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Original Research

Effect of medicated thread moxibustion on apoptosis of hippocampal neurons in rat models of chronic cerebral ischemic vascular dementia

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Abstract: To investigate the effect of medicated thread moxibustion onapoptosis in hippocampal neuronsin a rat model of chronic cerebral ischemic vascular dementia. A total of 40 male Wistar rats were randomly divided into normal group and sham-operated group (7 rats each), and rat model of chronic cerebral ischemic vascular dementia (14 rats). The model group rats were treated with medicated thread moxibustion two weeks after surgery, once a day, with one day break every six days, (24 times in all)and an observation period of 4 weeks. At the end of therapy, H&E staining was used to monitor changes in the neurons in CA1 area of the rat hippocampus. Changes in related indexes such as Bax, Bc1-2 and C-fos of neuron apoptosis in hippocampus CA1 area were determined by immunohistochemistry, while protein expression was semi-quantitatively assayed using imaging analysis technique. There was significant hippocampal neuronal necrosis six weeks after model establishment, but the necrosis was milder in rats in the medicated thread moxibustion group. Bax and C-fos were positively expressed and significantly higher in the hippocampus of chronic cerebral ischemic vascular dementia rats (model group) than in the medicated thread moxibustion group after treatment (p <0.01). The expression of Bcl-2 was increased in the medicated thread group after treatment, and was higher in the model group, but comparable to that in the sham-operated group (p=0.975>0.05). Medicated thread moxibustion alleviates hippocampal neuronal necrosis, inhibits neuron apoptosis in hippocampus CA1 area, protects nerves, and maintains relative equilibrium inBax/Bcl-2 through down-regulation of C-fos and Bax and up-regulation of Bcl-2. Thus, cell apoptosis-related pathway may be one of its mechanisms of action.

Key words: Chronic cerebral ischemia; Vascular dementia; Medicated thread moxibustion; Hippocampal neurons; Apoptosis.

Introduction

Chronic cerebral ischemia is a common pathological condition which is the primary cause of vascular dementia (1). Some patients already have decreased cerebral blood flow before they present with clinical manifestations of dementia. The pathological condition of dementia can also give rise to decreased cerebral blood flow. Indeed, the severity of dementia is associated with the degree of decline in cerebral blood flow (2). Many trials have demonstrated that simulated chronic cerebral ischemic environment harms the learning and memory ability of experimental rats, with up to 83 % incidence of dementia (3).

Hippocampus is mainly in charge of learning and memory function (4;5), and hippocampal neurons are responsible for processing, transmitting and storing up information. The greater the number of neurons, the stronger its capabilities in processing and storing up information, the better the ability to learn and remember is. It has been confirmed in living animal experiments and in vitro nerve cell culture studies that ischemia and hypoxia can lead to neuronal apoptosis (6). Ischemia and hypoxia in hippocampus may induce massive neuronal apoptosis, thereby resulting into cognitive disorder, which may be the pathological foundation of vascular dementia (7).

C-fos is a normal gene in human and animal central nervous system cells. It is an immediate early response gene that regulates cell growth, division, proliferation, differentiation and apoptosis and participates in learning and memory processes. Besides, it is inextricably bound up with fos protein in brain, cortex, hippocampus border and striatum. Under normal physiological condition, c-for is difficult to be detected due to its poor activity (8). When cerebral ischemia and hypoxia occur, c-fos will responds rapidly, and fos protein, its expression product, and growth factors are involved in promoting cell division and proliferation, but if c-fos is overexpressed, apoptosis will be induced (9;10).

Bcl-2 is one of main genes regulating apoptosis, whose protein family plays an essential role in apoptosis process (11).Bcl-2 protein expressed by Bcl-2 gene can inhibit apoptosis (12). Bax is another main gene regulating apoptosis. And abnormal increase of Bax protein expression can advance apoptosis (13). Bcl-2 and Bax are a pair of vital genes controlling apoptosis, and the change of their ratio regulates the process of apoptosis (14;15). Bax promotes apoptosis. When excessive apoptosis occurs, Bcl-2 comes forward and binds to Bax

to form a dimer, so as to weaken the effect of bax in advancing apoptosis and protect neurons (16).

Medicated thread moxibustion is a special type of moxibustion. However, its mechanism of action in treating vascular cognitive impairment (VCI) is unclear. The present study was designed to investigate its effect on neurons in the rat hippocampus CA1 area of chronic cerebral ischemic vascular dementia, and the mechanisms involved.

Materials and Methods

Experimental animals

A total of 40 male Wistar rats of clean grade (mean weight = 220±20g) were used in this study. The rats were obtained from Vital River Laboratory Animal Technology Co., Ltd. (SCXK (Jing) 2012-0001). The Animal Trial Center of Dongfang Hospital of Beijing University of Chinese Medicine provided a constant temperature and humidity environment (50-70%) where all the rats were fed adaptively for one week with standard food*ad libitum.*

All animals were kept in a pathogen-free environment and fed ad lib. The procedures for care and use of animals were approved by the Ethics Committee of Dongfang Hospital, Beijing University of Chinese Medicine, and all applicable institutional and governmental regulations concerning the ethical use of animals were followed. The approval number is 201619.

Main instruments and reagents

The instruments used and their suppliers/makers were: totally-enclosed automatic dehydrator (Asp-300, Leica, Germany); Tissue embedder (EG-1150, Leica, Germany); rotary paraffin slicer (RM-2255, Leica, Germany), andupright metallurgical microscope (BX51, Olympus, Japan).

The following reagents/kit were products of Beijing Zhongshang Golden Brige Biotechnology Co., Ltd.: Bcl-2 antibody (ZS-4920), Bax antibody (ZS-5260), C-fos antibody (ZS-520), SP-9000 secondary antibody kit, and DAB Color kit.

Animal grouping

Forty rats were randomly divided into various groups, of which the normal and sham-operated groups had 7 rats each, while the others were operated for model preparation. One week after model preparation, a total of 12 rats died from the side effects of surgical anesthetics, while the remaining 14 rats were successfully-built animal models. The 14 rats were randomly assigned to model group and medicated moxibustion group (7 rats per group). These two groups were observed along with the sham operated group and the normal group.

Model establishment

The dementia rat models were established through permanent bilateral common carotid artery amputation. The rats were anesthetized with 10% chloral hydrate (0.3g / kg). An incision was taken in the middle of the anterior neck of the rats. After exposing the blood vessels, the bilateral common carotid arteries were bluntly separated and No.1 suture was coiled to the posterior side of the blood vessel and pulled from its front end to make double ligations. Finally, the wound was sutured layer-by-layer from the inside to the outside. The rats in the sham-operated group were subjected tocommon carotid artery separation and exposure only i.e. there was no vascular ligation.

Intervention methods

Medicated thread moxibustion group

Acupoint selection: DU20 (*Baihui*), DU16 (*Fengfu*), ST36 (*Zusanli)* and GB20 (*Fengchi*) were used to *regulate mind* and *replenish marrow*, and ST36 was used to *tonify spleen* and *kidney*. Location of acupoints: By referring to the standard rat acupoint map in *Experimental Acupuncture and Moxibustion* (17), the acupoints were located based on ratanatomical and physiological features, as well as body shape. Manipulation: Rats placed in a fixer were shaved locally to expose the skin where acupoints are located. The shaved areas were sterilized with iodophor. One end of medicated thread 2 was ignited, and when the fire turned into a spark, the ignited end was pressed on the acupoint and then lifted quickly. If the rat squeaked and twisted its body, it was successful, which was one *Zhuang* (unit of moxibustion). Each point was moxibusted three times i.e. three *Zhuang*. The observation period lasted four weeks, during which they were moxibusted once a day, with one day break every six days (24 times in total).

Model group and sham-operated group

These two groups were fed food and water normally and grasped under same condition, but without any treatment.

Animal sacrifice and brain tissue collection

The rats were injected intraperitoneally with 10% chloral hydrate 10 min after the last medicated thread moxibustion. They were then fixed on operation board to let blood from left ventricle ----right auricular appendix. Normal saline (200 ml) was injected to wash it at the same time, followed by that 4% paraformaldehyde was injected for fixing. Their brain tissues wereexcised, fixed in 4% paraformaldehyde, andkept in a refrigerator at 4°C for 48 h.

Index determination

The removed hippocampus tissue was fixed with 4% paraformaldehyde for 48 hours and then rinsed with running water for 2 hours. After dehydrated using graded ethanol, the hippocampus tissue was placed in an embedding mold and then melted paraffin was poured into. When the paraffin wax solidified, the wax block was taken out, naturally cooled, and continuously sliced into sections of 5μm thickness on a paraffin slicer. Twenty slices cut from each rat were placed and flattened on water at 45 °C. The slices were hooked by slide glasses and dried and baked in a 45 ° C baking area for later use.

The brain tissue from rat hippocampus CA1 was stained with heamotoxylen-Eosin (H&E) for morphological observation. The expressions of bax, bcl-2 and c-fos in hippocampus CA1 area of chronic cerebral ischemic rat were determined using immunohistochemistry. If the interior of cells turned yellowish-brown, the expression was positive. The positive expressions

of Bax, Bcl-2 and C-fos in the hippocampus CA1 areas of the four groups were semi-quantitatively measured using OLYMPUS (BX51, Olympus, Japan) image analysis system. Ten visual fields were randomly selected from each slice for optical density measurement, and the mean optical density value was calculated.

Heamotoxylen-Eosin stain

Paraffin slices dewaxed using xylene and dehydrated using graded ethanol (the concentration was 95%, 95%, 90% and 85% in turn) were stained with aqueous solution of hematoxylin for 10 min, and stained with 0.5% erythromycin solution for 30 sec. Subsequently, the slices were re-dehydrated using ethanol which was replaced with xylene, sealed with neutral gum for use. Observation indexes: the arrangement, morphology and staining of neurons in rat hippocampus CA1 area were observed by light microscopy.

Immunohistochemistry

Paraffin slices dewaxed using xylene and dehydrated using graded ethanol (the concentration was 95%, 95%, 90% and 85% in turn) were rinsed with PBS buffer, bathed for heat antigen repair in 95 ° C water, and incubated in a greenhouse after adding goat serum. After primary antibody (rabbit anti-mouse polyclonal antibody, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) was added dropwise, separately, the slices were incubated at 4 °C overnight. After secondary antibody (goat anti-rabbit, SP9000) was added, the slices were incubated at 37 ° C, followed by horseradish enzyme-labeled streptavidin reagent (SA/HRP). Subsequently, the slices were implemented color development using DAB, re-dyed by hematoxylin, dehydrated using gradient ethanol. After using xylene to replace absolute ethanol, the slices were sealed with neutral gum, and then observed under light microscopy with the upright microscope OLYMPUS (BX51, Olympus, Japan) image analysis system.

Statistical analysis

Enumeration data are expressed as mean ± standard deviation ($\bar{x}$ ± SD). All data were analyzed using SPSS19.0. software. One-way analysis of variance was used for multiple group comparisons, while LSD test was used for paired-comparison between groups. When the variance was not uniform, Dunnett T3 test was used for comparison between groups. Differences were considered statistically significant at $p<0.05$.

Results

Morphology of hippocampus CA1 area neurons

In the normal group, cells lined up regularly with an integrated and regular morphology. Their nuclei were regular in shape, with clear edges. Cells in the sham-operated group were lined up regularly with relative neat morphology. The nuclei were regular in shape, with nucleolus located in the center of each cell. However, in the model group, the cells were lined up irregularly with an extremely irregular shape, and a large number of cell vacuoles were visible. The nuclei were incomplete due to fragmentation or dissolution or pyknosis, and nucleoli were irregular, withvarious shapes. In contrast, cells in the medicated thread moxibustion group were complete in shape, with few abnormal morphologies, but without neat array. Some nuclei with pyknosis were evident, but generally, the cell shape was regular (Figure 1).

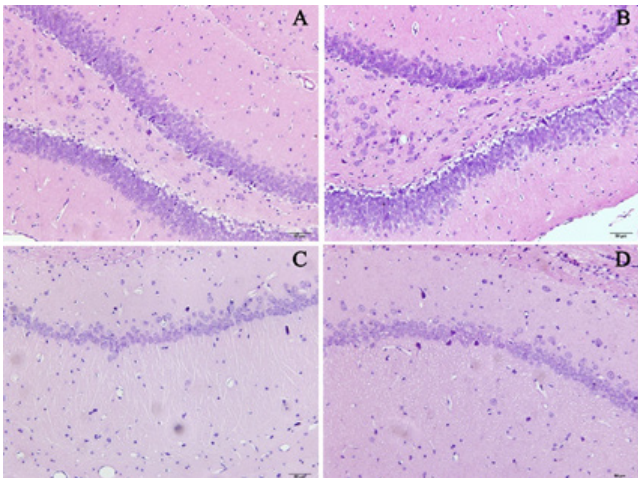

Figure 1. H&E staining of hippocampus CA1 area of groups ×20. A, normal group; B, sham group; C, model group; D, medicated thread moxibustion group.

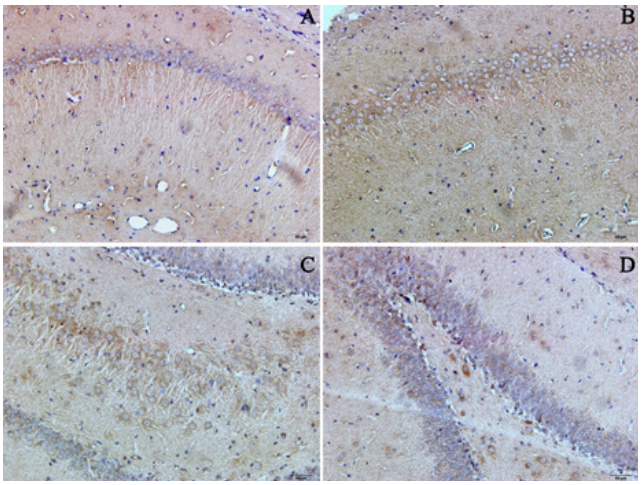

Figure 2. C-fos expression in rat hippocampus CA1 area of the groups ×10. A, normal group; B, sham group; C, model group; D, medicated thread moxibustion group.

Expressions of Bax, Bcl-2 and C-fos in hippocampus CA1 area of rats in each group

Some slightly stained C-fos-positive with scattered distribution were observed in the normal group (Figure 2A). The C-fos positive cells were slightly more in the sham-operated group than in the normal group, and they were scattered and stained lightly (Figure 2B). In the model group, C-fos-positive cells were crowded and numerous, and were distributed in irregular band (Figure 2C). The C-fos positive cells were scattered and lightly stained, and were few in the medicated thread moxibustion group (Figure 2D). There were statistically significant differences in mean optical density values amongst the four groups ($p = 0.000 <0.01$). Differences between paired groups were highly significant ($p <0.01$; Figure 3 and Table 1).

A small number of Bax-positive cells were present in the normal group. They were scattered and stained lightly, as shown in Figure 4A. The positive expression of Bax was slightly more in the sham-operated group than in the normal group, and it was distributed in a

Table 1. Optical density values of the expressions of Bax, Bcl-2 and C-fos in the hippocampus CA1 area of chronic cerebral ischemic rats (mean ±SD).

Group	Bax	Bcl-2	C-fos
Normal	4.695±0.468★	8.093±0.782△★	3.868±0.213△★
Sham-operated	6.491±0.225*☆★	4.144±0.177☆★	5.717±0.872*☆★
Model	9.999±0.865▲☆	1.703±0.075▲☆	10.882±0.319▲☆
Medicated thread moxibustion	5.425±0.258▲*	3.960±0.580▲△	9.499±0.715▲△*

▲medicated thread moxibustion group compared with the model group, $p< 0.05$; medicated thread moxibustion group compared with the sham-operated group, $p< 0.05$; △medicated thread moxibustion group compared with the normal group, $p < 0.05$; model group compared with the sham operation group, $p < 0.05$; ★sham operation group compared with the normal group, $p < 0.05$.

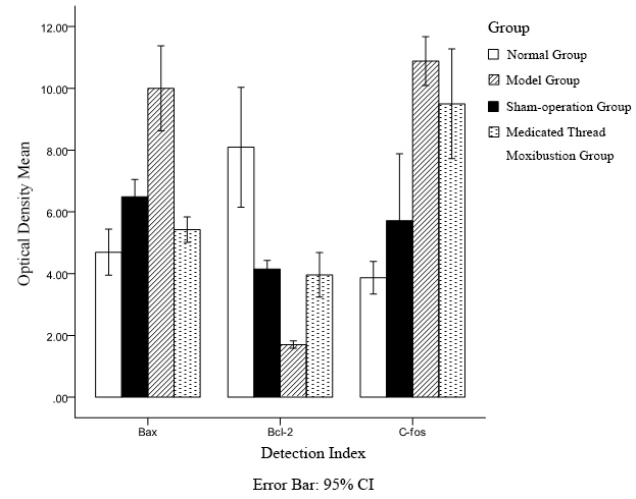

Figure 3. Comparison between groups of Bax, Bcl-2 and C-fos in each group.

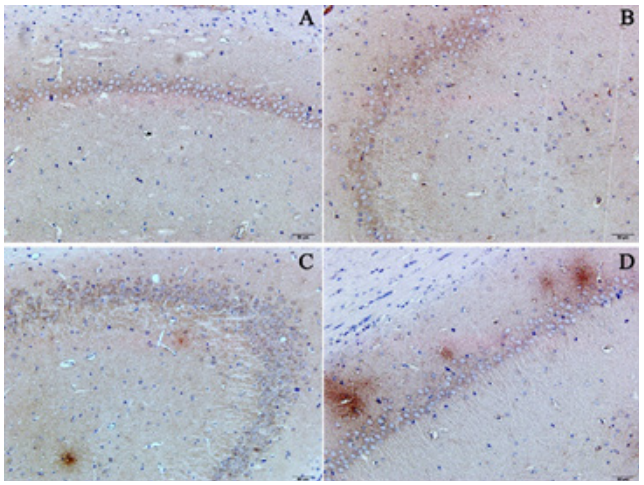

Figure 4. Bax expression in rat hippocampus CA1 area of the groups ×10. A, normal group; B, sham group; C, model group; D, medicated thread moxibustion group.

band shape and stained lightly (Figure 4B). In the model group, Bax-positive cells were concentrated and numerous, showing irregular zonal distribution (see Figure 4C). In the medicated thread moxibustion group, Bax positive cells were few, and were distributed in a band, and stained shallowly (Figure 4D). Statistical analysis of the average optical density values of the four groups showed statistically significant differences among them ($p = 0.000 < 0.01$). There was no significant difference between the normal group and the medicated thread moxibustion group ($p = 0.180 > 0.05$), but the differences between the other groups were significant ($p < 0.05$; Figure 3 and Table 1).

A large number of Bcl-2 positive cells were found in the normal group, which were densely distributed and stained deeply (Figure 5A). Positive expression of Bax was slightly less in the sham operation group than in the normal group, and the Bax positive cells were distributed in a band shape and stained deeply in the sham group (Figure 5B). As shown in Figure5C, few Bcl-2 positive cells were observed in the model group; they were scattered and stained shallowly. On the other hand, there were many Bcl-2 positive cells in the medicated thread moxibustion group. These cells were densely distributed in a band shape and stained deeply (Figure 5D). Results from statistical analysis of average optical density values showed statistically significant differences amongst the four groups ($p = 0.000 < 0.01$). There was no statistical difference between the sham-operated group and the moxibustion group ($p = 0.975 > 0.05$), but the differences between the other groups were significant ($p < 0.05$; Figure 3 and Table 1).

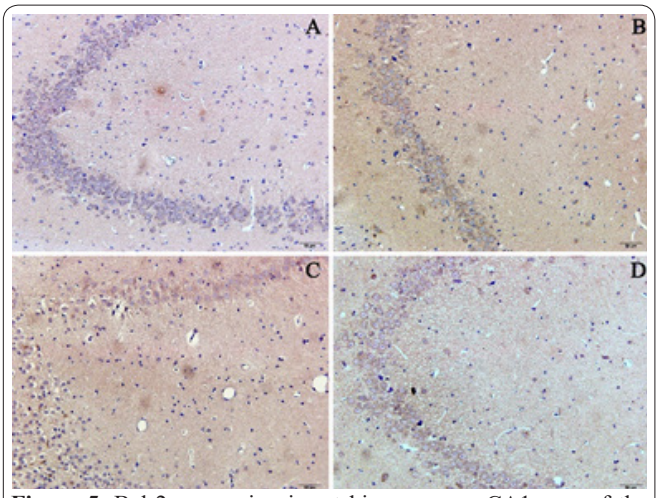

Figure 5. Bcl-2 expression in rat hippocampus CA1 area of the groups ×10. A, normal group; B, sham group; C, model group; D, medicated thread moxibustion group.

Discussion

Studies have found that chronic cerebral ischemia is the leading cause of vascular dementia (1). Chronic cerebral ischemia is closely related to vascular cognitive impairment. Some patients have decreased cerebral blood flow before the onset of clinical dementia symptoms. At the same time, the pathological state of dementia can also result in decreased cerebral blood flow, which degree is associated with the severity of dementia (2). Brain hippocampusis primarily responsible for learning and memory functions. The hippocampus is rich in hippocampal neurons which are small units of information processing, transmission and storage. The hippo-

campus is very sensitive to ischemia and hypoxia. Once ischemia and hypoxia occur, they are bound to affect neurons in the hippocampus. It has been confirmed in numerous animal studies and *in vitro* nerve cell culture that ischemia and hypoxia gives rise to neuronal apoptosis. Ischemia and hypoxia in the hippocampus cause massive amount of neuronal apoptosis, and the small units responsible for information processing, transmission and storage are destroyed, resulting in cognitive function impairment and dementia, which may be the pathological basis of VD (18). Hippocampal neuronal cells undergo apoptosis rather than cell necrosis, but this active death program is controlled by a series of chain reactions of genes, apoptotic factors, and related proteins. A variety of genes and factors such as Bcl-2, Bax, caspase-3, and C-fosare are involved in the regulation of apoptosis.

In the present study, a rat model of chronic cerebral ischemia and vascular dementia was established and used to study the effect of medicated thread moxibustion on pathological changes of neurons in the hippocampal CA1 area. The results have revealed that the neuronal cells in the hippocampus were degenerated 6 weeks after the operation, while those in the medicated thread moxibustion group were less degenerated. This indicates that rat hippocampal neurons are damaged in the internal environment of chronic cerebral ischemia, which is the pathological basis of learning and memory deterioration. The results also provide histological and pathological support for simulating chronic cerebral ischemia in a rat model of vascular cognitive impairment. Medicated thread moxibustion therapy can alleviate the damage due to chronic cerebral ischemia on hippocampal neurons.

The expressions of C-fos and Baxwere low in the hippocampus of rats in the normal group, but high in the model group, with significant differences in mean optical density values between the two groups. This indicates that the pro-apoptotic factors C-fos and Bax are at low levels under normal conditions. The immunohistochemical results from the model group revealed that the immediate response factor C-fos responded rapidly when ischemia and hypoxia occurred, so as to elevate its expression level and increase Bax factor synergistically, thereby enhancing apoptosis. Apoptosis in a large number of brain tissue neurons will inevitably give rise to a decline in learning and memory ability, especially for hippocampus neurons that are sensitive to ischemia and hypoxia. The results of the present study show that the expressions of C-fos and Bax in the hippocampus of rats were decreased after application of medicated thread moxibustion. Thus, the medicated thread moxibustion treatment down-regulated the expression of C-fos and Bax factors and inhibited the release of pro-apoptotic factors, thereby protecting the neurons. There was no significant difference in Bax gene expression between the normal group and the medicated thread moxibustion group, indicating that the medicated thread moxibustion down-regulated the level of Bax to that in normal rats, and reduced the over-release of pro-apoptotic factors, so as to protect the nerve cells.

There were differences in expression numbers and trends of Bcl-2 in each group, which also confirm its identity as attenuating apoptotic factor. In the hippocampus of normal rats, Bcl-2 was abundantly expressed so as to maintain the balance with Bax under normal apoptotic state. With onset of pathological state, the expression of Bcl-2 was decreased, relative to the normal, and the balance in the ratio of Bcl-2/Baxwas lost. The body itself releases Bcl-2 in a feedback manner, but since the released Bcl-2 binds to Baxto form a dimer which may not be detected through staining, the Bcl-2 level was lower in the model group than in the normal group. With medicated thread moxibustion, the expression of Bcl-2 in rat hippocampus was significantly increased, when compared with the model group, but comparable to that of the sham-operated group. This suggests that medicated thread moxibustion up-regulates Bcl-2 levels, inhibits apoptosis and protects neuronal cells.

Chronic cerebral ischemia damages the hippocampal neurons. This damage is one of the causes of learning and memory disorders. The results obtained in this study have shown that medicated thread moxibustion therapy inhibits the overexpression of the pro-apoptotic factor C-fos, inhibitsBax, up-regulates the protective factor Bcl-2, and controls cell apoptosis, thereby protecting hippocampal neurons. These may be some of the mechanisms of action of medicated thread moxibustion in mitigating chronic cerebral ischemia and vascular cognitive impairment.

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Interest conflict
There is no conflict of interest to be declared by the author.

Author's contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Jin Xianglan; Qin Weilan, Yan Yan, Liu Xuemei completed the study; Shen Wei, Liu Xiaohan, Ma Qingke, Wang Ying collected and analysed the data; Qin Weilan wrote the text and all authors have read and approved the text prior to publication.

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