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Basic Study

Effects of acupuncture plus mild hypothermia on apoptosis-related factors in rats with cerebral ischemia-reperfusion

针刺联合亚低温对脑缺血再灌注大鼠凋亡相关因子的影响

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Abstract

Objective: To investigate the effect of acupuncture plus mild hypothermia on neurological function impairment score, cerebral infarct size and apoptosis-related factors in cerebral ischemia reperfusion injury (CIRI) rats.

Methods: Sixty healthy male Sprague-Dawley (SD) rats were routinely reared for 1 week. Ten rats were randomly selected as the sham operation group and 10 rats as the blank control group, while the remaining 40 rats were subjected to preparing the middle cerebral artery occlusion (MCAO) model by modified filament occlusion method. The 40 MCAO rats were further randomly divided into a model group, an acupuncture group, a mild hypothermia group and an acupuncture plus mild hypothermia group, with 10 rats in each group. Rats in the sham operation group, the blank control group and the model group did not accept treatment except binding; rats in the acupuncture group received acupuncture treatment; rats in the mild hypothermia group received mild hypothermia treatment; rats in the acupuncture plus mild hypothermia group received acupuncture and mild hypothermia treatment. 72 h after the treatment, neurological function impairment score was performed; the infarct area ratio was determined by 2,3,5-tripheyl tetrazolium chloride (TTC) staining; apoptosis of brain cells was observed by TUNEL method; the expressions of Bcl-2, Bax and Caspase-3 were detected by immunohistochemistry.

Results: Compared with the blank control group and the sham operation group, the neurological function impairment score, cerebral infarct area ratio, apoptosis, and the expressions of Bax and Caspase-3 in the model group were significantly increased, while the expression of Bcl-2 was significantly decreased, and there were significant between-group differences (all $P<0.05$). After the treatment, there were statistically significant differences among the treatment groups in the neurological function impairment score, cerebral infarct area ratio and apoptosis in the ischemic side of rats, as well as the expressions of Bcl-2, Bax and Caspase-3 (all $P<0.05$), and from the figures, tables and statistical analysis it was found that a better tendency in the acupuncture plus mild hypothermia group than the acupuncture or mild hypothermia group.

Conclusion: Acupuncture plus mild hypothermia can protect the brain cells by improving neurological function impairment, decreasing cerebral infarct area ratio, reducing the number of apoptotic cells in the ischemic area and regulating the expressions of apoptosis related proteins to inhibit apoptosis.

Keywords: Acupuncture Therapy; Reperfusion Injury; Hypothermia, Induced; Brain Ischemia; Apoptosis; Caspases; Rats

【摘要】目的：探讨针刺联合亚低温方法对脑缺血再灌损伤(CIRI)大鼠神经功能缺损评分、脑梗死面积及细胞凋亡相关因子的影响。**方法：**将60只Sprague-Dawley (SD)健康雄性大鼠常规饲养1星期后随机选取10只入假手术组，10只入空白组,其余40只根据线拴法并改良以复制大脑中动脉缺血(MCAO)再灌注模型，待造模成功后再将40只大鼠随机分为模型组、针刺组、亚低温组和针刺联合亚低温组，每组10只。假手术组、空白组和模型组大鼠只捆绑不治疗，针刺组接受针刺治疗，亚低温组接受亚低温治疗，针刺联合亚低温组接受针刺和亚低温治疗。治疗72 h后进行神经功能缺损评分，使用氯化三苯基四氮唑(TTC)染色检测梗死面积比，TUNEL 法观察脑细胞凋亡情况，免疫组化检测Bcl-2、Bax、Caspase-3 的表达。**结果：**与空白组及假手术组比较，模型组大鼠神经功能缺损评分、梗死面积比、细胞凋亡、Bax、Caspase-3表达水平明显增高，Bcl-2表达水平显著下降，组间差异均有统计学意义

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(均 $P<0.05$)。治疗后神经功能缺损评分、梗死面积比、大鼠缺血侧细胞凋亡、以及 Bcl-2、Bax 和 Caspase-3 表达各治疗组间有统计学意义(均 $P<0.05$), 且从图、表或统计分析可发现针刺联合亚低温组有优于针刺组和亚低温组的趋势。**结论**：针刺联合亚低温治疗可通过改善神经功能缺损、减少脑梗死面积比、降低缺血区凋亡细胞数量及调整凋亡相关蛋白表达抑制凋亡，从而实现对脑细胞的保护作用。

【关键词】针刺疗法；再灌注损伤；低温，人工；脑缺血；细胞凋亡；半胱天冬酶；大鼠

【中图分类号】R2-03　　**【文献标志码】**A

Cerebral ischemia reperfusion injury (CIRI) refers to cerebral ischemia resulting in brain cell damage, while the ischemic injury will be further aggravated when blood supply returns to the damaged tissue, which is the main pathogenic cause of the cerebrovascular disease. Cerebrovascular disease has the characteristics of high morbidity, high disability rate and high mortality[1]. In previous studies we found that acupuncture at Dazhui (GV 14), Baihui (GV 20) and Shuigou (GV 26) could improve the neurological function impairment, reduce cerebral infarct area of middle cerebral artery occlusion (MCAO) in model rats, therefore, to protect the brain[2].

Mild hypothermia therapy is also one of the important protective measures of brain cells. Mild hypothermia treatment can significantly reduce brain damage after ischemia, and also play a role in promoting the recovery of brain function[3], and has been widely used in clinical practice.

Therefore, in this experiment, rat MCAO cerebral ischemia-reperfusion model was made by the filament occlusion method, and the effects of acupuncture plus mild hypothermia at Dazhui (GV 14), Baihui (GV 20) and Shuigou (GV 26) on Bcl-2, Bax, Caspase-3, apoptosis, neurological function impairment scores and cerebral infarct area ratio in CIRI rats were observed.

The experimental methods and results were represented as follows.

1 Materials and Methods

1.1 Materials

1.1.1 Experimental animals

Sixty SPF grade 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 (Hunan) 2013-0004.

1.1.2 Main reagents

Bcl-2, Bax and Caspase-3 kits (Wuhan Boster Biological Engineering Co., Ltd., China); TUNEL apoptosis in situ detection kit (KGA7025-A, KGA7025-B, KeyGENE BioTECH, China); 1.5% 2,3,5-tripheyl tetrazolium chloride (TTC) solution (C19H15, Well-Biology Co., Ltd., China); 4% paraformaldehyde (WB0401, Well-Biology Co., Ltd., China); DEPC H2O (WB0006, Well-Biology Co., Ltd., China); 10% chloral hydrate (Tianjin Kermel Chemical Reagent Co., Ltd., China); PBS solution (WB1001, Well-Biology Co., Ltd., China).

1.1.3 Main instruments

Digital thermometer (TES 1310 TYPE-K, Taiwan TES Electrical Electronic Corp., China); rectal temperature thermometer (Beijing Jinuotai Technology Development Co., Ltd., China); Huatuo brand cosmetic acupuncture needles (Suzhou Medical Supplies Co., Ltd., China); MIAS medical image analysis system (Beijing BUAA Tianhua Technology Co., Ltd., China).

1.2 Methods

1.2.1 Animal grouping

Sixty healthy SD rats were adaptively fed for 7 d at the Experimental Animal Center of Hunan University of Chinese Medicine. During the period, rats had free access to food and water. A week later, all the rats were randomly divided into 3 groups, a blank control group ($n=10$), a sham operation group ($n=10$) and a MCAO model group ($n=40$). After MCAO models were successfully prepared, the 40 MCAO rats were further randomly divided into a model group, an acupuncture group, a mild hypothermia group and an acupuncture plus mild hypothermia group, with 10 rats in each group. Five rats in each group were used for TTC staining, and the other five rats were used to detect Bcl-2, Bax, Caspase-3 and apoptosis cell number.

1.2.2 Model preparation

The model was prepared using the modified filament occlusion method developed by Longa EZ, *et al*[4]. 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 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

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skin. Two hours after the ischemia, carefully pulled out the occlusion line by about 8 mm, and 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 those in the model group. When the vital signs (breathing, heart rate) of the model animals were stable (1 h after the CIRI) and the neurological 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

The acupoints were located according to the *Experimental Acupuncture Science*[5], *Laboratory Animal Acupoint Atlas*[6], and simulation of human bone-length measurement method for acupoints. Dazhui (GV 14) is between the 7th cervical vertebra and the 1st thoracic vertebra, in the middle of the back; Baihui (GV 20) is at the center of the parietal bone; Shuigou (GV 26) is in the middle of rat's lip cleft and 1 mm below the nose tip.

1.2.4 Acupuncture method

Hwato brand cosmetic acupuncture needles (0.19 mm in diameter and 10 mm in length) were selected for the acupuncture therapy. Perpendicular needling was conducted by a depth of 3 mm at Dazhui (GV 14); subcutaneous needling was conducted at Baihui (GV 20) by 2 mm; oblique needling was conducted at Shuigou (GV 26) by 2 mm with the needle tip toward the nasal septum. Rats in the acupuncture group and acupuncture plus mild hypothermia group were given acupuncture therapy when the vital signs (breathing, heart rate) were stable (1 h after the CIRI), with needles manipulated every 15 min during the needle retaining, and the total needle retaining time was 30 min. The treatment was conducted once every 12 h for a total of 7 times.

1.2.5 Mild hypothermia therapy

Mild hypothermia method used in this study was the modified version of the one developed by Shu X[7] and Yin YH, *et al*[8].

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 the model rats became stable (1 h after the CIRI), rats in mild hypothermia group and 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 in sham operation and model groups were fastened and fixed for 30 min each time without any treatment, when all the rats, except those in the blank control group, came around and breathing, heart rate and other vital signs were stable. Rats in the acupuncture group only received acupuncture treatment; rats in mild hypothermia group only received mild hypothermia therapy; rats in acupuncture plus mild hypothermia group received both therapies.

1.3 Detection indicators and methods

1.3.1 Neurological function impairment score

After successful modeling of CIRI rats, when the vital signs (breathing, heart rate) of the model rats became stable (1 h after the CIRI), the neurological function impairment was evaluated immediately. After 72 h of treatment, the neurological function scores were further evaluated before the rats were sacrificed, using the 5-grade 4-point standard developed by Longa EZ, *et al*[4]. No neurological damage symptom was recorded as 0 point; contralateral forelimb of the embolized artery failed to straighten, when rat's tail was lifted 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. Rats with the score of 1 to 3 points were included in the experimental observation.

1.3.2 Cerebral infarct area ratio

After 72 h of continuous treatment, the rats were anesthetized with 10% chloral hydrate intraperitoneally. The brains were removed quickly by decollation and kept in 20 ℃ refrigerator for 5 min after rapidly rinsed in iced saline. Removed the polus frontalis and cut into 5 consecutive brain slices by 2 mm from the optic chiasm.

The sections were then quickly placed in 1.5% TTC solution and incubated for 15-30 min at 37 ℃ in dark and flipped once every 5 min, so that the sections could uniformly contact with the staining solution. After staining (normal tissue was red, infract area was white), the sections were placed in 10% formaldehyde in dark at 4 ℃ for 24 h. The pictures were obtained by a digital camera and transferred into the computer. The infract area of the biggest ischemic cross-section 'slice

A' was selected. The infarct area was represented as the percentage of the 'slice A' accounting for the total area of the brain. Scanned and calculated the infarct area and non-infarct area of the 'slice A' using MIAS medical image analysis system to determine the cerebral infract area ratio.

The infarct area percentage (IS %) of the 'slice A', indicating the degree of infarction, was calculated after correction with Swanson RA method[9].

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

1.3.3 TUNEL assay

Five rats in each group were randomly selected and anesthetized. The right side of the brain was removed quickly on ice by dislocation, 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 provided by KeyGENE BioTECH was strictly followed. Positive cells (apoptotic cells) were those with brown stained nuclei. Immunohistochemistry images of rat's brain tissues were collected by SONY camera, under 400 times amplification optical microscope, and analyzed using Biomias2001 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 (2.16 mm $\times$ 1.62 mm). Apoptotic cell numbers of different fields were calculated and averaged for statistical analysis. Apoptotic (positive) cells were counted by mouse click method[10].

1.3.4 Immunohistochemistry assay

Steps of immunohistochemical detection for Bcl-2, Bax and Caspase-3: conventional dewaxing was performed for the tissue sections; tissue sections were pre-treated according to the special requirements of the first antibody; incubated with 3% H_2O_2 deionized H_2O for 10 min to block the endogenous peroxidase activity; washed with PBS for 2 min $\times$ 3 times; incubated with the appropriate dilution of Bcl-2, Bax or Caspase-3 primary antibody at room temperature or 37 ℃ for 1-2 h or overnight at 4 ℃, respectively; washed with PBS for 2 min × 3 times; incubated with Polymer Helper at room temperature or 37 ℃ for 20 min and washed with PBS for 2 min $\times$ 3 times; incubated with Polyperoxidase-anti-mouse/rabbit IgG at room temperature or 37 ℃ for 20-30 min and washed with PBS for 2 min $\times$ 3 times; colored with DAB solution; rinsed with tap water and followed by counterstaining, dehydrating, transparent treatment and sealing film. Cytoplasm with brown particles was Bcl-2, Bax or Caspase-3 positive cells under light microscope (10 $\times$ 40). Positive cells were counted using the same method as used in TUNEL assay.

1.4 Statistical analysis

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 unfitting the normal distributions were statistically described by the median and quartile [M(Q)] and analyzed using the nonparametric tests. $P<0.05$ was used as the test standard.

2 Results

2.1 Influences of acupuncture plus mild hypothermia on neurological function impairment in CIRI rats

After successful modeling, rats in the model group all showed corresponding neurological function impairment, and the neurological function impairment score was significantly higher than that in the sham operation group and blank control group. The differences among groups were statistically significant ($P < 0.01$). There was no statistically significant difference in neurological function impairment between each modeling group before treatment ($P > 0.05$). Compared with the blank control group and sham operation group, the neurological function impairment scores in the model group and each treatment group were still significantly different ($P<0.01$) after 72 h of treatment. Compared with the model group, there was a significant difference in each treatment group ($P<0.05$). Compared with acupuncture plus mild hypothermia group, the difference was statistically significant in the acupuncture group ($P<0.05$), while there was no significant difference in mild hypothermia group ($P>0.05$), (Table 1).

Table 1. Comparison of neurological function impairment scores before and after treatment in each group ($\bar{x} \pm s$, point)

Group	n	Before treatment	72 h after treatment
Blank control	10	0.00 (0)	0.00 (0)
Sham operation	10	0.00 (0)	0.00 (0)
Model	10	1.50 (1)[1)2)]	1.50 (1)[1)2)]
Acupuncture	10	1.50 (1)[1)2)]	1.00 (0)[1)2)3)5)]
Mild hypothermia	10	2.00 (1)[1)2)]	1.00 (1)[1)2)4)]
Acupuncture plus mild hypothermia	10	2.00 (1)[1)2)]	0.00 (1)[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) $P<0.01$, compared with the acupuncture plus mild hypothermia group, 5) $P<0.05$

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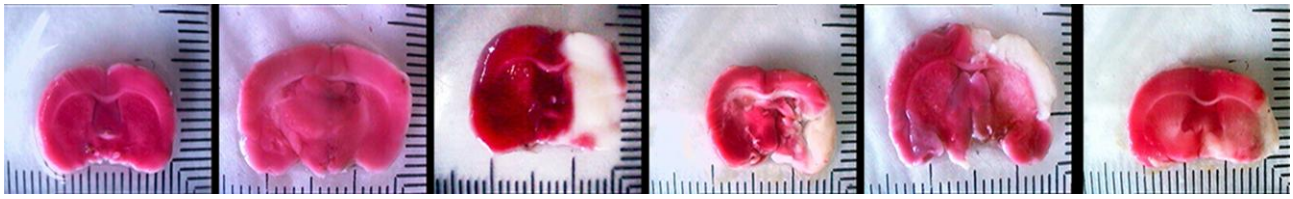
2.2 Influences of acupuncture plus mild hypothermia on cerebral infarct area ratio in CIRI rats

The brain tissue sections of each group were stained by TTC, the infarct area was white, and the normal area was red. Compared with the blank control group and sham operation group, the cerebral infarct area ratio in the model group, acupuncture group, mild hypothermia group increased significantly, the difference was statistically significant ($P<0.01$); statistically significant in acupuncture plus mild hypothermia group ($P<0.05$). Compared with the model group, the cerebral infarct area ratio was reduced in each treatment group, the difference was statistically significant ($P < 0.05$). Compared with acupuncture plus mild hypothermia group, the cerebral infarct area ratio was significantly different in the blank control group, sham operation group and acupuncture group ($P < 0.05$), and statistically significantly different in the model group and mild hypothermia group ($P<0.01$). Each treatment group showed a reduced infarct area ratio at varying degrees, and acupuncture plus mild hypothermia group was better than both acupuncture group and mild hypothermia group (Table 2, Figure 1).

Table 2. Comparison of cerebral infarct area ratio after treatment in each group ($\bar{X} \pm s$)

Group	*n*	Infarct area ratio
Blank control	5	0.00 (0)[6)7)]
Sham operation	5	0.00 (0)[6)7)]
Model	5	34.46 (9)[2)4)8)]
Acupuncture	5	19.79 (18.18)[2)4)6)7)]
Mild hypothermia	5	21.77 (9.55)[2)4)5)8)]
Acupuncture plus mild hypothermia	5	8.91 (13.39)[1)3)6)]

Note: Compared with the blank control group, 1) $P<0.05$, 2) $P<0.01$; compared with sham operation group, 3) $P<0.05$, 4) $P<0.01$; compared with model group, 5) $P<0.05$, 6) $P<0.01$; compared with acupuncture plus mild hypothermia group, 7) $P<0.05$, 8) $P<0.01$



A B C D E F

Figure 1. TTC staining of the infarct area in each group after treatment

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 Effects of acupuncture plus mild hypothermia on the apoptosis and Bcl-2, Bax, Caspase-3 positive cell numbers in CIRI rats

Cell apoptosis and Bcl-2, Bax, Caspase-3 staining were observed under a 10 × 40 magnification optical microscope. The number of positive apoptotic cells in the model group was the highest, showing a dense strip distribution. Compared with the model group, the positive apoptotic cells in other groups were less.

A large number of apoptotic cells with buffy or brown color, concentrated, dense, irregular shape and different sized nuclei appeared around the cerebral ischemia area of rats in the model group. Nuclei of the normal cells were stained blue. Compared with the blank control group and sham operation group, the number of positive cells of Bax, Caspase-3 and apoptosis in the model group increased, showing the massive and dense distribution; the number of Bcl-2 positive cells was decreased and the distribution of Bcl-2 positive cells was scattered, there was statistically significant difference among groups ($P<0.05$), while there was no statistically significant difference among acupuncture group, mild hypothermia group and acupuncture plus mild hypothermia group ($P>0.05$). Compared with the model group, there was statistically significant difference in the treatment groups ($P<0.05$). There was no statistically significant difference among the 3 treatment groups ($P>0.05$). However, the number of positive cells and the levels of Bax and Caspase-3 in the 3 treatment groups were significantly decreased, Bcl-2 expression was increased, and acupuncture plus mild hypothermia group showed a tendency to be superior to both acupuncture group and mild hypothermia group (Table 3, Figure 2-Figure 5).

Table 3. Comparing the number of apoptosis-related factors of rats in each group after treatment ($\bar{X} \pm s$)

Group	*n*	Apoptotic cells	Positive Bcl-2 cell	Positive Bax cell	Positive Caspase-3 cell
Blank control	5	76.00±11.59[5)]	66.75±19.89[6)]	49.88±21.21[6)]	40.50±13.15[6)]
Sham operation	5	77.63±14.52[5)]	60.75±28.32[6)]	53.00±28.90[5)]	56.50±26.47[6)]
Model	5	93.00±13.86[1)3)]	24.25±17.17[2)4)]	90.50±51.02[2)3)]	120.25±39.68[2)4)]
Acupuncture	5	67.63±11.54[6)]	48.00±27.19[5)]	58.00±22.32[5)]	57.75±23.68[6)]
Mild hypothermia	5	70.25±8.03[6)]	50.38±22.15[5)]	55.13±17.03[5)]	45.13±11.27[6)]
Acupuncture plus mild hypothermia	5	64.75±17.70[6)]	61.50±14.58[6)]	50.88±25.45[5)]	41.88±14.90[6)]

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

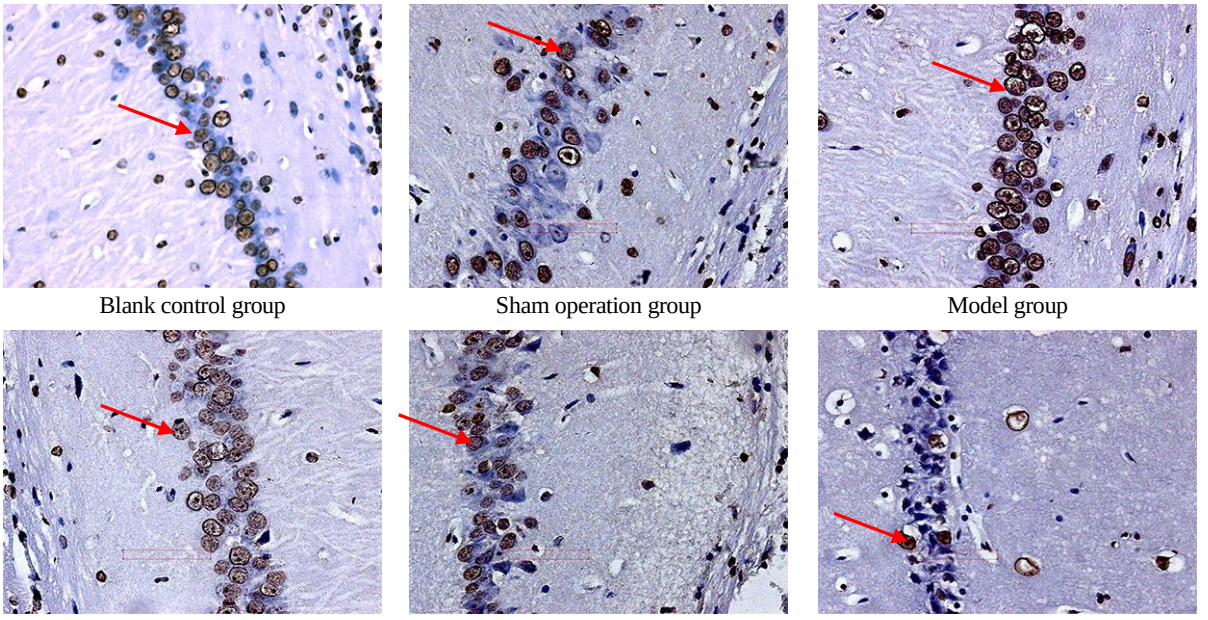


Figure 2. Positive apoptotic cells in each group after treatment

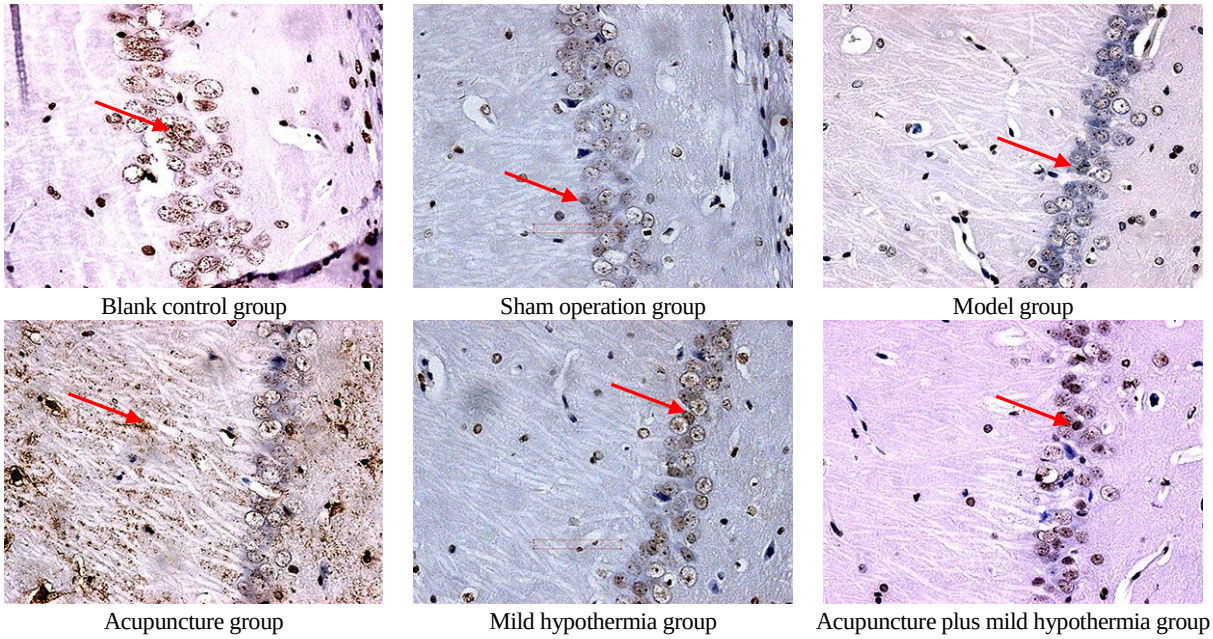


Figure 3. Positive Bcl-2 cells in each group after treatment

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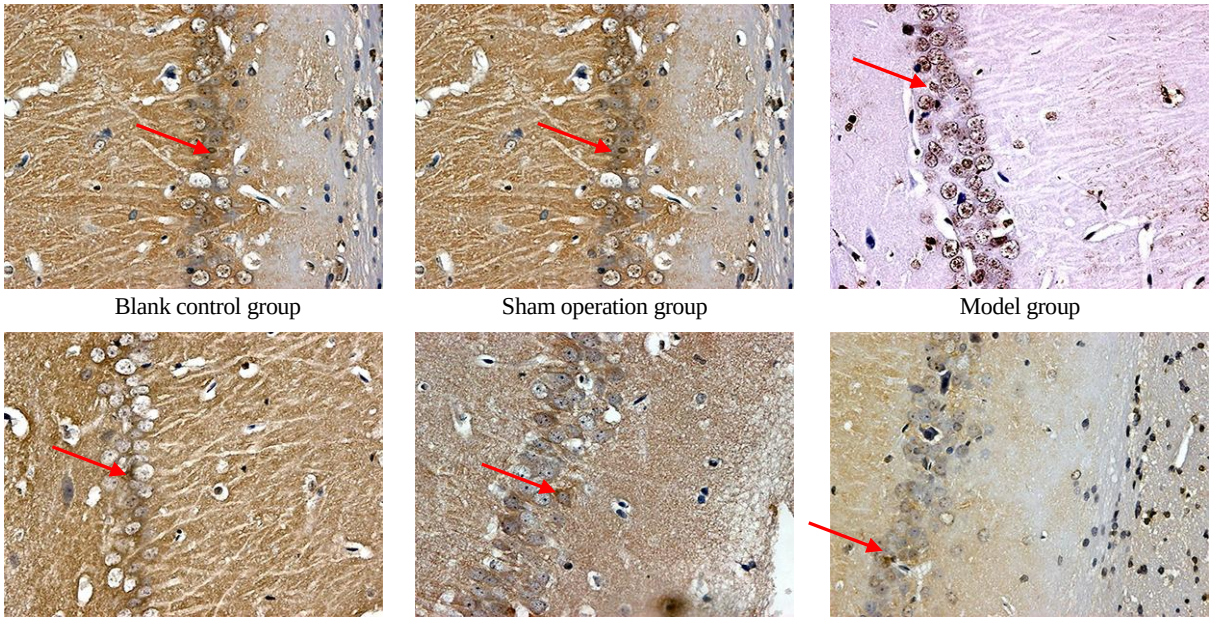

Figure 4. Positive Bax cells in each group after treatment

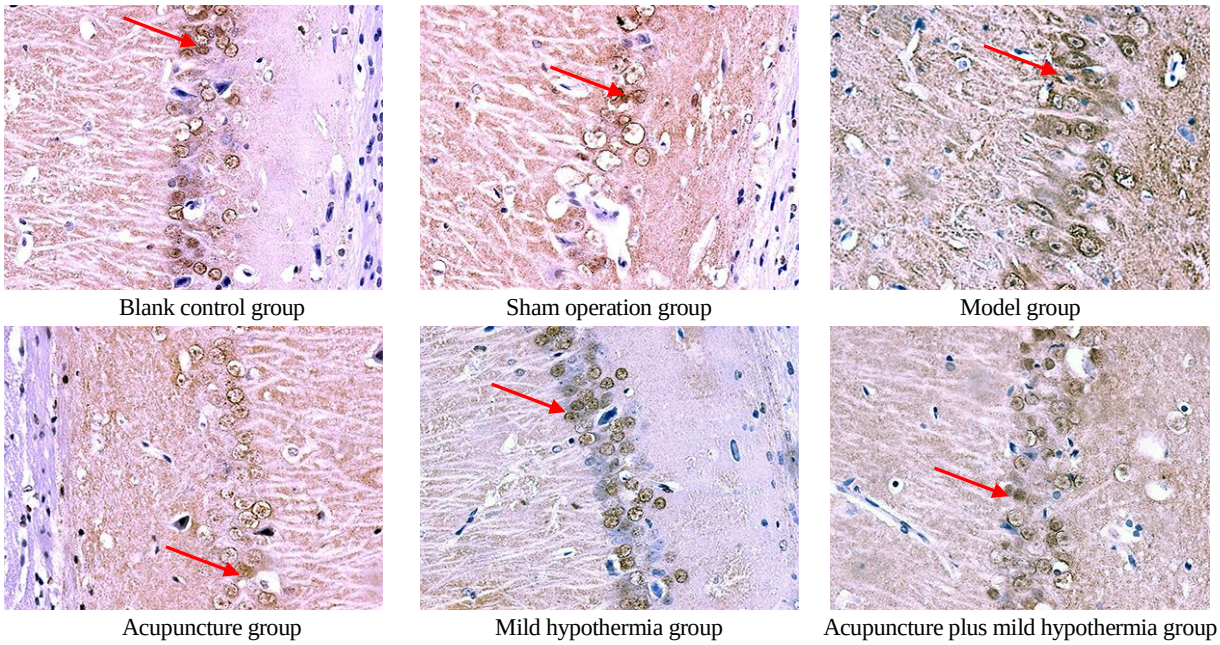

Figure 5. Positive Caspase-3 cells in each group after treatment

3 Discussion

Apoptosis is a mode of cell death under physiological conditions. Nucleated cells appear naturally cell death under certain conditions by activating their internal mechanisms to activate endogenous endonucleases. Studies showed that apoptotic cells emerged and was gradually increased after CIRI[11]. The signal pathways of apoptosis include apoptotic pathways mediated by death receptors and mitochondrial apoptotic pathways, namely, exogenous and endogenous apoptotic signaling pathways. Mitochondria are the most important initiator and executant of apoptosis. Bcl-2 family genes and Caspase family together involve in mitochondria-mediated 'endogenous pathway' in apoptosis. Bcl-2 family has many members, such as Mcl-1, NR-B, A1, Bcl-w, Bcl-x, Bax, Bad and Bim, which have both anti-apoptotic and pro-apoptotic effects. Wang believes that Bcl-2 can prevent the release of cytochrome c from the mitochondria, inhibit the subsequent occurrence of caspase activation and prevent the neuronal apoptosis[12]. Wu DH and others believe that, in the process of neuronal apoptosis after cerebral ischemia, Bcl-2 is an important apoptosis

suppressor gene, while Bax protein has a role in promoting cell apoptosis[10]. Caspase-3 is located at downstream of apoptosis and the necessary path of different apoptosis. Therefore, Caspase-3 plays a critical role in apoptosis[13].

The efficacy of acupuncture in the treatment of ischemic brain injury has been confirmed by many clinical reports and experimental studies[14-16]. The affected area in ischemic brain injury is the brain. Pang Y, *et al* suggested that 'Governor Vessel should be selected firstly for lesions in the brain', it is the main principle of selecting points to treat cerebral ischemic disease[17]. Selection of Baihui (GV 20), Dazhui (GV 14) and Shuigou (GV 26) from Governor Vessel in this experiment was because of the following reasons. Baihui (GV 20) is on the top of the head, also known as *San Yang Wu Hui* (joint of three yang meridians, liver meridian and Governor Vessel), where many meridians get together and it is linked to the brain. Therefore, Baihui (GV 20) can clear Governor Vessel and refresh brain; Dazhui (GV 14) is the hinge and key acupoint of the Governor Vessel. Stimulation to Dazhui (GV 14) can clear the Governor Vessel, smooth the qi and blood and affect the brain for blood-activating stasis-removing; Shuigou (GV 26) is between the nose and mouth, where the Governor Vessel and Hand/Foot Yangming Meridians meet. Acupuncture at Shuigou (GV 26) can adjust the yang qi of Governor Vessel to awake the brain and open the orifices. Zhang YC suggested that the protective mechanisms of acupuncture at Dazhui (GV 14), Baihui (GV 20) and Shuigou (GV 26) on cerebral ischemic injury in MCAO rats may be achieved by increasing blood flow of ischemic and injured brain tissues, and shortening the time of MAPK/ERK signaling pathway mediated cytoprotection, so as to protect and repair ischemic and injured brain tissue[18]. Wang T, *et al* found that acupuncture could improve the memory function of rats with multiple cerebral infarction by anti-neuron apoptosis. One of its anti-apoptotic mechanism is inhibiting Bax gene expression and increasing Bcl-2 gene expression[19].

According to unified international standards, 32-35 ℃ mild hypothermia is currently applied to brain protection research[20]. Mild hypothermia, as an important clinical treatment strategy for CIRI, could reduce free radical production[21], regulate phospholipase C-γ1[22], inhibit Caspase-3 expression[23], up-regulate Bcl-2 and down-regulate Bax[24]. Qi B, *et al* found that mild hypothermia played a role in brain protection by down-regulating the expression of Caspase-3[25]. Wu DH suggested that acupuncture and mild hypothermia treatment could improve Bcl-2 expression, reduce Bax expression, interfere with cerebral ischemia and play a protective effect on the neurons[10].

The results of this study showed that acupuncture group, mild hypothermia group and acupuncture plus mild hypothermia group could improve neurological function impairment of CIRI rats, reduce the cerebral infarct area ratio, increase the number of Bcl-2 positive cells, and reduce cell apoptosis, Bax and Caspase-3 positive cells. After the treatment, there were statistically significant differences in neurological function impairment score, infarct area ratio, apoptosis, and the number of Bcl-2, Bax and Caspase-3 positive cells among treatment groups ($P<0.05$). And from the graph, table and statistical analysis, we can see that acupuncture plus mild hypothermia group had a tendency to be superior to acupuncture group and mild hypothermia group.

Therefore, based on neurological function impairment score, cerebral infarct area ratio and apoptosis-related factors, we can determine whether acupuncture plus mild hypothermia therapy can produce a better efficacy than acupuncture or mild hypothermia treatment of stroke, and explore the mechanism of apoptosis to find the best way to treat CIRI and apply it to clinical practice for improving patient's quality of life as soon as possible.

Conflict of Interest

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

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Statement of Human and Animal Rights

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

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