Anti-inflammatory Effect of Heat-sensitive Moxibustion via the NF-κB Signaling Pathway on Cerebral Ischemia/Reperfusion Injury in Rats

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Abstract: Ischemic stroke is universally acknowledged as a common cause of long-term disability or even death. Suspended moxibustion, an indirect form of moxibustion, is when moxibustion is placed superficially over the skin without being in contact with it. Some researchers have used this method to treat stroke patients, but strong evidence of its therapeutic effectiveness is lacking. However, the effect of traditional suspended moxibustion has recently been improved with the development of heat-sensitive suspended moxibustion. Our previous studies showed that moxibustion for 35 min provided a more effective treatment strategy than moxibustion for 15 min, and moxibustion by 35 min with tail temperature increase had a better outcome than that without, however, the mechanism underlying the effect is not clear. In this study, we treated the stroke rats with moxibustion by 35min and divided them into non-heat sensitive moxibustion(NHSM) group and heat sensitive moxibustion (HSM) group according to difference in the tail temperature increase, then we compared the effect and investigated the mechanisms between NHSM and HSM. We found that HSM significantly decreased tail-flick latency, increased neurological function score, decreased infarct volume, reduced inflammatory cells, decreased the expression of inflammatory factor ICAM-1 and reduced the expression of NF-κB p65 and p-IKKα/β in rats with focal cerebral ischemia/reperfusion injury. Our experimental findings revealed that HSM exerted its anti-inflammatory and neuroprotective effects from MCAO-induced injury by decreasing the expression of the NF-κB signaling pathway.

Keywords: Auspended Moxibustion; Heat-sensitive moxibustion; Middle cerebral artery occlusion; Cerebral ischemia/reperfusion injury; Tail temperature; Tail-flick latency; Infarct volume; Inflammatory cells; CD11b; ICAM-1; NF-κB p65; p-IKKα/β; Traditional Chinese Medicine

Research Highlights: (1) Application of moxibustion at acupoint Dazhui (DU14) with tail temperature increase (HSM) significantly reduced focal cerebral ischemia/reperfusion injury.(2) Heat-sensitive moxibustion significantly decreased tail-flick latency, increased neurological function score, decreased infarct volume, reduced inflammatory cells (microglia) infiltration and decreased inflammatory factor ICAM-1 expression in rats with focal cerebral ischemia/reperfusion injury.(3) Heat-sensitive moxibustion significantly reduced the expression of NF-κB p65,p-IKKα/β in rats with focal cerebral ischemia/reperfusion injury.

Abbreviation:

MCAO: middle cerebral artery occlusion
IR: ischemia/reperfusion
HSM: heat-sensitive moxibustion
NHSM: non-heat-sensitive moxibustion
ICAM-1: intercellular adhesion molecule-1

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1. Introduction

Suspended moxibustion, an indirect form of moxibustion, is when moxibustion is placed superficially over the skin without being in contact with it. Similar to Chinese herbal medicine,[1] moxibustion is widely used in China and has been adopted by many for the treatment of various chronic diseases or symptoms as "deficient conditions" (weakness), including stroke patients, however, the results seems elusive.[2],[3] Recent developed "heat-sensitive" suspended moxibustion has been shown to be neuroprotective in stroke patients, even more so than traditional suspended moxibustion[4]. Traditionally, the duration of suspended moxibustion is 15 minutes, and an extended period of moxibustion (30–60 minutes) may provide an effective treatment strategy, which is one of the reasons for heat-sensitive moxibustion high efficiency.[4],[5] Our previous research showed that stroke rats treated with moxibustion for 35min had a better outcome than those for 15min.[6] Our lab further study found that some stroke rats with moxibustion for 40 min exhibited a definite tail temperature increase, while moxibustion for 15 min did not result in tail temperature increase; [7] and 40 min moxibustion with tail temperature increase was more efficient than that without[7]. These studies suggest that moxibustion's efficacy increases when distant heat is appeared. But the detail mechanism has not been explored.

Many complex factors are involved in the pathophysiology of stroke, including inflammation, disruption of cellular metabolism in the brain, excitatory amino acid toxicity, intracellular calcium overload, oxidative stress injury and nerve cell apoptosis.[8] It is well known that inflammation is an important pathological mechanism of cerebral infarction.[9],[10],[11],[12],[13],[14] The inflammatory response involves inflammatory cells and inflammatory mediators.[15] Elango et al[10] demonstrated that occlusion followed by reperfusion increased inflammatory cell activation and infiltration, pro-inflammatory cytokine(IL-1β, TNF-α, IL-6) and inflammation-related gene(intercellular adhesion molecule-1,ICAM-1) expression in the ipsilateral brain, which could result in the development of cerebral ischemia/reperfusion injury. ICAM-1 produced by endothelial cells regulates the leukocyte recruitment and participates in inflammatory events. [10],[14],[16] Myeloperoxidase abundantly presents in inflammatory cells (such as neutrophils and macrophages). [10],[16],[17] The main mediators of neuroinflammation are glial cells, and neuroinflammatory reaction is significantly influenced by the activation of microglia.[18] NF-κB is an important transcription factor involved in inflammatory responses and detected in almost all the cells including neuronal and microglial cells following ischemic injury or other inflammation-related diseases, [19],[20] when it is activated, it can promote the transcription of a variety of certain genes, releasing a series of pro-inflammatory cytokines, arachidonic acid derivatives, glutamate, and free radicals which contribute to inflammation and then lead to neuronal damage. Our previous experiment showed that there was a significant increase in myeloperoxidase activity, microglia infiltration and NF-κB protein level in cerebral cortex of rats following IR insult, [6],[21],[22] and 35-min moxibustion reduced myeloperoxidase activity, microglia infiltration and NF-κB protein level in cerebral cortex of rats following IR insult. [6],[21],[22]

However, it remains elusive whether suspended moxibustion for 35 min with tail temperature increase really has greater efficacy than that without, and does it exert its neuroprotective effect through anti-inflammation involving the NF-κB signaling pathway? Therefore, in the present study, we treated the stroke rat with moxibustion for 35min and divided them into non-heat-sensitive moxibustion group and heat-sensitive moxibustion group according to difference in the tail temperature increase. Then we examined the neuroprotective effects of suspended moxibustion, compared their efficacy between non-heat-sensitive moxibustion group and heat-sensitive moxibustion and investigated whether these anti-inflammatory effects were related to NF-κB signaling pathway in order to gain a clearer understanding of the molecular biological mechanisms.

2. Materials and Methods

2.1 Animals

Male Sprague-Dawley rats, weighing 220–280 g, were purchased from Hunan SJA Laboratory Animal Co., Ltd., Hunan, China. Animals were housed under controlled conditions (07:00–19:00 lighting, five rats/cage before MCAO and one rat/cage after MCAO, 50–60% humidity, and 22°C-25°C) with free access to water and food. The study was carried out in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals formulated by the Ministry of Science and technology of China (2006). All animal experiments were performed at the Experimental Animal Center of Jiangxi University of Traditional Chinese Medicine, Jiangxi, China.

A total of 80 rats were randomly divided into three groups: a sham-surgery group (n=11), a stroke model group (n=23), a moxibustion-treated group (n=46). Rats in a moxibustion-treated group were put a lit cigar made of mugwort above Dazhui acupoint for 35min and divided into NHSM group(the tail temperature after moxibustion treatment rises no more than 1℃ , or more than 1℃ but less than 2 times) and HSM group(the tail temperature rises more than 1℃ , and more than 2 times) according to difference in the tail temperature before and after moxibustion at 2h,24h, 48h and 72h(data not shown). 5 rats from the stroke only group, 9 rats from the moxibustion-treated group died of cerebral hemorrhage and subarachnoid hemorrhage after surgery, and 2 rats with 0 score, 2 rats with four scores from the stroke only group, and 5 rats with 0 scores, 4 rats with four scores from the moxibustion-treated group were excluded from the study. Therefore, a total of 53 rats were included in the final analysis. The experimental protocol is shown in Figure 1.

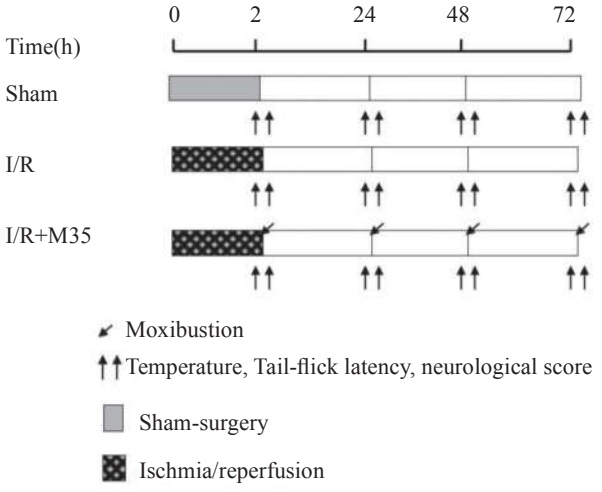


Figure 1. Experimental Design and Timelines

Sham: control animals underwent general anesthesia and sham surgical procedure without ischemia and reperfusion. I/R: ischemic-reperfusion animals underwent cerebral ischemia- reperfusion induced for 2 hours by transient middle cerebral artery occlusion (MCAO), followed by 3 days of reperfusion. I/R+M35: I/R animals with suspended moxibustion for 35 min. Slanted arrows: indicate the time of moxibustion administration. Double arrows indicate the time that tail temperature, rectal temperature, tail-flick latency and neurological function scores were conducted at 2h, 24h, 48h and 72h after surgery. At 3 days after surgery, TTC staining, HE staining and immunohistochemical staining were performed.

2.2 Focal Cerebral Ischemia/Reperfusion Injury

The focal cerebral ischemia/reperfusion injury model was induced for 2 hours by MCAO followed by three days of reperfusion in rats. All animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (3% (w/v)) at a dose of 40 mg/kg. Body temperature was maintained at 37.0 ± 0.5°C using a heat lamp and heating pad. MCAO was achieved using the intraluminal filament method, as described previously[6],[7],[21],[22] with minor modifications. Briefly, a midline neck incision was made, and the left common carotid artery, internal carotid artery and external carotid artery were isolated. The external carotid artery was ligated with 4-0 silk suture distal from the carotid bifurcation and the common carotid artery was ligated with 4-0 silk suture at the proximal end. Another 4-0 silk suture was tied loosely around the common carotid artery close to the carotid bifurcation. A piece of fishing line (Simago Fishing Tackle Company, Hangzhou, Zhejiang, China) 0.237 mm in diameter and 5 cm in length with a rounded tip was introduced into a small incision in the common carotid artery and gently advanced to the origin of the middle cerebral artery (20–22 mm from carotid bifurcation). The silk suture around the common carotid artery stump was tied tightly to prevent bleeding and to secure the fishing line. After 2 hours of occlusion, the fishing line was withdrawn to allow for reperfusion. Sham-surgery group rats were manipulated in the same way, but the middle cerebral artery was not occluded.

The following exclusion criteria are applied:

(1) Death within 72 hours after focal cerebral ischemia/reperfusion;

(2) Neurological severity score = 0 or ≥4 (0 hour after focal cerebral ischemia/reperfusion).
2.3 Suspended Moxibustion Treatment

Dazhui points (region in C7–T1), considered to be important for brain function,[23] were heated by suspended moxibustion using a moxibustion-cigar produced from mugwort (custom-made for use with animals, length 12 cm, diameter 0.6 cm, made in the Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine, Jiangxi, China.) at a height of approximately 3 cm over a hairless area of skin once a day for three days. Stimulation of moxibustion was performed at 2h after surgery and a duration of 35 minutes was employed. According to the difference of tail temperature before moxibustion and at 2h, 24h, 48d, 72d after moxibustion, moxibustion-treated rats were divided into the non-heat-sensitive moxibustion group and heat-sensitive moxibustion group.

2.4 Tail Temperature and Rectal Temperature

At 2h, 24h, 48h and 72h after surgery, tail temperature was measured before and after moxibustion at the point between the proximal third and the middle third of the whole tail in rats with focal ischemia-reperfusion injury using a digital thermometer (ST-1, Shanghai medical instrument factory, Shanghai, China.). In the meantime, rectal temperature was also measured using an electronic thermometer (T103, Bioland Medical equipment Co., Ltd., Shenzhen, China).

2.5 Tail-flick Latency Test

Before surgery and 2h, 24h, 48h and 72h after surgery, the latency of tail flick was measured to observe the effect of suspended moxibustion on pain threshold with rat/mice-tail light and thermalgesia algometer(YLS-12A, Jinan yiyan science technology development co., ltd., Jinan, China.).The basic principle of the tail-flick latency is that a light beam irradiates onto the tail, the tail temperature rises and produces pain. When pain intensifies to some extent that animals can't tolerate, the animals will move their tails away. For the tail flick test, the intensity of the power was set at 36W, rat was placed in a cylinder and the cylinder was put onto the fixed seat of the instrument. Then adjusted the cylinder position so that the tail between the middle third and the distal third of the tail was placed in the middle of the photoelectric pore, pressed the start button, the timer started, when the tail swings away, photoelectric switch turned off automatically, the timer ended. That was tail-flick latency. Each rat was twice measured.

2.6 Neurological Function Scores

At 2h, 24h, 48h and 72h after surgery, neurological assessment was performed according to Garcia[24] by an investigator who was blinded to the experimental groups, using a 18-point scale. The score given to each rat at the completion of the evaluation was the summation of all six individual test scores including spontaneous activity:

symmetry in the movement of four limbs, forepaw outstretching, climbing, body proprioception, response to vibrissae touch. The minimum neurological score is 3 and the maximum is 18.

2.7 Infarct Volumes of the Brain

3 days after surgery, rats were anesthetized with an intraperitoneal injection of 3% (w/v) sodium pentobarbital at a dose of 40 mg/kg, and their brains in sham, I/R and moxibution-treated group were removed. Shortly the brains were sectioned coronally into 2-mm slices and incubated in 1% (w/v) 2, 3, 5-triphenyltetrazolium chloride (TTC) in PBS solution at 37°C for 30 minutes, then the stained slices were photographed using a Nikon COOLPIX L1 camera (Nikon, Tokyo, Japan) and the areas of ipsilateral and contralateral hemispheres were measured using INFINITY ANALYZE software (Lumenera Corporation, Ottawa, ON, Canada). After correcting for edema, the infarct volumes were calculated as described previously measured for the ischemic lesion.[25] The ischemic lesion percentage of each slice was calculated as follows: corrected infarct volume (%) = [contralateral hemisphere volume − (ipsilateral hemisphere volume-infarct volume)]/the whole brain section volume ×100%.

2.8 Brain Histological Structures

After TTC staining and lesion area analysis, the slices were fixed in 10% (v/v) formalin, then embedded with paraffin(Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China), and the third TTC staining slice of each brain was used for hematoxylin-eosin(HE) staining. A series of adjacent 5-μm thick slices were sectioned in the coronal plane. Standard HE staining was performed, and histopathological changes were photographed with the use of image analysis system (Leica 2000; Leica Microsystems GmbH, Wetzlar, Germany).

2.9 Immunofluorescent Staining

3 days after surgery, the rats from each group were anesthetized with an intraperitoneal injection of 3% (w/v) sodium pentobarbital at a dose of 40 mg/kg and fixed with a PBS-buffered 4% paraformaldehyde solution(Sinopharm Chemical Reagent Co., Ltd., Beijing, China) by transcardial perfusion. Subsequently, the intact brains were removed, the cerebral cortices were separated and immersed in fixative 4 % paraformaldehyde for 2h, then they were transferred to 10%, 20%, 30 % sucrose in PBS solution respectively at 4°C until it sank.

Then the cerebral cortices were washed with PBS solution and sectioned at a thickness of 35 μm on a Leica CM1950 cryostat (Leica Biosystems Nussloch GmbH, Nussloch,Germany), all the sections were preserved in a -20˚C refrigerator. In order to quantify the number of positive cells, cortical sections from each brain were rinsed with PBS solution for three times, then the sections were incubated with 4 % goat serum for 2h and detected with rabbit antibody against CD11b (ab75476, diluted 1:1000, abcam, Cambridge, UK.) and rabbit antibody against p-IKKα/β(#2697, diluted 1:400, cell signaling technology Inc, Danvers, Massachusetts,USA.) at 4 °C overnight. After rinsing with PBS solution threetimes for 10 min, the tissue was incubated in a Alexa Fluor®568 goat anti-rabbit IgG (H+L) secondary antibody（A11011, diluted 1:500, Invitrogen, Carlsbad, CA, USA） at room temperature for 2 h. After the slices were washed in PBS solution 3 times for 10 min, they were mounted using ProLong Gold antifade reagent (P36931, Invitrogen, Carlsbad, CA, USA). The sections were then imaged with Leica microscope in conjunction with LAS V3.7 microscope software (Leica 2000; Leica Microsystems GmbH, Wetzlar, Germany). The expression of CD11b or p-IKKα/β protein was stained red, and the nucleus was stained blue. Three-five coronal brain sections from each brain and 3 brains from each group were used in order to quantify the percentage of positive cells. The percentage of positive cells was calculated as the number of CD11b- or p-IKKα/β-positive cells /the whole cells ×100% in per × 20 field separately.

2.10 Immunohistochemical Staining

3 days after surgery, the rats from each group were anesthetized with an intraperitoneal injection of 3% (w/v) sodium pentobarbital at a dose of 40 mg/kg and fixed with a PBS-buffered 4% paraformaldehyde solution(Sinopharm Chemical Reagent Co., Ltd., Beijing, China) by transcardial perfusion. Subsequently, the intact brains were removed; the cerebral cortices were separated and immersed in 10 % (v/v) formalin.

Then cerebral cortices were washed three times with PBS and sectioned at a thickness of 5 μm. The antigen retrieval of paraffin sections was performed with the high pressure after dewaxing and dehydration. Brain sections were firstly perforated in 3% triton solutions for 30 min at room temperature, and then washed three times with PBS solution for 10 min. The tissue was immersed in 1% H2O2 for 30 min to quench the endogenous peroxidase. After rinsing with PBS solution for three times, the sections were incubated with 5% goat serum for 30 min. Following incubation in serum, they were incubated with mouse monoclonal antibody against ICAM-1（SC-8439, diluted 1:15, Santa Cruz Biotechnology Inc, Dallas, Texas, USA）and rabbit polyclonal antibody against NF-κB p65 (sc-30080, diluted 1:50, Santa Cruz Biotechnology Inc, Dallas, Texas, USA) at 4°C overnight. After incubation,

the slice was rinsed in PBS solution 3 times for 5 min and then incubated in a peroxidase-conjugated goat anti-mouse secondary antibody (ZB-2305, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China.) and peroxidase-conjugated goat anti-rabbit secondary antibody (ZB-2301, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China.) indoor for 1h. Following another series of washing in PBS solution, the tissue was placed in a solution of 0.5 mg/ml diaminobenzidine (DAB) for 5-10 min until the desired staining intensity was achieved. Ultimately, the tissue was washed and mounted onto super frost glass slides and left to dry. The tissue was photographed with Leica microscope in conjunction with LAS V3.7 microscope software (Leica 2000; Leica Microsystems GmbH, Wetzlar, Germany). The ICAM-1 and NF-κB p65-positive cells were stained brown. Three-five coronal brain sections from each brain and 3 brains from each group were used in order to measure the average integrated optical density (IOD), and the average IOD of ICAM-1 and NF-κB p65-positive cells of the whole field(scale bar=50μm) was analyzed using the Image-ProPlus 5.0 software (Media Cybernetics, USA) separately.

2.11 Statistical Analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) 13.0 software. Quantitative data were expressed as mean ± standard error of the mean, SEM). Multiple group analysis was performed using one-way analysis of variance (ANOVA) followed by Fisher-LSD (least significant difference) post hoc or Dunnett's test for multiple pairwise comparisons. P values of less than 0.05 ($p < 0.05$) were considered statistically significant. All experiments and analysis were performed in a blinded manner, as the experimenters were not aware of the treatment conditions.

3. Results

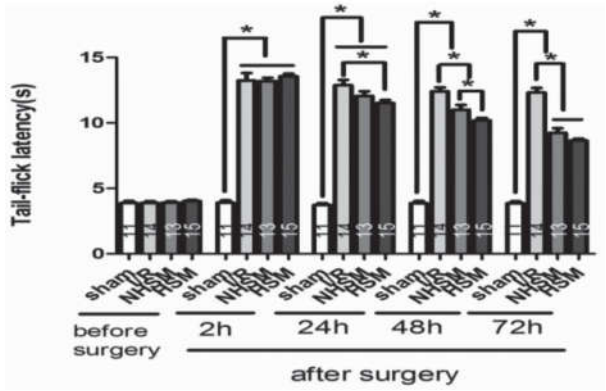
3.1 Heat-sensitive Moxibustion Improved Sensory Function and Increased Neurological Function Scores

Neurological behavioral evaluation was used to investigate whether HSM could reduce brain injury in rats, we determined sensory function and neurological function recoveries by assessing tail-flick latency and neurological function scores. All the tests were carried out in rats of sham, I/R and moxibustion-treated groups at various time points following IR insult.

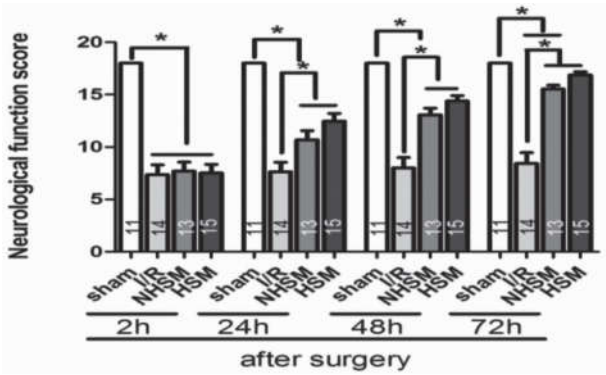
Tail-flick latency was used as one of the key standards in identification of potential therapeutics. In this study, we measured tail-flick latency before surgery, 2h, 24h, 48h and 72h after surgery in rats. Results showed that tail-flick latency didn't exhibit a significant difference among four groups before surgery (sham:3.885±0.140; I/R: 3.897±0.126; NHSM: 3.918±0.104; HSM:4.030±0.089), then after surgery tail-flick latencies in rat with focal cerebral ischemia/reperfusion injury(2h: 13.24±0.56; 24h: 12.88±0.40; 48h:12.41±0.30; 72h:12.32±0.35. $p < 0.05$) were longer than those of sham-surgical rats(2h: 3.901±0.175; 24h: 3.87±0.15; 48h:3.87±0.15; 72h: 3.88±0.13) at 2h, 24h, 48h and 72h. While suspended moxibustion could reduce tail-flick latency. At 24h, heat-sensitive moxibustion (24h: 11.52±0.23. $p < 0.05$) significantly reduced tail-flick latency compared to I/R group (24h: 12.88±0.40). With time, both non-heat-sensitive moxibustion (48h: 11:00±0.36; 72h: 9.25±0.35. $p < 0.05$)and heat-sensitive moxibustion(48h: 10:20±0.18; 72h:8.66±0.13. $p < 0.05$) significantly reduced tail-flick latency compared to I/R group(48h: 12:41±0.30; 72h:12.32±0.35) at 48h and 72h after surgery, and heat-sensitive moxibustion(48h: 10:20±0.18. $p < 0.05$) significantly reduced tail-flick latency compared to non-heat-sensitive moxibustion (48h: 11:00±0.36.) at 48h after surgery. These results indicate that HSM significantly improves sensory function after cerebral ischemia/reperfusion injury. (Figure 2a)

Similarly, neurological function scores were used to evaluate whether heat-sensitive moxibustion would improve neurological function. In this study, we assessed neurological function scores at 2h, 24h, 48h, and 72h of MCAO followed by reperfusion in rats according to Garcia et al (24). Results showed that the neurological function scores after surgery were as follows (2h: 7.36±3.50; 24h: 7.64±3.39; 48h: 8.00±3.72; 72h: 8.43±3.84. $p < 0.05$) in I/R group in comparison to 18 scores at all the above time points in sham group, which suggested that the rat model of focal cerebral ischemia/reperfusion injury was successful. Suspended moxibustion increased neurological function scores, rats treated with non-heat-sensitive moxibustion (24h: 10.69±3.12; 48h: 13.08±2.25; 72h: 15.54±1.27. $p < 0.05$) and heat-sensitive moxibustion (24h: 12.47±2.90; 48h: 14.40±1.96; 72h: 16.87±1.25. $p < 0.05$) at 24h, 48h, 72h exhibited a significant increase in neurological function scores compared to I/R group(24h: 7.64±3.39; 48h: 8.00±3.72; 72h: 8.43±3.84), but rats in non-heat-sensitive moxibustion group (2h: 7.69±3.12) and heat-sensitive moxibustion group(2h: 7.53±3.14) at 2h exhibited no significant in neurological function scores compared to I/R group. Heat-sensitive moxibustion(24h: 12.47±2.90; 48h: 14.40±1.96; 72h: 16.87±1.25.) increased more neurological function scores than non-heat-sensitive

moxibustion (24h: 10.69±3.12; 48h: 13.08±2.25; 72h: 15.54±1.27..) at 24h,48h and 72h after surgery, although there is no significant differrence.These results indicate that HSM improves neurological function after cerebral ischemia/reperfusion injury. (Figure 2b)



(a)



(b)

Figure 2. Effect of Heat-sensitive Moxibustion on Tail-flick Latency and Neurological Function Scores in Focal Ischemia-reperfusion Injury

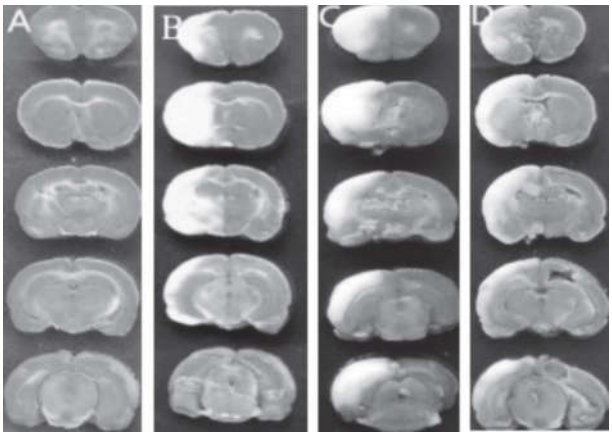
(a) Summary of tail-flick latency of the sham, I/R and moxibustion-treated group. Tail-flick latencies of all focal cerebral ischemia/reperfusion injury group were rosen at 2h after surgery compared with that of sham-surgical rats, while tail-flick latency in rats treated with suspended moxibuxtion exhibited a significant decrease at 48h and 72h, and tail-flick latency in rats treated with heat-sensitive moxibuxtion even exhibited a significant decrease at 24h compared to that in I/R group. Heat-sensitive moxibustion also exerted a significant decrease at 48h compared to non-heat-sensitive moxibustion .These results indicate that suspended moxibustion, especially heat-sensitive moxibustion improves sensory function following IR insult.

(b) Summary of neurological function scores of the sham, I/R and moxibustion-treated group. The neurological function scores of sham-surgery rats were 18 at all timepoints, and neurological function scores of all focal cerebral ischemia/reperfusion injury rats were reduced at 2h after surgery. However neurological function score in rats treated with suspended moxibuxtion exhibited a significant increase at 24h, 48h, 72h compared to that in I/R group. These results indicate that heat-sensitive moxibustion also improves neurological function following IR insult.

Notes: "*": $P < 0.05$. Sham: Sham-surgery group; I/R: cerebral ischemia/reperfusion injury group; NHSM: cerebral ischemia/reperfusion injury with non-heat-sensitive moxibustion group; HSM: cerebral ischemia/ reperfusion injury with heat-sensitive moxibustion.

3.2 Heat-sensitive Moxibustion Reduced Brain Infarct Volume

Cerebral infarct size was also used to evaluate whether heat-sensitive moxibustion could reduce brain injury in rats following IR insult. Infarct volume was measured from the sham or I/R group with or without moxibustion. Brain slices obtained from all groups were stained with TTC 3 days after IR to investigate the efficacy of heat-sensitive moxibustion. Results revealed that suspended moxibustion significantly reduced infarct size (NHSM group: 19.75%±0.59%; HSM group: 13.06%±1.08%. $P < 0.05$) at 3 days after I/R when compared to I/R group (26.09%±1.33%. $P<0.05$), and heat-sensitive moxibustion (13.06%±1.08%. $P < 0.05$) significantly reduced the infarct size when compared to non-heat-sensitive moxibustion group (19.75%±0.59%). These results demonstrate that heat-sensitive moxibustion significantly reduced brain damage following IR insult in rats. (Figure 3)



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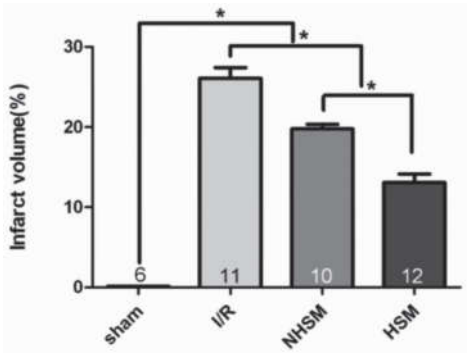


(b)

Figure 3. Effects of Heat-sensitive Moxibustion on Infarct Volume in Focal Cerebral Ischemia/Reperfusion Injury Rats

(a) Representative brain coronal slices stained with TTC. The ischemic area remained white, while the intact areastained red.

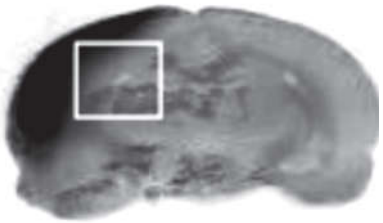
(b) Analysis of brain infarct volumes of the sham, I/R or moxibustion-treated group. Heat-sensitive moxibustion decreased brain infarct volumes of stroke rats in comparison with that of I/R group and non-heat-sensitive moxibustion group. So heat-sensitive moxibustion significantly protected the brain from cerebral ischemia/reperfusion injury.

Notes: "*": $P < 0.05$.

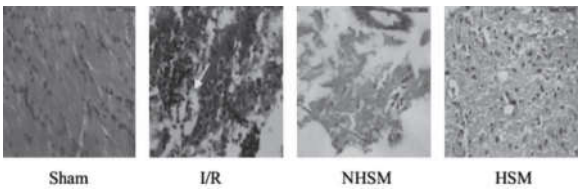
Sham(A): Sham-surgery group; I/R(B): cerebral ischemia/reperfusion injury group; NHSM（C）: cerebral ischemia/reperfusion injury with non-heat-sensitive moxibustion group; HSM（D）: cerebral ischemia/ reperfusion injury with heat-sensitive moxibustion.

3.3 Heat-sensitive Moxibustion Reduced the Presence of Inflammatory Cells in the Brain

Brain (ipsilateral) histological structure was used to evaluate whether heat-sensitive moxibustion could reduce brain injury and inhibit inflammation in rats following IR insult. Cerebral cortical slices from the sham or I/R group with or without moxibustion treatment were stained with HE (Figure 4a). Results revealed that there were damaged histological structure showing missing regions and many inflammatory cells in the cerebral cortices (ipsilateral) 3 days after surgery in the I/R group when compared to sham-surgery group. However, suspended moxibustion significantly protected the brain from cerebral ischemia/ reperfusion injury and reduced the presence of inflammatory cells; In contrast, outcomes with non-heat-sensitive moxibustion were not as good as outcomes with heat-sensitive moxibustion (Figure 4b). Further studies showed that cortical slices stained with immunofluorescence (IF) had higher percentage of CD11b-positive cells (I/R group: 41.72% ± 5.78%. $p < 0.05$) in the cerebral cortex (ipsilateral) when compared to sham-surgery group(1.16%±0.19%). However suspended moxibustion significantly reduced the percentage of CD11b-positive cells compared to I/R group, while the percentage of CD11b-positive cells in the heat-sensitive moxibustion group(14.38%±0.74%) were less than that in the non-heat-sensitive moxibustion group(29.00%±1.00%) (Figure 4c, 4d). These results indicate that heat-sensitive moxibustion significantly attenuated brain damage resulting from inflammatory reaction following IR insult in rats.



(a)



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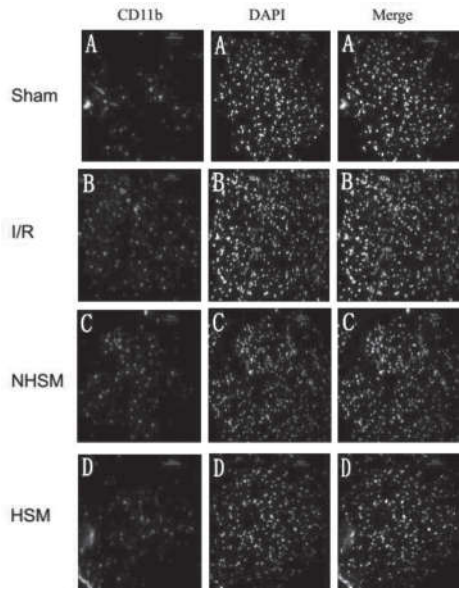

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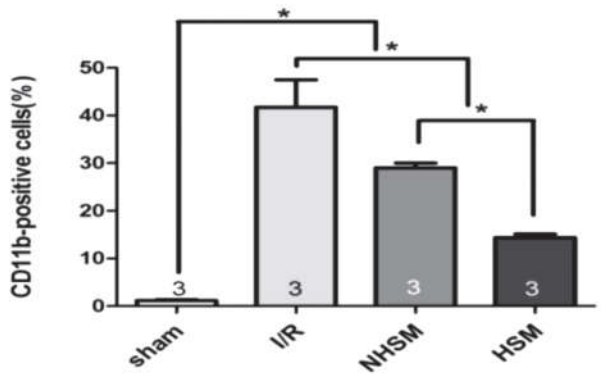


(d)

Figure 4. Effects of Heat-sensitive Moxibustion on Inflammatory Cells Infiltration in Focal Cerebral Ischemia/Reperfusion Injury Rats

(a) Rat brain coronal slice. Black box: the area for HE and immunohistochemical staining.

(b) Representative brain coronal sections stained with HE. Lots of inflammatory cells were in the cerebral cortices of I/R group in comparison to sham-surgery group. However, suspended moxibustion attenuated cerebral ischemia/reperfusion injury and significantly reduced the presence of inflammatory cells following IR insult. In contrast, outcomes in non-heat-sensitive moxibustion group were not as good as outcomes in heat-sensitive moxibustion group. Scale bars corresponds to 50μm. Green arrows indicates the inflammatory cells.

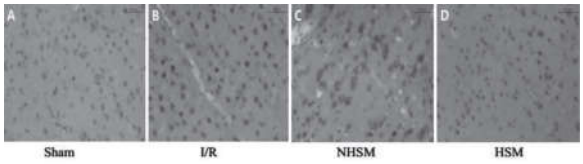
(c) Double-immunofluorescent staining of CD11b and nucleus in cerebral cortex. CD11b-positive cells stained red, while nuclei stained blue. Scale bars correspond to 50μm.

(d) Summary of CD11b-positive cells of sham, I/R and moxibustion-treated cerebral cortex. Heat-sensitive moxibustion decreased the percentage of CD11b-positive cells 3 day following IR insult in comparison to that of I/R group and non-heat-sensitive moxibustion group.

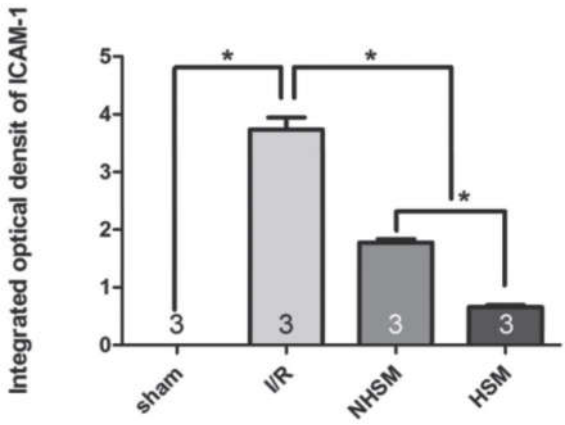
Notes: "*": $P < 0.05$. Sham(A): Sham-surgery group; I/R(B): cerebral ischemia/reperfusion injury group; NHSM (C) : cerebral ischemia/reperfusion injury with non-heat-sensitive moxibustion group; HSM (D) : cerebral ischemia/reperfusion injury with heat-sensitive moxibustion.

3.4 Heat-sensitive Moxibustion Reduced the Expression of ICAM-1 in the Cerebral Cortex

Intercellular adhesion molecule-1 (ICAM-1), an inflammation-related factor, produced by endothelial cells regulates the leukocyte recruitment and participates in inflammatory events. In this study, we compared the levels of the inflammation-associated proteins from ipsilateral cerebral cortices among sham, I/R and moxibustion-treated groups. As shown in Figure 5a, 5b, the average integrated optical density (IOD) of ICAM-1-positive cells in cerebral cortices was high in I/R group (3.7340±0.2094. $P < 0.05$) 3 days after surgery in comparison to that in sham-surgery rats (0.0002±0.0000). However, suspended moxibustion for 35 min significantly decreased the average integrated optical density (IOD) of ICAM-1-positive cells ($P<0.05$) in comparison to that in I/R group, and heat-sensitive moxibustion (0.6585±0.0353. $P<0.05$) decreased even more so than non-heat-sensitive moxibustion (1.7750±0.0598). These results indicated that heat-sensitive moxibustion significantly decreased inflammatory factor after the IR procedure, partly accounting for reduction of inflammatory cells in the cortical cortex.



(a)



(b)

Figure 5. Effects of Heat-sensitive Moxibustion on the Expression of ICAM-1 in Focal Cerebral Ischemia/Reperfusion Injury Rats

(a) Representative immunohistochemical staining of ICAM-1 protein. ICAM-1 was stained brown. Scale bars correspondsto 50μm.

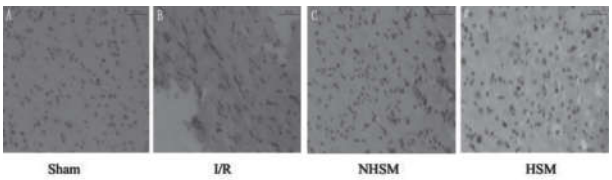
(b) Summary of the averageIOD analysis of ICAM-1 proteinin cerebral cortex of sham, I/R and moxibustion-treated group. The average (IOD of ICAM-1protein

was significantly high in the cerebral cortex 3 days following IR insult in comparison to that of sham-surgery group. However, suspended moxibustion significantly reduced the average IOD of ICAM-1 protein. In contrast, the average IOD in heat-sensitive moxibustion group were much lower than that in non-heat-sensitive moxibustion group. So heat-sensitive moxibustion significantly decreased the expression of ICAM-1 proteins following IR insult.

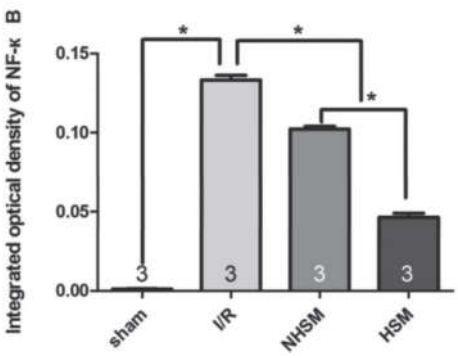
Notes: "*":P < 0.05. .Sham(A): Sham-surgery group; I/R(B): cerebral ischemia/reperfusion injury group; NHSM (C): cerebral ischemia/reperfusion injury with non-heat-sensitive moxibustion group; HSM (D) : cerebral ischemia/ reperfusion injury with heat-sensitive moxibustion

3.5 Heat-sensitive Moxibustion Reduced the Expression of NF-κ B and p-IKKα/β in the Cerebral Cortex

NF-κB is a pleiotropic transcription factor participating in inflammation. To understand whether neuroprotective effects of heat-sensitive moxibustion are mediated through suppressing NF-κB signaling pathway, we compared the levels of NF-κB signaling pathway proteins in cerebral cortex 3 days following IR insult among sham, I/R and moxibustion-treated groups. As shown in Figure 6a, 6b, the average IOD of NF-κB was high in cerebral cortices in I/R group (0.1334±0.00287.P < 0.05) in comparison to that in sham-surgery group (0.0012±0.0001). However, suspended moxibustion for 35 min significantly decreased the average IOD of NF-κB-positive cells (P < 0.05), and heat-sensitive moxibustion(0.0466±0.0025.P<0.05) decreased even more so than non-heat-sensitive moxibustion (0.1023±0.0016). Similarly, as shown in Figure 6c, 6d, p-IKKα/β protein level significantly increased in cerebral cortices in I/R group (53.71% ± 3.54%, p < 0.05) in comparison to that in the sham group (2.19%±0.28%). However, suspended moxibustion for 35 min significantly reduced p-IKKα/β protein level (p < 0.05) in comparison to that in the I/R group, and heat-sensitive moxibustion (15.76%±0.66%, p < 0.05) reduced more p-IKKα/β protein level than non-heat-sensitive moxibustion (32.83%±0.74%), possibly as a result of the reduced presence of inflammatory cells in the cerebral cortex.



(a)



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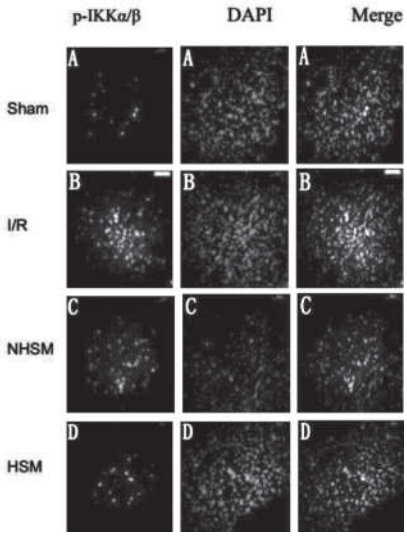

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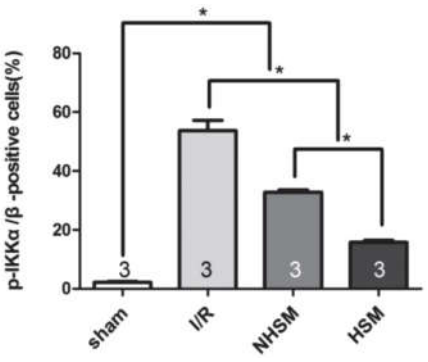

(d)

Figure 6. Effects of Heat-sensitive Moxibustion on the Expression of NF-κB and p-IKKα/β in Focal Cerebral Ischemia/Reperfusion Injury Rats

(a) Representative immunohistochemical staining of NF-κB protein 3 day following IR insult. NF-κB was stained brown. Scale bars correspond to 50μm.

(b) Summary of the average IOD analysis of NF-κB protein in cerebral cortex of sham, I/R and moxibustion-treated group. The average IOD of NF-κB proten was significantly high in the cerebral cortex 3 days following IR insult in comparison to that of sham-surgery group. However, suspended moxibustion significantly reduced the verage IOD of NF-κB protein. In contrast, the average IOD in heat-sensitive moxibustion group were much lower than that in non-heat-sensitive moxibustion group. So heat-sensitive moxibustion significantly decreased the expression of NF-κB proteins following IR insult.

(c) Double-immunofluorescent staining of p-IKKα/β and nucleus in cerebral cortex. P-IKKα/β-positive cells stained red, while nucleus stained blue. ×200

(d) Summary of p-IKKα/β-positive cells of sham, I/R model and moxibustion-treated cerebral cortex. Heat-sensitive moxibustion decreased the expression of p-IKKα/β proteins 3 day following IR insult in comparison to that in I/R group and non-heat-sensitive moxibustion group.

Notes: "*": $P < 0.05$. Sham(A): Sham-surgery group; I/R(B): cerebral ischemia/reperfusion injury group; NHSM (C): cerebral ischemia/reperfusion injury with non-heat-sensitive moxibustion group; HSM (D) : cerebral ischemia/ reperfusion injury with heat-sensitive moxibustion.

4. Discussion

In this study, we reported that suspended moxibustion for 35 min with tail temperature increase (i.e HSM):

(1) Significantly improved sensory function and increased neurological function scores after cerebral ischemia-reperfusion injury in rats;

(2) Significantly protected the brain from cerebral ischemia/reperfusion injury;

(3) Significantly reduced the presence of inflammatory cells and CD11b-positive cells in the cerebral cortices;

(4) Significantly decreased the expressions of inflammation-related factor ICAM-1 in the cerebral cortices;

(5) Reduced the protein level of p-IKKα/β /NF-κB signaling pathway in the cerebral cortices. Our findings suggest that HSM protects brain from cerebral ischemia-reperfusion injury, which is largely mediated by anti-inflammation involved the decreased expression of p-IKKα/β /NF-κB signaling pathway during cerebral ischemia-reperfusion injury.

Suspended moxibustion is an important treatment method in Traditional Chinese Medicine. The previous controlled trials have demonstrated the enhancement of recovery in stroke patients by 15-minute traditional suspended moxibustion on the fixed acupoints,[26],[27] however, other studies had different results.[2],[3] The newly developed application of "heat-sensitive" suspended moxibustion substantially improved upon the effect of traditional suspended moxibustion.[28] The theoretical basis for heat-sensitive moxibustion is that the acupoints which are activated from the resting state to the sensitized state by a pathological process become heat sensitive. Clinic practices demonstrated that moxibustion placed above a heat-sensitive acupoint can achieve effect namely "less stimulation, greater effect", so the efficacy of moxibustion is significantly improved.[29] Acupoint, according to the core viewpoint and theory of ancient Chinese medicine, represents the certain area of the body surface which can respond to disease. Usually, an acupoint has two states, the "resting" state and the "sensitized" state; selecting the sensitized acupoints is the key to improving efficacy.[29] There are a variety of types of sensitized acupoints; the heat-sensitive type is a new one among these.

Is an acupoint heat-sensitive or not? According to the results of clinical studies for more than 20 years by professor Chen Rixin et al[28], moxibustion above heat-sensitive acupoint can show 6 kinds of moxibustion sensation. If one of six sensations is produced, the acupoint is thought to be heat-sensitive. Among these six sensations, one is heat transduction, it means the heat of moxibustion point could transduct along a line to the distance. As the locations of acupoints are arranged along with meridians, moxibustion applied above the heat-sensitive acupoint penetrates deeply into the body and can transduct the heat to distant regions along the meridians. When such distant heat occurs, the acupoint is called heat-sensitive one, and the efficacy of moxibustion increases.[30] So in this experiment we divided moxibustion-treated mice into NHSM and HSM group according to the difference of tail temperature before moxibustion and after moxibustion.

Additionally, the amount of moxibustion is a key technology when applying heat-sensitive suspended moxibustion. When other factors are constant, duration of suspended moxibustion is a critical factor for improving the efficacy of moxibustion.[5],[7],[28] Generally, distant heat first appears on the patient at about 15 minutes into suspended moxibustion treatment, after the 15 minute time point, a rapid increase in distant heat is exhibited in the patients and maintained until the end of the treatment, usually 30 min to one hour.[31] Long-term clinical practice prove that the duration of heat-sensitive suspended moxibustion ranges from 30 minutes to 1 hour, which is usually longer

than conventional moxibustion (usually around 15 minutes), and find that prolonged application of moxibustion above certain acupoints can often induce not only internal heat-sensation but also physically detectable elevated temperature at distant locations away from the suspended moxibustion acupoint. One study showed that a 40-minute suspended moxibustion caused a measurable tail temperature increase in some MCAO rats while 15 minute treatments didn't have this effect,[7] and the occurrence rate of tail temperature increase was 54.1%.[7] Our previous study showed that suspended moxibustion for 35 minutes, but not 15 minutes, for 3 days markedly alleviated MCAO-induced infarct volume and neuronal loss, as determined using TTC, HE staining when compared with the stroke only group.[6] Among rats with suspended moxibustion for 35 minutes, those with tail temperature increase had a better outcome than those without[21]. Consistent with the previous report, our result showed that the occurrence rate of tail temperature increase was 53.6% in 35-min moxibustion group. These evidences collectively showed that prolonged moxibustion is capable of stimulating the heat-sensitive acupoints and gradually transfers the heat to a distant area, and moxibustion with tail temperature increase had a better efficacy than that without. This study showed that suspended moxibustion for 35 minutes with tail temperature increase for 3 days markedly improved sensorimotor function, reduced infarct volume and alleviated neuronal loss, as determined using neurobehavioral evaluation, TTC, HE staining when compared with the stroke only group and suspended moxibustion for 35 minutes without tail temperature increase. These results indicates that moxibustion placing above the heat-sensitive acupoint achieve a better neuroprotective effect than moxibustion placing above non- heat-sensitive acupoint.

Inflammation plays a pivotal role in the pathologic and physiological processes in cerebral ischemia-reperfusion injury, accumulation of data has revealed that cerebral ischemia-reperfusion injury is associated with a marked inflammatory reaction that contributes to the evolution of the tissue injury.[10],[11],[12],[13],[14] During brain ischemia/reperfusion insult, inflammatory responses are initiated involving infiltration into the brain of inflammatory cells and the production of inflammatory cytokines, i.e., inflammatory cells and inflammatory mediators play an important role in the brain inflammatory response to I/R, which has been observed during ischemic pathology in experimental animal models of stroke.[10],[11],[12],[13],[14] Myeloperoxidase, abundantly present in inflammatory cells (such as neutrophils and macrophages), produces hypochlorous acid from hydrogen peroxide and chloride ions during the respiratory burst of neutrophils (abundance of reactive oxygen species). Hypochlorous acid is highly cytotoxic and has been demonstrated to damage CNS tissue during inflammation.[32] Further, myeloperoxidase activity assay has been successfully used to confirm inflammatory cell activation and recruitment in brain after MCAO.[10],[16],[17] Microglia is resident immunocompetent and phagocytic cells in the CNS, which play a critical role in the event of infection, hypoxemia, ischemia, and neurodegeneration in the central nervous system (CNS).[18] CD11b is the marker of microglia/macrophages, our previous experiment showed that there was a significant increase in CD11b-positive cells in cerebral cortex of IR model rats in comparison to those in sham-surgery group.[21] Microglial cells activation exacerbates ischemic injury in the brain.[33],[34],[35] Intercellular adhesion molecule-1 (ICAM-1) produced by endothelial cells regulates the leukocyte recruitment and participates in inflammatory events.[10],[14],[16] It is well known that an excessive inflammatory reaction can damage target cells and tissue. Therefore, inhibition of inflammatory responses provided an attractive therapeutic strategy.[10],[11],[12],[13],[14] Our previous study also showed that 35 min moxibustion reduced myeloperoxidase activity in comparison with 15 min moxibustion,[6] and 35 min moxibustion with tail temperature increase significantly decreased the CD11b-positive cells in comparison with 35 min moxibustion without tail temperature increase,[21] which suggested that prolonged moxibustion with distal heat had a more potent effect. In this study, we compare the anti-inflammatory effects between moxibustion for 35 min with tail temperature increase and that without. Our results demonstrated that 35 min suspended moxibustion treatment with tail temperature increase reduced inflammatory cells infiltration and the percentage of CD11b-positive cells, decreased the average IOD of inflammation-related factor ICAM-1-positive cells. The results indicated that 35 min moxibustion with tail temperature increase markedly reduced inflammatory reaction, which partly contributed to the neuroprotective effect of heat-sensitive moxibustion.

It is well known that NF-κB is a master regulator of inflammatory signaling pathways and involved in cerebral ischemia-reperfusion injury. It has been known that NF-κB exists in almost all the cells.[19] In the central nervous system (CNS), NF-κB activity is detected in both neuronal and glial cells following ischemic injury.[20] Then inflammatory cells activation triggers rapid transcription-factor NF-κB signal transduction cascades, mediating expression of inflammatory mediators during pathophysiological changes after brain ischemia injury.[12] The most common

form of NF-κB, a dimeric transcription factor, is a heterodimer composed of Rel A (p65), namely NF-κB (p65). Normally NF-κB p65 protein is quiet in cells, residing in the cytoplasm as a complex with inhibitory IκB that mask their nuclear localization signal. Upon brain ischemia, ischemia/reperfusion injury et al, IκB is phosphorylated by p- IKKα/β and proteolytically degraded, resulting in the translocation of NF-κB p65 to the nucleus and production of inflammatory factors. Following I/R injury NF-κB p65 expression was upregulated,[13],[14],[22],[23] so inhibition of NF-κB activation exerted anti-inflammatory and neuroprotective effects.[13],[14],[22],[23] Thus, we investigated the potential effects of heat-sensitive moxibustion on the expression of NF-κB signaling pathway. We observed that the protein level of p- IKKα/β and NF-κB p65 were high in the cerebral cortices following IR injury, while heat-sensitive moxibustion significantly decreased the expression of p- IKKα/β and NF-κB p65, consistant with the previous report.[33] We concluded that decreasing expression of the NF-κB signaling pathway partly accounted for the anti-inflammatory effect of heat-sensitive moxibustion due to reducing the production of inflammatory factors and decreasing infiltration of inflammatory cells.

5. Conclusion

In summary, our study demonstrated that heat-sensitive moxibustion significantly provided beneficial effects on cerebral ischemia/reperfusion injury by decreasing the inflammatory cells such as microglia, reducing inflammatory factors like ICAM-1and lowering the expression of NF-κB signal transduction pathway. Although there is currently no clear physiological correlate to this theory; in addition, it remains unknown what the relevant properties of the moxibustion heat stimulus are responsible for the therapeutic effects. However, the clinical evidences support the notion that moxibustion may improve physiological conditions through regulation of the homeostasis of the body. TCM claims that moxibustion functions by restoring the balance and flow of "vital energy" through sensitized acupoints, thus we suppose that under pathological conditions, the sensitized acupoints serve as the switch that controls the complicate internal regulatory system towards to dysfunction. Moxibustion applied upon sensitized acupoints can fully activate the regulatory system and enhance the internal ability to correct the imbalance of the internal regulation by inducing rapid, precise and optimal responses to harmful stimuli, and then to enhance treatment efficacy.

To sum uponclusion, the present study showed that heat-sensitive moxibustion exerts its neuroprotective effects against inflammation with the involvement of decreasing expression of NF-κB signaling pathway. These findings will further encourage heat-sensitive moxibustion to be applied in the clinic stroke field.

Acknowledgments

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