EFFECTS OF ACTIVATING BLOOD CIRCULATION TO DISSIPATE BLOOD STASIS ON THE EXPRESSION OF HMGB1 AND PKA/CREB IN RATS WITH CEREBRAL HAEMORRHAGE

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ABSTRACT

In this study, the expression of high mobility group box protein B1 (HMGB1), protein kinase A (PKA) and cyclic adenosine response element binding protein (CREB) in rats with activated blood circulation and removal of blood stasis (ICH) was investigated. A total of 180 healthy male Wistar rats were divided into a model group (n=60), an acupuncture group (n=60) and a sham operation group (n=60) by the random number table method. Each group was divided into five subgroups based on different time points, 6 h, 12 h, 24 h, 72 h and 7 d, with 12 rats in each group. Rats in the model group were injected with autologous blood to establish a cerebral haemorrhage model. Rats in the acupuncture group were treated with acupuncture "Baihui" to penetrate "convoluted temples" after establishing the cerebral haemorrhage model. Rats in the sham operation group received the same operation as the model group but no blood injection. The neurological function score and the expression levels of CD36, HMGB1, PKA and CREB in brain tissues were observed. The neurological function score for the sham operation group was significantly lower than that for the model group, and the difference was statistically significant (P<0.05). The neurological function score for the acupuncture group was significantly lower than that for the model group 24 hours after operation (P < 0.05). The expression levels of CD36 and HMGB1 in brain tissues of rats in the model group were significantly higher than those in the sham operation group (P<0.05). After 12 hours, the expression of CD36 in brain tissues of the model group was significantly lower than that in brain tissues of the acupuncture group (P < 0.05). After 12 hours, the expression of HMGB1 in brain tissues of the model group was significantly lower than that in brain tissues of the acupuncture group (P < 0.05). After 12 hours, the expression of P-PKA in brain tissues of the model group was significantly higher than that in brain tissues of the acupuncture group at each time point (P<0.05). The expression of P-CREB in the model group was significantly higher than that in the sham operation group at each time point (P < 0.05). The expression levels of P-CREB in the acupuncture group at 6 h, 12 h, 24 h, 72 h and 7 d were significantly higher than those in the model group (P<0.05). The results show that the method of activating blood circulation and removing blood stasis can improve the neurological function score, promote the expression of CD36 protein and inhibit the expression of HMGB1 protein. It could simultaneously also activate the PKA / CREB pathway, increase the expression of PKA and CREB proteins, alleviate brain injury and promote nerve repair in rats with cerebral haemorrhage.

Keywords: Activating blood circulation to dissipate blood stasis, cerebral haemorrhage, HMGB1, PKA/CREB.

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Introduction

Intracerebral haemorrhage (ICH) refers to non-traumatic intraparenchymal haemorrhage caused by vascular rupture and accounts for 10-20% of all cerebrovascular accidents (CVA)(1-2). Many epidemiological surveys have shown that the worldwide acute mortality rate is 30-40% every year. Many factors can trigger ICH, and the underlying mechanism is complex. Perneckzy R et al. reported that ICH is mainly related to vascular ageing, diabetes, hypertension, hyperlipidaemia and other cerebrovascular diseases(3). After the majority of cases are initially treated, patients are often left with sequelae, such as speech disorders, cognitive disorders, and dyskinesias, that seriously affect their quality of life. Liu S et al. found that the blood circulation and phlegm method can improve the symptoms of cerebral haemorrhage and microcirculation, promote haematoma absorption, and reduce the disability rate and is an important measure for the treatment of acute cerebral haemorrhage(4). However, there are few studies concerning the effects of high
mobility group protein B1, protein kinase A and cyclic adenosine monophosphate response element binding protein expression. This study sought to investigate the effects of promoting blood circulation and removing blood stasis on the expression of HMGB1, PKA, and CREB in rats with cerebral haemorrhage in order to provide an experimental and theoretical basis for clinical treatment.

Materials and methods

Experimental animals and grouping

A total of 180 healthy male Wistar rats (qualified license number: SCXK 2015-0001), weighing 280-320 g, were purchased from Beijing Weitong Li-hua Experimental Animal Technology Co., Ltd. Rats were housed in animal laboratory ventilation cages (6 rats per cage), maintained at constant temperature (20-23°C) and humidity (50-60%), and received 12 hours of light per day. All rats were fed the same type of sterile feed without water restriction, fasting 12 hours before surgery and given no water for 6 h prior to surgery. The rats were divided into a model group (n=60), an acupuncture group (n=60) and a sham operation group (n=60) by the random number table method. According to time points, each group was divided into five subgroups (12 in each group), including 6 h, 12 h, 24 h, 72 h, and 7 d. There were no significant differences in the physical data of the three groups (P value over 0.05). This study was approved by the Animal Protection Association and the Medical Ethics Committee.

Reagents and materials

Rabbit anti-human CD36 polyclonal antibody and rabbit anti-human HMGB1 polyclonal antibody were purchased from Clonetech. RIPA tissue protein lysate was purchased from Beijing Baiyek Biotechnology Co., Ltd. Rabbit anti-CREB polyclonal antibody was purchased from AMRESCO. Rabbit anti human PKA polyclonal antibody was purchased from OmegaBiotek. TBS buffer and electrophoresis buffer were purchased from Changchun Bona Biotechnology Co., Ltd. 10% chloral hydrate and 10% ammonium persulfate were purchased from Shanghai Xinran Biotechnology Co., Ltd. Iodine phenol and gentamycin were purchased from Dongguan Walixi Chemical Co., Ltd. The acupuncture needles were purchased from Guangzhou Kangmai Medical Instrument Co., Ltd. The microplate reader (AU5800) was purchased from Since Shenzhen Shengxinkang Technology Co., Ltd. The stereo locator (RD1617-1ss) was purchased from Stoelting.

Preparation of experimental model

Model group: the rats were anaesthetized with 10% chloral hydrate. After anaesthesia, the rats were fixed in the prone position with a stereotactic positioner. The heads of the rats were depilated and disinfected with iodophenol. Skin incisions along the top of the head were made to expose the peristeum and pre-halogen points. The peristeum and pre-halogen points were opened 3.5 mm adjacent to the pre-halogen points and 0.2 mm behind the pre-halogen points.

Sterilized rat tail blood (50μl) was injected into the caudate putamen nucleus of the mice. Gentamycin injection was dripped into the incision on the head and sealed with a toothpick. The skin of the head was sutured and disinfected, and the rats were returned to their cages. After the rats regained consciousness, screening for successfully established mode mice was performed according to the Berderson scoring system(5). If the number of rats in the same group was insufficient, the same batch of rats was selected for remodelling and supplementation.

Sham operation group: no blood was injected during the preparation of the rat cerebral haemorrhage model.

Acupuncture group: after preparing the rat cerebral haemorrhage model, acupuncture intervention was performed on the pathological side with 0.25 mm×15 mm Baihui and Qufu points (insert needle 0.8 inches, leave needle for 30 minutes, needle once every 5 minutes during needle retention).

Detection method

The protein expression levels of CD36, HMGB1, P-PKA and P-CREB in rat brain tissues were determined by Western blot analysis. At the corresponding time points, 6 rat brain tissue samples were taken from each subgroup. RIPA tissue protein lysate solution was added; the tissues were shaken and pulverized, allowed to stand for 30 min, and centrifuged, and then the supernatant was collected and stored at -80°C for subsequent analysis. Protein concentration, electrophoresis, membrane transfection, and blocking were performed in turn. Primary antibody was added, and incubation was carried out at 4°C overnight. Secondary antibody was added, and incubation was carried out at 25°C for 2 h.
After washing, exposure and development were performed, and the grey value of the band was analysed with a Quantity-one gel imaging analysis system.

Neurological function score: the neurological function of the rats was evaluated by the Berder-son score (score 0 to 3, lower scores indicate better neurological function), as shown in Table 1.

### Table 1: Rat neurological function score.

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Observed parameters
The neurological function scores of the rats in each group, and the expression levels of CD36, HMGB1, PKA and CREB in the brain tissues of each group.

Statistical methods
The data were statistically analysed using SPSS version 22.0 software. Utilizing repeated measures, analysis of variance was used to perform comparisons between the data. The difference was determined to be statistically significant at a p<0.5.

Results

### Neurological function score in each group
The scores of neurological function in the sham operation group were significantly lower than those in the model group (p<0.5). After model establishment for 24 h, the scores of neurological function in the acupuncture group were significantly lower than those in the model group (p<0.5) (Figure 1 and Table 2).

### Table 2: Neurological function scores of each group (n=12, x̅ ± s).

<table>
<thead>
<tr>
<th>Group</th>
<th>Modulus (number of animals)</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
<th>72 h</th>
<th>7 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation</td>
<td>60</td>
<td>0.00±</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Model</td>
<td>60</td>
<td>1.66±</td>
<td>0.50*</td>
<td>2.34±</td>
<td>2.48±</td>
<td>2.58±</td>
</tr>
<tr>
<td>Acupuncture</td>
<td>60</td>
<td>1.59±</td>
<td>0.48</td>
<td>2.08±</td>
<td>1.88±</td>
<td>1.68±</td>
</tr>
</tbody>
</table>
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### Figure 1: Neurological function score in each group.

The expression level of CD36 in brain tissues of rats in each group
The expression of CD36 in the model group was significantly higher than that in the sham operation group at each time point (p<0.05), and the expression of CD36 in the brain tissues of the model group was significantly lower than that in the brain tissues of the acupuncture group 12 hours after model generation (p<0.05) (Figure 2, Table 3).

### Table 3: The expression level of CD36 in brain tissues of each group (n=12, x̅ ± s).

<table>
<thead>
<tr>
<th>Group</th>
<th>Modulus (number of animals)</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
<th>72 h</th>
<th>7 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation</td>
<td>60</td>
<td>5623.68±868.37</td>
<td>5894.08±788.58</td>
<td>5741.52±814.53</td>
<td>5633.27±752.06</td>
<td>5652.61±819.49</td>
</tr>
</tbody>
</table>
| Model         | 60                          | 8498.32±882.09* | 11359.39±695.28* | 13360.59±627.19* | 15401.88±791.44* | 16381.95±750.00*
| Acupuncture   | 60                          | 9589.83±789.49 | 13711.31±856.30* | 18603.18±661.31** | 20315.96±888.89* | 23402.58±805.41** |

Note: compared with the sham operation group, "p<0.05; compared with the model group, "p<0.05."
The expression level of HMGB1 in brain tissues of rats in each group

The expression level of HMGB1 in brain tissues of the model group was significantly higher than in the sham operation group at each time point (p <0.05). The expression level of HMGB1 in brain tissues of the model group was significantly higher than that in the brain tissues of the acupuncture group 12 hours after model generation (p<0.05) (Figure 3 and Table 4).

The expression level of P-PKA in brain tissues of rats in each group

In the model group, the expression of P-PKA increased 6 hours after operation but decreased significantly 12 hours, 24 hours and 72 hours after operation. The expression of P-PKA in brain tissues of 7 day-model rats was significantly higher than that in brain tissues of sham-operated rats (p<0.05), and the expression of P-PKA in brain tissues of rats in the acupuncture group 6 hours post-operation was significantly higher than that in the brain tissues of the sham-operated rats (p<0.05). The expression of P-PKA in brain tissues of rats 12 and 24 hours after operation was significantly lower than that in the brain tissues of rats 72 hours after operation. The expression of P-PKA was significantly higher in the acupuncture group than in the model group (p<0.05). The expression of P-PKA was slightly lower in the 7 day–model rat brain tissues, and the expression of P-PKA in the acupuncture group was significantly higher than that in the model group at 6, 12, 24 and 72 hours (p<0.05) (Figure 4).

The expression level of P-CREB in brain tissues of rats in each group

In the model group, the expression of P-CREB increased 6 hours after operation but decreased significantly 12 hours, 24 hours and 72 hours after operation. The expression of P-CREB in brain tissues of rats 7 days after operation was significantly higher than that in the brain tissues of rats in the sham operation group (p<0.05). After 6 hours, the expression of P-CREB began to increase; the expression of P-CREB decreased significantly at 12 hours. The expression of P-CREB increased gradually at 24 hours, 72 hours and 7 days. The expression of P-CREB in the acupuncture group was significantly higher than that in the model group at 6 hours, 12 hours, 24 hours, 72 hours and 7 days (p<0.05).
Discussion

As the blood pressure rises suddenly, brain aneurysms and brain arteriovenous malformations often result in cerebral artery rupture and haematoma, producing clinical manifestations related to dysfunction of local nerves in the brain, such as dysphagia, gaze palsy, speech disorders, bilateral sputum stagnation, headache and dizziness. The main clinical treatment principles are to reduce intracranial pressure, control hypertension and reduce brain oedema, in order to reduce the disability rate and associated complications and save the lives of affected patients. Activating blood circulation and removing blood stasis is reportedly effective for the treatment of cerebral haemorrhage, which can improve nerve function, lower blood pressure and shorten the course of disease. The acupuncture method is commonly used to promote blood circulation and remove blood stasis. After needle insertion, blood stasis can be directly removed and eliminated, and meridians can be dredged. It has also been reported that early use of acupuncture in mice with cerebral haemorrhage could promote haematoma absorption and reduce sequelae. The present study found that the neurological function scores in the model group were significantly higher than those in the sham-operated group. Twenty-four hours after model establishment, the neurological function scores of the model group were significantly higher than those of the acupuncture group, which is largely consistent with previous reports. This shows that promoting blood circulation and removing blood stasis can significantly improve nervous system function, reduce brain oedema and improve the quality of life of affected rats.

Babiker MYA et al. reported that CD36 polymorphism was closely related to cerebral vascular diseases, which was particularly relevant for the prediction of ICH. CD36 is an important member of class B scavenger receptors (SR), and CD36 is expressed in many cell types, including dendritic cells, macrophages, monocytes, vascular endothelial cells, haematopoietic cells, and others. CD36 participates in the absorption of haematomas, atherosclerosis (AS) formation and other processes and is a scavenger receptor for endocytosed low-density lipoprotein (ox-LDL) on the surface of macrophages. The expression level of CD36 is regulated by several inflammatory factors. Liu J et al. found that inflammatory reactions can aggravate ICH and downregulate the expression of CD36, thus affecting the absorption of haematomas and aggravating brain injury. The level of CD36 protein in rat brain tissues was detected by Western blot analysis. Our results showed that the expression level of CD36 in the model group at each time point was significantly higher than that in the sham operation group. After model establishment for 12 h, the expression level in the model group was significantly higher than that in the sham operation group. The expression of CD36 in brain tissue of the model group was significantly lower than that in the acupuncture group, indicating that promoting blood circulation and removing blood stasis could promote the expression of CD36 after ICH, reducing neuronal damage and protecting brain tissue. Recent studies have confirmed that acupuncture and moxibustion can affect the expression of HMGB1-positive cells after intracerebral haemorrhage. HMGB1, a DNA-binding protein, is widely expressed in eukaryotic cells and has three unique domains: A, B and C. As NF-κB, ERK1/2, p38MAPK, JAK/STAT and other signal transduction pathways are activated, the release of HMGB1 is promoted. The B region exerts its pro-inflammatory function, the A region plays an anti-inflammatory role, and the C region binds to other proteins to regulate DNA and HMGB1 affinity. Matsuda A et al. found that when the murine HMGB1 gene was knocked out, the inflammatory response was inhibited, further confirming that HMGB1 is closely related to the inflammatory reaction. It has also been reported that HMGB1 can stimulate endothelial cells (ECs) to increase the expression of cell adhesion molecule (CAM) and inflammatory cytokines, such as IL-1β, TNF-α and IL-6. The plasma level of this protein in patients with cerebral ischaemia increased to 13 times greater than the normal level within 24 hours after acute onset, suggesting that HMGB1 is involved in the pathological process of cerebral ischaemia. In this study, the expression of HMGB1 in the brain tissues of the model group was significantly higher than that in the brain tissues of rats in the sham-operation group at each time point, and the difference was statistically significant (P<0.05). After 12 hours of modelling, the expression of HMGB1 in the brain tissues of the model group was significantly higher than that in the brain tissues of rats in the acupuncture group, indicating that promoting blood circulation and removing blood stasis could inhibit the expression of HMGB1 after ICH, thereby inhibiting the HMGB1-mediated inflammatory response, reducing brain damage and protecting brain tissue.
Related studies have shown that regulation of PKA/CREB signalling has a significant effect on ICH treatment. The PKA/CREB pathway is involved in protein synthesis and is an essential signal transduction pathway for inducing and maintaining long-term memory potentiation (LTP), which is closely related to learning and memory. PKA mainly exists in the cytosol, plasma membrane and nuclear membrane of mammalian cells, and consists of two catalytic subunits (C subunits) and two regulatory subunits (R subunits). Cyclic adenosine monophosphate (cAMP) can dissociate the R subunits and C subunits by binding to R subunits, thus releasing the C subunit. The activated PKA C subunit can promote the phosphorylation of several protein serine or threonine residues in cells, thereby altering protein activity and further affecting gene expression. Ernest P et al. found that the activation of CREB in rats with ICH could enhance neuronal activity, which is related to the regulation of downstream gene transcription by CREB16. It has also been reported that Buyang Huanwu Decoction can increase the level of cAMP, the expression of PKA protein and the DNA binding activity of CREB in vascular dementia (VD) model rats, enhancing the role of the PKA/CREB pathway and thereby improving cognitive impairment, learning and memory impairment in rats17. Therefore, the PKA/CREB pathway may function as a neuroprotective mechanism against cerebrovascular diseases.

The results of Western blot analysis showed that the expression of P-PKA and P-CREB in the rat brain tissues in the model group increased 6 hours after operation. The local inflammatory reaction in the brain tissues after acute ICH, the phosphorylation of the PKA protein and the increase in PKA protein expression under the stimulation of inflammatory factors may have promoted the phosphorylation of downstream CREB protein, thereby enabling the phosphorylation of PKA protein. PKA and CREB jointly promote nerve repair. However, the expression of P-PKA and P-CREB in brain tissues of rats decreased significantly with time after the operation (12 h, 24 h and 72 h). On the 7th day after the operation, the stimulation to the brain tissue was reduced, the haematoma was absorbed, and the nerve function was gradually restored; therefore, the expression of P-PKA and P-CREB increased. At the same time, the expression of P-PKA in the acupuncture group was significantly higher than that in the model group at 6 h, 12 h, 24 h and 72 h post-operation, and the expression of P-CREB in the acupuncture group was significantly higher than that in the model group. This may be related to the impressive self-healing observed in rats. This further suggests that the method of activating blood circulation and removing blood stasis can activate the PKA/CREB pathway, increase the expression of the PKA and CREB proteins, alleviate brain injury in rats, and promote nerve repair.

In conclusion, activating blood circulation and removing blood stasis could improve neurological function scores, promote the expression of the CD36 protein and inhibit the expression of the HMGB1 protein. This resulted in activation of the PKA/CREB pathway, increased expression of PKA and CREB proteins, alleviation of brain injury and promotion of nerve repair in rats with cerebral haemorrhage.

References


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