Effect of acupuncture at Fengchi (GB 20) on the activity of myosin light chain kinase in the middle meningeal artery of migraine modeled rats


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Abstract

OBJECTIVE: To study the effect of acupuncture at Fengchi (GB 20) on the activation of myosin light chain kinase (MLCK) in the middle meningeal artery of migraine modeled rats.

METHODS: Forty-four clean grade healthy female Sprague-Dawley (SD) rats were randomly divided into four groups: the control group, blank control group, Fengchi (GB 20) acupuncture group, and Fengchi (GB 20) prevention group. Neurogenic inflammation of these rats was induced by electrical stimulation. The γ-32P infiltration method was then used to detect MLCK activation in the middle meningeal artery, and immunocytochemistry was applied to detect the structural protein expression of MLCK.

RESULTS: The migraine model was successfully established in the rats. Compared with the control group, MLCK activation was significantly decreased in the blank control group (P < 0.01).

CONCLUSION: The activation of MLCK in the middle meningeal artery was increased by acupuncture at Fengchi (GB 20), indicating its effectiveness in preventing and curing on acute migraine attacks.

INTRODUCTION

Migraine is a type of headache disorder; its mechanism is associated with neurogenic inflammation. Stimulation of the trigeminal nerve endings around blood vessels causes the release of substance P, calcitonin gene-related peptide (CGRP), and other vascular-active substances. These substances have a strong dilatory effect on blood vessels; CGRP has the strongest effect on blood vessel expansion, and it can also induce intracranial allergic pain in those that are sensitive to it. Headaches are actually caused by vasodilation and central sensitization. Myosin light chain kinase (MLCK) and protein kinase C are both involved in the process of vascular contraction and expansion, with the MLCK signal pathway playing the principal role. Ca^2+/calmodulin (CaM)-dependent MLCK was the first protein discovered that depends on CaM kinase. Ca^2+/CaM-dependent MLCK is also an important protein in the inositol triphosphate, Ca^2+/CaM transduction pathway; MLCK phosphorylates the regulatory myosin light...
The 44 rats (individual weight (265 ± 15) g) were randomized into four groups using the method of simple random sampling; SAS software was used to generate random numbers that were implemented using the central randomization system. The four groups were: a normal control group, a blank control group, a Fengchi (GB 20) prevention group; each group contained 11 rats.

**Materials and Methods**

**Materials**

Clean grade, healthy, 9-week-old, female Sprague-Dawley rats (n = 44) each weighing (265 ± 15) g were obtained from Weitong Lihua Animal Experimental Technology Co., Ltd., Beijing, China, No. SCXK (Jing) 2006-0009. CaM was purchased from Abcam company (Cambridge, MA, USA). γ-32P was purchased from Beijing Forest Biological Engineering Co., Ltd. The other reagents used were domestic.

The rat stereotaxic instrument was purchased from Narihige Co., (Tokyo, Japan). The dental laboratory bench drill was purchased from NHK, Japan. The Sigma 3K30 ultracentrifuge and Wallac 1450 liquid scintillation analyzer were produced by PerkinElmer, Massachusetts, USA.

**Animal selection and grouping**

The 44 rats [individual weight (265 ± 15) g] were randomly divided into four groups using the method of simple random sampling; SAS software was used to generate random numbers that were implemented using the central randomization system. The four groups were: a normal control group, a blank control group, a Fengchi (GB 20) acupuncture group, and a Fengchi (GB 20) prevention group; each group contained 11 rats.

**Establishing of migraine modeled rats**

Each rat was anesthetized by intraperitoneal injection of 10 % chloral hydrate from Beijing wo hai global Technology Co., Ltd. (Beijing, China). The anesthetized rat was then fixed onto a device with its skull exposed. An incision 2.8-3.2 mm long was made in the anterior fontanelle retrusion of 3.2-3.4 mm. The dental laboratory bench drill was used to drill two 1-mm diameter holes, exposing the dura mater over the sagittal sinus as a stimulus hole. The metal electrodes of the tandem-wound electrode stimulator from Japan NSK Co., Ltd. (Tokyo, Japan) were then used to stimulate the dura. The electrical stimulation was a 16 Hz, square pulse of 10-min duration, with a current strength of 0.5-1 mA. The above procedures were carried out at room temperature under aseptic conditions in a quiet environment free from bright lights.

**Acupuncture treatment method**

No treatment was given to the normal control group. For the blank control group: no treatment on it after modeling. For acupuncture Fengchi (GB 20) group: while modeling successful, immediately acupuncturing their Fengchi (GB 20) for 2 min, and then retaining needles for 20 min. For Fengchi (GB 20) prevention group: first Needling Fengchi (GB 20) for 2 min, retaining for 20 min, then modeling.

The acupuncture point Fengchi (GB 20) was located according to human standards and transposed to the analogous anatomic positions in the rat. Acupuncture was performed on rats in the two treatment groups using 0.5-inch (25-mm) Huatuo brand stainless steel needles from Suzhou Medical Appliance Factory (Suzhou, China). An even reinforcing-reducing method was used; the needle was thrust in 1 cm at Fengchi (GB 20), and twisted and turned 180 degrees at a frequency of 120 times/min. The needle was continuously twisted for 2 min, and then retained for 20 min. The rats in the Fengchi (GB 20) acupuncture group were needled at Fengchi (GB 20) prevention group were needled at Fengchi (GB 20) for 2 min, with the needles retained for 20 min. The rats in the Fengchi (GB 20) prevention group were needled at Fengchi (GB 20) for 2 min, the needles were retained for 20 min, and then electrical stimulation was applied.

**Specimen preparation**

After the above-mentioned acupuncture procedure was completed, immediately cut off the rats’ heads and removed the brains. The scalps were incised, and the skulls were cut using a saw. Blunt dissection at the bottom of the dura mater was conducted until the foramen magnum was reached.

The middle meningeal artery was removed and quickly placed into a 0.1 multiple phosphate buffer solution (MPBS) (pH 7.4) + 10 % formaldehyde solution (including 1/1000 diethylpyrocarbonate) and kept for three consecutive days.

The middle meningeal artery was placed in liquid nitrogen for cold storage, and then transferred to a freezer at - 80 °C to await further testing.

**Determination of MLCK protein expression**

Specimens were embedded in paraffin; then three slices (thickness: 6 μm) of adjacent tissue section were made, dewaxed, and put into water. Distilled water (30 mL) was added to the tissue sections for 10 min at room temperature to prepare for the addition of fresh 0.3 % hydrogen peroxide (H2O2); sections were washed with...
distilled water three times. Citrate buffer (10 mL) at about 92-95 °C was added and left for 15 min. The specimens were then placed in phosphate saline buffer (10 mL) (PBS) (pH 7.4) three times for 5 min each time. Normal serum was added in a dropwise fashion (1:100), and left for 20 min at room temperature. The first antibody (1:200) was added at 4 °C and incubated overnight. PBS (pH 7.4) was used to wash the specimens for 2 min, repeated with fresh PBS three times. Biotin-labeled secondary antibody was then added at 37 °C for 20 min. PBS (pH 7.4) was again used to wash the specimens for 2 min, repeated with fresh PBS three times. Tris-HCl buffer solution (TBS) 1:100 dilution with Strept Avidin-Biotin Complex (SABC) complex was then added at room temperature for 20 min. PBS (pH 7.4) was again added and used to wash the specimens for 5 min, repeated with fresh PBS three times. Diaminobenzidine (DAB) - Hydrogen peroxide (H₂O₂) developing, hematoxylin redyeing, dehydration, washing for 3 times, mixture with distyrene, plasticizer and xylene (DPX) sealing piece, Specimens were then observed and photographed with a high-powered microscope. An MPIAS-1000 multimedia pathology imaging analysis system (Champion image Co., Ltd., Wuhai, China) was used to determine the positive reactant area of protein expression.

**Determination of activation of MLCK**
Preparation of MLC:

1. Preparation of reaction liquid 50 µL, including 5 µL 10 +0.1% BSA analysis buffer, 5 µL CaM (5 µg/µL), 30 µL MLC, 5 µL γ-32P-ATP and 5 µL γ-32P-ATP in 25 °C 20 min for further use. Took 40 µL of the reaction liquid and dropped it on chromatography paper, allowing it to dry naturally.

We Used 75 mM phosphoric acid to wash the paper three times, then washed in 95 % ethanol for 5 min, then drying with an electric hairdryer. The chromatography paper was then put in 5 mL scintillation liquid overnight, and counted the cpm (count per minute) value.

**Statistical analysis**
All experimental data are expressed as mean ± standard deviation (̅x ± i). SAS software version 8.2 (SAS Institute, Chicago, IL, USA) was used for data analysis. Analysis of variance was performed. P < 0.05 was considered statistically significant.

**RESULTS**
Through measure the positive reactant area and count cpm value to analyse protein expression. The normal control group’s middle meningeal artery shown a highest MLCK expression level, but the level of MLCK of the blank control group dramatic decline, after acupuncture Fengchi (GB 20), the level of MLCK rise again, and relieve headaches on Acupuncture Fengchi (GB 20) group and Fengchi (GB 20) prevention group (Table 1).

Compared with the normal control group, the MLCK expression in the blank control group was significantly decreased (P < 0.01). This indicates that acute migraines might be associated with a decrease in MLCK in the CGRP signal system activities. Acupuncture at Fengchi (GB 20) caused an obvious increase in the middle meningeal artery MLCK activation in the neurogenic migraine model in rats.

The microscopic observations showed that the middle meningeal artery from the normal control group rats had a higher level of MLCK expression, while the blank control group had significantly decreased MLCK expression. When compared with the blank control group, both the Fengchi (GB 20) acupuncture group and the Fengchi (GB 20) prevention group showed varying degrees of increases in MLCK expression volume, distribution, and coloring (Figure 1). Compared with the normal control group, the MLCK expression in the blank control group was significantly decreased (P < 0.01). In contrast, there was no difference in MLCK expression between the normal control group and both the Fengchi (GB 20) prevention group and the Fengchi (GB 20) acupuncture group (P > 0.05). However, the MLCK expression in both treatment groups was significantly different compared with the blank control group (P < 0.05).
and further working as a second messenger; this facilitates Ca\(^{2+}\) from the cytoplasm to the membrane structure in the cell structure to block the flow of Ca\(^{2+}\) into cells, and the intracellular Ca\(^{2+}\) concentration to drop, and eventually leading to MLCK inactivation that is dependent on CaM, and cause actomyosin ATPase inactivation, bringing about vasodilatation.\(^{15}\) MLCK plays an important role in smooth muscle contraction and relaxation. MLCK can catalyze 19 serine residues of myosin light chain phosphorylation, then ATP enzyme in Myosin Activity increase and Muscle contraction.\(^{16}\) It is therefore considered that MLCK regulates the contraction and relaxation of vascular smooth muscle. When migraine occurs, the signaling pathway mediated through MLCK in vascular smooth muscle contraction is restrained, thus causing severe diastole of blood vessels, causing symptoms including throbbing head pain, nausea and vomiting.

Acupuncture at Fengchi (GB 20) has been shown to be effective at relieving migraine headaches in clinical practice. A series of experimental studies has confirmed that acupuncture Fengchi (GB 20) in migraine modeled rats has an effect on relaxation of blood vessels on the middle meningeal artery. This information leads us to the following questions: are the effects of contraction and relaxation of blood vessels induced by acupuncture at Fengchi (GB 20) and the increase in activation of MLCK caused by a calcium-dependent mechanism or a non-calcium-dependent mechanism? Is the activation of MLCK concentration-dependent? What is the relationship between the two? Finally, what role do CGRP and 5-hydroxytryptamine play in starting the activation of MLCK in migraines? Future studies are warranted to investigate these questions further.

### REFERENCES


### Table 1 Comparison of MLCK expression and activation in rats from different groups (cpm/mg protein, \(\bar{x} \pm s\))

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Comparison of MLCK area ((\mu m^2))</th>
<th>Activation of MLCK</th>
<th>Scintillation count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>11</td>
<td>114±17</td>
<td>260480±49251</td>
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<tr>
<td>Blank control</td>
<td>11</td>
<td>65±13(^*)</td>
<td>103174±17962</td>
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<tr>
<td>Acupuncture Fengchi (GB 20)</td>
<td>11</td>
<td>86±18(^*)</td>
<td>172226±3310(^*)</td>
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<tr>
<td>Fengchi (GB 20) prevention</td>
<td>11</td>
<td>80±21(^*)</td>
<td>133766±24021(^*)</td>
<td></td>
</tr>
</tbody>
</table>

Notes: normal control and blank control groups were received no treatment. Fengchi (GB 20) acupuncture group: while the model was established, acupuncture was given immediately at Fengchi (GB 20) for 2 min, with needles retained for 20 min, then modeling. MLCK: myosin light chain kinase.

\(^*\)P < 0.01, compared with normal control group. \(^*\)P < 0.05, compared with blank control group. \(^*\)P < 0.01, compared with the normal control group. \(^*\)P < 0.05, compared with the blank control group.