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Basic Study

Effect of electroacupuncture on expressions of VEGF and CD31 in MCAO model rats

电针对 MCAO 模型大鼠 VEGF 和 CD31 表达的影响

Chen Guo (陈果), Xiang Juan (向娟), Ouyang Li-zhi (欧阳里知), Song Jin (宋瑾), Xue Zhi-hui (薛智慧), Li Hong-liang (李洪亮), Li Tie-lang (李铁浪)
School of Acupuncture, Moxibustion & Tuina, Hunan University of Chinese Medicine, Changsha 410208, China

Abstract
Objective: To investigate the effect of electroacupuncture (EA) at Ganshu (BL 18) and Shenshu (BL 23) on vascular endothelial growth factor (VEGF) and platelet endothelial cell adhesion molecule-1 (PECAM-1)/CD31 around the cerebral infarction focus in middle cerebral artery occlusion (MCAO) rats and the possible mechanism, thus to provide a new strategy for the treatment of cerebral ischemic stroke by acupuncture.
Methods: A total of 180 healthy male Sprague-Dawley (SD) rats were randomly divided into a sham operation group, a model group, an acupoint group and a non-acupoint group, 45 rats in each group. MCAO model was established using the modified line-embolus method in all rats except for those in the sham operation group; rats in the acupoint group were treated with EA at Ganshu (BL 18) and Shenshu (BL 23); rats in the non-acupoint group were treated with EA at the control points; rats in other 2 groups were only subjected to bundling without treatment. Ten rats in each group were randomly selected on the 3rd day, the 14th day and the 21st day after acupuncture stimulation to test the neurological function impairment. The expression levels of CD31 and VEGF were also detected.
Results: Compared with the model group and non-acupoint group, the neurological function score of the acupoint group was decreased at each time point, and the differences were statistically significant (P<0.05, P<0.01). The expressions of VEGF and CD31 in each group were the lowest on the 3rd day, reached the peak on the 14th day and still remained at high level on the 21st day. And the differences among groups were statistically significant both on the 14th day and the 21st day (P<0.05, P<0.01). Compared with the model group and the non-acupoint group, the expressions of VEGF and CD31 in the acupoint group were increased, and the differences were statistically significant (all P<0.05).
Conclusion: EA at Ganshu (BL 18) and Shenshu (BL 23) can significantly improve the neurological function score of MCAO model rats, and shows protective effect on cerebral ischemia. The protective mechanism may be related to the up-regulation of CD31 and VEGF expression around the cerebral infarction focus in the MCAO model rats and induction of angiogenesis.
Keywords: Acupuncture Therapy; Electroacupuncture; Point, Ganshu (BL 18); Point, Shenshu (BL 23); Brain Ischemia; Infarction, Middle Cerebral Artery; Vascular Endothelial Growth Factors; Rats

【摘要】目的：探讨电针肝俞和肾俞对大脑中动脉梗塞(MCAO)模型大鼠脑梗死灶周围血管新生相关因子血管内皮生长因子(VEGF)、血小板-内皮细胞粘附分子(PECAM-1)/CD31的影响及可能机制，为针刺治疗脑缺血中风提供新方案。**方法：**将180只健康雄性Sprague-Dawley (SD)大鼠随机分为假手术组、模型组、穴位组和非穴组，每组45只。除假手术组外，其余各组大鼠均采用改良线栓法制备MCAO模型；穴位组予电针肝俞和肾俞治疗，非穴组予电针非穴点治疗，其余两组大鼠只捆绑，不治疗。在MCAO术后针灸刺激的第3 d、14 d及21 d三个时间点各组随机抽10只大鼠测试大鼠神经缺损症状；同时检测CD31和VEGF的表达量。**结果：**与模型组和非穴组比较，穴位组各时相的神经功能评分降低，组间差异具有统计学意义(P＜0.05, P＜0.01)。各组大鼠VEGF和CD31的表达在第3 d时最低，于14 d达高峰，第21 d仍维持在较高水平，各组第14 d与第21 d比较均有统计学意义(P＜0.05, P＜0.01)。与模型组及非穴组比较，穴位组各时相VEGF和CD31的表达升高，组间差异具有统计学意义(均P＜0.05)。**结论：**电针肝俞和肾俞能明显改善MCAO模型大鼠神经功能评分，对脑缺血有保护作用，保护机制可能与电针上调MCAO模型大鼠梗死灶周围CD31和VEGF表达，诱导血管新生有关。

【关键词】针刺疗法；电针；穴，肝俞；穴，肾俞；脑缺血；大脑中动脉梗塞；血管内皮生长因子；大鼠
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Author: Chen Guo, 2014 grade master degree candidate
Corresponding Author: Li Tie-lang, M.D., professor.
E-mail: litielang810@163.com

Ischemic cerebrovascular disease (ICVD) is a disease with cerebral ischemia and necrosis caused by blood flow decrease due to a variety of factors, thereby affecting neurological function[1], accounting for 75%-85% of all cerebrovascular diseases. ICVD is characterized by high morbidity and mortality, greatly threatening lives and health of patients, and has become the hot field of medical research[2-3]. Current ICVD treatment is mainly to improve the blood supply to damaged brain tissue to restore the neurological function. Acupuncture plays an important role in inhibiting inflammatory response and apoptosis, thus promoting nerve and vascular regeneration[4]. It is one of the main methods to treat ICVD confirmed by evidence-based medicine[5]. Our previous study found that electroacupuncture (EA) at Ganshu (BL 18) and Shenshu (BL 23) could increase the neuronal density in hippocampal CA1 area and cortical Ⅲ-Ⅳ layer, causing cerebrovascular dilatation and increase of blood flow[6].

Other studies have confirmed that vascular endothelial growth factor (VEGF) and platelet endothelial cell adhesion molecule-1 (PECAM-1)/CD31 play an important role in angiogenesis[7-8]. This study aimed to observe the effect of EA at Ganshu (BL 18) and Shenshu (BL 23) on the expressions of VEGF and CD31 in middle cerebral artery occlusion (MCAO) model, and provide experimental evidence for treatment of ICVD.

1 Materials and Methods

1.1 Locations of acupoints and control points[9]

Ganshu (BL 18): Below the spinous process of the 9th thoracic vertebra, and 7 mm away on both sides.

Shenshu (BL 23): Below the spinous process of the second lumbar vertebra, and 7 mm to the spine.

Locations of points in non-acupoint group: 1 cm away from Ganshu (BL 18) and Shenshu (BL 23).

1.2 Laboratory equipment and reagents

Hwato Brand sterile acupuncture needles (specifications: 0.30 mm in diameter and 25 mm in length, Suzhou Medical Products Factory Co., Ltd., China); G6805-Ⅱ type EA instrument (Qingdao Xin Sheng Industrial Co., Ltd., China); AlphaEaseFC scanner (EPSON, Japan); dehydrator (Wuhan Junjie Electronics Co., Ltd., China); 79-1 magnetic stirrer (Changzhou Aohua Instrument Co., Ltd., China); pathology slicer (Shanghai Leica Instrument Co., Ltd., China); CD31 antibody (Santa, USA); RIPA lysate (Beijing Huayueyang Biology Company, China); SABC immunohistochemistry kit (Shanghai Meixuan Biotechnology Co., Ltd., China).

1.3 Model preparation and judgment

MCAO model was established using the method of insertion line-embolus into the right common carotid artery according to the literature[10]. Rats were anesthetized by intraperitoneal injection of 3.5 mL/(kg·bw) 10% chloral hydrate. About 1.0-1.5 cm longitudinal incision was performed at 0.3 cm right from the midline of the neck, followed by blunt separation with ophthalmic tweezers. The common carotid artery and external carotid artery were ligated. The line-embolus with a diameter of 0.26 mm was inserted into the internal carotid artery from the common carotid artery till encountered resistance. The insertion depth was (18±0.5) mm. After successful insertion of the line-embolus, the common carotid artery was tightly ligated. The redundant line-embolus was cut off and the wound was sutured. For anti-inflammation, penicillin was administrated by intraperitoneal injection for 3 d. Rats were fed separately for observation. Rats in the sham operation group only received surgery to expose the carotid artery, ligation of the common carotid artery, without line-embolus.

Evaluation of the model: The 5-point system developed by Longa EZ and others was used to determine the success or failure of the model 2 h after the operation when the vital signs were stable[11]. 0 point stood for no neurological impairment symptoms; 1 point indicated that the left forepaws were unable to fully stretch; 2 points indicated incessant turning to left; 3 points indicated toppling to the left; 4 points indicated that rats were unable to walk with decreased consciousness (rats scored 1-3 points were included in the experiment, and the rest were rule out). After successful modeling, rats in each group were treated accordingly.

1.4 Laboratory animals and grouping

A total of 180 clean-grade male Sprague-Dawley (SD) rats, 10-12 weeks old, body weight 220-250 g, were purchased from the Animal Experimental Center of Hunan University of Chinese Medicine [certificate number: SYXK (Hunan) 2013-0005]. During the experiment, all the interventions followed the guidance of *Guiding Opinions on the Treatment of Experimental Animals* issued by the Ministry of Science and Technology.

All rats were fed with sufficient standard diet with free access to water. Rats were kept at temperature of 20-25 ℃ and relative humidity of 50%-70%, on a 12-hour light, 12-hour dark diurnal variation. After adaptive feeding for 1 week, rats were randomly divided into four groups by the random number table, i.e. a model group, a sham operation group, an acupoint group and a non-acupoint group, 45 rats in each group. Rats in the model group, the acupoint group and the non-acupoint group were used to prepare MCAO model.

Sham operation group: Rats in the sham operation group only received ligation of common carotid artery without line-embolus. Rats only received binding without treatments during experiment.

Model group: Rats in the model group only received binding without treatments.

Acupoint group: 2 h after operation, rats in the acupoint group received behavioral scoring, and then received EA treatment. Bilateral Ganshu (BL 18) and Shenshu (BL 23) were selected. Needles (0.30 mm in diameter and 25 mm in length) were inserted into the points for 6 mm and then connected to G6805-II type EA instrument. The same pair of output electrodes was connected to the two points on the same side with sparse-dense wave (10 Hz/50 Hz) and the intensity of 1-3 mA until limbs appeared tremor[12-13]. EA stimulation lasted 30 min each time, once a day. Fifteen rats were randomly selected to collect samples on day 3, day 14 and day 21.

Non-acupoint group: Rats were fixed to the plate and received acupuncture at non-acupoint points. Control points of Ganshu (BL 18) and Shenshu (BL 23) were marked after positioning; other procedures were same as those in the acupoint group. Rats in the sham operation and model group were fixed on the plate for 30 min without acupuncture, once a day, and other procedures were same as those in the acupuncture group.

1.5 Observed items and methods

1.5.1 Behavioral scoring

The neurological function impairment scoring was conducted 2 h, 3 d, 14 d and 21 d after MCAO[11]. The highest score was set as 11. The higher scores indicated severer behavioral disorder.

1.5.2 The volume ratio of cerebral infarction

The volume ratio of cerebral infarction was measured by 2, 3, 5-triphenyltetrazolium chloride (TTC) staining. Rats in each group were treated with 10% chloral hydrate. The brain tissues were isolated and frozen at –20 °C in a refrigerator for 20-30 min. Five successive slices with 2 mm in thickness were prepared from the anterior pole of the forehead.

The brain slices were placed in a culture dish containing 1% TTC in a thermostat, followed by incubation at 37 ℃ for 30 min, and flipped once at 15 min. After colored, the slices were fixed in 4% paraformaldehyde for 12 h and taken pictures. The volume ratio of cerebral infarction was calculated by Image-Pro-Plus software. The volume ratio of cerebral infarction ＝ (Volume of cerebral hemisphere on the normal side － Volume of normal cerebral hemisphere on the infracted side)÷Volume of cerebral hemisphere on the normal side × 100%.

1.5.3 Expression of CD31 around the infarction focus

Expression of CD31 around the infarction focus was detected by immunohistochemistry. The left ventricle of anesthetized rats was perfused and rinsed with saline, and then perfused with 4% paraformaldehyde to fix for 45-60 min. After perfusion, brain tissues were separated and fixed in 4% paraformaldehyde solution, embedded in paraffin, and then sliced.

1.5.4 Expression of VEGF around infarction focus

Expression of VEGF around infarction focus was detected by immunoblotting. Brain tissues of rats anesthetized with 10% chloral hydrate were separated from optic chiasma to 0.4 cm away from the optic chiasma in the right hemisphere, stored in a 1.8 mL cryopreserved tube at –80 ℃. The film was scanned and archived after fixed and developed. Optical density values of target bands were obtained by the Alpha software.

1.6 Statistical processing

The SPSS 19.0 version statistical software was used for statistical analysis. Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$) and compared by one-way ANOVA when the normal distribution and variance were satisfied; and the inter-group difference was examined with the least significant difference (LSD) method; nonparametric test was used when the normal distribution or variance homogeneity was not satisfied. $P<0.05$ indicated that the difference was statistically significant.

2 Results

2.1 Zea-Longa score

The Zea-Longa score can be used not only as a criterion for MCAO model success but also can reflect rat neurological function. The results showed that the difference of Zea-Longa score 2 h after MCAO between the model group and the sham operation group was statistically significant ($P<0.01$), which indicated that the model was successful. There were no statistically significant differences in the scores among the other groups except for the sham operation group (all $P>0.05$), indicating that the experimental groups were homogeneous (Figure 1).

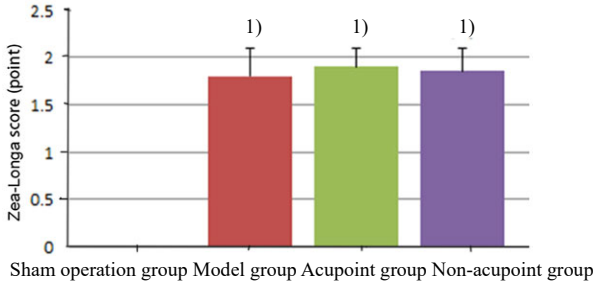

Figure 1. Zea-Longa score in each group after 2 h of MCAO

Note: Compared with the sham operation group, 1) $P<0.01$

2.2 Assessment of neurological function impairment

The neurological function scores of the three modeling groups were the highest on the 3rd day after operation, indicating that the neurological function impairment was the most serious; the scores were gradually decreased on the 14th and 21st day after operation; differences between the 3rd day and the

14th or 21st day after operation in the same group were statistically significant ($P < 0.05$, $P < 0.01$). Difference between the acupoint group and the model group or the non-acupoint group was statistically significant ($P<0.05$, $P<0.01$), (Table 1).

2.3 Expressions of CD31 and VEGF around the cerebral infarction focus

Expressions of CD31 and VEGF in each group were the lowest on the 3rd day after operation and reached a peak on the 14th day after operation. The expressions of CD31 and VEGF remained at a high level on the 21st day after operation, though showing slight decrease. Expressions of CD31 and VEGF on the 3rd day after operation were statistically significant different versus those both on the 14th day and 21st day after operation in the same group ($P<0.05$, $P<0.01$). The expressions of CD31 and VEGF in the acupoint group at each time point were significantly higher than those in the model group and non-acupoint group ($P<0.05$, $P<0.01$), (Table 2 and Table 3).

Table 1. Comparison of neurological function impairment scores at different time points among groups ($\bar{x}$ ±s, point)

Group	*n*	Postoperative day 3	Postoperative day 14	Postoperative day 21
Sham operation	10	0	0	0
Model	10	7.81±0.92	7.44±0.84	6.65±0.97[2)]
Acupoint	10	6.35±1.06[4)5)]	4.98±1.10[1)4)6)]	3.61±1.58[2)3)4)6)]
Non-acupoint	10	7.54±1.27	6.56±1.08	5.64±0.97[2)]

Note: Compared with the 3rd day after operation in the same group, 1) $P<0.05$, 2) $P<0.01$; compared with the 14th day after operation in the same group, 3) $P<0.01$; compared with the model group at the same time point, 4) $P<0.01$; compared with the non-acupoint group at the same time point, 5) $P<0.05$, 6) $P<0.01$

Table 2. Comparison of CD31 expressions at different time points in rats among groups ($\bar{x}$ ±s)

Group	*n*	Postoperative day 3	Postoperative day 14	Postoperative day 21
Sham operation	5	4.63±1.03	10.05±0.53[1)]	6.73±0.75[1)2)]
Model	5	4.78±1.03[3)]	12.85±0.63[1)3)]	8.53±4.72[1)2)3)]
Acupoint	5	4.79±1.93[3)4)]	20.09±0.83[1)3)4)5)]	19.23±1.21[1)2)3)4)6)]
Non-acupoint	5	4.78±1.83[3)4)5)]	20.08±0.57[3)4)]	18.18±1.55[2)3)4)]

Note: Compared with the 3rd day after operation in the same group, 1) $P<0.05$; compared with the 14th day after operation in the same group, 2) $P<0.01$, 3) $P<0.01$; compared with the sham operation group at the same time point, 3) $P<0.01$; compared with the model group at the same time point, 4) $P<0.01$; compared with the non-acupoint group at the same time point, 5) $P<0.05$, 6) $P<0.01$

Table 3. Comparison of rat VEGF expression among groups at different ischemia stages ($\bar{x}$ ±s)

Group	*n*	Postoperative day 3	Postoperative day 14	Postoperative day 21
Sham operation	5	57.24±6.85	59.28±7.73[1)]	58.36±5.72[1)2)]
Model	5	60.25±7.03[3)]	61.38±7.72[1)3)]	59.85±5.63[1)2)3)]
Acupoint	5	80.98±7.83[3)4)]	90.45±7.81[1)3)4)5)]	90.31±4.83[1)2)3)4)6)]
Non-acupoint	5	63.23±5.85[3)4)5)]	68.18±3.5[3)4)]	65.54±3.57[2)3)4)]

Note: Compared with the 3rd day after operation in the same group, 1) $P<0.05$; compared with the 14th day after operation in the same group, 2) $P<0.01$; compared with the sham operation group at the same time point, 3) $P<0.01$; compared with the model group at the same time point, 4) $P<0.01$; compared with the non-acupoint group at the same time point, 5) $P<0.05$, 6) $P<0.01$

3 Discussion

Ischemic stroke belongs to stroke in traditional Chinese medicine (TCM). The fundamental pathogenesis is the deficiency of liver and kidney. Therefore, regulating and reinforcing the liver and kidney should be the fundamental rules to treat this disease. In TCM, it is believed that liver stores blood, and liver blood is the material basis for brain marrow formation; the kidney stores essence, and kidney essence is an important material to reinforce brain. Ganshu (BL 18) and Shenshu (BL 23) are the Back-Shu points of the liver and kidney, where infuse the essence of the two organs into the acupoints. The Bladder Meridian goes into the brain and brings the kidney essence, liver blood and other essence to brain and marrow, and calms the liver to stop the wind. According to the modern medical view, Ganshu (BL 18) and Shenshu (BL 23) are located in the surface projection area of thoracic/abdominal sympathetic trunk and spinal cord, where the spinal nerve passes through[14]. EA at Ganshu (BL 18) and Shenshu (BL 23) can improve

blood flow in ischemic penumbra brain tissues by increasing of the concentration of vascular growth factor in cerebral ischemia area.

The goal in treating ICVD is to build collateral circulation by vascular anastomosis to improve cerebral ischemia. VEGF is currently the most powerful angiogenic factor. It can promote the establishment of collateral circulation to increase perfusion and oxygen, as well as reduce brain edema in the impaired brain tissues; VEGF is also a neurotrophic factor, and can directly repair the damaged nerve tissue, promote growth of neurons and glial cells. Neuroprotection is activated by the α7 receptor signaling pathway after cerebral ischemia, and the expression levels of α7 receptor, hypoxia-inducible factor-1 (HIF-1α), VEGF and its receptor in cerebral ischemic region are increased. Therefore, the extracellular mass of endothelial cells is changed to promote the proliferation, migration and angiogenesis[15-17]. A study has shown that acupuncture could increase the concentration of VEGF in rats with cerebral ischemia[18]. CD31 is a specific marker of endothelial progenitor cells. It is a member of the immunoglobulin superfamily with about 130 kd, expressed in vascular endothelial cells, platelets and white blood cells. A study has found that CD31 is involved in the process of vascular regeneration, with the repair function for ischemic injury and predictive value for cardiovascular and cerebrovascular disease[19]; at the same time, CD31 has an antiapoptotic effect by activating or inhibiting signal proteins in the signaling pathway, such as B-cell lymphoma-2 (Bcl-2), cysteinyl aspartate specific proteinase (Caspase)[20]. A study has shown that EA can significantly improve the expression of CD31 in MCAO rats[21].

Our current results showed that the score of neurological function impairment in each group was decreased gradually and to the lowest on the 21st day. The decrease of neurological function score was more obvious in acupoint group compared with that in the model group and non-acupoint group at each time point, indicating EA at Ganshu (BL 18) and Shenshu (BL 23) can promote the repair of neurological function in rats. Few VEGF and CD31 were expressed in the cerebral vascular endothelial cells of the brain tissues in the model group. The expressions of VEGF and CD31 in the brain tissues of the acupoint group were significantly higher than those of the other groups. The expressions of VEGF and CD31 were increased gradually with time in each group and reached the highest on the 14th day, suggesting that angiogenesis was active at this time point. This was slightly decreased on 21st day, while still at a high level, which may be due to the decreased need for the angiogenesis. The expressions of VEGF and CD31 in the acupoint group were increased and significantly different from those in the other groups, suggesting that EA at Ganshu (BL 18) and Shenshu (BL 23) effectively promotes the angiogenesis in ischemic area of the brain.

In summary, our current data showed that EA at Ganshu (BL 18) and Shenshu (BL 23) has a protective effect on brain injury in MCAO rats, which may be caused by the increase of VEGF and CD31, and the promotion of the function recovery of brain tissue with ischemic lesions. Protective effect produced by EA may be related to a variety of endogenous protective factors, and the specific mechanism is worth further study.

Conflict of Interest

There was no potential conflict of interest in this article.

Acknowledgments

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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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Translator: Yang Yan-ping (杨燕萍)

