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Efficacy of suspended moxibustion in stroke rats is associated with a change in tail temperature

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Abstract

Suspended moxibustion-produced heat can transfer from the acupoint to other sites of the body. The suspended moxibustion should be terminated when clinical propagated sensation disappears, because this implies that the quantity of moxibustion is sufficient. We wanted to investigate if this phenomenon also occurs in experimental animals. In the present study, a rat model of stroke was established and treated with suspended moxibustion at *Dazhui* (DU14) for 60 minutes. Results showed that the increase in tail temperature began at 15 minutes after suspended moxibustion and decreased gradually at 40 minutes. In addition, neurological function was significantly improved in stroke rats with tail temperature increase following suspended moxibustion, and this effect was associated with significantly reduced tumor necrosis factor α and interleukin 1β mRNA. However, there was no significant difference between 40- and 60-minute suspended moxibustion. The findings indicate that elevated tail temperature began to decrease at 40 minutes after suspended moxibustion, and further suspended moxibustion was not useful in the recovery of stroke rats.

Key Words

neural regeneration; traditional Chinese medicine; acupuncture and moxibustion; suspended moxibustion; stroke; tail temperature; tumor necrosis factor-alpha; interleukin-1 beta; grants-supported paper; neuroregeneration

Research Highlights

(1) Tail temperature increased after 15-minute suspended moxibustion, but decreased after 40 minutes in stroke rats.
(2) Suspended moxibustion with tail temperature increase improved neurological deficit better than did moxibustion without tail temperature increase.
(3) Continuing suspended moxibustion could not further strengthen its efficacy when tail temperature began to decrease.
(4) This finding is in accordance with the rules of heat-sensitive moxibustion, which states that moxibustion should be terminated when the propagated sensation disappears, as this implies that the quantity of moxibustion is sufficient.

Rixin Chen and Zhimai Lv contributed equally to this work.

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INTRODUCTION

Suspended moxibustion is an important treatment method in traditional Chinese medicine in which moxibustion is placed superficially over the skin but does not make contact. It has been widely used to treat various diseases, such as stroke. Although strong evidence regarding its therapeutic efficacy is lacking[1], it has been suggested that if the duration of the suspended moxibustion is appropriately extended and a phenomenon called “distant heat” is produced, then the efficacy of the suspended moxibustion is significantly strengthened[2]. The phenomenon of distant heat has been demonstrated in a clinical setting, where moxibustion heat is transferred from the original moxibustion acupoint to other areas of the body[3]. We have also found a similar reaction to the phenomenon of distant heat when observing tail temperature increase during 40-minute suspended moxibustion on the *Dazhui* (DU14) acupoint in a rat model of transient middle cerebral artery occlusion[4]. In traditional Chinese channel theory, *Dazhui* and the tail are both on the *DU* channel (Governor Vessel) and stimulating *Dazhui* could provide heat for the tail through the channel[5]. Transient middle cerebral artery occlusion rats with tail temperature increase were found to recover better than those without. Furthermore, temperature increased at about 15 minutes, and peak temperature was maintained until the end of the 40-minute treatment[4]. However, two issues remained: how long will tail temperature increase last if the duration of suspended moxibustion is increased, and is it useful for recovery of transient middle cerebral artery occlusion rats if suspended moxibustion continues after tail temperature increase begins to decrease. The present study was designed to resolve these issues, and investigated the mRNA expression of two important inflammatory mediators, tumor necrosis factor-α and interleukin-1β in the cortex of stroke rats. The study could help deepen the understanding of the underlying biological mechanisms of this moxibustion technique.

RESULTS

Quantitative analysis of experimental animals

Of 120 rats used, 30 were randomly assigned to the sham-surgery (n = 10) and ischemia (n = 20) groups, both of which underwent suspended moxibustion for 3 days. The ischemia group was subdivided into non-tail temperature increase (temperature change less than 1°C) and tail temperature increase (temperature change more than 1°C) according to tail temperature changes. The remaining 90 rats were used to establish transient middle cerebral artery occlusion models and assigned to the ischemic control (n = 15), suspended moxibustion for 15 (M15; n = 15), 40 (M40; n = 30) and 60 minutes (M60; n = 30) groups. The M40 and M60 groups were further divided into two subgroups according to tail temperature changes in 7 days, a non-tail temperature increase subgroup (temperature change ≤ 1°C in 7 days: M40-non-tail temperature increase group, n = 13; M60-non-tail temperature increase group, n = 14) and a tail temperature increase subgroup (temperature change > 1°C in 7 days: M40-tail temperature increase group, n = 14; M60-tail temperature increase group, n = 15). In the first part of experiments, two rats were excluded (one died and one failed in establishing the model). In the second part of experiments, two rats from the ischemic control group died, two from the M15 group failed to establish the model, one from the M40 group died, two failed in model establishment, and 1 from M60 died. Finally, 28 rats in the first part of the experiment and 82 in the second part of the experiment were included in the final analysis.

Tail temperature change following suspended moxibustion

In the first part of experiments, tail temperature began to quickly increase immediately after suspended moxibustion. At about 5–10 minutes, the temperature reached a relatively stable level, but was an increase of less than 1°C on average. The tail temperature remained unchanged in nine rats from the ischemia group and all the control rats (non-tail temperature increase) throughout the treatment session. However, the other nine transient middle cerebral artery occlusion rats exhibited tail temperature increase (more than 2°C on average). Furthermore, the tail temperature in the rats with tail temperature increase increased to a peak value at around 15 minutes, and peak temperature was maintained until 40 minutes. Up to 50 minutes, tail temperature decreased to a level similar to that of the non-tail temperature increase or sham-surgery rats. Results were similar during the next 3 consecutive days (Figure 1). According to the change in tail temperature, we selected 15, 40 and 60 minutes as the suspended moxibustion duration in subsequent experiments. In the second part of the experiments, no rats exhibited tail temperature increase in the ischemic control or M15 groups. There were 15 rats from the M40 and 16 from M60 groups which exhibited tail temperature increase.

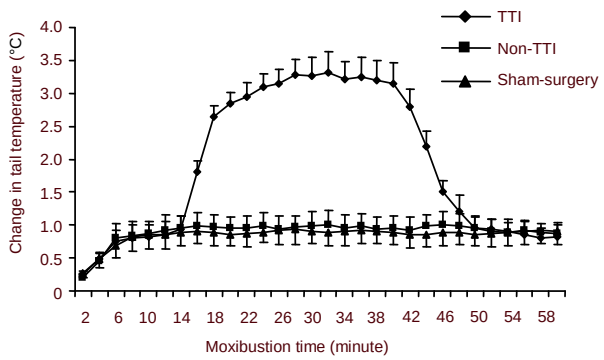

Figure 1 Change in tail temperature induced by 60-minute suspended moxibustion in the first part of the experiments.

Because the changes in tail temperature were similar across the three consecutive testing days, data from the first day were presented as representative findings. Data are expressed as mean ± SD. TTI: Tail temperature increase.

Suspended moxibustion significantly improved the neurological function of rats

To investigate the efficacy of suspended moxibustion of different durations, we examined the neurological deficit scores following transient middle cerebral artery occlusion in the second part of the experiment. Results revealed that the neurological deficit scores were significantly reduced in the M40-tail temperature increase group and the M60-tail temperature increase group at 3 days after reperfusion compared with the ischemic control and the M15 groups ($P < 0.05$). The neurological deficit scores were further ameliorated in the M40-tail temperature increase and M60-tail temperature increase groups at 7 days after reperfusion, compared with the ischemic control, M15, M40-non-tail temperature increase and M60-non-tail temperature increase groups ($P < 0.05$). However, there was no significant difference between M40-tail temperature increase and M60-tail temperature increase groups at 1, 3 or 7 days. The neurological deficit scores at 7 days were significantly reduced in the M40-non-tail temperature increase and M60-non-tail temperature increase groups compared to the ischemic control and M15 groups ($P < 0.05$), but scores were similar between the M40-non-tail temperature increase and M60-non-tail temperature increase groups. The M15 group did not exhibit any protective effect compared to the ischemic control (Figure 2).

Suspended moxibustion suppressed cortical tumor necrosis factor-α and interleukin-1β mRNA expression in tail temperature increase rats

To investigate the level of inflammation in the cortex following transient middle cerebral artery occlusion, tumor necrosis factor-α and interleukin-1β mRNA was measured using real time PCR at the inflammatory peak (the 1 or 3 days testing point)[6]. Results showed that the M40-tail temperature increase ($P < 0.01$) or M60-tail temperature increase ($P < 0.01$) group attenuated the increase of tumor necrosis factor-α and interleukin-1β mRNA caused by transient middle cerebral artery occlusion at 1 day post ischemia, while other treatment groups had no effect on these inflammatory mediators compared to the ischemic control group. At 3 days post ischemia, both tumor necrosis factor-α and interleukin-1β mRNA levels were reduced in M40-non-tail temperature increase, M60-non-tail temperature increase, M40-tail temperature increase and M60-tail temperature increase groups, while M15 had no effect on tumor necrosis factor-α and interleukin-1β mRNA compared to ischemic control group. The suppression of tumor necrosis factor-α and interleukin-1β mRNA in the M40-tail temperature increase and M60-tail temperature increase groups was more evident than in the M40-non-tail temperature increase or M60-non-tail temperature increase groups at 3 days post ischemia. In addition, there was no significant difference in tumor necrosis factor-α and interleukin-1β mRNA expression between M40-tail temperature increase and M60-tail temperature increase groups at 1 or 3 days. Low expression of tumor necrosis factor-α and interleukin-1β mRNA was detected in the sham-surgery group (Figure 3).

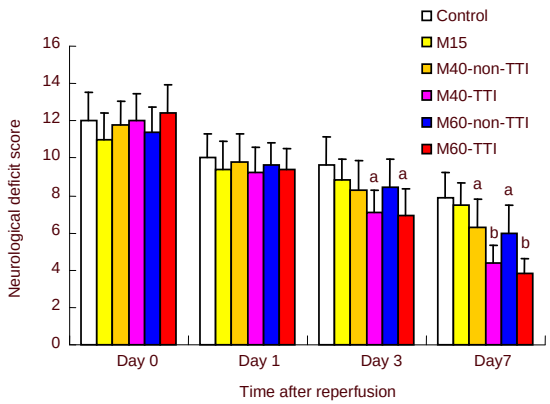

Figure 2 Effect of suspended moxibustion on neurological deficit scores in rats undergoing transient middle cerebral artery occlusion.

Neurological deficit scores were graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18). Data are presented as mean ± SD. [a]$P < 0.05$, *vs.* ischemic control and M15 groups; [b]$P < 0.05$, *vs.* ischemic control, M15, M40-non-TTI and M60-non-TTI groups using one-way analysis of variance.

M15, M40, M60: Suspended moxibustion for 15, 40 and 60 minutes; TTI: tail temperature increase.

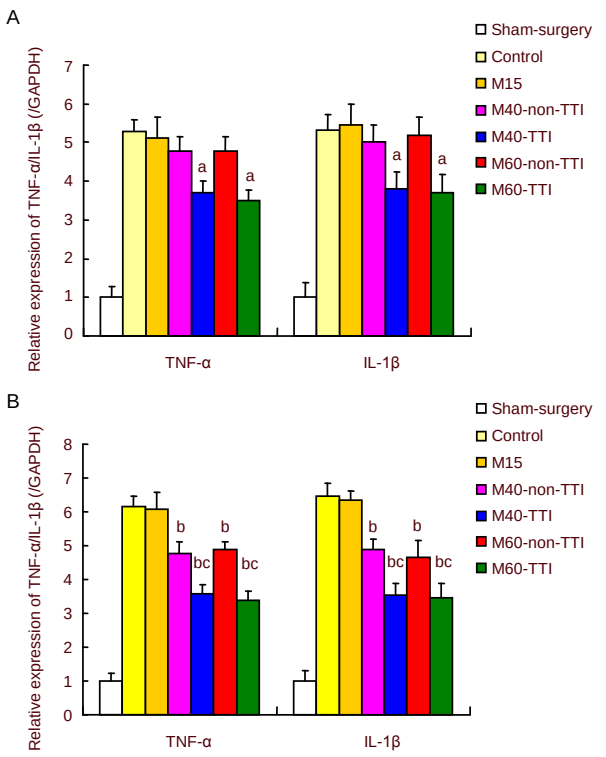

Figure 3 Suppression of ischemia-induced upregulation of tumor necrosis factor (TNF)-α and interleukin (IL)-1β mRNA in rats undergoing transient middle cerebral artery occlusion.

Expression of TNF-α and IL-1β mRNA at 1 (A) and 3 days post ischemia (B). $^{a}P < 0.01$, *vs*. ischemic control, M15, M40-non-TTI and M60-non-TTI groups; $^{b}P < 0.01$, *vs*. ischemic control and M15 groups; $^{c}P < 0.01$, *vs*. M40-non-TTI and M60-non-TTI groups using one-way analysis of variance. Data are presented as mean ± SD.

M15, M40, M60: Suspended moxibustion for 15, 40 and 60 minutes; TTI: tail temperature increase.

DISCUSSION

In the first part of experiments, we selected 60 minutes as the suspended moxibustion duration so that the observing time was long enough to study the regular pattern of tail temperature variation. Results showed that the tail temperature of the sham-surgery rats and nine transient middle cerebral artery occlusion rats reached a relatively stable level but less than 1°C on average at about 5–10 minutes. Stable temperature was maintained until the end of the 60-minute suspended moxibustion treatment. However, the other nine transient middle cerebral artery occlusion rats exhibited tail temperature increase. It was revealed that tail temperature increase did not appear in sham-surgery rats. This indicates that tail temperature increase is relevant to stroke. This seems to be consistent with the fact in clinical settings that distant heat induced by suspended moxibustion is highly relevant to the pathological process of diseases[2]. However, why did the nine transient middle cerebral artery occlusion animals not exhibit a marked tail temperature increase? As we discussed previously[2, 4], acupoint stimulation can cause distant heat at different locations in subjects who are afflicted with the same disease. Acupoints other than *Dazhui* were stimulated in the nine transient middle cerebral artery occlusion rats in order to produce tail temperature increase. Our previous study showed that 40–60% of the stroke rats that received suspended moxibustion on *Dazhui* exhibited a tail temperature increase[4]. Therefore, we utilized double the number of transient middle cerebral artery occlusion rats than the sham-surgery group so that the number of non-tail temperature increase subjects or tail temperature increase subjects selected from the suspended moxibustion group was almost the same as the sham-surgery group. The above explanations can also be applied to the second part of the experiments.

Prior to the second part of experiments, we had carefully studied the variation of tail temperature in rats with tail temperature increase. Tail temperature increased quickly at about 15 minutes but declined after 40 minutes. Hence, we selected 15, 40 and 60 minutes as the suspended moxibustion duration in the second part of studies, in which we confirmed the key role of tail temperature increase in improving suspended moxibustion efficacy. The application of suspended moxibustion for 15 minutes is considered the stage of meridian-*Qi* excitation[2], so it is not enough to induce tail temperature increase. This is exactly the reason why 15-minute suspended moxibustion has no effect on the recovery of rats. Although the efficacy of M40-non-tail temperature increase or M60-non-tail temperature increase was not as strong as the M40-tail temperature increase or M60-tail temperature increase group, a therapeutic effect was still observed compared to the M15 group. These findings revealed that the duration of suspended moxibustion was important to therapeutic efficacy. However, this does not show that the longer the duration of suspended moxibustion session, the better the effects, because the effects also depend on whether tail temperature increase appears. Our data showed that the efficacy of suspended moxibustion without tail temperature increase for 40 or 60 minutes seemed to be limited, while suspended moxibustion with tail temperature increase significantly strengthened the efficacy of suspended moxibustion. In addition, there was no difference in efficacy of suspended moxibustion with tail temperature increase between 40 and 60 minutes. This

suggests that continuing suspended moxibustion after 40 minutes of treatment was useless for recovery from transient middle cerebral artery occlusion when tail temperature began to decline. It seemed that suspended moxibustion for 40 minutes was more suitable for recovery from transient middle cerebral artery occlusion because it took less curative time and achieved a similar effect as 60-minute treatment. These observations are useful for guiding the use of clinical suspended moxibustion.

It is widely known that tumor necrosis factor-α and interleukin-1β in the brain increase and exacerbate ischemic brain injury following ischemia[7]. This study showed that suspended moxibustion for 40 or 60 minutes with tail temperature increase significantly reduced the expression levels of tumor necrosis factor-α and interleukin-1β mRNA induced by transient middle cerebral artery occlusion compared with the other treatment groups. Furthermore, there was no difference in tumor necrosis factor-α and interleukin-1β mRNA between M40-tail temperature increase and M60-tail temperature increase groups at 1 or 3 days. This result indicates that suspended moxibustion at the Dazhui acu-point for 40 or 60 minutes with tail temperature increase can create an anti-inflammatory effect.

In summary, after suspended moxibustion at Dazhui in transient middle cerebral artery occlusion rats, the tail temperature began to increase significantly at around 15 minutes but decreased after 40 minutes. Suspended moxibustion with tail temperature increase significantly strengthened the efficacy of suspended moxibustion in stroke rats, which was associated with suppression of tumor necrosis factor-α and interleukin-1β mRNA. However, continuing suspended moxibustion could not increase its efficacy when the tail temperature began to decrease.

MATERIALS AND METHODS

Design

Arandomized, controlled, animal study.

Time and setting

This experiment was performed at the Laboratory Animal Center, Jiangxi University of Traditional Chinese Medicine, China from April to May 2011.

Materials

Animals

A total of 120 adult, male, Sprague-Dawley rats, 8 weeks old, weighing 220–250 g, of clean grade were purchased from the Shanghai Laboratory Animal Resources Center (license No. SCXK (Hu) 2003-0002), Shanghai, China. The rats were maintained in a cage at room temperature 22 ± 2°C, with controlled humidity 60 ± 5% and 12-hour day/night cycle, with a maximum of five rats per cage. All experimental studies were performed in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

Instrument

Moxa stick, weighing 6 g, 12 cm in length, 0.6 cm in diameter, was prepared by the Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine, China.

Methods

Preparation of experimental stroke model in rats

Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (3%) at a dose of 30 mg/kg. Core body temperature was monitored using a rectal probe (Shenchao Transducer Co., Ltd, Shenzhen, Guangdong Province, China) and maintained at 37 ± 0.5°C by a heating lamp and a heating pad. Middle cerebral artery occlusion was performed using the intraluminal filament method as described previously[8]. After 2 hours of occlusion, the fishing line advanced to the origin of the middle cerebral artery was unclamped to allow reperfusion. Adequacy of vascular occlusion and reperfusion was assessed by Laser Doppler Monitoring (PeriFlux 5000, Perimed AB, Stockholm, Sweden) of cerebral cortical perfusion. Regional cerebral blood flow in the middle cerebral artery territory was reduced to < 20% of baseline after advancing the fishing line to the origin of the middle cerebral artery, and reconstituted to > 60% of baseline after removal of the fishing line. Rats dying within 24 hours after surgery, or displaying a neurological score of 0 or subarachnoid hemorrhage (as macroscopically assessed), were excluded from the final analysis.

Suspended moxibustion treatment

Stimulation of suspended moxibustion was performed 6 hours after reperfusion on the first day. A special cage in which the rat could maintain a comfortable position and the motion was restricted was used while testing. The cage was convenient for performing suspended moxibustion. Room temperature was maintained at 25 ± 2°C for the entire experimental process. The acupoint *Dazhui* (at C_7–T_1), which is very important for brain function[5], was heated by suspended moxibustion using a moxa (used specifically for animals) at approximately

3 cm (held by hand) over the hairless skin once a day for 3 days (in the first part of the experiments) or 7 days (in the second part of the experiments).

Tail temperature measurement

The midpoint tail temperature of rats was recorded once every 2 minutes precisely by an electro-digital thermometer (Shanghai Medical Instrument Factory, Shanghai, China) during suspended moxibustion treatment. The testing environment was maintained quiet, and room temperature was maintained at 25 ± 2°C. Rats were placed in a cage for 30 minutes before the experiment was started.

Assessment of neurological function

In the second part of experiments, neurological assessment, using a modified neurological severity scale (as described previously[9]), was performed at 0, 1, 3 and 7 days after transient middle cerebral artery occlusion by an investigator who was blinded to the experimental groups. In brief, scores on this scale were derived by evaluating animals for hemiparesis, sensory deficits, beam balance tests, absent reflexes, and abnormal movement. Neurological deficit scores were graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18). One point was awarded for the inability to perform a task or the lack of a reflex.

Real time PCR analysis for tumor necrosis factor-α and interleukin-1β mRNA expression in rat cortex

Rats were sacrificed by cervical dislocation. Infarcted cortex was separated from the brain. Total RNA from cortex was reverse-transcribed into cDNA using oligo (dT) 18 primers and Avian Myeloblastosis reverse transcriptase (Gibco, Carlsbad, CA, USA). Real-time PCR was performed in the presence of a fluorescent dye (Evagreen, BIOTIUM, Hayward, CA, USA). The primer sequences used in this study were as described previously[10]:

Primer	Sequence	Product size (bp)
Tumor necrosis factor-α	Forward: 5'- CCC AGA CCC TCA CAC TCA GAT-3' Reverse: 5'- TTG TCC CTT GAA GAG AAC CTG-3'	215
Interleukin-1β	Forward: 5'- CAC CTT CTT TTC CTT CAT CTT TG-3' Reverse: 5'- GTC GTT GCT TGT CTC TCC TTG TA-3'	241
GAPDH	Forward: 5'- TGC CAA GTA TGA TGA CAT CAA GAA G-3' Reverse: 5'- AGC CCA GGA TGC CCT TTA GT-3'	80

The PCR protocol consisted of 5-minute enzyme activation at 95°C, followed by 45 cycles of 20-second denaturation at 95°C, 30-second annealing at 55°C and 23-second extension and fluorescence measurement at 72°C. Relative absorbance of mRNA was calculated after normalization to GAPDH ribosomal RNA and determined using crossing point analysis of log/linear plots of fluorescence/cycle number. The experiments were performed at least three times. ABI 7500 PCR system (ABI Carlsbad, CA, USA) was used.

Statistical analysis

Data were presented as mean ± SD and analyzed using one-way analyses of variance with *post hoc* Newman-Keuls multiple range tests for multiple groups. SPSS 13.0 (SPSS, Chicago, IL, USA) was used for analysis. A value of $P < 0.05$ was considered statistically significant.

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Author contributions: Rixin Chen and Mingren Chen designed and carried out the study, and supervised the project. Zhimai Lv and Dangdang Huang established the models. Zhimai Lv, Dangdang Huang and Fan Yi performed the moxibustion, temperature measurement, neurological assessment and real-time PCR. Rixin Chen and Zhimai Lv collected and analyzed the data, discussed the interpretation of the results, and wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest: None declared.

Ethical approval: All experimental procedures involving the use of animals were approved by the Animal Use and Care Committee for Jiangxi University of Traditional Chinese Medicine.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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