Research Square

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Effects of Massage on Neuro-Vascular Regulation and Apoptosis in Rabbits with Cervical Spondylosis of the Vertebral Artery Type

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Research Article

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Abstract

Objective

To explore the therapeutic mechanisms of massage for cervical spondylosis of vertebral artery type (CSA) from the effects of sympathetic neurotransmitter changes on vertebral artery blood flow and apoptosis.

Methods

Forty rabbits were randomly divided into a normal group, model group, electroacupuncture (EA) group, and massage group, with 10 rabbits in each group. The CSA rabbit model was established by neck injection of sclerosing agent in all groups except the normal group. In the EA group, the left “Fengchi” (GB 20) and the 3rd-5th cervical vertebrae (C3-5) “Jiaji” (EX-B2) were selected for EA treatment. In the massage group, pushing manipulation with one finger was performed at 0.5 cm to the left side of the C3-5 spinous process and the tip of the transverse process. The vertebral artery blood flow was detected by laser Doppler. The levels of serum neuropeptide Y (NPY) and norepinephrine (NE) were determined by ELISA. The pathological morphological changes of vertebral arteries were observed by HE staining. The apoptosis of vertebral arteries and cerebella were detected by Tunel assay. The protein expressions of CHOP, Bcl-2, and Bax in vertebral arteries and cerebella were detected by Western blot.

Results

Vertebral artery blood flow was significantly decreased in all rabbits after modeling. Massage increased vertebral artery blood flow, decreased serum levels of NPY and NE which secreted by sympathetic nerves, improved vertebral artery lumen narrowing, intimal thinning, and mesenteric smooth muscle cell alignment. Moreover, these effects were superior to that in the EA group. In addition, the massage group significantly reduced the apoptotic index, decreased the CHOP and Bcl-2 associated X protein (Bax) protein expressions, and increased the B-cell lymphoma-2 (Bcl-2) protein expression in vertebral arteries and cerebella relative to the model group.

Conclusion

The treatment of CSA has a neuro-vascular regulatory mechanism. Massage can decrease the release of sympathetic neurotransmitters that constrict blood vessels and mitigate apoptosis induced by excessive endoplasmic reticulum stress (ERS) due to sympathetic excitation, so as to improve vertebral artery blood flow and serve as a treatment for CSA.

1. Introduction

Cervical spondylosis of the vertebral artery type (CSA) is mainly caused by the stimulation of the vertebral arteries, resulting in insufficient blood supply, with dizziness as the primary symptom[1]. There are many theories about the pathogenesis of CSA, one of which is based on the Barré-Liéou syndrome hypothesis [2]: pathological changes in the cervical spine stimulate sympathetic nerve fibers, affecting the blood flow in the vertebral artery and causing a lack of blood supply to the brain, resulting in a series of symptoms such as dizziness, tinnitus, and headache, namely the "sympathetic dysregulation theory". Some studies have shown that there are many sympathetic nerve fibers distributed on the ligamentum flavum (LF), which may be the anatomical basis for the development of cervical vertigo [3]. A large body of literature confirmed that sympathetic nerves were closely associated with inadequate blood supply to the vertebral arteries [4–6]. Our previous findings [7] also indicated that two sympathetic neurotransmitters, neuropeptide Y (NPY) and endothelin-1 (ET-1), are closely related to changes in vertebral artery blood flow. Therefore, there may be a neuro-vascular regulatory mechanism in the treatment of CSA. In addition, sympathetic blockade or inhibition may reduce the degree of apoptosis [8, 9]. Massage can increase the blood flow of muscle and skin, increase the secretion of relaxation hormones, and produce the effect of inhibiting sympathetic nerve activity [10, 11]. Therefore, This study aimed to investigate the mechanisms associated with massage treatment of CSA through the effects of sympathetic neurotransmitter changes on vertebral artery blood flow and apoptosis.

2. Materials And Methods

2.1. Animals

A total of 40 healthy New Zealand male large-eared white rabbits, 6 months old, body mass (2.0 ± 0.5) kg, were purchased from Hubei Yi-zhi-cheng Biotechnology Co., Ltd. Animal license No: SCXK (Hubei) 2016-0020. During the experiment, the room temperature was kept constant at 23–25 ℃, the humidity was 60%, and ultraviolet light was disinfected regularly. Free ingestion of water and standard feed were used.

2.2. Main reagents and instruments

Sclerosing agent: Anti-Hemorrhoid Injection (Ji'an Yisheng Pharmaceutical (Jilin) Co., Ltd, 180620); NPY Rabbit ELISA Kit (Yuanye Biotech (Shanghai) Co., Ltd, E20180101A); NE Rabbit ELISA Kit (Elabscience Biotech (Wuhan) Co., Ltd., E-EL-0047c); Hematoxylin Stain Kit (Solarbio Technology (Beijing) Co., Ltd, G1005); Apoptosis Detection Kit (Vazyme Biotech (Nanjing) Co.,Ltd, A111-01); CHOP Rabbit Polyclonal Antibody (Bioss Biotech (Beijing) Co., Ltd, bs-20669R); Bax Rabbit Monoclonal Antibody (Invitrogen (USA), MA5-32031); Bcl-2 Mouse Monoclonal Antibody (invitrogen, Lot No. MA1-12246) Ltd., Lot No. MA1-12246); GAPDH rabbit polyclonal antibody (Good Here Biotech (Hangzhou) Co., Ltd, AB-P-R001); HRP-labeled sheep anti-rabbit secondary antibody (BOSTER Biological Technology (Wuhan) Co., Ltd, BA1054). Disposable sterile acupuncture needle, size 0.25mm×25mm (Tianxie Acupuncture Instruments (Suzhou) Co., Ltd).

KWD-808 Pulse Acupuncture Therapy Instrument (Yingdi Electronic Medical Equipment (Changzhou) Co., Ltd); PeriFlux5000 Laser Doppler Instrument (Sweden, Perimed); Epoch Enzyme Labeling Assay

(BioTeK); RM2016 Rotating Pathology Slicer (Leica, Germany); BX53 Biological Microscope (Olympus, Japan); Vertical Electrotransformer (Liuyi Instrument Factory (Beijing)).

2.3. Moulding

The rabbit model replication of CSA referred to the description by Miao et al. [12]. The rabbits were firstly shaved from the back of the occiput to the neck on the left side, disinfected with local iodophor, local anesthesia with 1% lidocaine 2 ml, injected anti-hemorrhoid sclerosing agent 10 ml on the lateral side of the 3rd-5th cervical vertebrae (C3-5) transverse process on the left side of the rabbits. The needle was advanced to reach the edge of the transverse process, and no blood was drawn back, then the injection was pushed in, and the operation was repeated once at the 2nd and 4th weeks. This resulted in scar tissue compression around the vertebral artery in rabbits and local palpable striae, which led to vertebral artery compression and cervical sympathetic irritation, leading to a rabbit CSA model. Blood flow in the left vertebral artery was measured by a PeriFlux 5000 laser Doppler before and after modeling to verify successful modeling.

2.4. Grouping

The experimental animals were randomly divided into normal group, model group, electroacupuncture (EA) group, and massage group, with 10 rabbits in each group.

Normal group: without any treatment, normal feeding.

Model group: normal feeding after CSA modeling.

EA group: After successful modeling, the rabbits were fixed on the treatment table, the left "Fengchi" (GB 20, oblique stabbing 0.5-0.8cm downward) and the left C3-5 "Jiaji" (EX-B2, straight stabbing 0.5-0.8cm) were selected as the treatment points. After feeding, the needles were connected to the EA instrument using the following method: a pair of electrodes were connected to "Fengchi" and C3 "Jiaji", and another pair of electrodes were connected to the C4 "Jiaji" and C5 "Jiaji", which were subjected to dilatational wave with a frequency of 2-100Hz and a voltage of 2-4V. Keep the needles for 20 min, once a day, 10 days for a course of treatment, rest 1 day for the next course of treatment, a total of 2 courses of treatment.

Massage group: After successful modeling, the rabbits were fixed on the treatment table. “Pushing manipulation with one finger” was performed at 0.5 cm to the left side of the C3-5 spinous process and the tip of the transverse process, operation duration was 20 minutes. Massage treatment course was the same as EA group. The “Pushing manipulation with one finger” method is a very important Chinese massage technique that uses wrist activities to drive the thumb joint flexion and extension activities, and the force is continuously applied to the treatment area through the end of the thumb finger. The massage operators can ensure the consistency of the massage treatment by strict training and testing of the massage manipulation tester, maintain the massage strength at 0.5 kg and the massage frequency at 60 times/min.

2.5. Indicators detection

2.5.1. Vertebral artery blood flow detection

Blood flow in the vertebral artery of the intracranial segment was measured by a PeriFlux 5000 laser Doppler [7]. Before, 1 week, 2 weeks, and 3 weeks after treatment, a probe was placed at the occipital window behind the left posterior occipital ridge of the rabbit to measure the number of red blood cells passing through the window per unit time, and thus the blood flow at the site was understood.

2.5.2. Sample Collection

After all sessions were completed, the rabbits were anesthetized intravenously with 3% sodium pentobarbital (30 mg/kg) and 5–8 ml of venous blood was collected from the ear marginal vein. The supernatant was separated by centrifugation and stored at -80℃. After collecting the blood, the vertebral artery and cerebellum of rabbits were removed and cut in half, and stored in 4% paraformaldehyde solution and − 80℃ refrigerator, respectively.

2.5.3. ELISA

The levels of serum NPY and norepinephrine (NE) were determined by ELISA. The specific operation process shall be carried out according to the kit instructions.

2.5.4. HE staining

The paraffin sections thickness of the left vertebral arteries were 4 μm. Staining was performed according to the HE kit operating instructions. The histopathological morphology of the left vertebral arteries were observed under a light microscope.

2.5.5. Tunel

Apoptosis in rabbit vertebral arteries and cerebella were detected by tunel assay. Paraffin sections were made from vertebral arteries and cerebella and then stained according to the operation procedure of the tunel kit. The nuclei were stained with DAPI. The images were collected by observation under a fluorescence microscope. Five unduplicated 400x microscopic fields were randomly taken and the apoptotic index was calculated. Apoptotic index (%) = (number of apoptotic cells / total number of cells) × 100%.

2.5.6. Western blot

The protein expressions of CHOP, Bcl-2 associated X protein (Bax), and B-cell lymphoma-2 (Bcl-2) were detected by western blot analysis. Protein was extracted from 50 mg vertebral artery and cerebellum in each rabbit and quantified using the BCA kit. Protein solution was denatured by boiling and then subjected to SDS-PAGE and membrane transfer. 5% skimmed milk powder was used to close the parallel primary antibody (1:1000 for CHOP, Bax, and Bcl-2 protein, 1:5000 for GADPH protein) and HRP-labeled

secondary antibody (1:50000), incubated and then developed and fixed. The grayscale value of the film was analyzed by BandScan.

2.6. Blind design

Assignment of animals, experimental procedures, and data analysis were performed by different personnel.

2.7. Statistical methods

SPSS22.0 software was used for data analyse, and the measurement data were expressed as mean ± standard deviation. One-way ANOVA was performed for comparison of data between multiple groups obeying normal distribution. The comparison of vertebral artery blood flow at different time points was analyzed by ANOVA with repeated measurements. For pairwise comparisons between groups, LSD method was used when the variance was equal, and Dunnett's T3 method was used when the variance was unequal. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Comparison of vertebral artery blood flow among groups

Before treatment, vertebral artery blood flow was significantly lower in the post-modeling groups compared to the normal group ($P < 0.01$), indicating successful modeling. After treatment with both EA and massage, vertebral artery blood flow improved, but the increase in blood flow was more pronounced in the massage group after 1 and 3 weeks of treatment ($P < 0.01$). The change of vertebral artery blood flow in each group of rabbits had a time effect ($F = 130.508$, $P < 0.01$), that is, the improvement of vertebral artery blood flow in the EA and massage groups had an upward trend with the extension of time, and the upward trend was higher in the massage group than in the EA group ($P < 0.01$) (Fig. 1).

3.2. Comparison of serum sympathetic neurotransmitter levels among groups

The serum NPY and NE levels were significantly increased in the post-modeling groups compared to the normal group ($P < 0.01$). After treatment with EA and massage, both NPY and NE decreased significantly, and the decrease was more obvious in the massage group ($P < 0.01$) (Fig. 2).

3.3. Comparison of histomorphological changes of vertebral artery in various groups (HE staining, ×200)

The lumen of the vertebral arteries were large, the intima were smooth and structurally intact, and the mesenteric smooth muscle cells were clearly aligned and well arranged in the normal group. The lumen of the vertebral arteries were significantly narrowed, the intima were thinned, the smooth muscle cells in

the middle membrane were reduced and disorganized, and some elastic fibers were broken in the model group. The luminal narrowing, intimal thinning, and mesenteric smooth muscle cell alignment of the vertebral arteries showed different degrees of improvement in the EA and massage groups compared with the model group. Among them, the massage group improved better than the EA group (Fig. 3).

3.4. Comparison of apoptosis in vertebral arteries and cerebella among groups

The apoptotic indices in the vertebral arteries and cerebella were significantly increased in the post-modeling groups compared to the normal group ($P < 0.01$) and significantly decreased after treatment with EA and massage ($P < 0.01$). The improvements of apoptotic indices in the massage group were better than that in the EA group ($P < 0.01$) (Fig. 4).

3.5. Comparison of CHOP, Bax, and Bcl-2 protein expression in vertebral arteries and cerebella among groups

After molding, CHOP and Bax protein expressions were significantly increased in the vertebral arteries and cerebella ($P < 0.01$), Bcl-2 protein expression was significantly decreased in the vertebral arteries ($P < 0.01$) and cerebella ($P < 0.05$). After treatment with EA and massage, CHOP and Bax protein expressions were significantly lower in the vertebral arteries and cerebella ($P < 0.01$), and Bcl-2 protein expression was significantly higher in the vertebral arteries ($P < 0.05$). Compared with the EA group, CHOP and Bax protein expressions were significantly reduced ($P < 0.01$) and Bcl-2 protein expression was significantly raised ($P < 0.01$) in both vertebral arteries and cerebella in the massage group (Fig. 5).

4. Discussion

Degeneration of the cervical spine can cause biomechanical imbalances in the cervical spine [13] and direct compression of the vertebral basilar artery (VBA) [14]. It can also stimulate the cervical sympathetic nerves leading to inadequate blood supply of VBA [2], and then cause symptoms of vertigo. The anterior cervical decompression and fusion procedure can improve the biomechanical imbalance caused by cervical degeneration and remove the posterior longitudinal ligament (PLL) containing the sympathetic nerves, thus effectively relieving vertigo symptoms [15, 16]. Therefore, vertigo symptoms can be efficiently alleviated by improving biomechanics and suppressing sympathetic nerves.

Massage is an external Chinese medicine treatment method that applies suitable mechanical force to specific parts of the human body, and converts the mechanical force into electrical signals through surface receptors and transmits them to the central nervous system in the form of nerve impulses, ultimately playing a therapeutic role. Back and hand massage can relax subjects and produce sympathetic inhibition in the form of lower heart rate and diastolic blood pressure[17, 18]. Our previous study[7] also confirmed the possible changes in sympathetic excitability during massage to improve vertebral artery blood supply. In related clinical studies[19, 20], massage therapy significantly improved

the symptoms of vertigo in patients and was more effective than acupuncture and betahistine. Our results (Fig. 1) suggested that massage treatment improved the blood flow of the vertebral artery, and this improvement becomes more pronounced with time. The pathological results (Fig. 3) showed that the morphology and cellular arrangement of the vertebral artery were also improved in the massage group, and these improvements were superior to those in EA group. This indicated that massage had the advantage in promoting soft tissue recovery compared with EA. The improvement of soft tissue balance was conducive to the reestablishment of cervical spine balance. The restoration of cervical spine balance reduced the compression state of blood vessels and nerves, thus playing a long-term and stable role in increasing blood volume.

NE is a well-known vasoconstrictor, an important neurotransmitter secreted by sympathetic nerves, which can affect some arterial diseases by inhibiting the proliferation of vascular smooth muscle cells (VSMCs) [21, 22]. It has been shown that stimulation of the cervical sympathetic ganglia could increase NE levels and decrease basilar artery (BA) blood supply, whereas adrenergic receptor blockade could inhibit this effect [4]. One study also found in a CSA rabbit model [23] that stimulation of the cervical sympathetic ganglion secretes large amounts of NPY and tyrosine hydroxylase (TH), resulting in spasms and reduced blood supply to the vertebral artery. NPY is a co-transmitter released from the postsympathetic ganglia, especially at higher levels of sympathetic excitation [24]. Elevated sympathetic stress leads to increased NPY and NE release [25]. NPY and NE have a synergistic effect, jointly regulating vascular tone and potentiating NE-mediated vasoconstriction [26]. Therefore, the neurotransmitters NPY and NE secreted by cervical sympathetic nerves are closely related to vertebral artery blood flow. The results indicated that (Fig. 2) that massage treatment significantly decreased the release of two important sympathetic transmitters, NPY and NE, while improving vertebral artery blood flow. This may be due to the massage relaxing the soft tissues of the neck and reducing sympathetic arousal, thereby improving the blood flow of the vertebral artery, which also suggested that there may be a neuro-vascular regulation mechanism in the treatment of CSA.

It has been found [27, 28] that by inhibiting sympathetic excitability, serum NE levels can be reduced and the p38MAPK signaling pathway can be inhibited, causing intracellular reactive oxygen species (ROS) levels are reduced, thereby reducing the degree of endoplasmic reticulum stress (ERS) in the cell. Stellate ganglion blockade could also reduce ERS overactivation in arterial tissue by inhibiting sympathetic excitability, thereby improving vascular calcification [29].The most important protective pathway of ERS is the unfolded protein response (UPR) [30], however, excessive ERS can lead to an imbalance in the regulatory capacity of the UPR and promote CHOP protein activity, which in turn induces apoptosis [31]. The results of the present study demonstrated (Figs. 2, 5) that massage treatment reduced sympathetic neurotransmitter release along with reduced CHOP protein expression in vertebral arteries and cerebella, suggesting that massage can mitigate excessive activation of ERS through the UPR pathway by weakening sympathetic excitability.

Overexactivation of ERS will lead to the onset and development of apoptosis [32, 33]. Many studies in recent years [34–36] have shown that the process of apoptosis was closely related to the treatment of

cervical spondylotic myelopathy (CSM) and cervical spondylotic radiculopathy (CSR). However, there are relatively few studies on the relationship between apoptosis and CSA. As seen in the present study (Figs. 1,4), the lower the proportion of apoptotic cells, the more obvious the improvement of vertebral artery blood supply, so apoptosis may be a potential therapeutic target for CSA. Bax and Bcl-2 are often used to evaluate the extent of apoptosis [37, 38]. During apoptosis, the role of BAX is to induce apoptosis, contrary to bax, Bcl-2 inhibits cell apoptosis by forming a dimer with Bax) [39, 40]. Therefore, the key to determine apoptosis and survival is the ratio of Bcl-2 to Bax protein expression. The present study (Figs. 2,5) showed that massage treatment not only reduced the release of sympathetic neurotransmitters, but also increased the Bcl-2/Bax ratio in vertebral arteries and cerebella, indicating that massage can attenuate apoptosis induced by excessive ERS due to sympathetic excitation.

In summary, there may be a neuro-vascular regulatory mechanism in the treatment of CSA, and massage can improve vertebral artery blood flow and play a role in the treatment of CSA by relaxing the soft tissues of the neck, relieving the irritation of the sympathetic nerves caused by abnormal structure of the neck, reducing the release of sympathetic transmitters that constrict blood vessels, and alleviating apoptosis induced by excessive ERS due to sympathetic excitation. The changes in sympathetic excitability in this study were judged indirectly by its transmitter release, and further exploration can be done by adding the observation of heart rate, blood pressure, pupil, and monitoring of sympathetic discharge activity in subsequent studies. In addition, the process of apoptosis induced by ERS is more complex and involves multiple signaling pathways, which is one of the directions to explore the mechanism of massage for CSA in the future.

Abbreviations

CSA: cervical spondylosis of vertebral artery type; EA: electroacupuncture; NPY: neuropeptide Y; NE: norepinephrine; LF: ligamentum flavum; ET-1: endothelin-1; Bax: Bcl-2 associated X protein ; Bcl-2: B-cell lymphoma-2; VBA: vertebral basilar artery; PLL: posterior longitudinal ligament; VSMCs: vascular smooth muscle cells; BA: basilar artery; TH: tyrosine hydroxylase; p38MAPK: p38-mitogen-activated protein kinase; ROS: reactive oxygen species; ERS: endoplasmic reticulum stress; UPR: unfolded protein response; CSM: cervical spondylotic myelopathy ; CSR: cervical spondylotic radiculopathy.

Declarations

Ethics approval and consent to participate

Animal feeding, modeling and all experimental processes were conducted in the Animal Experiment Center of Anhui University of Chinese Medicine, and were approved by the Institutional Animal Ethics Committee (No. 2022006). The study was to go ahead following the ARRIVE guidelines for animal experiments.

Consent for publication

Not applicable.

Availability of data and materials

The data and materials used during the study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that there are no conflicts of interest to disclose.

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Authors' contributions

Chao Wang and Junchen Zhu designed the study. Hui Xu completed the experimental part. Statistical analysis was accomplished by Yingzong Xiong. Chao Wang and Su Yi drafted the manuscript. Yingchun Li participated in the production of the figures. All authors read and approved the final manuscript.

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Not applicable.

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Figures

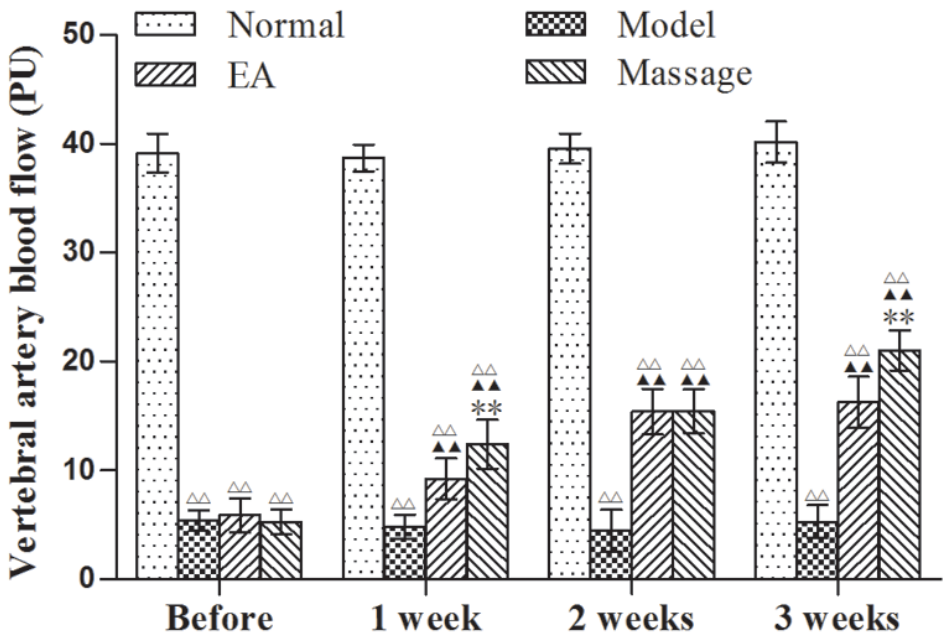

Figure 1

Effect on vertebral artery blood flow in each group. $^{\triangle\triangle}P<0.01$ versus the normal group; $^{\blacktriangle\blacktriangle}P<0.01$ versus the model group; $^{**}P<0.01$ versus the EA group. (n=10).

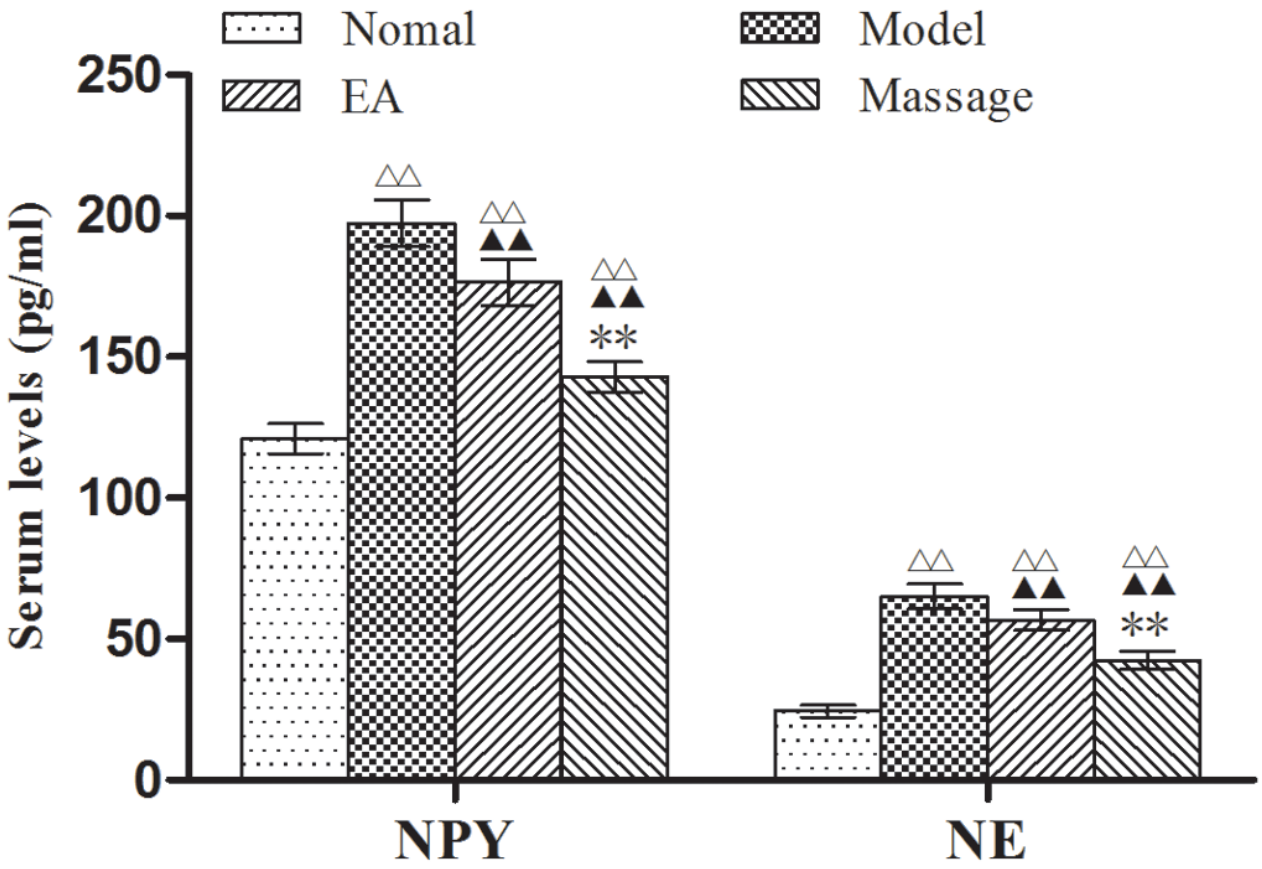

Figure 2

Effect on the serum levels of NPY and NE in each group. $^{\triangle\triangle}P<0.01$ versus the normal group; $^{\blacktriangle\blacktriangle}P<0.01$ versus the model group; $^{**}P<0.01$ versus the EA group. (n=10).

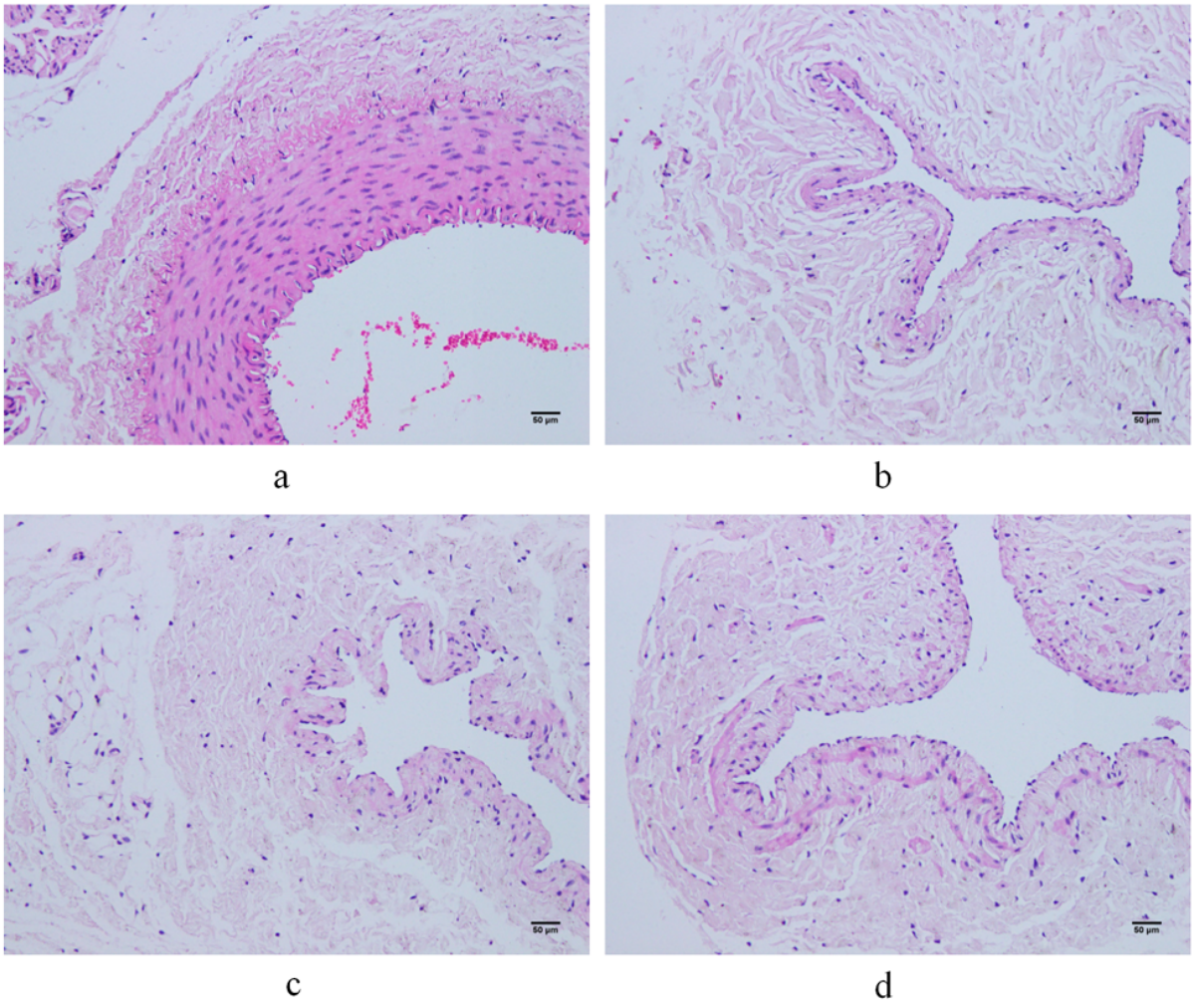

Figure 3

Effect on the histomorphological changes of vertebral arteries in the groups of normal (a), model (b), EA (c), massage (d). (HE, ×200).

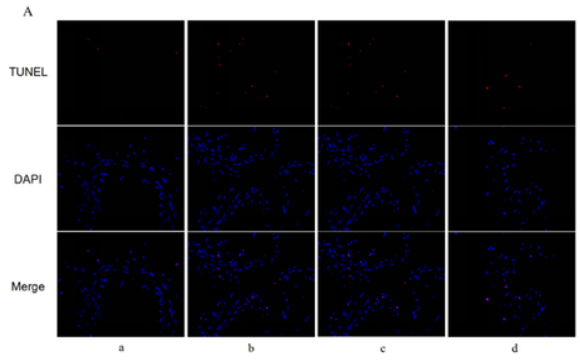

Figure4A

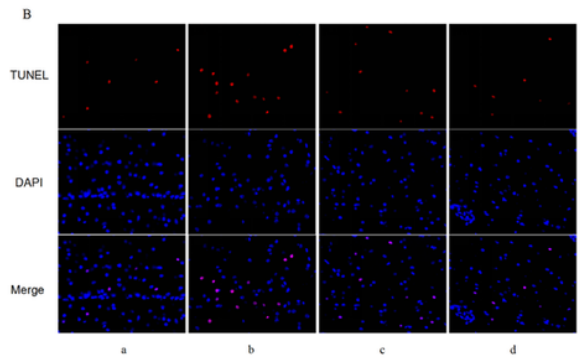

Figure4B

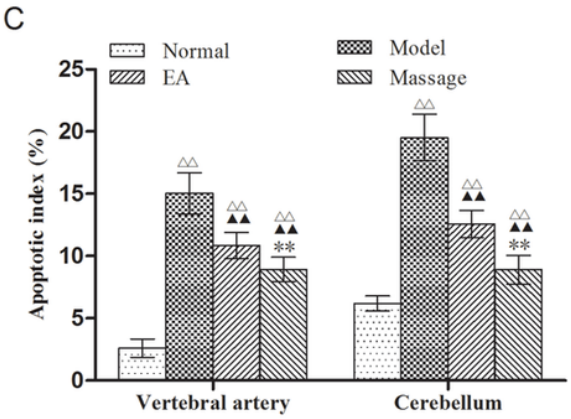

Figure4C

Figure 4

Apoptosis of vertebral arteries (A) and cerebella (B) in groups of normal (a), model (b), EA (c), massage (d) (Tunel, ×400). Apoptotic index of vertebral arteries and cerebella in each group (C). $^{\triangle\triangle}P<0.01$ versus the normal group; $^{\blacktriangle\blacktriangle}P<0.01$ versus the model group; $^{**}P<0.01$ versus the EA group. (n=10).

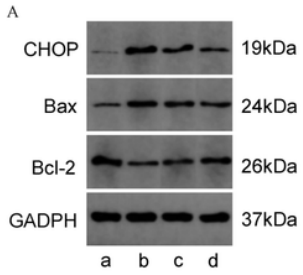

Figure5A

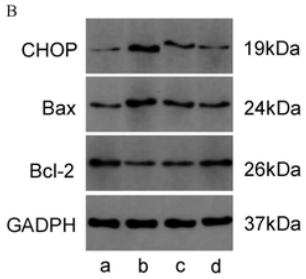

Figure5B

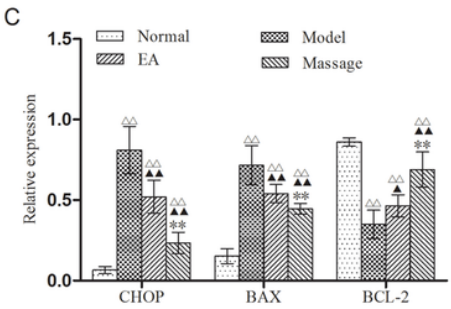

Figure5C

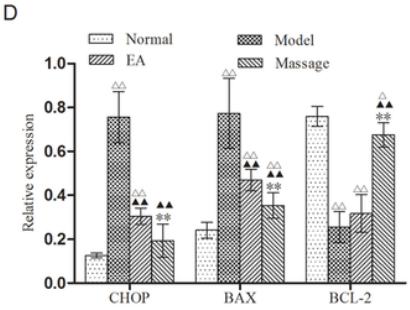

Figure5D

Figure 5

The protein levels of CHOP, Bax, Bcl-2, and GADPH in vertebral arteries (A) and cerebella (B). (a) Nomal group, (b) Model group, (c) EA group, (d) Massage group. The protein loading intensity was calculated with GADPH as an internal control in the vertebral arteries (C) and cerebella (D). $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ versus the normal group; $^{\blacktriangle}P<0.05$, $^{\blacktriangle\blacktriangle}P<0.01$ versus the model group; $^{**}P<0.01$ versus the EA group. (n=10).