Effects of laser needle-knife therapy on vertebroarterial morphology and protein expression of PI-3K, AKT and VEGF in the carotid artery in a rabbit model of cervical spondylotic arteriopathy

Fang Liua,b, Wei Wei a,c, Jianhua Fang c, Yuanyuan Wub, Qifei Zhang b, Cunxin Wanga, Lihong Ye a, Min Liua

a Hangzhou Red Cross Hospital, Hangzhou 310003, China
b Zhejiang Chinese Medical University, Hangzhou 310053, China
c Affiliated Hangzhou First People’s Hospital, Zhejiang University School of Medicine, Hangzhou 310006, China

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Abstract
Purpose: To explore how the use of a laser needle-knife affects vertebroarterial morphology and protein expression of PI-3K, AKT and VEGF in the carotid artery of a rabbit model of cervical spondylotic arteriopathy (CSA), and to determine its primary treatment mechanism.

Methods: The CSA rabbit model consisted of 30 rabbits with CSA that were randomly divided into a model group (n = 10), an acupuncture group (n = 10) and a laser needle-knife group (n = 10) plus a further 10 wild type rabbits placed into a normal group. Rabbits in the acupuncture group were treated by needling the Fengchi (GB 20) and Jiaji (EX-B2) acupoints, while those in the laser needle-knife group were treated with a laser needle-knife on the Jiaji (EX-B2) near the spinous process of the fifth cervical vertebra, the rabbit in both groups immobilized during treatment. Rabbits in the other two groups received no treatment, but were immobilized for a similar duration. The morphology of the right vertebral artery and the distribution of pore size before and after treatment were compared using BET and SEM, and the protein expression of PI-3K, AKT and VEGF in the carotid artery of the four groups measured using Western blot analysis.

Results: The pore diameter and specific surface area of the right vertebral artery increased after treatment, as did the number of micropores. Compared with the normal group, the protein expression of PI-3K, AKT and VEGF in the carotid artery of the model group was significantly lower (P < 0.05), while that of the laser needle-knife group was significantly higher (P < 0.05 and P < 0.01). In addition, rabbits from the acupuncture and laser needle-knife groups demonstrated significantly higher levels of protein expression of PI-3K and VEGF in the carotid artery compared with the model group (P < 0.05).

Conclusions: By promoting micrangium hyperplasia within the vertebral artery of rabbits with CSA, treatment with a laser needle-knife modified the protein expression of PI-3K, AKT and VEGF, suggesting that laser needle-knife therapy possibly treats CSA though these signaling pathways.

1. Introduction

Cervical spondylotic arteriopathy (CSA) is caused by insufficient blood supply resulting from compression or stimulation of the vertebral artery, mainly manifesting as dizziness, paroxysmal headache of the cervicoppital or occipitoparietal areas, tinnitus, or visual or hearing loss (Kaya et al., 2018; Bozkurt et al., 2018; Policha et al., 2018). It is categorized as “dizziness” and “headache” in traditional Chinese medicine (TCM). As stated in the Discussion on the Most Important and Abstruse Theory by Su Wen, “all wind diseases characterized by tremor and dizziness are associated with
the liver” (Ge et al., 2017; Khan et al., 2018; Peng et al., 2016). Zhu Danxi advocated the theory that dizziness is caused by phlegm and fire and proposed the view that “dizziness occurs when deficiency of qi is combined with fire”.

At present, CSA is principally treated using acupuncture, moxibustion, manipulation, Chinese herbs, needle-knife, traction or acupoint injection (Ding et al., 2012; Guan et al., 2017; Moon et al., 2017). It has been confirmed by a number of relevant studies that laser needle-knives, which are an improved version of a traditional needle-knife, significantly improves blood circulation, diminishes inflammation and alleviates pain when used in clinical practice. Results of previous studies we have conducted suggest that use of a laser needle-knife effectively improves the hemodynamics in patients with CSA (Liu et al., 2017). In order to establish its mechanisms of action, the aim of this paper was to compare the therapeutic effects of laser needle-knife therapy and acupuncture on CSA through animal experiments and to further confirm the possible mechanisms of treatment (Alnaim and Almaz, 2017; Kevrekidis et al., 2018; Ocek et al., 2017).

2. Material and methods

2.1. Experimental animals

A total of 40 healthy general-grade adult rabbits (equal numbers of male and female) weighing 2.50 ± 0.2 kg were obtained and housed in the Experimental Animal Center of Zhejiang Chinese Medical University.

2.2. Model construction

The CSA rabbit model was established using methods described by Hu et al. (2005). Briefly, blood was collected from the central artery of the ear of a donor rabbit and mixed at a 1:1 ratio with 25% normal saline to prepare 9 mL of coagulated blood. The coagulated blood was injected into the right splenius cervicis muscle and the intramuscular and intermuscular gaps of the interspinalis muscles of unrelated rabbits. Prior to, and 2 weeks following the injection, all rabbits underwent color Doppler ultrasound imaging. Rabbits with >10 cm/s reduction in blood flow velocity in the right vertebral arteries were selected as satisfying the requirements of the CSA model.

2.3. Grouping and treatment

Forty rabbits were randomly divided into 4 groups by reference to a random number table, a normal control group, a model group, an acupuncture group and a laser needle-knife group, with 10 rabbits in each. CSA was induced in the model, acupuncture and laser needle-knife groups, but not in the normal control group. Rabbits that failed the induction of CSA were removed from their respective group 2 weeks after initiating formation of the model.

Normal control group (JK): CSA was not induced, and rabbits were immobilized in a similar manner to those in the acupuncture group during treatment.

Model group (JC): CSA was induced, and rabbits were immobilized in a similar manner to those in the acupuncture group during treatment.

Acupuncture group (JA): Treatment began 2 weeks post-induction of CSA. Acupuncture points: fengchi point and cervical jiaji points (C3-C7). Acupuncture method: #30 filiform needles (1.5 in.) were directly inserted into the 6 acupuncture points described above using a twirling reinforcement-reduction technique. Once placed, insertion of the needles was maintained for 30 min, with appropriate adjustments made every 10 min. Treatment was provided once two days for a total of 10 treatments. Rabbits were examined by color Doppler ultrasound on day 20 of the treatment, after which they were euthanized.

Laser needle-knife group (JN): Treatment began 2 weeks post-induction of CSA. Procedures: (1) Position: rabbits were immobilized in a surgical container in a prone position with cervical flexion. Jiaji points on both sides of the C5 spinous process, identified from the spinous process and vertebrae, were selected as the targets of treatment, and based on the acupuncture point map of the experimental animal. (2) Skin disfection: the skin of rabbits was disinfect using conventional iodophore. (3) Rabbits were given local anesthetic with 1% lidocaine. (4) The skin was cut, peeled and loosened by laser needle-knife until no more nodules or blockage was identified. He-Ne laser radiation was applied to the needle at an output voltage of 200 mW in beam expanding mode with on-off output (Zeredo et al., 2007). Treatment was 30 min in duration, the site of treatment covered using an adhesive bandage after removal of the needle. The wound was kept dry for the subsequent 24 h. Treatment was given every 10 days for a total of 3 treatments. The rabbits were evaluated using color Doppler ultrasound on day 20 of the treatment, after which they were euthanized.

2.4. Main reagents and instruments

The following instruments were used during the study: an He-Ne laser therapeutic instrument (JH30C, Shanghai Jiading Photo-electric Instrument Co., Ltd.); a needle-knife (Hanzhang Needle-knife HZ, Beijing Zhuoyue Huayou Medical Equipment Co., Ltd.); needles for acupuncture (Wujiangshi Jiachen Acupuncture Instrument Co., Ltd., 0.25x40mm); scanning electron microscope (Hitachi SU11510, Japan), Physiosorption Apparatus (American Micromeritics ASAP2020); Microplate Reader (SpectraMax Plus 384, Molecular Devices Inc.); Electrophoresis System (Mini-Protean Tetra System, Bio-Rad) and a gel imaging instrument (Chemidoc XRS + System, Bio-Rad). The following antibodies were used: (1) AKT: bs-6951R, Bioss. (2) PI3K: BA1352-2, Boster. (3) VEGF: ab46154, Abcam. (4) GAPDH: b36703, MultiSciences. (5) Goat anti-Rabbit IgG: GAR0072, MultiSciences. (6) Goat anti-Mouse IgG: GAM007, MultiSciences.

2.5. Observation index and method

2.5.1. Effects of laser needle-knife on pore size distribution of the vertebral artery

The vertebral arteries and microvessels at the joints between the fourth and fifth, and the fifth and sixth cervical vertebrae were removed under aseptic conditions after the rabbits had been sacrificed and placed into culture dishes following rinsing with Hank’s solution. The connective tissue on the outermost layer and the outer membrane were gently removed with ophthalmic scissors and tweezers. The exposed vessels were placed into 15 mL centrifuge tubes and processed as follows: they were firstly incubated in 6–8 mL trypsin at a concentration of 1.25 g/L supplemented with penicillin-streptomycin solution for 4 h. They were then washed 3 times with PBS and placed in sterile deionized water for 12 h prior to permeabilization with 1% Triton X-100 solution for 24 h. Finally, the vessels were incubated in 1% SDS for 24 h, followed by 3 washes with PBS prior to rinsing with Hank’s solution for 4 h before preservation. All the procedures described above were conducted in a thermostatic oscillator at 37 °C with sustained oscillation. The blood vessels were cleaned gently with running water to remove residual tissue debris, then dried naturally in air. Degasification was performed at room temperature for 8 h and the absorption-desorption of high purity nitrogen was measured using a static volumetric method comprising a liquid nitrogen cold trap at a temperature of 77 K.
2.5.2. SEM images of the various samples

Treated samples were mounted on copper stubs using conductive double-sided tape, sprayed with a layer of gold coating under vacuum conditions and evaluated using a SU1510 scanning electron microscope (Hitachi).

2.5.3. Effects of laser needle-knife on protein expression of PI-3 K/AKT/VEGF in the carotid artery of CSA rabbits

All specimens were preserved in a −80 °C freezer. Tissue samples were removed and placed in 1.5 mL centrifuge tubes then washed once with PBS. An appropriate quantity of lysate was added and the tubes placed on ice for 30 min, during which time the tube was agitated several times. The tissue extracts were centrifuged at 3000 rpm for 15 min at 4 °C, after which the supernatant was transferred to a fresh 1.5 mL centrifuge tube for preservation at −80 °C. Protein concentration was quantified using a bicinchoninic acid (BCA) assay followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to a PVDF membrane which was then submerged in 1x TBST buffer then carefully washed for 5 min while shaking. The PVDF membranes were then incubated with one of the following primary antibodies at 4 °C: AKT (bs-6951R, Bioss) diluted 1:1000; PI3K (BA1352-2, Boster) diluted 1:500; VEGFA (ab46154, Abcam) diluted 1:1000 or GAPDH (ab36703, MultiSciences) diluted 1:5000. After overnight incubation, each PVDF membrane was transferred into 1x TBST buffer, slowly washed for 20 min while shaking, 3 times. Each membrane was then incubated with the appropriate secondary antibody, either goat anti-rabbit IgG (GAR0072, MultiSciences) or goat anti-mouse IgG (GAM007, MultiSciences), both diluted 1:5000, for 1 h while shaking at room temperature. Each PVDF membrane was washed then placed on preservative film for enhanced chemiluminescence (ECL). Equal volumes of solutions A and B from an ECL kit were mixed, poured on the membrane surface and then imaged in a gel imaging instrument and developed in chemical photosensitive mode.

2.6. Statistics

The database of results was statistically analyzed using SPSS version 17.0 software. Results were presented as mean ± standard deviation (±s). Two-tailed analysis was adopted for all statistical tests with single factor analysis of variance (ANOVA) for data that was normally distributed with equal variances. P < 0.01 or P < 0.05 indicated statistical significance.

3. Results

3.1. Effects of laser needle-knife on pore size distribution of the vertebral artery of CSA rabbits

As shown in Table 1, compared with the normal group, the specific surface area of the model group decreased significantly, while the pore diameter increased, suggesting poorer blood circulation within the vertebral artery of the rabbits in the model group.

This result is consistent with the discussion of Seas of Ling Shu that “when the sea of marrow is insufficient, it causes dizziness and tinnitus”. After treatment using acupuncture or laser needle-knife, the specific surface area of the vertebral artery of the CSA rabbits increased, while the pore diameter decreased, indicating an increase in the number of micropores.

3.2. Effects of laser needle-knife on morphology of the vertebral artery in CSA rabbits

Following the completion of treatment, the surfaces of the vertebral arteries of rabbits in each group were evaluated using SEM (Fig. 1). In rabbits in the normal control group, as shown in Fig. 1-a, numerous folds were present on the surface of the decellularized matrix, indicating good nutrient and energy transport in the vertebral arteries where CSA was absent. Conversely, rabbits in the model group exhibited arteries that were more occluded with fewer folds on the vascular surface, suggesting that CSA had resulted in reduced blood and nutrient supply in the vertebral arteries (Fig. 1-b). After CSA rabbits had been treated with acupuncture (Fig. 1-c), a low level of capillary hyperplasia was observed on the vertebral arterial surface, suggesting improved microcirculation, increased blood flow volume and a reduction in the symptoms of CSA. Similarly, CSA rabbits treated with a laser needle-knife had greater capillary hyperplasia on the vertebral artery surface with an increased number of micropores (Fig. 1-d, e). Given that micropores are beneficial for the transport of nutrients, these findings suggest that the mechanism of action of laser needle-knife in the treatment of CSA may be associated with an increase in micropores.

3.3. Effects of laser needle-knife on the protein expression of PI-3K, AKT and VEGF in the carotid arteries of CSA rabbits

The protein expression of PI3K in the carotid artery specimens is displayed in Fig. 2. There were no significant differences in protein expression (*P > 0.05) between the normal, model and acupuncture group, but there was a significant increase in protein expression in the laser-knife group (*P < 0.05), and there was no significant change in model and acupuncture group.

There were no significant differences in AKT protein expression in any group compared with normal rabbits (*P > 0.05). Protein expression in the acupuncture group was slightly higher and lower in the laser-knife group, but neither difference was significant (*P > 0.05 for both).

Compared with normal group, the VEGF protein expression was significantly increased in acupuncture and laser-knife groups (*P < 0.05), and the VEGF protein expression of model group was decreased significantly (*P < 0.05). However, no significantly difference was found between acupuncture and laser-knife group.

4. Discussion

A large number of medical experts hold the opinion that the majority of cases of CSA, where the brain is affected, represent a deficiency syndrome, whose mechanism is that of a shortage of qi combined with blood stasis, a deficiency at the origin and excess in superficiality. Dizziness occurs as a result of failure of the clear yang to increase because of the lack of blood and under-nourishment of the brain due to blood stasis, under-nourishment of the channels and collaterals, obstructed vessels, and failure of deficient kidney qi and yang to drive blood circulation. In addition, as the kidneys store essence, deficiency at the kidneys leads to a failure of bone marrow generation, which also serves as a key factor for the development of CSA.

| Group               | Specific surface area (m²/g) | Pore diameter (mm) |
|---------------------|------------------------------|--------------------|
| Normal group (JK)   | 21.7                         | 2.9                |
| Model group (JC)    | 17.8                         | 3.7                |
| Acupuncture group (JA) | 19.4                      | 3.4                |
| Laser needle-knife group (JN) | 21.5                   | 3.2                |
Compared with traditional acupuncture using metal needles, laser acupuncture is a non-invasive and painless therapy with no risk of infection (Zhong et al., 2017). By exploring its effects on acute postoperative pain, which were found to be similar to those of electro-acupuncture, Zeng et al. (2018) established that the mechanism was related to the protein expression of mitogen-activated protein kinase (pERK and pp38), inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF). Through investigation of its effects on neuralgia, Butkovic and Tot (2017) found that laser needle-knife was an effective method of relieving hyperalgesia and paralgesia. According to research conducted by Ohta et al. (2017) in which the effects of laser electro-acupuncture on protein synthesis in mouse skeletal muscles were investigated, laser radiation was believed to significantly inhibit the protein expression of MSTN and increase the phosphorylation of P70S6K within the mTOR/S6 signaling pathway. As described by Jittiwat (2007), who studied the impact of using a laser needle-knife on brain injury, cortical oxidation and the quantity of superoxide dismutase in the mitochondria of animals at the site of focal ischemic stroke, the volume of cerebral infarction reduced in the case of cerebral ischemia due to a decrease in oxidative stress. Laser needle-knife therapy, essentially through a process of energy transformation and transmission, increases local temperature, dilating blood vessels, promoting angiogenesis, improving blood circulation, restoring hemodynamic balance and accelerating metabolism after the tissues have absorbed a certain number of photons and converted the light energy of laser radiation to thermal energy. Theoretically, a laser needle-knife is the optimal therapy for CSA considering that its primary target is vertebrobasilar insufficiency which leads to a decrease in regional cerebral blood flow.

Phosphatidylinositol 3-kinase (PI-3K) is a form of lipid kinase in the cytoplasm which catalyzes the phosphorylation of D3 phosphatidylinositol. Type I PI3K comprises the regulatory subunit p85 and catalytic subunit p110. Akt is a direct target protein downstream of PI3K, a highly conserved serine-threonine kinase, composed of 480 amino acid residues. The powerful angiogenic factor VEGF specifically acts on vascular endothelial cells and serves to promote vascular proliferation and angiogenesis by...
activating its ligand VEGFR-2 in a paracrine manner. Hori et al. (2017) found that VEGF could significantly induce tumor angiogenesis and metastasis. It was shown by Ved et al. (2013) that VEGF (165)b possesses the capability to relieve diabetic retinal vascular dysfunction. Research conducted by Fu et al. (2013) discussed the effects of itraconazole to block the maturation of blood vessels. On the basis of the studies described above, it can be confirmed that VEGF is closely associated with vascular proliferation.

5. Conclusions

As established by the results of this study, the mechanism of compound therapy of laser needle-knife for the treatment of CSA probably utilizes the PI3K-AKT-VEGF signaling pathway which increases the number of microvessels and micropores, resulting in a reduction in vertebroarterial hemodynamic abnormalities caused by CSA and promoting the supply of local blood.

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Fig. 2. Effects of various treatments on the protein expression of PI-3K, AKT and VEGF in the carotid arteries of CSA rabbits, in comparison with normal rabbits.

References

Alnaim, L., Almaz, S., 2017. A study of barriers and facilitators of clinical practice guidelines implementation among physicians. Indian J. Pharm. Sci. 79 (6), 923–929.

Bozkurt, F., Kose, C., Sari, A., et al., 2018. An inverse approach for automatic segmentation of carotid and vertebral arteries in CTA. Expert Syst. Appl. 93, 358–375.

Butkovic, D., Tot, O.K., 2017. Laser acupuncture treatment of neuropathic pain in a boy with brain tumour. Complement. Ther. Med. 35, 53–56.

Ding, Q.M., Yan, M.R., Zhou, J., et al., 2012. Clinical effects of innovative tuina manipulations on treating cervical spondylosis of vertebral artery type and changes in cerebral blood flow. J. Tradit. Chin. Med. 32 (3), 388–392.

Fu, C.L., Gerber, S., Castillo, R., et al., 2013. Overcoming the resistance to antiangiogenic therapy: itraconazole blocks blood vessel maturation and synergizes with anti-VEGF therapy. Cancer Res. 73 (8–1), 5084.

Ge, S., Liu, Z., Furuta, Y., Peng, W., 2017. Characteristics of activated carbon remove sulfur particles against smog. Saudi J. Biol. Sci. 24 (6), 1370–1374.

Guan, Q., Chen, L., Long, Y., et al., 2017. Iatrogenic vertebral artery injury during anterior cervical spine surgery: a systematic review. World Neurosurg. 106, 715–722.

Hori, Y., Ito, K., Hamamichi, S., et al., 2017. Functional characterization of VEGF- and FGF-induced tumor blood vessel models in human cancer xenografts. Anticancer Res. 37 (12), 6629–6638.

Hu, X.M., Zheng, Z., Yang, S.T., et al., 2005. Establishment of a cervical spondylotic arteriopathy rabbits model by static blood blocking collaterals. J. Sichuan Tradit. Chin. Med. 23 (12), 19–22.

Jittiwat, J., 2007. Laser acupuncture at GV20 improves brain damage and oxidative stress in animal model of focal ischemic stroke. J. Acupunct. Meridian Stud. 10 (5), 324–330.

Kaya, A., Ak gol, G., G ulkesen, A., et al., 2018. Cerebral blood flow volume using color duplex sonography in patients with fibromyalgia syndrome. Arch. Rheumatol. 33 (1), 66–72.

Kevrekidis, D.P., Minarikova, D., Markos, A., Malovecka, I., Minarik, P., 2018. Community pharmacy customer segmentation based on factors influencing their selection of pharmacy and over-the-counter medicines. Saudi Pharm. J. 26 (1), 33–43.

Khan, A., Jan, G., Khan, A., Jan, F.G., Danish, M., 2018. Evaluation of antioxidant and antimicrobial activities of berigeria ciliata sternb (rhizome) crude extract and fractions. Pakistan J. Pharm. Sci. 31 (1), 31–35.

Liu, F., Wei, W., Yang, G.Y., et al., 2017. Therapeutic effects and finite element analysis of a combined treatment using laser needle-knife with supine repositioning massage on patients with cervical spondylotic vertebral arteriopathy. Int. J. Pattern Recogn. 31 (11), 1757008.

Moon, K., Albuquerque, F.C., Cole, T., et al., 2017. Stroke prevention by endovascular treatment of carotid and vertebral artery dissections. J. Neurointerv. Surg. 9 (10), 952–957.

Ozek, L., Senec, U., Ilgezdi, I., Ozcelik, M.M., Zorlu, Y., 2017. Acute ischemic stroke in patients with cancer: risk factors, clinical and imaging outcomes. Acta Medica Mediterranea 33 (4), 601–606.

Ohta, M., Hosokawa, Y., Hatano, N., et al., 2017. Efficacy of femtosecond lasers for application of acupuncture therapy. Laser Med. Sci. 32 (9), 2167–2171.

Peng, W., Lin, Z., Wang, L., Chang, J., Gu, F., Zhu, X., 2016. Molecular characteristics of illicium verum extractives to activate acquired immune response. Saudi J. Biol. Sci. 23 (3), 348–352.

Policha, A., Baldwin, M., Lee, V., et al., 2018. Clinical significance of reversal of flow in the vertebral artery identified on cerebrovascular duplex ultrasound. J. Vasc. Surg. 67 (2), 568–572.

Polonga, F., Eubanks, G., 2017. Laser acupuncture-induced analgesic effect and molecular alterations in an incision pain model: a comparison with electroacupuncture-induced effects. Laser Med. Sci. 33 (2), 295–304.

Zeredo, J.L., Sasaki, K.M., Toda, K., 2007. High-intensity laser for acupuncture-like stimulation. Laser Med. Sci. 1, 37–41.

Zhong, F.L., Kong, Z.L., Xu, G.Y., et al., 2017. High stability and robustness of a novel laser acupuncture theranostic device. Microelectron. Reliab. 78, 401–405.