Effects of Electroacupuncture on Expressions of Angiogenesis Factors and Anti-angiogenesis Factors in Brain of Experimental Cerebral Ischemic Rats after Reperfusion

Ma Jingxi 马瑞曦 & Luo Yong 罗勇

The First Affiliated Hospital, Chongqing University of Medical Sciences, Chongqing 400016, China

Objective: To explore the mechanism of electroacupuncture (EA) in improving ischemic stroke. Methods: The Wistar rat model of focal cerebral ischemia-reperfusion was prepared by the thread embolism method. The rats were randomly divided into a normal group, a model group, and an EA group. EA was given at bilateral “Hegu” (LI 4) in rats of the EA group. Expression of the vascular endothelial growth factor (VEGF) mRNA was detected with hybridization in situ, and expressions of the angiogenin-1 (Ang-1) and endostatin proteins were detected with the immunohistochemical method. Results: As compared with the normal group, the expressions of VEGF mRNA, Ang-1 protein and endostatin protein significantly increased in the model group (all \(P<0.05\)); and when compared with the model group, the EA group showed even more significant increase in expressions of the VEGF mRNA and Ang-1 protein (both \(P<0.05\)), but with an obvious decline in the increase of expression of endostatin protein (\(P<0.05\)). Conclusions: EA can promote angiogenesis in brain of experimental cerebral ischemic rats after reperfusion probably through up-regulating the expression of angiogenesis factors and down-regulating the expression of anti-angiogenesis factors.

After cerebral ischemia, restoring blood supply to the ischemic region as quick as possible to save the dying neurons, neuroglial cells and vascular endothelial cells is the key link for treatment of ischemic cerebrovascular diseases. In the past, the relevant researches were stressed on the neurons with less attention paid to the vascular endothelial cells. In fact, besides the conventional methods, such as dilating the blood vessels, the anti-coagulant therapy and the thrombolytic therapy, the measures taken for stimulating the genesis of blood capillaries may open up a new way. After focal cerebral ischemia, the newly formed collateral blood vessels can obviously improve perfusion of the surrounding tissues, and can promote recovery of the nervous functions.\(^1\) The studies of recent years have found that dynamic changes of the angiogenesis factors and anti-angiogenesis factors may jointly influence formation of the new blood vessels after cerebral ischemia. The present study is designed to investigate the focal cerebral ischemia-reperfusion and electroacupuncture (EA) on expressions of the vascular endothelial growth factor (VEGF), angiogenin (Ang-1) and endostatin in the rat brain, and to observe dynamic changes of the angiogenesis factors and anti-angiogenesis factors, so as to further expound the mechanism of EA for improving ischemic stroke.

MATERIALS AND METHODS

Grouping of the animals

The rats were randomly divided into a normal group (\(n=6\)), a focal cerebral ischemia-reperfusion model group (model group, \(n=42\)), a focal cerebral ischemia-reperfusion plus electroacupuncture group (EA group, \(n=42\)). The latter two groups were respectively subdivided into the 2-h, 12-h, 24-h, 3-d, 7-d, 14-d and 21-d reperfusion groups (\(n=6\)) after 1-h focal cerebral ischemia. No any treatment was given...
to the rats of the normal group.

Preparation of the rat model

Ninety adult male healthy Wistar rats, weighing 250-300 g, were supplied by the Center for Experimental Animals, Daping Hospital, the Third Military Medical University (Certificate No:SCXK 20020002). The focal cerebral ischemia-reperfusion rat model was prepared by thread embolism of the right middle cerebral artery. The rats were anesthetized with intraperitoneal injection of 3.5% chloral hydrate (1 mL/100 g body weight) and fixed in a dorsal position. Then, the common carotid artery, external carotid artery and the extracranial segment of the internal carotid artery were exposed in order via a medial incision on the neck. A ring-shaped incision was made at the end of external carotid artery, and a polyurethane-treated embolus nylon thread in a diameter of 0.23 mm was inserted 17-20 mm deep into the intracranial segment of the internal carotid artery. A feeling of resistance indicated that the thread thrombus head had reached the starting part of the middle cerebral artery with the occurrence of cerebral ischemia. One hour later, the thread thrombus was withdrawn to the end of the external carotid artery, and reperfusion was realized.

Stimulation methods and parameters of EA

In the EA group, bilateral “Hegu” (LI 4) were selected in reference to The Acupoint Atlas for Experimental Animals drawn by The Experimental Acupuncture and Moxibustion Committee, Chinese Association of Acupuncture and Moxibustion. 45 min after focal cerebral ischemia, EA was given at bilateral “Hegu” (LI 4) for 15 min each time. In the groups with reperfusion for over 1 day, EA was given once a day for 7 days at most. After that, the rats were fed naturally with no special treatment given.

EA stimulation parameters: the sparse-dense wave $F_1=40$ Hz, $F_2=60$ Hz, and no-load output voltage 1.5V.

Sampling

At the stipulated reperfusion time for each group, the rats were anesthetized, the left ventricle was rapidly washed by perfusion of 200 ml saline, and fixed with rapid and then slow perfusion of 500 ml 4% paraformaldehyde plus 0.1% DEPC, followed by decapitation with the brain taken and the paraffin sections prepared. The surrounding tissues of the ischemic region were observed and the adjacent sections were taken respectively for hybridization in situ and immunohistochemical examination.

Detection of VEGF mRNA with hybridization in situ

The VEGF mRNA in situ hybridization kits used were made by Wuhan Boshide Company. Processing of the pre-hybridization: After deparaffin, the sections were processed with a 3% citric acid-pepsin solution for 3 min, and then fixed with 1% paraformaldehyde (prepared with pH7.4 0.1 mol/L PBS, added with 0.1% DEPC). Hybridization: Drip pre-hybridization solution for a 6-h pre-hybridization at 39°C, and then drip appropriate amount of hybridization solution, cover with an in situ hybridization coverslip to keep overnight at 41°C. Washing after hybridization: Wash with 2XSSC, 0.5XSSC, 0.2XSSC, respectively. Drip the blocking solution, the biotinylated rat anti-digoxin antibody, SABC, and the biotinylated peroxidase in order, followed by DAB coloration, dehydration, clearing and mounting.

Immunohistochemical examination of Ang-1 and endostatin proteins

After deparaffin of the sections, antigen heat repair for 15 min at 92-98°C, drip blocking solution, drip respectively 1: 100 rabbit anti-rat Ang-1 antibody (Wuhan Boshide Company) and 1:100 rabbit anti-rat endostatin antibody (Wuhan Boshide Company) to keep overnight at 4°C, and drip SABC, followed by DAB coloration, dehydration, clearing and mounting.

Judgment and statistical processing

Count the positive cells under visual field (X400). The cells with cytoplasm stained brown-yellow was regarded as the positive ones. 3 non-continuous sections were randomly selected from each rat, and the mean of 10 non-continuous visual fields taken
from each section was recorded. The data were expressed as $\bar{x} \pm s$, the Excel software was used for $t$-test, and $P<0.05$ was regarded as the statistically significant difference.

RESULTS

VEGF mRNA in situ hybridization

In the normal group, a few positive cells were found in the cortex, internal capsule, and hippocampus. In the model group, VEGF mRNA expression significantly increased 2h after reperfusion ($P<0.01$) and reached to the peak 24h after reperfusion ($P<0.01$), which was still kept at a high level 7d after reperfusion ($P<0.01$). The diffuse distribution of the positive cells was found in the cortex round the ischemic necrotic region. And the brown-yellow cytoplasma was found in the nerve cells and vascular endothelial cells of the lateral ventricle and the dentate gyrus of hippocampus. As compared with the model group, VEGF mRNA increased more significantly in the EA group 2h, 12h after reperfusion ($P<0.01$) and reached to the peak 24h after reperfusion ($P<0.01$); and it still showed a statistically significant increase 14d after reperfusion ($P<0.05$). See Table 1.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>2h</th>
<th>12h</th>
<th>24h</th>
<th>3d</th>
<th>7d</th>
<th>14d</th>
<th>21d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>6</td>
<td>$4.08\pm0.85$</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Model</td>
<td>6</td>
<td>$7.22\pm1.32^*$</td>
<td>$14.42\pm3.44^*$</td>
<td>$25.23\pm4.22^*$</td>
<td>$20.29\pm3.45^*$</td>
<td>$11.24\pm2.56^*$</td>
<td>$7.47\pm1.72^*$</td>
<td>$3.92\pm0.24$</td>
</tr>
<tr>
<td>EA</td>
<td>6</td>
<td>$13.23\pm4.32^*$</td>
<td>$31.25\pm2.92^*$</td>
<td>$40.63\pm5.14^*$</td>
<td>$35.98\pm3.17^*$</td>
<td>$16.92\pm2.45^*$</td>
<td>$10.01\pm2.14^*$</td>
<td>$4.03\pm1.08$</td>
</tr>
</tbody>
</table>

Note: As compared with the normal group, *$P<0.01$; as compared with the model group, $^\Delta P<0.05$.

Immunohistochemical detection of Ang-1 protein

In the normal group, there was almost no the expression of positive cells in the brain tissue. In the model group, the Ang-1 protein expression was not significantly different to that of the normal group 2h and 12h after reperfusion ($P>0.05$); but it increased 24h after reperfusion ($P<0.01$), reached to the peak 14d after reperfusion ($P<0.01$), and did not return to the normal range 21d after reperfusion ($P<0.05$). The expression significantly increased in the cortex round the ischemic necrosis region. The choroids of lateral ventricle, ependymal cells and cytoplasma of endothelial cells of the peripheral small blood vessels were blown-yellow in color. A great number of neurons and gliocytes in the dentate nucleus of hippocampus showed very strong positive expression.

In the EA group, the Ang-1 protein expression increased from 2h to 21d after reperfusion as compared with that of the normal group, and it showed even more significant increase as compared with that of the model group, (the expression positions were same as those of the model group). As compared with the normal group, the expression began to increase 2h after reperfusion ($P<0.05$), it even more significantly increased 12h after reperfusion ($P<0.01$), and still kept a higher level 21d after reperfusion ($P<0.01$). When compared with the model group, it significantly increased 2h after reperfusion ($P<0.05$) and reached to the peak 14d after reperfusion ($P<0.01$), and still showed a significant difference 21d after reperfusion ($P<0.05$). See Table 2.

Immunohistochemical detection of endostatin protein

In the normal group, a less expression of endostatin protein was found in the hippocampus and striatum. In the model group, the endostatin protein expression began to increase 2h after reperfusion ($P<0.01$), reached to the peak 12h after reperfusion ($P<0.01$), and still showed significant difference 3d after reperfusion ($P<0.01$), but with no obvious difference 7d after reperfusion ($P>0.05$). The endostatin protein was expressed mainly in the ischemic penumbra,
including the surroundings of lateral ventricle, hippocampus and striatum, but with a less expression in the cortex; and it was expressed mainly in the vascular endothelial cells, but with less expression in the nerve cells.

As compared with the normal group, in the EA group, the endostatin protein expression increased 2h after reperfusion ($P<0.01$), but the increase was much lower and with no significant difference 3d after reperfusion ($P>0.05$). When compared with the model group, the endostatin protein expression was significantly inhibited within 3d after reperfusion, and it still showed a declining tendency in the following days though there was no obvious difference ($P>0.05$). See Table 3.

### Table 2. The Ang-1 protein expressions ($\bar{X} \pm s$)

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>2h</th>
<th>12h</th>
<th>24h</th>
<th>3d</th>
<th>7d</th>
<th>14d</th>
<th>21d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>6</td>
<td>5.15±1.03</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Model</td>
<td>6</td>
<td>5.72±0.75</td>
<td>6.32±1.72</td>
<td>8.72±2.31**</td>
<td>10.37±2.53**</td>
<td>13.08±2.93**</td>
<td>20.19±3.12**</td>
<td>8.15±2.45*</td>
</tr>
<tr>
<td>EA</td>
<td>6</td>
<td>7.23±1.24*</td>
<td>8.92±2.50**</td>
<td>11.42±2.32**</td>
<td>18.72±3.17**</td>
<td>17.14±2.45**</td>
<td>28.17±4.54**</td>
<td>11.22±1.57**</td>
</tr>
</tbody>
</table>

Note: As compared with the normal group, **$P<0.01$, *$P<0.05$; as compared with the model group, $^\Delta$P<0.01, $^\#$P<0.05.

### Table 3. The endostatin protein expressions ($\bar{X} \pm s$)

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>2h</th>
<th>12h</th>
<th>24h</th>
<th>3d</th>
<th>7d</th>
<th>14d</th>
<th>21d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>6</td>
<td>4.15±1.97</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Model</td>
<td>6</td>
<td>24.82±4.06*</td>
<td>28.64±3.17*</td>
<td>23.30±3.05*</td>
<td>15.79±3.26*</td>
<td>5.32±1.45</td>
<td>3.72±1.33</td>
<td>4.09±2.17</td>
</tr>
<tr>
<td>EA</td>
<td>6</td>
<td>14.52±1.33*</td>
<td>21.32±3.24*</td>
<td>15.61±5.17*</td>
<td>6.51±2.55*</td>
<td>3.45±1.62</td>
<td>4.05±2.15</td>
<td>3.81±1.86</td>
</tr>
</tbody>
</table>

Note: As compared with the normal group, *$P<0.01$; as compared with the model group, $^\Delta$P<0.01, $^\#$P<0.05.

### DISCUSSION

Expression of the angiogenesis factors

Angiogenesis after cerebral ischemia can be regulated by a series of angiogenesis factors and anti-angiogenesis factors. The angiogenesis factors, including specific VEGF family and Ang family of the endothelial cells, have the positively regulative action. They are interrelated each other and jointly regulate various links of angiogenesis. The VEGF/VEGFR system may possibly play a key role in initiating angiogenesis and is the central link for regulating and controlling angiogenesis after cerebral ischemia. VEGF is a specific mitogen and chemo-tactic factor of the endothelial cells. In the present study, the expression of angiogenesis factors increased after cerebral ischemia. VEGF mRNA began to increase 2h after reperfusion, reached to the peak 24h after reperfusion, and still showed significant difference 14d after reperfusion; while Ang-1 protein began to increase 24h after reperfusion, reached to the peak 14d after reperfusion, and did not return to the normal range 21d after reperfusion. It is indicated that VEGF may possibly play a role mainly at the early stage and metaphase, while Ang-1 show the effect at the metaphase and later stage, with a crossed and overlapped acting time. The activity of Ang-1 began to increase later than that of VEGF, which may help to increase branches of the blood vessels, and promote the remodeling, maturity and stability of blood vessels. The different time for VEGF and Ang-1 expressions may be the process for VEGF to activate endogenous angiogenesis at the early stage of cerebral ischemia with the increase of vascular permeability and the destruction of blood-brain barrier function; while Ang-1 has an inhibitory action on migration of the nascent endothelium, it mediates maturity of the blood vessels and prevents the blood-brain barrier from being over destroyed.

As compared with the model group, in the EA group, VEGF mRNA increased 2h after reperfusion, reached to the peak 24h after reperfusion, and still showed a statistically significant difference 14d after reper-
fusion, indicating that the increase of VEGF mRNA was more obvious in the EA group and lasted longer. Ang-1 protein in the EA group showed a significance increase 2h after reperfusion as compared with that of the normal group; and when compared with the model group, in the EA group, Ang-1 protein began to increase 2h after reperfusion, reached to the peak 14d after reperfusion, and still showed significant difference 21d after reperfusion, indicating that Ang-1 protein increased earlier and more obviously in the EA group. Most of the previous studies on focal cerebral ischemia-reperfusion were aimed at the early stage. In the present study, the period of experiment was prolonged to 21 days, so we can see a whole process of the expressions for angiogenesis factors. And the EA stimulation was given only for 7 days at most, but the expression level of angiogenesis factors was still higher than that of the model group, showing a lasting effect of EA.

In the present study, the expressions of VEGF and Ang-1 were found mainly around the ischemic regions involving both the nerve cells and the vascular endothelial cells, indicating that the angiogenesis factors may possibly act simultaneously on the nerve cells and the vascular endothelial cells, mediating the interaction of nerves and blood vessels, and playing an important role in repair of the nerve system and the blood vessel system after cerebral ischemia. The “nerve-vessel unit” is mainly composed of the nerve cells, the vascular endothelial cells, the astrocytes, and their surrounding extracellular matrixes as well, which should be comprehensively considered for treatment of cerebral ischemia. This concept emphases the interaction and dynamic balance between the cells, and between the cells and matrixes, comprehensively reflecting the pathological and physical processes for cerebral ischemia. Whether or not the actions of VEGF and Ang-1 are correlated with the “nerve-vessel unit”, and how the EA stimulation influence the “nerve-vessel unit” need to be further studied.

**Expression of the anti-angiogenesis factors**

In the past, in the study of angiogenesis after cerebral ischemia, more attention was paid to the angiogenesis factors, but not enough to the anti-angiogenesis factors. The anti-angiogenesis factors include endostatin and angiostatin, which have the negative regulating effects to antagonize the action of the angiogenesis factors. Endostatin may induce apoptosis of the endothelial cells via multiple ways, and it can inhibit proliferation and migration of the endothelium and block activation of the VEGF receptor. The ability of angiogenesis depends on dynamic balance between the angiogenesis factors and anti-angiogenesis factors, so regulating the balance between them may help promote or inhibit the growth of blood vessels. At present, most of the studies for endostatin are focused on inhibition of the tumor growth. The endostatin expression after cerebral ischemia has not been reported yet. In the present study, the endostatin expression significantly increased 2h after cerebral ischemia and reached to the peak 12h after reperfusion in the model group, which basically conforms to the time of apoptosis of the endothelial cells; but it showed no significant difference 7d after reperfusion. Endostatin inhibits angiogenesis at the early stage (<24h) after cerebral ischemia. The above studies suggest that a decreased expression of the anti-angiogenesis factors at the early stage (<24h) may possibly contribute to angiogenesis. In the EA group, the endostatin protein expression increased within 24h after reperfusion (P<0.01), but the increase was significantly inhibited as compared with the model group (P<0.01 or 0.05), indicating that EA can significantly decrease the early expression of anti-angiogenesis factors.

**Effects of EA on the angiogenesis factors and anti-angiogenesis factors**

The previous studies suggested that EA at “Baihui”
(GV 20) and “Shuigou” (GV 26) could stimulate the expression of angiogenesis factors after cerebral ischemia, and promote the neogenesis of blood vessels and the recovery of nervous functions. 7-9 EA promoting angiogenesis after cerebral ischemia is possibly related with its bidirectional regulating effects on expression of the vascular factors, i.e., up-regulating the expression of angiogenesis factors and down-regulating the expression of anti-angiogenesis factors. It is found in the present study that after EA, the expression of angiogenesis factors was increased, while the expression of anti-angiogenesis factors was inhibited.

At present, for therapeutic angiogenesis, the corresponding recombinant proteins or genes are usually introduced into the ischemic tissues to stimulate neogenesis of the collateral capillary vessels. But clinically, it is not easy to control this exogenous “supplementary” mechanism. We think that EA may possibly activate an endogenous angiogenesis with a better regulation and effect.

REFERENCES