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Basic Study

Effect of electroacupuncture at Neiguan (PC 6) and Baihui (GV 20) on CHOP and caspase-12 gene expressions in rats after ischemia-reperfusion injury

电针内关、百会对缺血再灌注损伤大鼠 CHOP 和 caraspase-12 基因表达的影响

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Abstract
Objective: To investigate the effects of electroacupuncture (EA) at Neiguan (PC 6) and Baihui (GV 20) by observing the changes of CCAAT/enhancer-binding protein(C/EBP) homologous protein (CHOP) and caspase-12 gene expressions in rats after cerebral ischemia-reperfusion injury (IRI), and explore whether the apoptosis pathway of endoplasmic reticulum stress (ERS) is involved in the protective mechanisms of EA.
Methods: Sixty rats were randomly assigned to five groups (12 in each group): a normal control group (group A), a sham-operation group (group B), an operation group (group C), an Edaravone group (group D) and an EA group (group E). The cerebral IRI rat model was induced by middle cerebral artery occlusion (MCAO) using intraluminal monofilament. 2,3,5-triphenyl tetrazolium chloride (TTC) staining was adopted in the measurement of cerebral infarction volume. Real-time polymerase chain reaction (RT-PCR) was used to determine the mRNA expressions of CHOP and caspase-12.
Results: Compared with group A and group B, the volume of cerebral infarction and mRNA expressions of CHOP and caspase-12 in group C, group D and group E were increased, with statistical significances (P<0.05 or P<0.01); compared with group C, the volume of cerebral infarction and mRNA expressions of CHOP and caspase-12 in group D and group E were decreased significantly (P<0.05 or P<0.01); there were no significant differences between group D and group E in comparing the above items (P>0.05).
Conclusion: EA at Neiguan (PC 6) and Baihui (GV 20) can effectively suppress the volume of cerebral infarction. Furthermore, the underlying mechanism of EA at Neiguan (PC 6) and Baihui (GV 20) is possibly related to the down-regulation of CHOP and caspase-12 mRNA expressions, so as to decrease cell apoptosis.
Keywords: Acupuncture Therapy; Electroacupuncture; Point, Neiguan (PC 6); Point, Baihui (GV 20); Brain Ischemia; Reperfusion Injury; Apoptosis; Rats

【摘要】目的：通过观察 CHOP 和 Caspase-12 基因表达在脑缺血再灌注大鼠体内的变化探讨电针内关、百会的效应以及电针保护性机制是否与内质网应激介导的凋亡通路有关。**方法**：将 60 只大鼠随机分为 5 组，每组 12 只，即正常对照组(A)、假手术组(B)、模型组(C)、依达拉奉组(D)、电针干预组(E)。采用线栓法结扎大脑中动脉制备大鼠局灶性脑缺血再灌注模型，2,3,5-氯化三苯基四氮唑(TTC)染色测定脑梗死体积，RT-PCR 法测定缺血侧顶叶大脑皮层 CHOP、caspase-12 mRNA 表达变化。**结果**：与正常组(A)和假手术组(B)比较，模型组(C)、依达拉奉组(D)和电针组(E)脑梗死体积百分比、CHOP、caspase-12 mRNA 表达均显著增高(P＜0.01 或 P＜0.05)；与模型组(C)比较,依达拉奉组(D)和电针组(E)脑梗死体积、CHOP、caspase-12 mRNA 表达均显著降低(P＜0.01 或 P＜0.05);与依达拉奉组(D)比较,电针组(E)脑梗死体积、CHOP、caspase-12 mRNA 表达无显著差异(P＞0.05)。**结论**：电针内关、百会可以有效降低大鼠脑梗死体积,其脑保护潜在机制可能与下调凋亡因子 CHOP、caspase-12 mRNA 表达，从而抑制神经细胞凋亡相关。
【关键词】针刺疗法；电针；穴，内关；穴，百会；脑缺血；再灌注损伤；凋亡；大鼠
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Ischemic apoplexy accounts for 75%-85% of cerebral vascular disease (CVD), which has become a common and frequently encountered disease for a long time and endangers human health. After cerebral ischemia, some parts of vessels can recover naturally or through antithrombotic treatment; however, reperfusion may lead neural apoptosis or necrosis in ischemic and the surrounding area. Neural apoptosis induced by cerebral ischemia-reperfusion injury (IRI) may be a delayed-

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death, and the number of apoptotic cells increases with the extension of ischemia time.

At present, the mechanism of neural cell apoptosis induced by IRI has not been fully elucidated. However, the basic research and clinical treatment of IRI have achieved a great progress. In 1993, it was reported that neuronal protein synthesis inhibition (PSI) was found in the whole or focal cerebral ischemic reperfusion model[1]. And one of the reasons for PSI is the signal change of eukaryotic translation initiation factor (EIF), such as EIF2 and EIF3[2]. Recent research shows that neuronal apoptosis is caused by various pathways through endoplasmic reticulum stress (ERS) induced by cerebral IRI[3-4]. ERS is due to the broken balance of calcium or disordered protein folding. In the beginning, ERS is a self-protective mechanism in cells. However, when cells are subjected to severe and prolonged ERS, proapoptotic factors CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) and caspase-12 are induced[5-8]. Therefore, these factors play a vital role in cell apoptosis after ischemic reperfusion and were thus selected to explore the apoptosis mechanism in our research.

In traditional Chinese medicine (TCM), electro-acupuncture (EA) has been regarded as an effective method in treating patients under IRI[9]. It was found that acupuncture can be an outside-signal to adjust genes related to apoptosis in ischemic reperfusion rats. Acupuncture can not only inhibit apoptosis-related genes but also promote protective genes after cerebral ischemic reperfusion[10]. Neiguan (PC 6) and Baihui (GV 20) can promote the flow of qi and blood, unblock and activate the meridians and collaterals[11], which is the principle of acupuncture treatment for ischemic reperfusion. That is why those points were selected to discuss one of the protective mechanisms of EA in treating cerebral ischemic reperfusion.

Therefore, our research is to observe the protective effects of EA through stimulating Neiguan (PC 6) and Baihui (GV 20) points and to see whether one of the protective mechanisms of EA is related to apoptosis pathway of ERS through observing changes of CHOP and caspase-12 gene expressions in rats with cerebral ischemic reperfusion.

1 Materials and Methods

1.1 Animal grouping and experiment procedure

Sixty clean healthy male Sprague-Dawley (SD) rats, 10-12 weeks, weighing 230-280 g, were from Animal Center of Hunan University of Chinese Medicine (certificate number: Xiangyidongzi 20-002). They were caged in an environment with free access to food and water, room temperature at 18-25 ℃, relative humidity 50%-60%, and the cages were cleaned once a day. They were randomly assigned to five groups by design of randomized block-experiment, 12 rats in each group. Rats in the normal control group (group A) were only tied up, while rats in the sham-operation group (group B), operation group (group C), Edaravone group (group D) and EA group (group E) were developed to be model of middle cerebral artery occlusion (MCAO). The neurological function severity score[12] was adopted to verify the success of MCAO model, when rats woke up and their vital signs were stable. Afterwards, the different intervening measures were used: group C was injected with normal saline into rat's tail; group D with Edaravone injection at 3 mg/(kg·bw); group E was given EA at Neiguan (PC 6) and Baihui (GV 20), 30 min for a session. The neurological function severity was scored again respectively 24 h, and 48 h later for each group, and EA treatment was performed once afterwards for group E. The neurological function was scored for the fourth time after 72 h, followed by sacrifice after anesthesia and collection of brain on ice plate. 2,3,5-triphenyl tetrazolium chloride (TTC) staining method was adopted to measure the volume of cerebral infarction. Real-time polymerase chain reaction (RT-PCR) was adopted to measure the mRNA expressions of CHOP and caspase-12. In each group, six rats were used for TTC and the rest for PCR. All the procedures in this research were approved by the Laboratory Animals Management Committee of Hunan University of Chinese Medicine.

1.2 Model preparation

By referring the related literature, ischemic reperfusion rat model was established by MCAO[10]. After anesthesia by intraperitoneal injection of 10% Chloral hydrate [35 mg/(kg·bw)], rats were fixed in a supine position to separate respectively the left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA). When a vascular clamp was applied at the bifurcation of the CCA into the ECA and ICA, a small incision was made at the end of ECA. Inserted a piece of intraluminal monofilament into the incision [by about (18.5±0.5) mm in group C, D and E, while about 8-10 mm in group B]. The rat was put into a cage alone after fine suture and disinfection. The monofilament was pulled out 2 h after the operation. The rat was kept warm by a lamp through the whole process.

1.3 Edaravone injection method

Edaravone injection 10 mg (5 mL) (trade name: Bicun; lot number: H20031342; specification: 5 mL) was diluted with 5 mL physiological saline into 1 mg/mL Edaravone injection for tail vein injection in group D at a dose of 3 mg/(kg·bw).

1.4 Points locations and acupuncture methods

The points were located according to *Experimental Acupuncture Science*[13]. Neiguan (PC 6): on the palmar aspect of forearm, between the radius and ulna, 3 mm away from the palmar wrist crease. Baihui (GV 20): on

the head, the midpoint of parietal bone. Filiform needles of 0.30 mm in diameter and 10 mm in length were used. Neiguan (PC 6) was perpendicularly needled by 2 mm and Baihui (GV 20) was obliquely needled by 2 mm towards the anterior. After insertion, the needles were connected to EA apparatus (Hwato SDZ-Ⅱ). Two pairs of poles were applied, with one of the negative poles was connected to the left Neiguan (PC 6) and the other negative pole to Baihui (GV 20); the two positive poles were connected to the rat's tail. Sparse-dense wave (10 Hz, 50 Hz) was selected, with moderate stimulation intensity to produce a slight vibration. The EA stimulation lasted 30 min.

1.5 Observation items and detection methods

1.5.1 TTC staining

TTC staining was adopted to measure the volume of cerebral infarction, performed by following the instructions of kits. The normal tissue stained red, while the infarction tissue stained white, and then the tissues were fixed by 10% formaldehyde phosphate buffer.

Medical image analysis system (MIAS, produced by Beihang Company, China) was used for calculation.

Cerebral infarction percentage (%) = Sum of the infarction area on each brain slice ÷ Sum of total areas × 100%.

1.5.2 CHOP and caspase-12 mRNA expressions

RT-PCR was used to detect the mRNA expressions of CHOP and caspase-12 (Table 1). PCR primers design of CHOP and caspase-12 were supplied by Sangon Biotech (Shanghai) Co., Ltd.

Target gene and housekeeping gene of each sample were examined by RT-PCR respectively. According to the DNA standard curve with drawing gradient dilution, the results of concentration related to target gene and housekeeping gene of each sample were generated directly (Table 1).

Table 1. PCR detection

Gene	Primer sequence	Annealing (℃)	Product length (bp)
GAPDH	F: 5'GGAAAGCTGTGGCGTGAT3' R: 5'AAGGTGGAAGAATGGGAGTT3'	60	308
CHOP	F: 5'ACCTTCACTACTCTTGACCCTGC3' R: 5'CTCCATTCTCCTGCTCCTTCTC3'	60	223
Caspase-12	F: 5'AATGGAGGTAAATGTTGGAGTG3' R: 5'CCAATCACGAGAACATAGCTTC3'	60	112

1.6 Statistical analysis

All the data were processed by SPSS 17.0 version statistical software. The measurement data in normal distribution were analyzed by one-way ANOVA. The variance was then checked. Data with homogeneity of variance were analyzed by the least significant difference (LSD) and Student-Newman-Keuls (SNK); otherwise, Tamhane's T2 or Dunnett's T3 would be used. Data in abnormal distribution were analyzed by K independent samples. $P<0.05$ was considered as a statistical significance.

2 Results

2.1 Volume of cerebral infarction percentage (VCIP)

There was no significant difference between group A and group B in comparing VCIP ($P>0.05$); compared with group A and group B, VCIP in group C, group D and group E increased significantly ($P<0.05$ or $P<0.01$); compared with group C, VCIP in group D and group E decreased significantly ($P<0.05$ or $P<0.01$); there was no significant difference between group D and group E in comparing VCIP ($P>0.05$), (Table 2).

It suggests that MCAO model was successfully developed in group C, D and E, because VCIP increased significantly compared to the normal samples. EA at Neiguan (PC 6) and Baihui (GV 20) can effectively reduce VCIP, equivalent to Edaravone.

Table 2. Comparison of VCIP ($\bar{x}\pm s$, %)

Group	n	VCIP
A	6	0.000±0.000
B	6	0.000±0.000
C	6	29.749±2.696[1)2)]
D	6	22.372±3.302[1)2)3)]
E	6	23.641±3.450[1)2)3)]

Note: Compared with group A, 1) $P<0.01$; compared with group B, 2) $P<0.01$; compared with group C, 3) $P<0.01$

2.2 CHOP and caspase-12 mRNA expressions

After the intervention, compared with group A, there were no significant difference between group A and group B in comparing the mRNA expressions of CHOP and caspase-12 ($P>0.05$); compared with group A and group B, the mRNA expressions of CHOP and caspase-12 in group C, group D and group E increased significantly ($P<0.05$ or $P<0.01$); compared with group C, CHOP and caspase-12 mRNA expressions in group D and group E decreased significantly ($P<0.05$ or $P<0.01$); there were no significant differences between group D and

group E in comparing the mRNA expressions of CHOP and caspase-12 ($P>0.05$), (Table 3).

It suggests that ERS has been induced after IRI[14]. EA at Neiguan (PC 6) and Baihui (GV 20) can down-regulate CHOP and caspase-12 mRNA expressions, so as to decrease cell apoptosis, which is equivalent to the function of Edaravone.

Table 3. Comparison of CHOP and caspase-12 mRNA expressions ($\bar{x}\pm s$)

Group	*n*	CHOP	Caspase-12
A	6	17.355±0.916	23.383±0.744
B	6	18.448±0.590	23.690±0.445
C	6	21.401±1.262$^{2)4)}$	26.475±1.094$^{2)4)}$
D	6	19.904±0.805$^{2)4)5)}$	24.660±0.675$^{1)3)6)}$
E	6	19.973±1.013$^{2)4)5)}$	24.871±0.330$^{2)4)6)}$

Note: Compared with group A, 1) $P<0.05$, 2) $P<0.01$; compared with group B, 3) $P<0.05$, 4) $P<0.01$; compared with group C, 5) $P<0.05$, 6) $P<0.01$

3 Discussion

According to TCM classification, apoplexy is divided into two types: stroke of meridian-collateral and stroke of Zang-fu. The stroke of meridian-collaterals is excessive syndrome and mainly manifested by hemiplegia, slurred speech and deviation of the mouth corner. The pathogenic factors of acute stage in ischemic apoplexy include deficiency of qi and stagnation of blood, stagnation in vessels and collaterals, and blocking in the brain[15]. Therefore, the effective methods are to promote the flow of qi and blood, clear and activate the channels and collaterals.

Baihui (GV 20) is an intersection connecting brain and five meridians, i.e. the Governor Vessel, Bladder Meridian, Triple Energizer Meridian, Gallbladder Meridian, and Liver Meridian. Thus, it can adjust yin and yang, tranquilize liver-wind, supplement essence and marrow, reinforce qi and nourish blood, and resuscitate consciousness[16]. It's found that EA at Baihui (GV 20) can promote the synthesis of nitric oxide (NO) in blood and brain, improve microvascular self-discipline exercise, improve microcirculation and the activity of superoxide dismutase (SOD), decrease lipid peroxide (LPO) in brain tissue to reduce oxidative damage, and reduce calcium-overload, benignly regulate abnormal metabolism of central neurotransmitters, and then reduce brain cell necrosis or apoptosis[17]. There is a good regulation function in the neuropeptide Y (NPY) and calcitonin gene-related peptide (CGRP) in the super early time of acute cerebral ischemia[18]. Neiguan (PC 6) belongs to the Pericardium Meridian, working to connect the Yin Link Vessel, balance yin and yang, adjust heart and resuscitate consciousness. Neiguan (PC 6) can obviously improve the clinical symptoms of ischemic heart disease, adjust heart rate and regulate blood pressure in dual-directional way[19]. EA at Neiguan (PC 6) can effectively correct enzyme metabolism disorders caused by ischemia and improve cerebral energy metabolism so as to achieve the neuroprotective effect[20-21].

The endoplasmic reticulum (ER) is the central intracellular organelle in the secretory pathway[22]. It is responsible for protein translocation, protein folding, and protein post-translational modifications. Perturbations in ER function, a process named ER-stress, trigger the unfolded protein response (UPR), a tightly orchestrated collection of intracellular signal transduction reaction designed to restore protein homeostasis. The UPR is distinguished by the action of three signaling proteins named inositol-requiring protein-1α (IRE1α), protein kinase RNA (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6)[23]. PERK-elF2α-ATF4 is a main pathway of expression of CHOP protein[24]. When cells are subjected to severe and prolonged ER stress, a proapoptotic factor CHOP/GADD153, a member of the C/EBP family of transcription factor, is induced. It's reported that CHOP can increase the sensitivity to ER stress by decreasing Bcl-2 expression and enhanced oxidant injury[25]. It's also reported that expression of CHOP mRNA significantly increased in some part of cell nuclei from the pyramidal cell of striatum and region 1 of hippocampus (CA1) area after cerebral ischemia reperfusion[26]. The level of transcripts of CHOP (GADDl53) increased to two times 6 h after ischemic reperfusion[27], six times after 48 h in hippocampus[28], after 12-24 h reached a peak level[29]. In addition, the number of apoptotic cell decreased, when lacking CHOP gene, rats were adopted for cerebral IRI experiment[30].

Caspase-12 belongs to a family of enzymes called caspases and is localized to ER and activated by ER stress, including disruption of ER calcium homeostasis and accumulation of excess proteins in ER[31]. Excessive ERS can trigger cellular apoptosis through the activation of caspase-12, which resides on the outside of ER membrane, and is cleaved and activated during ERS. As an initiator caspase, caspase-12 triggers the activation of caspases-9, -7, and -3 in a cytochrome c and Apaf-1-independent manner. Caspase-12 knock-out cells are resistant to ERS-induced apoptosis. The synthesis and activity of caspase-12 happen during 5-23 h after ischemic reperfusion through the immunohistochemical staining and the location of caspase-12 is in accordance with apoptosis-neurocyte[32]. When ischemic reperfusion is prolonged, the activity of protective factor GRP78 is inhibited by caspase-12[22]. Furthermore, it's found that the apoptosis area reduced in the brain induced by ERS in rats without gene of caspase-12[33]. Therefore, it is effective to block caspase-12 to inhibit apoptosis induced by ERS.

Edaravone is a new effective neuroprotective agent in treating acute cerebral ischemia. It can protect vascular endothelial cells and neurons against the oxidative stress[34]. Edaravone can improve the symptoms of neurological impairment and cause the inhibition of delayed neuronal cell death in the rodents induced by transient forebrain ischemia or focal cerebral ischemia[35-36]. Thus, Edaravone was selected as the positive control in this study.

In conclusion, EA at Neiguan (PC 6) and Baihui (GV 20) points can effectively suppress the volume of cerebral infarction percentage. Furthermore, the underlying mechanism of EA at Neiguan (PC 6) and Baihui (GV 20) points is possibly related to the down-regulation of CHOP and caspase-12 mRNA expressions so as to decrease cell apoptosis.

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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