Effect of Electroacupuncture at Acupoints of the Governor Vessel on Aquaporin-4 in Rat with Experimental Spinal Cord Injury

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This study is to investigate the effects of electroacupuncture at acupoints of the Governor Vessel (GV) on aquaporin-4 (AQP-4) expression and on functions of the hind limbs in the rat of spinal cord injury. The functions of the hind limbs were detected with BBB scale on the 1d, 3d, 7d and 21d after the spinal cord injury, respectively, and AQP-4 expression in the spinal cord was determined with immunohistochemical method and analyzed quantitatively with image analyzer. The results indicated that on the 1d after the spinal cord injury, increased AQP-4 expression can be seen significantly in both the gray matter and the white matter of the injured spinal cord, and it reached the peaks on the 3d after the spinal cord injury in both the electroacupuncture group and the spinal cord injury group. However, AQP-4 express was significantly decreased in the electroacupuncture group as compared with that in the control group on 7d, 14d and 21d (P<0.05 or P<0.01). The decrease of AQP-4 expression almost went with the improvement of the neurological function, which suggested that electroacupuncture at the acupoints of the Governor Vessel can inhibit edema of the spinal cord to alleviate the secondary spinal cord injury by means of decreasing the AQP-4 expression after the spinal cord injury, so as to protect the residual normal spinal cord tissues and promote the rebuilding of nervous tissues.

Aquaporin-4 (AQP-4), also called mercury-insensitive water channel (MIWC),\(^1\) is a specifically selected water channel. AQP-4 distributes mainly in the central nervous system, including the brain and the spinal cord.\(^2\) It is found in the study on AQP-4 expression in the brain of chick embryo that the expression of AQP-4 is correlated with the maturity and functions of the blood-brain (spinal cord) barrier.\(^3\) However, at present it is not clear whether AQP-4 expression at physiological and pathological states of the nervous system is functional, or whether it is beneficial to the removal or exacerbation of liquid formation in edema. This study is designed to determine the effects of electroacupuncture at acupoints of the Governor Vessel on AQP-4 expression of the injured spinal cord so as to explain the pathological mechanism of secondary spinal cord injury and open a new thinking for inhibiting the mechanism to protect the residual nervous tissue of the spinal cord, and provide a theoretical support for electroacupuncture at acupoints of the Governor Vessel to treat spinal cord injury, such as traumatic paraplegia.

Materials and Methods

1. Materials

Experimental animal: Healthy normal grade Sprague-Dawley rats, weighing 200-250g, equal female and male number, with no pregnancy, were supplied by The Experimental Animal Center, Guangzhou University of TCM.

Main reagents: Chloral hydrate (Shanghai Chemical Reagent Company, China Medicine Group), Rabbit anti-aquaporin-4 antibody, SABC immunohistochemical staining reagent kit and DAB
coloration reagent kit (Wuhan Boshide Biological Co. Ltd.), Mayer Campeachy (Sigma Company).

2. Methods

Grouping: The rats were divided into 3 groups: a normal control group (A), a spinal cord injury group (B) and an electroacupuncture group (C), 50 rats in each group. The rats in each group were again divided into 5 subgroups, 1d, 3d, 7d, 14d and 21d, 10 rats in each subgroup.

Modified Allen spinal cord injury rat model: The rats were free access to food and water. The rats were anesthetized with intraperitoneal injection of 10% chloral hydrate (0.3ml/100g). A median incision of 5-6cm in length with the spinous process of T12 as the center was made at the back, then the skin, subcutaneous tissues and muscles were incised and the soft tissue was separated from the attached spinous process and the vertebral lamina. The spinal process and the whole vertebral lamina of T12, and the up and low half vertebral lamina of T11 and T13 were biting-removed, and the vertebral canal was open till the pedicle of vertebral arch to fully expose the spinal cord 10 mm in length with integrity of dura mater. A substance of 10 gram fell freely from a fixed height of 10 mm with inducing injury energy of 100gcm, to make a rat model of incomplete collision injury. A successful model was marked by rebound flutter of both hind limbs and spastic vibration of the tail. Finally, the incised tissues were sutured layer by layer.

Electroacupuncture: After the modeling, a filiform needle was inserted respectively into Dazhui (GV 14) and Mingmen (GV 4) for a depth of 0.5cm. Dazhui (GV 14) was connected with the anode and Mingmen (GV 4) with the cathode of an electroacupuncture stimulator and stimulation was given for 3 min, with the frequency of 20Hz, continuous pulse current, and an intensity of inducing regular contraction and vibration of both hind limbs. Afterwards, electroacupuncture was given once daily, for 7 days (one therapeutic course). The rats were then sacrificed at 1d, 3d, 7d, 14d and 21d after the spinal cord injury, respectively.

Sampling and section of the spinal cord: at 1d, 3d, 7d, 14d and 21d after the spinal cord injury, the rats were anesthetized with intraperitoneal injection of 10% chloral hydrate. Thoracotomy was performed to expose the heart, then a No.12 perfusion needle was inserted into the aorta along the left ventricle, and the right auricle was rapidly broken with a scissor. After that, the blood was rinsed out with perfusion of the aorta of the left ventricle with 200 ml saline of 37°C until reddish liquid flowed out from the right auricle, followed by rapid perfusion with 250ml of 0.1mol/L phosphate buffer (PH=7.4) containing 4% paraformaldehyde at 4°C for fixation, until the head, the neck and the tail became hardened. The thoracic and lumbar segments were then immerged in the above fixation liquid at 4°C for 24-48h.

3. Observation of Indexes

Detection of neurological function (BBB scale): The animal was kept active at the central area of activity range as more as possible and investigated with the BBB scale for 4 min.

Determination of AQP-4: The paraffin-embedded spinal tissues were cut with paraffin microtome in 5 μm sections and AQP-4 expression was detected with immunohistochemical staining method. Routine deparaffin and dehydration of the sections were carried out, and then sections were immerged in a 3% H2O2 solution for 10 min at room temperature to inactivate endogenous peroxidase, and washed with double distilled water for 3 times. Complex enzyme digestive solution was dripped on the section. After 2 min at room temperature, it was repaired for 10 min with microwave low fire, followed by washing (3 min × 2 times) with 0.1MPBS (PH7.4). Antigen repair liquid -I was dripped on the section, and after 2 min at room temperature, microwave low fire repair was carried out for 10 min, and the section was
washed 3 min × 2 times with 0.1MPBS (PH7.4). The normal goat serum sealing solution was added at room temperature, and 30 minutes later, the superfluous liquid was removed with no washing. Then the first antibody, rabbit anti-AQP-4 antibody (1:200) was added for over night in a refrigerator at 4°C; and washed (2 min × 3 times) with 0.1MPBS (PH7.4). The second antibody, biotinylated sheep anti-rabbit antibody was dripped at room temperature, and 30 minutes later, it was washed (2 min × 3 times) with 0.1MPBS (PH7.4). SABC (third antibody) was then added at room temperature, and 30 min later, it was washed (5min × 4 times) with 0.1MPBS(PH7.4). DAB coloration was conducted under the condition of light-free and it was then fully washed clean with distilled water (5min × 4 times) to stop the reaction, followed by light counter staining with Mayer campeachy, dehydration, hyalinization, mounting with neutral resin.

Control of immunohistochemical staining: In the sections of the same group, the rabbit anti-AQP-4 antibody was replaced respectively by the normal goat serum and PBS for incubation, and other procedures were the same as mentioned above so as to detect the specificity of AQP-4 immunoreaction.

Integral Optic Density (IOD): The sections were observed under an Olympus optical microscope. 12 sections were taken from each rat, and IOD of each section was analyzed with a pathological image analysis system (3.0) made by Beijing Aviation and Space University. The mean of the 12 sections was regarded as IOD of AQP-4 of the spinal cord of this rat.

4. Statistical Method and Data Processing

All of the data obtained were entered into a computer and analyzed statistically by one-way analysis of variance with SPSS10.0 statistical software, and the results were expressed as mean(x) ± standard difference (s). α =0.05 was regarded as significant difference and α =0.01 as very significant difference.

Results

1. Effect of electroacupuncture at acupoints of the Governor Vessel on neurological functions of the hind limbs in the rat of spinal cord injury (see Table 1 and Fig 1).

On the 1st day after the spinal cord injury, the rats in the spinal cord injury group (group B) and the electroacupuncture group (group C) showed paralysis of the hind limbs; on the 3d and 7d after the spinal cord injury, the function in the electroacupuncture group started to improve, with a significant difference as compared with the spinal cord injury group (P<0.05); on the 14d and 21d, rats in the electroacupuncture group could walk on a flat ground while those in the spinal cord injury group still have difficulty in walking.

2. Expression of AQP-4 in the injured spinal cord (See Table 2)

A certain number of AQP-4 expression was found in both the gray matter and the white matter of the spinal cord in the normal control group (group A) on 1d, 3d, 7d, 14d and 21d, with the cell membrane of the positive cells showed brown-yellow. The positive cells mainly distributed on the membrane of neurogliocytes and the angle plate membrane (one of important components of blood-brain barrier) close to the end of process of astrocytes of capillary, and the expression also was found on the membrane of the neurogliocytes (oligodendrocyte cell is main) surrounding the medullary sheath of the white matter and the limiting membrane of the glia. However, almost no expression was found on the membrane of the neuron.

1d after the spinal cord injury, brown-yellow immunomarker on the neuroglia membrane in the gray matter and the white matter of the spinal cord in the electroacupuncture group and the spinal cord injury group increased significantly, with significantly differences as compared with that in the
control group ($P<0.05$); The expression of AQP-4 reached to their peak values on 3d in both the two groups, and the expression in the white matter was stronger than that in the gray matter, and a weak positive expression was found on the membrane of a few neurons, with a very significant difference as compared with the normal control group ($P<0.01$); 7d, 14d and 21d after the spinal cord injury, there were very significant differences between the electroacupuncture group and the spinal cord injury group in the expression of AQP-4, and the expression of AQP-4 on 21d was close to the normal.

**Table 1. Comparison of BBB scores of the functions of the hind limbs among the 3 groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Days after operation (d, $\bar{x} \pm s$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>A</td>
<td>50</td>
<td>19.40±0.21</td>
</tr>
<tr>
<td>B</td>
<td>50</td>
<td>3.96±0.27**</td>
</tr>
<tr>
<td>C</td>
<td>50</td>
<td>4.12±0.30**</td>
</tr>
</tbody>
</table>

Note: Compared with the control group (A), *$P<0.05$, **$P<0.01$; Compared with the spinal cord injury group (B), *$P<0.05$, **$P<0.01$.

Fig.1 Comparison of BBB scores of behind limbs at different time among the 3 groups

**Table 2 Comparison of IOD of AQP-4 immunohistochemical staining among the three groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Days after operation(d, $\bar{x} \pm s$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>A</td>
<td>50</td>
<td>96.74±25.38</td>
</tr>
<tr>
<td>B</td>
<td>50</td>
<td>194.33±58.42*</td>
</tr>
<tr>
<td>C</td>
<td>50</td>
<td>170.56±29.27*</td>
</tr>
</tbody>
</table>

Note: Compared with the control group (A) *$P<0.05$, **$P<0.01$; Compared with the spinal cord injury group (B), *$P<0.05$, **$P<0.01$.

**Discussion**

TCM holds that traumatic paraplegia induced by spinal cord injury belongs to the category, "paraplegia", "flaccidity syndrome", but according to principles of the meridian theory, it should belong to "injury of the Governor Vessel". Therefore, injury of vertebrae is a phenomenon and the injury of the Governor Vessel is essence for the patient of traumatic paraplegia. Action of acupuncture at the Governor Vessel can reach the affected area, which conforms with the statement, "It is necessary to treat
the origin of a disease". So, for treatment of paraplegia, the Governor Vessel should be first selected. Xu’s holds that one of the mechanism of electroacupuncture at the Governor Vessel for treatment of traumatic paraplegia is possibly improvement of local blood microcirculation, changes of flow of cerebral spinal fluid, alleviation of the pressure of edema and hematoma in the part of the spinal cord injury, and reduction of the factors of inducing exacerbation of successive injury of the spinal cord. Therefore, in order to explore the mechanism, in the present study electroacupuncture at the Governor Vessel is used for treatment of experimental spinal cord injury in the rat.

The medical circles have been paying great attention to AQP-4, the newest study achievement in nervous sciences in recent 10 years. With deep study on molecular structure of AQP-4, its transporting mechanism and distribution in tissues, particularly, its specific distribution in the brain and the spinal cord of the central nervous system is correlated with formation and maturity of the blood-brain barrier, pathological studies of AQP-4 are of more significance. Verkman, et al. reported in 2000 that water content in the brain tissue and the swelling of the foot process of astrocytes adhering to the surroundings of capillary could be greatly reduced in local ischemic apoplexy induced by acute water intoxication and embolism of mesencephalic arteries in the rat of removed AQP-4 gene. This is indicated that antagonism against AQP-4, a key role in water-liquid transportation in the central nerve, can provide a new treatment way for relieving cerebral edema. It can be found in the experimental spinal cord injury tissue with HE staining that after spinal cord injury in the rat, the whole spinal tissue including the white matter and the gray matter, showed very obvious swelling within a short time, and the blood-brain barrier had been destroyed possibly. As compared with this, edema of the spinal tissue in the electroacupuncture group was milder. Thus, it can be deduced that electroacupuncture eliminates edema possibly by means of regulating expression of AQP-4.

In the experiment, there was a significant difference of neurological functions between the electroacupuncture group and the spinal cord injury group on 3d and 7d after the spinal cord injury, and a very significant difference on 14d and 21d. This almost paralleled with the decrease of AQP-4 expression after electroacupuncture treatment, and it also possibly indicated that electroacupuncture can reduce AQP-4 expression after spinal cord injury, inhibiting edema of the spinal cord to eliminate the successive injury of the spinal cord, so as to protect the survival normal spinal tissue and promote rebuilding of the nervous tissue. In the experiment, it also found that one of pathological mechanisms of edema due to the successive injury of the spinal cord is the injury inducing expression of AQP-4 on the membrane of the astrocyte constituting blood-brain barrier, which promotes water-liquid to rapidly pass through the capillary and get into the space of nervous tissues, and produce an abnormal expression of AQP-4 in a few of neurons.

Reference

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