, 5) $P<0.01$

2.2 Influences of acupuncture plus mild hypothermia on rat's cerebral infarction area after CIRI

After 72 h, the normal brain tissue was red while the infarction region was white, after TTC staining of the rat brain tissue sections. No infarction lesions were found in the blank control group and the sham operation group. White infarction lesions were obvious in the model group, indicating the successful modeling (Figure 1, the arrows indicated the infarction locations).

Compared with the blank control group and the sham operation group, the infarction area ratios in the model group, the acupuncture group, the mild hypothermia group, and the acupuncture plus mild hypothermia group were all significantly increased after 72 h. Differences between groups were all statistically significant ($P<0.01$). Compared with the model group, the infarction area ratios in the acupuncture group, the mild hypothermia group and the acupuncture plus mild hypothermia group were all decreased, differences between groups were all statistically significant ($P<0.05$ or $P<0.01$); there were no statistically significant differences among the treatment groups ($P>0.05$), (Table 2).

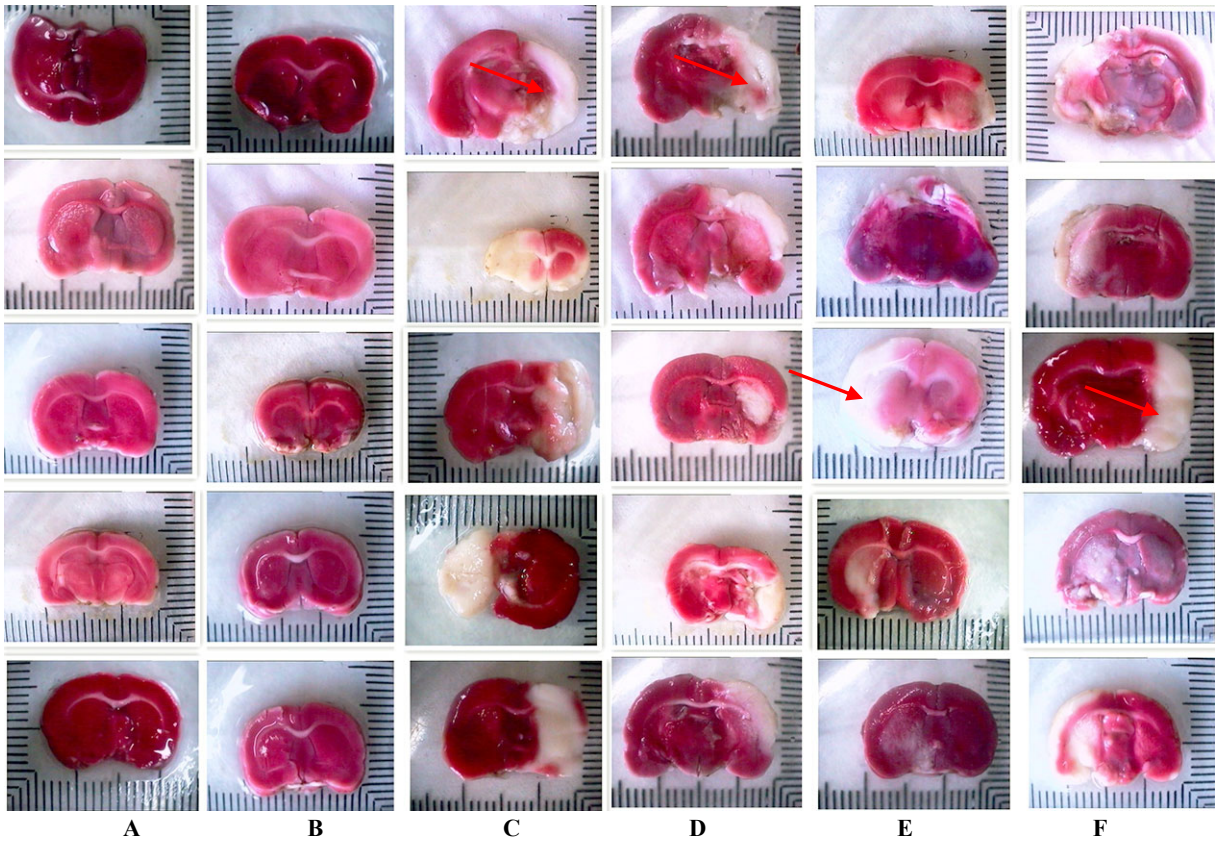


Figure 1. TTC staining of the infarction area in each group (Note: A=Blank control group; B=Sham operation group; C=Model group; D=Acupuncture group; E=Mild hypothermia group; F=Acupuncture plus mild hypothermia group)

2.3 Influences of acupuncture plus mild hypothermia on apoptosis of the cerebral ischemia side after CIRI

Five rats in each group, two slices of each rat, and the average of 5 different microscopic high-power (HP) fields in the hippocampus of the affected side of each slice were selected. This resulted in 10 data of each group. Removed the highest and the lowest numbers in each group, and the remaining 8 data were used for

statistical analysis. Apoptotic nuclei appeared yellow brown or dark brown under the microscope after TUNEL staining; the normal cell nuclei appeared blue after hematoxylin counterstaining.

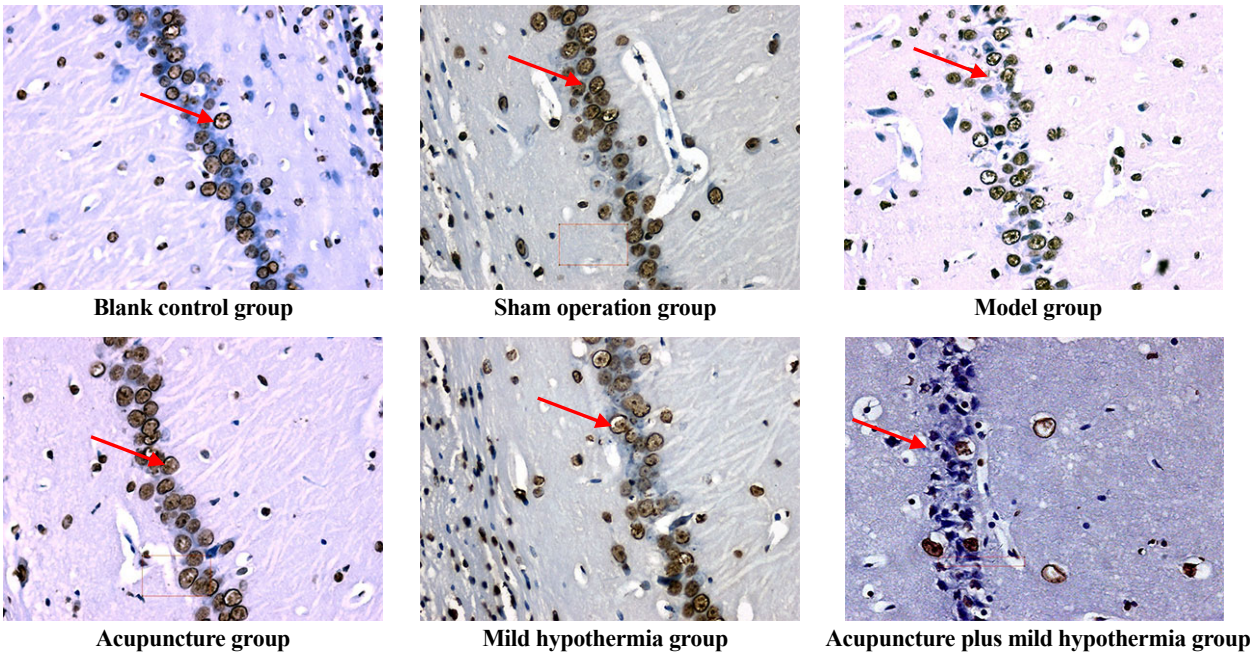
After 72 h, only a few apoptotic cells in the rat brain tissues of the blank control and the sham operation groups; there was a large number of brown nuclei in the model group, indicating a large number of apoptotic cells (Figure 2, the arrows indicated the apoptotic cells).

After 72 h, compared with the blank control and sham operation groups, apoptotic cell numbers in the model group were increased significantly; differences between groups were statistically significant ($P<0.05$). Compared with the model group, apoptotic cell numbers in each treatment group were decreased; differences between groups were statistically significant ($P < 0.01$). The differences were not statistically significant among the treatment groups ($P>0.05$), (Table 3).

Table 2. Comparing the ischemic infarction area ratio in each group [M (Q), %]

Group	*n*	Infarction area ratio after 72 h
Blank control	5	0 (0)
Sham operation	5	0 (0)
Model	5	34.46 (9.00)[1)2)]
Acupuncture	5	19.79 (18.18)[1)2)3)]
Mild hypothermia	5	21.77 (9.55)[1)2)]
Acupuncture plus mild hypothermia	5	8.91 (13.39)[1)2)4)]

Note: Compared with the blank control group, 1) $P<0.01$; compared with the sham operation group, 2) $P<0.01$; compared with the model group, 3) $P<0.05$, 4) $P<0.01$



Blank control group **Sham operation group** **Model group**

Acupuncture group **Mild hypothermia group** **Acupuncture plus mild hypothermia group**

Figure 2. Nerve cell staining in ischemia-side rat brain tissue of each group (TUNEL, ×400)

Table 3. Comparing apoptotic cell numbers in rat's ischemia side of each group ($\bar{x}$ ±*s*)

Group	*n*	Number of apoptotic cells after 72 h
Blank control	5	76.00±11.59
Sham operation	5	75.75±15.34
Model	5	92.00±12.94[1)2)]
Acupuncture	5	70.13±11.32[3)]
Mild hypothermia	5	70.25±8.03[3)]
Acupuncture plus mild hypothermia	5	64.75±17.70[3)]

Note: Compared with the blank control group, 1) $P<0.05$; compared with the sham operation group, 2) $P<0.05$; compared with the model group, 3) $P<0.01$

2.4 Influences of acupuncture plus mild hypothermia on protein expression of phosphorylated Raf1, MEK2 and ERK1/2 in rat's cerebral ischemia hippocampus tissues after CIRI

Western blot assay was use to detect the protein expressions of p-Raf1, p-MEK2 and p-ERK1/2 of rat's cerebral ischemia hippocampus tissues in each group. 72 h after the rat's CIRI, protein expressions of p-Raf1, p-MEK2 and p-ERK1/2 in the model group were significantly increased compared with those in the sham operation group; while compared with the model group, those were decreased in each treatment group (Figure 3).

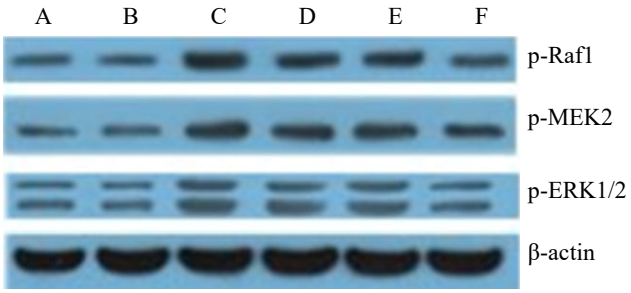


Figure 3. Raf-1, MEK-2, ERK-1/2 and β-actin protein expressions in ischemia-side rat's brain tissue of each group (Note: A=Blank control group, B=Sham operation group, C=Model group, D=Acupuncture group, E=Mild hypothermia group, F=Acupuncture plus mild hypothermia group)

Compared with the blank control group and the sham operation group, p-Raf1, p-MEK2 and p-ERK1/2 protein expressions in rat's hippocampal tissues were increased in the model group, the acupuncture group, the mild hypothermia group and the acupuncture plus mild hypothermia group after CIRI, and the differences between groups were statistically significant ($P<0.05$ or $P<0.01$); compared with the model group, p-Raf1, p-MEK2 and p-ERK1/2 protein expressions in rat's hippocampal tissues in the treatment groups were decreased, and the differences between groups were statistically significant (all $P<0.01$); compared with the acupuncture group, p-Raf1, p-MEK2 and p-ERK1/2 protein expressions in rat's hippocampal tissues were decreased in the acupuncture plus mild hypothermia group, and the differences were statistically significant ($P<0.01$ or $P<0.05$), while the p-Raf1, p-MEK2 and p-ERK1/2 protein expressions in rat's hippocampal tissues in the mild hypothermia group showed slight changes, the intra-group differences were not statistically significant ($P>0.05$); compared with the mild hypothermia group, p-Raf1, p-MEK2 and p-ERK1/2 protein expressions in rat's hippocampal tissues were decreased in the acupuncture plus mild hypothermia group, the differences were statistically significant ($P<0.01$), (Table 4).

Table 4. Comparing phosphorylated Raf-1, MEK-2 and ERK-1/2 protein expressions in ischemia-side hippocampus tissues in each group ($\bar{x}\pm s$)

Group	*n*	p-Raf1	p-MEK2	p-ERK1	p-ERK2
Blank control	3	0.28±0.04	0.30±0.01	0.24±0.01	0.31±0.01
Sham operation	3	0.28±0.02	0.29±0.01	0.22±0.02	0.29±0.04
Model	3	0.57±0.03[2)3)]	0.56±0.04[2)3)]	0.48±0.04[2)3)]	0.56±0.03[2)3)]
Acupuncture	3	0.50±0.01[2)3)4)]	0.46±0.03[2)3)4)]	0.37±0.04[2)3)4)]	0.43±0.01[2)3)4)]
Mild hypothermia	3	0.49±0.03[2)3)4)]	0.45±0.02[2)3)4)]	0.38±0.04[2)3)4)]	0.45±0.04[1)3)]
Acupuncture plus mild hypothermia	3	0.40±0.03[2)3)4)6)7)]	0.39±0.02[2)3)4)6)7)]	0.31±0.02[1)3)4)5)7)]	0.37±0.03[1)3)4)5)7)]

Note: Compared with the blank control group, 1) $P<0.05$, 2) $P<0.01$; compared with the sham operation group, 3) $P<0.01$; compared with the model group, 4) $P<0.01$; compared with the acupuncture group, 5) $P<0.05$, 6) $P<0.01$; compared with the mild hypothermia group, 7) $P<0.01$

3 Discussion

Acupuncture treatment is a therapeutic tool with the purpose of treating disease by stimulating acupoints to dredge the meridians and regulate Zang-fu organs[8]. The basic pathogenesis of stroke is yin and yang imbalance, qi and blood disorders, thus invading the brain. The disease is located in the brain. The Governor Vessel is in the middle of the back, up to Fengfu (GV 16), into the brain and to the top of head, closely associated with the brain. Therefore, treatment should focus on the Governor Vessel. Shuigou (GV 26), Baihui (GV 20) and Dazhui (GV 14) are acupoints on the Governor Vessel, commonly used in the treatment of stroke. As an important treatment for CIRI, mild hypothermia therapy can reduce free radicals production[9], regulate phospholipase C-γ1[10], inhibit the expression of Caspase-3[11] and up-regulate Bcl-2 and down-regulate Bax[12]. Studies confirmed that, if the body temperatures of patients, with acute ischemic cerebrovascular disease, were higher than normal, when they were hospitalized, the disability rate and mortality in patients were significantly increased[13]. Down-regulating the body temperatures of patients in the early stage of CIRI could effectively decrease the disability rate and mortality.

CIRI can lead to nerve cell death; necrosis is the main pathological factor in the central area; apoptosis is the main pathological factor in the ischemic penumbra. If given treatment in the effective time, the ischemic penumbra can be restored, and this is of important significance for reducing apoptosis. Apoptosis can be regulated by ERK1/2, the related kinase in the MAPK/ERK pathway. Studies have shown that ERK1/2 phosphorylation induced in spinal cord in ischemia-

reperfusion injury rats could mediate apoptosis of damaged neurons[14].

Ras/Raf/MEK/ERK cell signaling pathway is the cascade reaction caused by connection of guanosine triphosphate (GTP) binding protein to receptor tyrosine kinase and cytoplasmic protein kinase, belongs to the MAPK pathway. The core of MAPK pathway is the protein kinase reaction chain, and composed of MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK. Raf, MEK and ERK are respectively from the MAPKKK, MAPKK and MAPK. Phosphorylation of Raf-1 activates the downstream substrate MEK-2; the activated MEK-2 phosphorylates and activates the downstream substrate ERK; the phosphorylated ERK then activates other downstream substrates, which can regulate a variety of cellular responses[15]. There are two subtypes of ERK, therefore, ERK protein bands show two sub-bands of ERK1 and ERK2, with molecular weights of 44kD and 42kD. After CIRI, Ras/Raf/MEK/ERK cell signaling pathway is activated and then regulates the cell differentiation and apoptosis. Apoptosis involves in the normal development and aging process *in vivo* and is a protective response during aging or injury[16-17], which is accomplished via apoptosis signal transmitted by different signaling pathways[18-19]. However, apoptosis can cause dysfunction in the body, when the cell numbers drop to a certain level due to the apoptosis. Appropriate treatment can reduce apoptosis[18]. How to effectively reduce the neuronal apoptosis and increase nerve cell survival after CIRI, has become an important topic[20].

Results of this experiment showed that acupuncture treatment, mild hypothermia, and acupuncture plus mild hypothermia therapy all could improve neurologic injury, decrease cerebral infarction area and apoptosis, and down-regulate the phosphorylated Raf-1, MEK-2 and ERK1/2 after CIRI in rats, when the initiation of the treatment was immediately after the rat models were successfully established and the vital signs were stable. Acupuncture plus mild hypothermia therapy was better than acupuncture treatment in reducing the neurologic injury score, which showed certain significance for the guidance of improving stroke symptoms.

Our results showed that the acupuncture treatment and mild hypothermia therapy had no statistically significant differences in down-regulation of phosphorylated Raf-1, MEK-2 and ERK1/2 in CIRI rats, suggesting that acupuncture treatment and mild hypothermia therapy had no significant difference in regulating MAPK/ERK pathway. However, compared with the acupuncture treatment or mild hypothermia therapy, acupuncture plus mild hypothermia therapy could significantly down-regulate phosphorylated Raf-1, MEK-2 and ERK1/2, and the differences between groups were statistically significant, indicating that acupuncture plus mild hypothermia therapy should be better than acupuncture treatment or mild hypothermia therapy in reducing phosphorylation of Raf-1, MEK-2 and ERK1/2.

Therefore, we could conclude that one of the mechanisms of acupuncture, mild hypothermia, acupuncture plus mild hypothermia in the treatment of CIRI, may be through reducing the phosphorylation of Raf-1, MEK-2 and ERK1/2, and apoptosis. The acupuncture plus mild hypothermia group was better than the acupuncture group and the mild hypothermia group, which was probably caused by the additive or synergistic effects of acupuncture treatment and hypothermia therapy further down-regulated the phosphorylated Raf-1, MEK-2 and ERK1/2. However, some studies showed that one of the protective mechanisms of acupuncture on nerve cells in cerebral ischemia rats may be through improving the expression of phosphorylated ERK[21], which is distinct from the present experiment results. Therefore, further studies on the changes of the MAPK/ERK pathway in CIRI are necessary, to clarify the specific proteins involved and provide more theoretical basis for the clinical practice.

Conflict of Interest

The authors declared that there was no potential conflict of interest in this article.

Acknowledgments

This work was supported by National Natural Science Foundation of China (国家自然科学基金项目, No. 81303051); Traditional Chinese Medicine Science Research Planning Project of Hunan Province (湖南省中医药科研计划项目, No. 201471); Province and Ministry Co-construction Key Laboratory for Internal Medicine of Traditional Chinese Medicine of the Education Ministry of China (中医内科学省部共建教育部重点实验室, No. ZYNK201501).

Statement of Human and Animal Rights

The treatment of animals conformed to the ethical criteria in this experiment.

Received: 14 December 2015/Accepted: 26 January 2016

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