Bloodletting at Jing-well points decreases interstitial fluid flow in the thalamus of rats

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OBJECTIVE: To investigate the changes in the neuronal microenvironment of the middle cerebral artery (MCA) territory induced by Jing-well points bloodletting acupuncture (WPBA) and to explore the neuroprotective mechanism of WPBA in stroke.

METHODS: Adult male Sprague Dawley (n = 32) rats were randomly divided into four groups of eight animals each: WPBA-thalamus group (WT), WPBA-caudate nucleus group (WC), sham-control thalamus group (ST) and sham-control caudate nucleus group (SC). Animals in the WT and WC groups received 2 μL of the extracellular tracer gadolinium-diethylene triamine pentaacetic acid (Gd-DTPA) injected into the thalamus or caudate nucleus, respectively, and 12 Jing-well points in the distal ends of the rats' digits were used for WPBA. Although 2 μL of Gd-DTPA was injected into the thalamus or caudate nucleus, respectively, and 12 Jing-well points in the distal ends of the rats' digits were used for WPBA. Although 2 μL of Gd-DTPA was injected into the thalamus or caudate nucleus, respectively, and 12 Jing-well points in the distal ends of the rats' digits were used for WPBA.

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clearance rate, (7.47 ± 3.15) × 10−1/s (P = 0.009); half-life, (1.52 ± 0.13) h, P = 0.000). By contrast, no significant changes in brain extracellular space and interstitial fluid flow parameters were detected in the caudate nucleus after WPBA (P = 0.649). In addition, no differences in the morphology of the brain extracellular space or the final distribution of the traced brain interstitial fluid were demonstrated between the WT and WC groups (P = 0.631, P = 0.970, respectively).

CONCLUSION: The WPBA decreased the speed of the local thalamic ISF flow in rats, which is assumed to be a beneficial protection by down-modulated the metabolic rate of the attacked neurons under stroke.

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Key words: Bloodletting; Extracellular space; Gadolinium ethoxybenzyl DTPA; Interstitial fluid; Magnetic resonance imaging

INTRODUCTION

Jing-well points bloodletting acupuncture (WPBA) is an ancient therapy in traditional Chinese medicine that has been used to treat stroke. The positive therapeutic effects observed following WPBA include a reduction in swelling and the removal of the obstruction in the meridian-collateral channels.12 Recent evidence also indicates that WPBA effectively promotes blood circulation and modulates ion channel activity on neuronal membranes.13 According to Traditional Chinese Medicine theory, meridians and collaterals compose a network of passages through which energy or Qi circulates and on which most acupuncture points are distributed.14 Therefore, the network is equivalent neither to the vascular system nor to the nervous system. The extracellular space (ECS), surrounding neurons and capillaries in the brain tissue, has long been neglected in anatomical studies. However, by using a newly developed method based on magnetic resonance imaging (MRI), the interstitial fluid (ISF) flow can be dynamically visualized with the tracer gadolinium-diethylene triamine pentaacetate acid (Gd-DTPA) injected into the space.9,12 In the present study, changes in the neuronal microenvironment were quantitatively measured after WPBA. Two sites commonly damaged during stroke were selected as the regions of interest, the thalamus and the caudate nucleus, which were supplied by the middle cerebral artery.13 The neuroprotective mechanism of WPBA against cerebral stroke injury is discussed based on the findings.

METHODS

Animals

Thirty-two healthy, age-matched, adult male Sprague Dawley rats (250-300 g) of SPF grade were supplied by the laboratory animal center of Peking University Health Science Center (Certificate of quality No. SCXK [jing] 2011-0012). The experimental protocols were approved by the Ethics Committee of Peking University Health Center (No. LA2012-016).

Animal treatment and grouping

Thirty-two rats (n = 8 per group) were randomly divided into the following four groups by using a random number table: WPBA-thalamus group (WT), WPBA-caudate nucleus group (WC), sham-control thalamus group (ST), and sham-control caudate nucleus group (SC). Animals in the WT and WC groups received 2 µL of Gd-DTPA injected into the thalamus or caudate nucleus, respectively, and 12 Jing-well points in the distal ends of their digits were used for WPBA. Although 2 µL of Gd-DTPA was injected into the thalamus or caudate nucleus, respectively, and 12 Jing-well points were placed in the digits were used for WPBA, no acupuncture or bloodletting was performed.

Drugs and reagents

The Gd-DTPA solution was prepared using 10 mmol/L of Gd-DTPA (Magnevist; Bayer Schering Pharma AG, Berlin, Germany) diluted with 0.9% NaCl solution (Double-Crane Pharmaceutical Company Limited, China Resources, Beijing, China).

Intraparenchymal microinjection of Gd-DTPA into the brain

The skin overlying the calvaria of anesthetized rats was shaved and disinfected using iodinated alcohol. An incision was made on the scalp along the sagittal suture, from the midpoint of interaural line to the middle of the interocular line, and the skull was exposed by scraping away the skin and underlying tissues. The rats were placed in a stereotaxic instrument (Lab Standard Stereotaxic-Single, Stoelting Co., Wood Dale, Illinois, USA), and 2 µL of Gd-DTPA was administered by intracranial microinjection into the thalamus or caudate nucleus over 10 min. The stereotaxic coordinates for the thalamus were bregma − 2 mm, lateral − 2 mm, vertical − 5.5 mm, and those for the caudate nucleus were bregma+1 mm, lateral − 3 mm, and vertical − 4.5 mm. Rats were kept on a heating pad set at 38 ± 0.5 ℃ to maintain body temperature and the core temperature was monitored with a rectal thermometer.

Jing-well points bloodletting acupuncture

The 12 Jing-well points used for bloodletting were located in the distal ends of the digits and at the acupuncture points of Shaoshang (LU 11), Shangyang (LI 1), Zhongchong (PC 9), Guanchong (TE 1), Shaoc...
The bloodletting procedure was conducted by two physicians who applied pressure to restrict blood flow and to increase the visibility of veins. The vein was then swiftly pricked using a three-edged needle to a superficial depth of 2.5 mm, a few drops of blood were allowed to escape, and the point was pressed with sterile cotton until the bleeding stopped.

**MRI scanning**

Prior to MRI scans, all rats were anesthetized by intraperitoneal injection of a combined solution of pentobarbital sodium (26.58 mg/kg; Sigma, St. Louis, MO, USA), ethanol (427.5 mg/kg; Beijing Chemical Works, Beijing, China), chloral hydrate (127.5 mg/kg; Beijing Chemical Works, Beijing, China), magnesium sulfate (63.6 mg/kg; Beijing Chemical Works, Beijing, China), and propylene glycol (1.014 mL/kg; Beijing Chemical Works, Beijing, China). The anesthetic solution was administered every 2 hand the body temperature was maintained between 36.0 and 37.0 °C during the scanning process. A 3.0 T MRI system (Magnetom Trio, Siemens Medical Solutions, Erlangen, Germany) interfaced with an eight-channel wrist coil was used with a T1-weighted three-dimensional magnetization-prepared rapid acquisition gradient echo sequence. The rats underwent an MR pre-scan before tracer injection. Sequential MR scans were performed at 15, 30 min, 1, 2, 3, 4, 6, and 7 h after the tracer injection and bloodletting was completed in the WT and WC groups, and after the tracer injection in the ST and SC groups. The acquisition parameters were as follows: echo time, 3.7 ms; repetition time, 1500 ms; flip angle, 12°; inversion time, 900 ms; field of view, 267 mm; voxel, 0.5 × 0.5 × 0.5 mm³; matrix, 512 × 512; number of averages, 2; phase encoding steps, 96. The acquisition time for each rat was 290 seconds.

**MR image processing**

Image post-processing was performed using a self-made software package based on Matlab (MathWorks, Natick, MA, USA). A mutual information-based method was used for deformable image registration. The pre-scanned images were subtracted from post-scanned images. The signal attenuation (ΔSI) was automatically measured from the subtracted images and was converted to Gd-DTPA concentration (ΔSI) was automatically measured from the subtracted images and was converted to Gd-DTPA concentration. The pre-scan images were used to calculate the distribution volume ratio (Vd) of Gd-DTPA, which is a proportion of the Gd-DTPA distribution volume to the whole brain volume. The half-life of Gd-DTPA was calculated by the relationship $t_{1/2} = \ln 2/\text{clearance rate}$.

**Statistical analysis**

The experimental data were measured and calculated using Matlab software, without subjective factors. All numerical data are presented as the mean ± standard deviation ($\bar{x} \pm \sigma$). Statistical analysis was performed using SPSS statistical software, version 13.0 (IBM SPSS Statistics for Windows, Armonk, New York, USA). One-way analysis of variance followed by individual comparisons of means was used for the comparison of different grouped data. Paired-samples t tests were used to compare two groups of data. $P$ value less than 0.05 was considered to be significant.

**RESULTS**

**Drainage and the final distribution of the Gd-DTPA tracer in the brain ISF after WPBA**

WT versus ST: the accumulation of the Gd-DTPA-enhanced signal in the brain ISF was visualized as a hyper-signal intensity on MR images. The tracer was locally cleared, and no difference was found for the final maximal distribution of the traced ISF between the WT and ST groups. The enhancement was attenuated to normal in 2-3 h for the ST group, whereas the clearance took 6 h in WT group (Figure 1).

![Figure 1 Representative coronal views of Gd-DTPA clearance in the thalamus of the rat brain](image)

Enhanced MR signal intensity is represented as white. A: Gd-DTPA clearance in the Jing-WPBA-thalamus group (2 μL of Gd-DTPA injected into the thalamus and 12 Jing-Well points used in the distal ends of the digits for WPBA). B: Gd-DTPA clearance in the representative sham-control (ST group: 2 μL of Gd-DTPA injected into the thalamus, without WPBA). Gd-DTPA: gadolinium-diethylene triamine pentaacetic acid; WPBA: well points bloodletting acupuncture; MR: magnetic resonance imaging.

We found that $k’$ in the WT group is significantly lower than that in the ST group, and that $t_{1/2}$ in the WT group is significantly lower than that in the ST group.
DISCUSSION

The present study demonstrated that WPBA effectively decreases the speed of the ISF flow in the thalamus, without altering local tortuosity.

The brain ISF is contained in a channel system surrounded by membranes of neural cells and capillaries. In the present study, we employed the in vivo tracer-based MRI method to detect the dynamic functional signal from the deep brain ISF, which is developed by Han HB and his colleagues. Due to the high resolution and feasibility to measure both the morphological and physiological parameters in the brain tissue channel, the method is so far the most optimal method to study the brain ISF flow and shows great potential for testing the meridian-collateral theory.17 Theoretically, both neural cells and capillaries influence the ISF flow. However the vascular compartment occupies only 3%-5% of the brain volume,14 and the Gd-DTPA tracer used in our study is an extracellular contrast agent with an extremely low permeability to the blood-brain barrier.15-21 Therefore, it is reasonable to assume that the decreased brain ISF flow detected in our present study occurred mainly in the interstitial space rather than the vascular compartment. By contrast, neural cells occupy 70%-80% of the brain volume. Thus, the cellular components are not significantly different between the two groups (Table 1).

Table 1 Structural and physiological parameters in the thalamus of the bloodletting and control groups (\(\bar{x} \pm s\))

<table>
<thead>
<tr>
<th>Group</th>
<th>D* (× 10^-11 mm^2/s)</th>
<th>(\lambda)</th>
<th>k (× 10^-11/s)</th>
<th>t_1/2 (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3.36±0.93</td>
<td>1.29±0.32</td>
<td>7.47±3.15</td>
<td>1.52±0.13</td>
</tr>
<tr>
<td>ST</td>
<td>3.28±0.67</td>
<td>1.28±0.17</td>
<td>14.40±4.50</td>
<td>0.81±0.03</td>
</tr>
</tbody>
</table>

Notes: WT: Jing-well points bloodletting acupuncture-thalamus group (2 µL of Gd-DTPA injected into the thalamus and 12 Jing-Well points in the distal ends of the digits used for WPBA); ST: sham-control thalamus group (2 µL Gd-DTPA injected into the thalamus, without bloodletting). Gd-DTPA: gadolinium-diethylene triamine pentaacetic acid; WPBA: well points bloodletting acupuncture; D*: effective diffusion coefficient; \(\lambda\): tortuosity; k: clearance rate of the tracer; t_1/2: half-life; P value: comparison of WT and ST groups.

Table 2 Brain structural and physiological parameters in the caudate nucleus of the bloodletting and control groups (\(\bar{x} \pm s\))

<table>
<thead>
<tr>
<th>Group</th>
<th>D* (× 10^3 mm^2/s)</th>
<th>(\lambda)</th>
<th>k (× 10^-3/s)</th>
<th>t_1/2 (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WC</td>
<td>3.37±0.93</td>
<td>1.31±0.52</td>
<td>7.90±2.10</td>
<td>1.47±0.24</td>
</tr>
<tr>
<td>SC</td>
<td>3.38±1.08</td>
<td>1.31±0.31</td>
<td>7.84±4.18</td>
<td>1.46±0.56</td>
</tr>
</tbody>
</table>

Notes: WC: Jing-WPBA-caudate nucleus group (2 µL of Gd-DTPA injected into the caudate nucleus and 12 Jing-well points in the distal ends of the digits used for WPBA); SC: sham-control caudate nucleus group (2 µL Gd-DTPA injected into the caudate nucleus, without bloodletting). Gd-DTPA: gadolinium-diethylene triamine pentaacetic acid; WPBA: well points bloodletting acupuncture; D*: effective diffusion coefficient; \(\lambda\): tortuosity; k: clearance rate of the tracer; t_1/2: half-life; P value: comparison of WC and SC groups.
partment is likely to contribute more to the decrease in brain ISF flow. Because neuronal nutrients and the metabolic waste are transported via the brain ISF, its flow rate should be strongly correlated with the metabolic rate of the neurons or their excitatory state. According to our previous results, the ISF drainage rate in the thalamus was faster than that in the caudate nucleus or olfactory bulbs, consistent with previous results demonstrated using 18F-fluorodeoxyglucose. A lower metabolic rate induced by either anti-excitation or injection of anesthetic drugs provides beneficial protection for ischemic neurons, a finding that also supports the assumption that the slowdown in the ISF drainage rate was caused by a regional down-modulation of the metabolic rate, which potentially offers neuroprotection during an ischemic or hemorrhagic attack.

The biophysical property of the brain interstitial space is the other key factor for understanding the ISF flow speed. According to the results in present study, the tortuosity of the ECS was not changed with WPBA, indicating that the slowdown in the ISF flow was not caused by local tortuosity but by decreased secretion or changes in the brain ISF drainage route. Most previous studies have demonstrated that the ISF clears the brain by following the lymphatic drainage of the head and neck, and a close connection between brain ISF and cerebrospinal fluid (CSF) cycles has been proposed. The results of all these aforementioned studies suggest a potential connection between central brain ISF flow and peripheral tissue interstitial channels.

The Jing-well point bloodletting therapy is not a simple treatment. It can lead to at least four biological results: bleeding, point acupuncture, pain stimulation, and the release of local interstitial fluid. Most previous analyses examining the mechanism of acupuncture therapy effects on stroke were focused on bleeding. However, during bloodletting therapy, blood and interstitial fluid are simultaneously released. Originally, stroke was thought to be caused by a viscous wind that penetrated the brain center and disrupted brain function, with the wind’s turbulence generating heat and phlegm stagnation in blood. The heat was thought to be discharged by the blood released at Jing-well points because blood was considered a vehicle for removing excess heat. However, it is currently known that the peripheral blood is the same blood that circulates throughout the entire body; thus, there is no reason to expect that bleeding removes excess heat. In addition to blood, bloodletting acupuncture releases a small amount of ISF in the extremities. Because the lymphatic route is an ISF drainage passage, the correlation between the lymphatic system and the bloodletting Jing-well points needs further investigation.

In conclusion, the effect of WPBA to reduce the brain ISF flow rate in the thalamus provides a new perspective in understanding the mechanism of Jing-well points bloodletting acupuncture therapy in stroke.

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