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The therapeutic effect and mechanism of Chinese medicine *Xuan-Yun-Ding* on posterior circulation ischemia with vertigo in a rabbit model

Liu Chunhua[1], Li Zhixiong[2*], Wu Dahua[3], Yuan Chunyun[4], Deng Haoqin[5], Yuan Sisi[4], Liu Fang[6] and Xiao Yao[7]

[1]College of Integrated Traditional Chinese and Western Medicine, Hunan University of Chinese Medicine, Changsha, Hunan, China
[2]Medical School of Hunan University of Chinese Medicine, Changsha, Hunan, China
[3]Department of Encephalopathy, Affiliated Hospital of Integrated Traditional Chinese and Western Medicine, Hunan University of Chinese Medicine, Changsha, Hunan, China
[4]Department of Geriatrics, Affiliated Hospital of Integrated Traditional Chinese and Western Medicine, Hunan University of Chinese Medicine, Changsha, Hunan, China
[5]Department of Traditional Chinese Medicine, 458th Hospital of Chinese People's Liberation Army, Guangzhou, Guangdong, China
[6]Liu Zuyi's Medical Master's Studio, Hunan Academy of Traditional Chinese Medicine, Changsha, Hunan, China
[7]Department of Traditional Chinese Medicine of Hunan Province People's Hospital, Changsha, Hunan, China

Abstract: The aim of research is to unveil the mechanisms of the beneficial effects of XYD on PCIV in a rabbit model. 40 New Zealand white rabbits were randomly divided into 5 groups,including normal control group (NC), model control group (MC), low-dose of XYD group (LXYD), high-dose of XYD group (HXYD) and Yang-Xue-Qin-Nao group (YXQN). PCIV rabbit model was established by feeding high-fat diet companied with paravertebral sclerotherapy and rotation exercise. The general observation, step-down test, rheoencephalogram, blood tests, histopathological detection and the plasma concentration of the effective component of XYD were investigated. After pharmacological intervening, the step-down time, REG, PL, IPL, blood viscosity, the levels of blood lipids, CRGP were significantly improved. Moreover, the vertebral artery showed the reduced stenosis of arterial lumen and less proliferation of fibrous tissue in the arterial wall in the LXYD, HXYD and YXQN group. Based on the LC-MS detection, the blood concentrations of puerarin in the LXYD and HXYD group were significantly increased after pharmacological intervening. XYD could ameliorate the symptoms of vertigo, Qi-deficiency and blood stasis in PCIV rabbits via effectively regulating the levels of blood lipids and vasoactive substances, decreasing blood viscosity, increasing CBF and protecting vestibular function.

Keywords: Posterior circulation ischemia, vertigo, *Xuan-Yun-Ding,* puerarin, Chinese medicine.

INTRODUCTION

Vertigo, a subtype of dizziness, indicates a sense of disorientation and exhibits an erroneous self-/object-motion sensing or a positional illusion caused by dynamic or static spatial disorientation, resulting in the mismatches within vestibular, visual and somatosensory systems (Blum and Kasner, 2015; Whitman, 2018). About 20% of patients with ischemic strokes will suffer from vertigo and imbalance which are the most common symptoms of posterior circulation ischemia (PCI) (Choi *et al*., 2016). Although patients with PCI usually have various neurologic symptoms, there is still a part of cases with PCI only presenting with isolated vertigo (Chen *et al*., 2018). Therefore, early diagnosis and treatment of PCI vertigo (PCIV) are very important due to significantly different prognoses between benign and malignant vertigo-related causes (Lee and Kim, 2015).

Currently, the comprehensive treatment for stroke-related vertigo is extremely necessary, including the pharmacological combination of thrombolytic therapy, sedatives, antiemetics, corticosteroid and vestibular suppressants, endovascular or surgical treatment (Karatas, 2011). However, the management of PCIV is further complicated by the complex anatomical structures and physiological function of posterior-circulation territories (Schneider and Olshaker, 2012). Traditional Chinese medicine (TCM) has shown beneficial effects on aging-related chronic diseases, especially treating cerebral ischemia, via dilating blood vessels, suppressing platelet aggregation, inhibiting ischemia-reperfusion injury and reducing anoxic damage (Chen, 2012). According to the theoretical system of TCM, vertigo is caused by a loss of nourishment in the upper orifices resulting from pathogenic wind, heat, phlegm, deficiency, or stasis (Deng *et al*., 2015). From our long-term TCM clinical experience, PCIV is a kind of vertigo characterized by Qi-deficiency and blood-stasis syndrome. Thus, based on a well-known Chinese herb prescription *Buyang Huanwu* decoction (BHD) (Mu *et al*., 2014), we have created a new TCM prescription *Xuan-Yun-Ding* (XYD) which is composed of eleven kinds of Chinese herbs, including *Huangqi*, *Gegen*, *Danggui*, *Danshen*, *Chuanxiong*, *Danpi*, *Dilong*, *Dangshen*, *Shanzha*, *Jixueteng* and *Chishao*. XYD has been functionally characterized by Qi supplement and improving blood

Corresponding authour: e-mail: li_zhixiong0098@sina.com

circulation, and widely used for the treatment of PCIV in the clinical practice.

However, the mechanism of XYD therapeutic effects on PCIV is still unclear. Therefore, in the present study, we established a rabbit model of PCIV by feeding high-fat diet companied with paravertebral sclerotherapy and rotation exercise. Subsequently, the therapeutic effects of XYD on PCIV were investigated based on the general observation, step-down test, rheoencephalogram (REG), brainstem auditory evoked potential (BAEP), hemorheology test, blood tests of lipids, endothelin-1 (ET-1) and calcitoningene-related peptide (CGRP), histopathological detection. Finally, the plasma concentration of the effective component of XYD (puerarin) was measured by high performance liquid chromatograph-mass spectrometry (HPLC-MS). In summary, the aim of present research is to unveil the mechanisms of the beneficial effects of XYD on PCIV in a rabbit model.

MATERIALS AND METHODS

Animal grouping and modeling

All experiments were performed following the guidelines of Animal Use and Care Committee. New Zealand white rabbits (male, Specific Pathogen Free, body weight 2.0±0.3 kg, age 3-4 months) were obtained from Experimental Animals Center of Hunan TCM University and maintained in a standard specific pathogen free animal lab with a controlled temperature of 25±1°C, humidity of 55±10%, and a regulated 12-h light/dark cycle.

After one-week adaptive feeding, 40 rabbits were divided into 5 groups (n=8) randomly, including normal control group (NC), model control group (MC), low-dose of XYD group (LXYD), high-dose of XYD group (HXYD) and *Yang-Xue-Qin-Nao* group (YXQN). Rabbits in the NC group were provided with normal diet for 6 weeks and rabbits in the MC, LXYD, HXYD and YXQN groups were provided with high-fat diet (1 g cholesterol, 15 g egg yolk powder, 5 g lard oil and 79 g basic feedstuff per 100 g high-fat feed) for 6 weeks. Meanwhile, weekly paravertebral sclerotherapy with the injection of 10 mL sclerosing agent at the lateral side of C3-5 left transverse processe and weekly rotation exercise (90 r/min, 5 min) were used for modeling in the MC, LXYD, HXYD and YXQN groups. Sclerosing agent was prepared by a mixture (1:1 w/w) of Xiaozhiling (Beijing Double Crane Pharmaceutical Co. Ltd, China) and lidocaine (Shanxi Jinxin Shuanghe Pharmaceutical Co. Ltd, China).

A rating scale for PCIV rabbit model was showed in the Table 1. After 6-week modeling, rabbits in the LXYD, HXYD and YXQN groups were given by intragastric administration of low-dose XYD (7.66 g/kg·d, 20mL, Hunan Zhenxing Chinese Herbal Pieces Industrial Co., Ltd, China), high-dose XYD (15.32 g/kg·d, 20mL) and YXQN (0.575 g/kg·d, 20mL, Tianjin Tasly Pharmaceutical Co. Ltd, China) for 14 days, respectively. Moreover, rabbits in the NC and MC group were given distilled water (20mL) via intragastric administration for 14 days.

Experimental tests for vertigo

Experimental tests for vertigo included the s*tep-down test*, eheoencephalogram (REG) and brainstem auditory evoked potential (BAEP).

First, the step-down test was used to examine the passive avoidance conditioned reflex (He *et al*., 2014). This experiment equipment was composed of a Plexiglas box (60 cm × 12 cm × 18 cm) with an electrified copper grid base and an electrically insulating platform (12 cm × 5 cm). The rabbits received the training of stimulation avoidance and established a conditioned reflex for escaping from electric shock (36 V alternating current). After rotation exercise, the escape latency (time taken to stay on the platform for 10 s) was recorded. Secondly, the cerebral blood flow was measured by using the REG-IG2 automatic analyzer (Hunan Yilin Technology Co., Ltd, Changsha, China). Briefly, the rabbits were placed in a special fixator after shearing. Needle electrodes were implanted in the forehead, mastoid processes and occipital protuberance. The REG were detected in the bipolar derivation with a measuring frequency of 90 kHz. Several main indicators of REG, including the wave amplitude (Ω), area (S, Ω·S) and velocity (V, Ω/S^2), were recorded.

Finally, BAEP data were recorded by using the Neuropack M1 MEB-9200/9300 (Nihon Kohden, Japan). After shearing, the rabbits were anesthetized and placed in a special fixator. Briefly, Fpz was used as ground electrode, recording electrodes were implanted at Cz and reference electrodes were implanted at A1 and A2. Alternating polarity clicks with 0.1 ms duration were given in the unilateral ear and the stimulus (10 Hz, 80dB) was separately presented in both ears. Each response was recorded after receiving ×100,000 amplification, filtration at 100-3,000 Hz and stacks in runs of 1,000 sweeps. Two examinations of each ear were performed to prove the repeatability of waves and tracing overlapping. The computer-based electrodiagnostic system was used to record the values of peak latencies (PL) and interpeak latencies (IPL) (Cai *et al*., 2012; Ji and Zhang, 2014).

Blood tests of hemorheology, lipids, ET-1, CGRP and Concentration of puerarin After 8 hours fasting, 8 mL blood was collected from auricular vein. Among them, 2 mL blood adding anticoagulant (0.2 mL, 12500 u/mL heparin) was used for measuring blood viscosity by LBY-N6B hemorheological detector (Beijing Precil Instrument Co., Ltd, Beijing, China); 2 mL blood without adding

Table 1 Rating scale for PCIV rabbit model

Criteria	Symptoms		Score
Qi-deficiency	Mental state	Malaise, resistance to capture	3
		Lassitude, less resistance to capture	5
		Somnolence, no resistance to capture	7
	Feces	Less amount and small granule	3
		Less amount and semisolid	5
		Loose stool and unformed	7
Blood-stasis	Ear	Vascular collapse, skin temperature decreasing	10
	Lip	Dark red or dark purple	10
	Hemorheology	All shear rates increasing	10
	Blood lipid	Levels of total cholesterol, triglyceride and low density lipoprotein increasing	10
Vertigo	BAEP	Abnormal wave I-V pattern, prolongation of PL and or IPL	10
	Step-down test	Time lengthening 6s	10
	REG	The blood supply of vertebrobasilar artery system is reduced by 20%	10
Result	Scores of Qi-deficiency ≥ 8, Blood-stasis ≥ 20 and Vertigo≥ 20 indicate modeling successfully.		

BAEP: brainstem auditory evoked potential; REG: rheoencephalogram; PL: peak latencies; IPL: interpeak latencies.

anticoagulant was used for measuring total cholesterol (TC), triglyceride (TG) and low-density lipoprotein (LDL) by C800 automatic biochemical analyzer (Abbott, USA); 2 mL blood was used for measuring the levels of ET-1 and CGRP by enzyme-linked immunosorbnent assay kits(ELISA, Hunan Weier Bio-Technology Co., Ltd, China); 2 mL blood was used for measuring the concentration of puerarin by high performance liquid chromatograph-mass spectrometry (Agilent 1260 HPLC-MS, Agilent, USA). Puerarin analysis was performed using the following mass spectrometry parameters: gas temp 300; gas flow 10 L/min; nebulizer 20 psi; sheath gas temp 350; sheath gas flow 11; VCap 3000; Nozzle Voltage 1000 V; Fragmentor 280; Skimmer 65. After centrifugation, 0.2 mL serum was added into 1.8 mL acetonitrile followed by 3 min vortex concussion. Subsequently, the sample was centrifuged at 10,000 rpm for 8 min followed by a filtration via a 0.22 μm membrane. 20 μL samples were prepared for the HPLC-MS analysis. Furthermore, 2, 5, 10, 20, 50 and 100 ng/mL mixed standard solution was respectively injected, and the calibration curves were built by plotting the peak area versus the concentration (ng) of each analyte. Diluted standard solutions were injected into chromatographic system to calculate the limit of detection (LOD) and the limit of quantification (LOQ) under the present conditions.

Histopathological examination

After a survival period of 8 weeks, all examinations were completed and the rabbits were deeply anesthetized with 20% urethane (1 g/kg) and perfused through the left ventricle with 0.9% saline followed by 4% paraformaldehyde. 5-7 mm vertebral artery was dissected out carefully, post-fixed with 10% formaldehyde for 4 h, and then stored in 30% sucrose overnight. The samples were embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). The sections of vertebral artery were observed under an optical microscope (Olympus CX31, Japan). Subsequently, the brains were removed, the positions of vestibule nerve and cochlear nerve were located, nuclei vestibularis (2×2 mm) was dissected from dorsolateral side of medulla oblongata and fixed into 2.5% glutaraldehyde solution for 24 h. This was followed by secondary fixation with 2% osmium tetroxide for 2 h and a graded series of ethanol-based dehydration ending with propylene oxide. Epon resin was used for sample embedding, then ultra-thin sections (about 50 nm) were prepared by using LKB-III ultramicrotome. The sections were observed using HT7700 high-resolution transmission electron microscope (Hitachi, Japan).

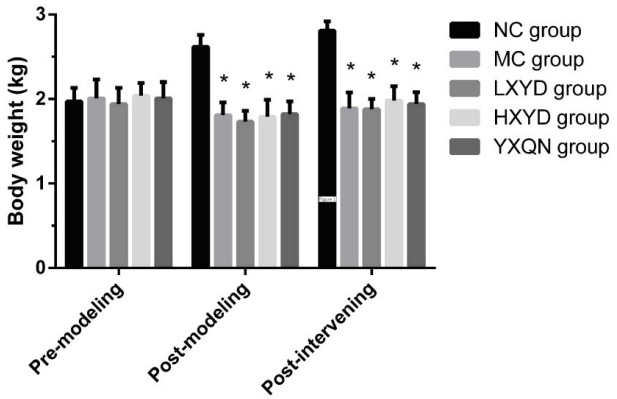

Fig. 1: Body weight values of rabbits from all five groups at the timepoints of pre-modeling, post-modeling and post-intervening. *P<0.05 compared with NC group.

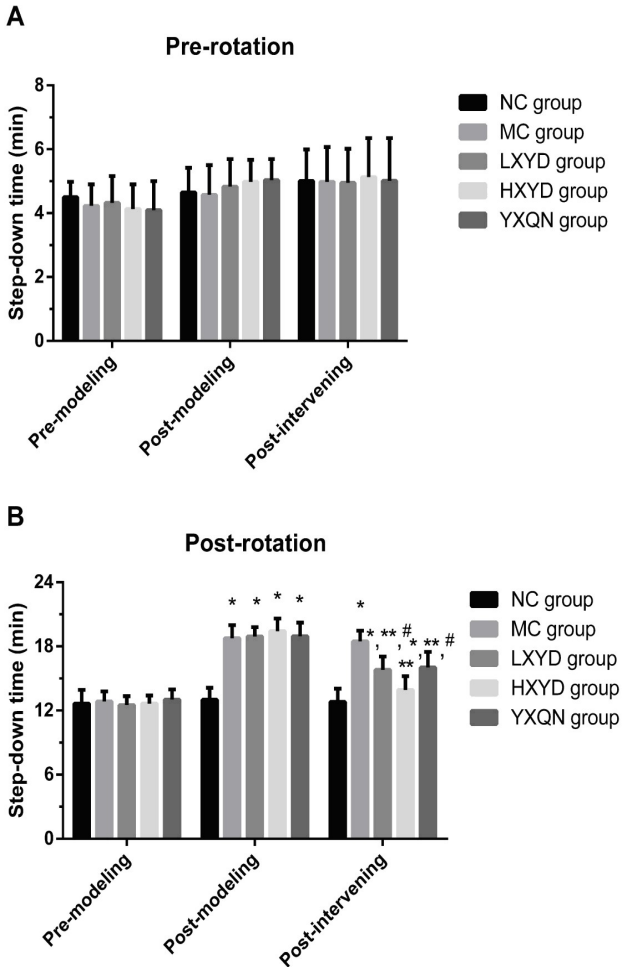

Fig. 2: Step-down tests of rabbits from all five groups before (A) and after rotation (B). *P<0.05 compared with NC group, **P<0.05 compared with MC group, #P<0.05 compared with HXYD group.

Ethical approval

This work was supported by the Ethic committee of the Medical School of Hunan University of Chinese Medicine.

STATISTICAL ANALYSIS

All data were analyzed by using SPSS 18.0 (IBM Software, Armonk, NY, USA) and were exhibited as the mean±standard deviation (SD). The data were analyzed using one/two-way analysis of variance (ANOVA). Firstly, normality and homogeneity of variance tests were examined. If the variances were equal, least-significant difference tests were employed. If the variance were unequal, Tamhane tests were used. As to data with non-normal distribution, Kruskal-Wallis tests were used. A value of P<0.05 was considered statistically significant.

RESULTS

General observations

General observations, including body weight values and changes of PCIV symptoms, were used for evaluating the general condition of rabbits from all five groups. As shown in fig. 1, no significant differences were found within body weight values among all five groups before modeling (P>0.05). After modeling, the body weight values of rabbits from MC, LYXD, HYXD and YXQN group were significantly lower than that of rabbits from NC group (P<0.05), however, there were no significant differences among these four groups (P<0.05).

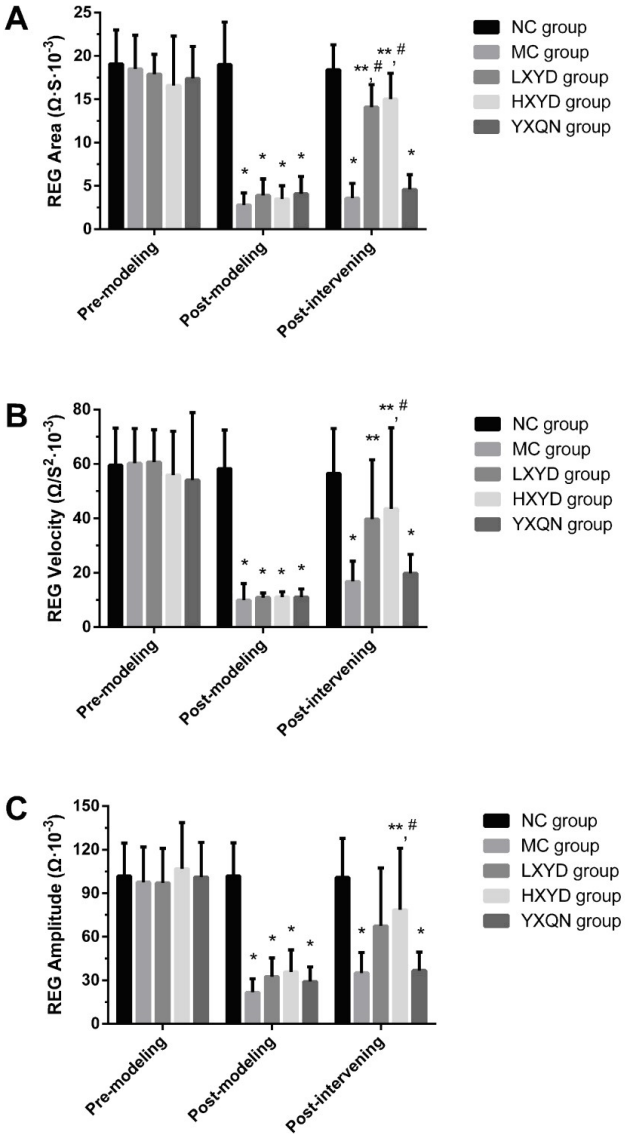

Fig. 3: REG area (A), velocity (B) and amplitude (C) of rabbits from all five groups at the timepoints of pre-modeling, post-modeling and post-intervening. *P<0.05 compared with NC group, **P<0.05 compared with MC group, #P<0.05 compared with YXQN group

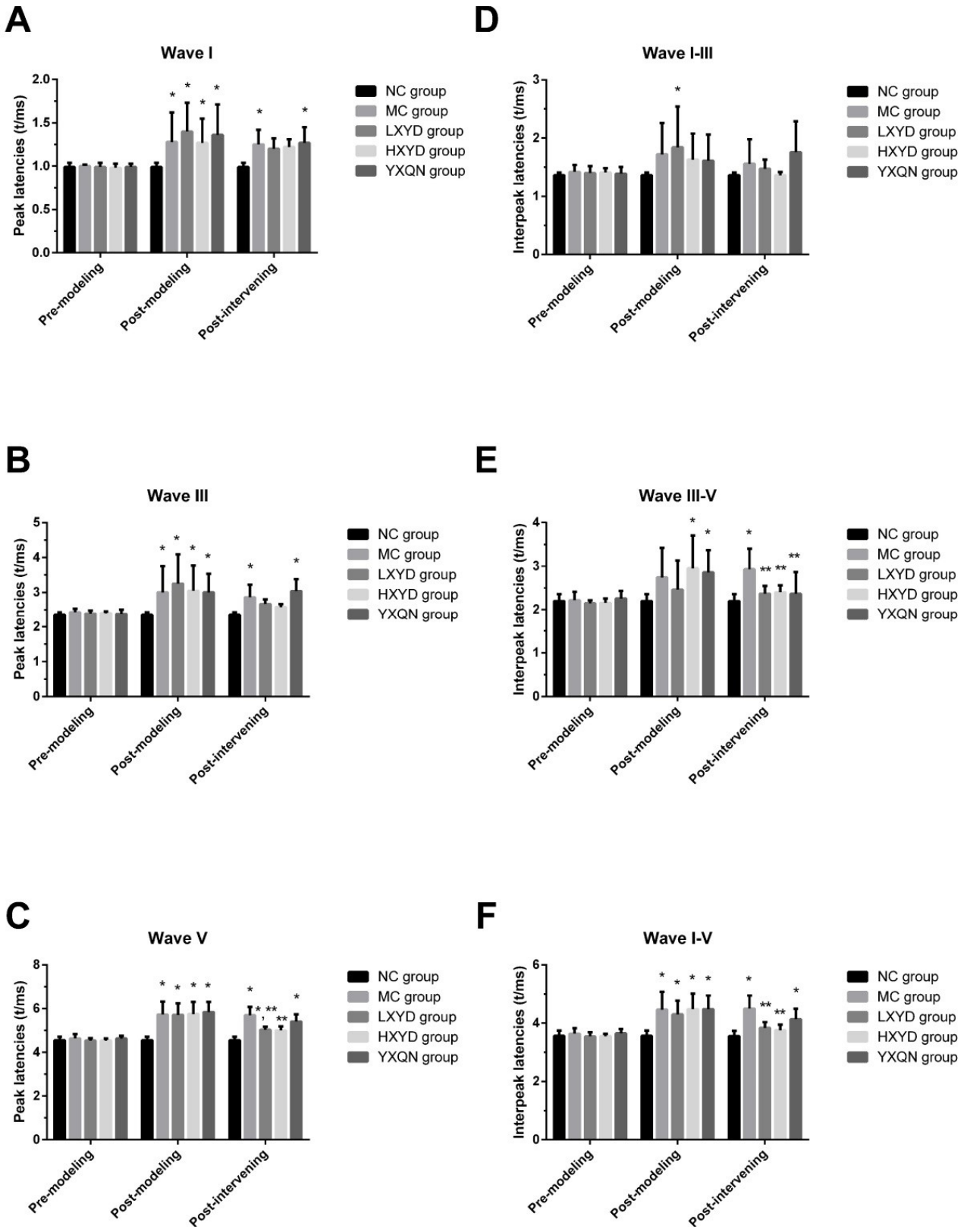

Fig. 4: BAEP changes of rabbits from all five groups at the timepoints of pre-modeling, post-modeling and post-intervening, including peak latencies of wave I (A), III (B) and V (C), interpeak latencies of wave I-III (D), III-V (E) and I-V (F). *P<0.05 compared with NC group, **P<0.05 compared with MC group.

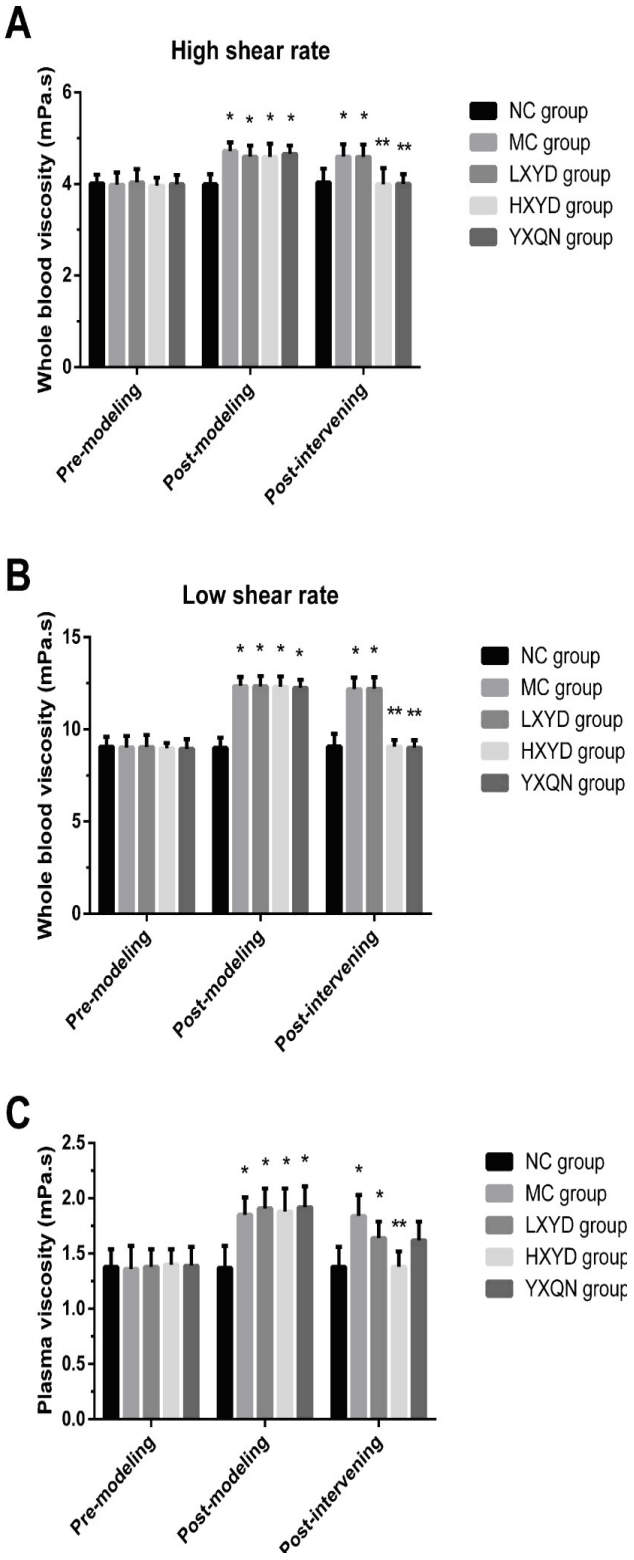

Fig. 5: Hemorheology tests of rabbits from all five groups at the timepoints of pre-modeling, post-modeling and post-intervening, including whole blood viscosity at high shear rate (A) and low shear rate (B) and plasma viscosity (C). *P<0.05 compared with NC group, **P<0.05 compared with MC group.

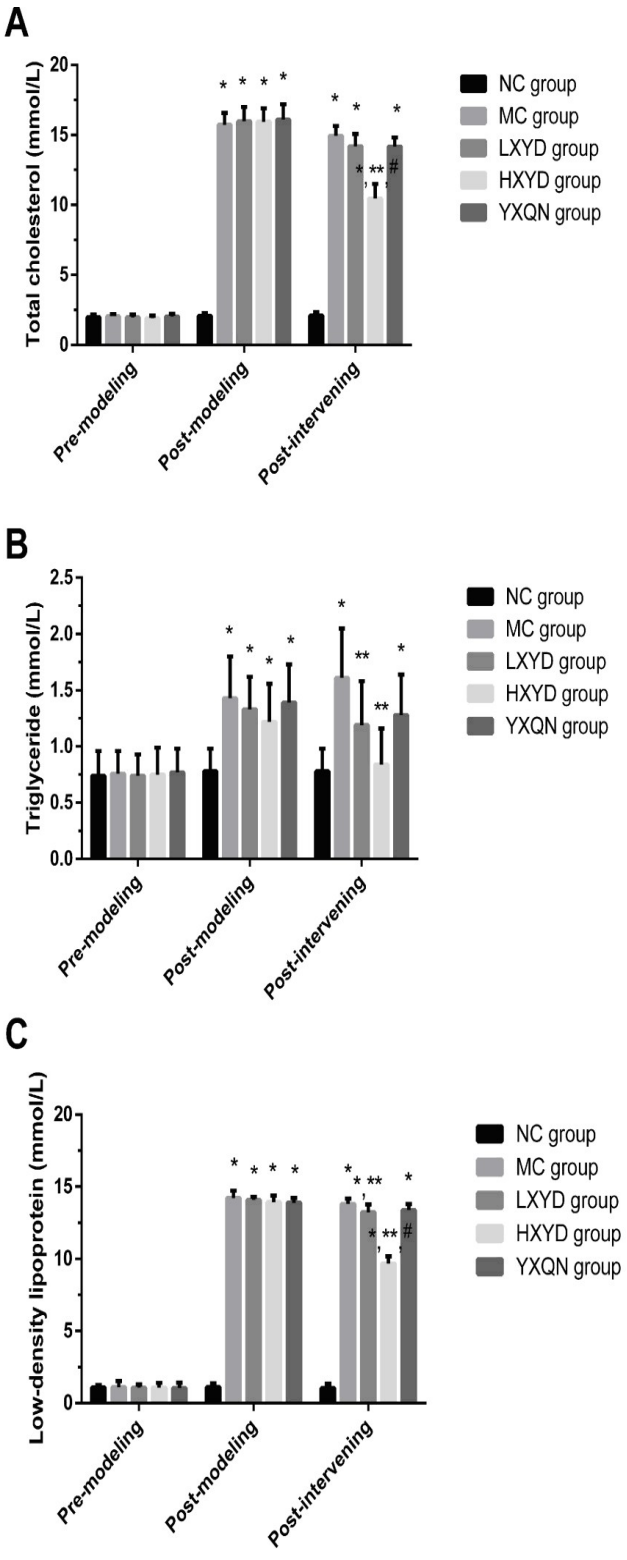

Fig. 6: Blood lipids levels of rabbits from all five groups at the timepoints of pre-modeling, post-modeling and post-intervening, including total cholesterol (A), triglyceride (B) and low-density lipoprotein (C). *P<0.05 compared with NC group, **P<0.05 compared with MC group, #P<0.05 compared with YXQN group.

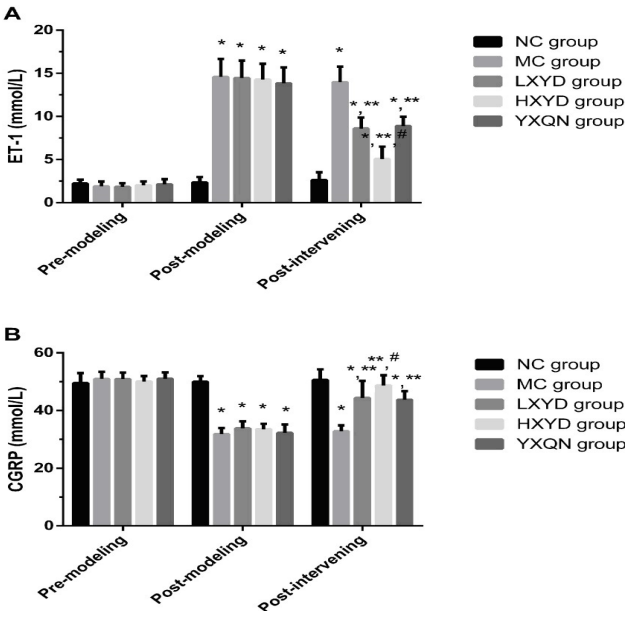

Fig. 7: The effect of XYD and YXQN on the levels of ET-1 (A) and CGRP (B), post-modeling and post-intervening, including total cholesterol (A), triglyceride (B) and low-density lipoprotein (C). *P<0.05 compared with NC group, **P<0.05 compared with MC group, #P<0.05 compared with YXQN group.

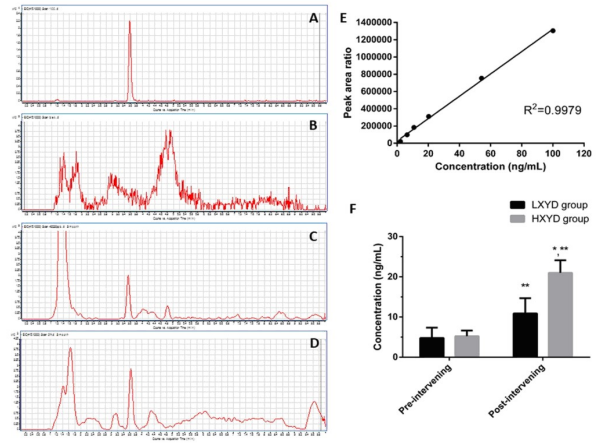

Fig. 8: The plasma concentration of puerarin in the YXD-treated rabbits. (A) extracted ion chromatogram of puerarin sample, (B) extracted ion chromatogram of blank plasma, (C) extracted ion chromatogram of blank plasma with puerarin (20ng/mL), (D) extracted ion chromatogram of plasma from YXD-treated rabbits, (E) the calibration curve of puerarin, (F) blood concentrations of puerarin before and after treatment of YXD. *P<0.05 compared with LXYD group, **P<0.05 compared with pre-intervening.

Similarly, the body weight values of rabbits from MC, LYXD, HYXD and YXQN group were significantly lower than that of rabbits from NC group (P<0.05) after pharmacological intervening, however, no significant differences were found among these four groups (P<0.05). As to general conditions, rabbits in the NC group exhibited the smooth, warm fur, flexible activities, 100 g/d soft and formed feces; rabbits in the other groups exhibited lackluster, sparse fur, less activities, and decreasing amount of feces. After pharmacological intervening, the general condition of rabbits in the MC group rarely changed, the rabbits in the YXQN group showed loose stool and unformed feces; however, the rabbits in the LYXD and HYXD exhibited the normal fur, feces and activities.

Step-down test

As to rabbits possessed passive avoidance conditioned reflex, the step-down time after rotation was significantly higher than that before rotation due to the disability of controlling body movement. As shown in fig. 2A, there were no significant differences of step-down time within five groups before rotation (P>0.05). However, the step-down time of MC, LXYD, HXYD and YXQN groups were significantly higher than that of NC group after rotation at the timepoint of post-modeling (P<0.05). After pharmacological intervening, the post-rotation step-down time of LXYD, HXYD and YXQN groups were significantly lower than that of MC group (P<0.05); the post-rotation step-down time of LXYD and YXQN groups were significantly higher than that of NC group (P<0.05); there was no significant difference of post-rotation step-down time between HXYD group and NC group (P>0.05); the post-rotation step-down time of LXYD and YXQN groups were significantly higher than that of HXYD group (P<0.05). *REG*

The main indexes of REG, including amplitude (Ω), area (S, Ω·S) and velocity (V, Ω/S^2), were used to evaluate the status of cerebrovascular and cerebral blood supply. Specifically, amplitude and area were used to measure the amount of cerebral blood flow (CBF) based on the positive correlation with CBF and decreasing amplitude or area indicated the cerebrovascular stenosis, spasticity or obstruction; velocity was used to measure the variation rate of CBF which indicated the stability of CBF. As shown in fig. 3A, there were no significant differences of REG area within five groups before modeling (P>0.05). However, the REG area of MC, LXYD, HXYD and YXQN groups were significantly lower than that of NC group after modeling (P<0.05). After pharmacological intervening, the REG area of LXYD and HXYD groups were significantly higher than that of MC and YXQN group (P<0.05); the REG area of MC and YXQN groups were significantly lower than that of NC group (P<0.05). As shown in fig. 3B, there were no significant differences of REG velocity within five groups before modeling (P>0.05). However, the REG velocity of MC, LXYD, HXYD and YXQN groups were significantly lower than that of NC group after modeling (P<0.05).

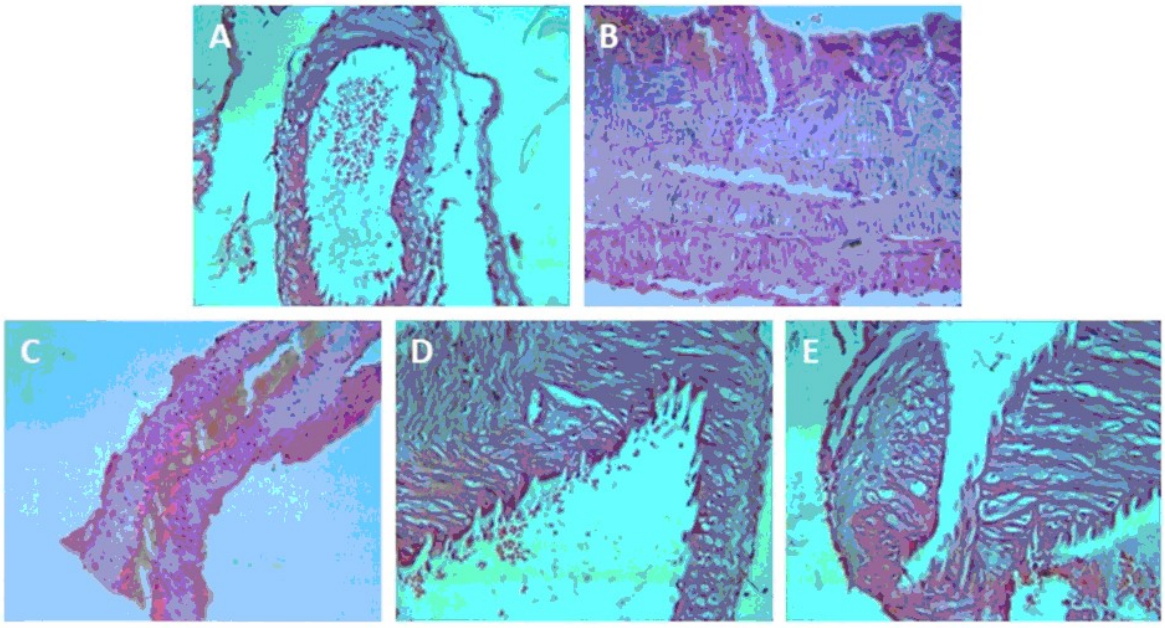

Fig. 9: The H&E staining of vertebral artery sections after pharmacological intervening within the NC (A), MC (B), LXYD (C), HXYD (D) and YXQN (E) group with 400× magnification.

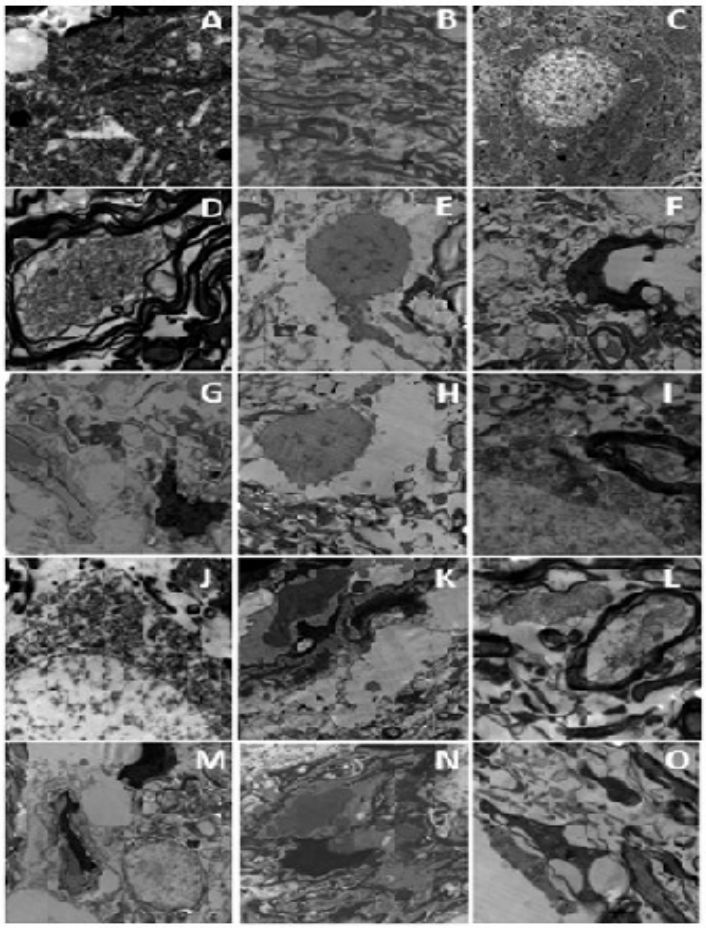

Fig. 10: TEM test of nuclei vestibularis after pharmacological intervening within the NC (A-C), MC (D-F), LXYD (G-I), HXYD (J-L) and YXQN (M-O) group with 6000× magnification.

After pharmacological intervening, the REG velocity of LXYD and HXYD groups were significantly higher than that of MC group (P<0.05); the REG velocity of MC and YXQN groups were significantly lower than that of NC group (P<0.05); the REG velocity of HXYD group was significantly higher than that of YXQN group (P<0.05). As shown in fig. 3C, there were no significant differences of REG amplitude within five groups before modeling (P>0.05). However, the REG amplitude of MC, LXYD, HXYD and YXQN groups were significantly lower than that of NC group after modeling (P<0.05). After pharmacological intervening, the REG amplitude of HXYD group was significantly higher than that of MC and YXQN group (P<0.05); the REG velocity of MC and YXQN groups were significantly lower than that of NC group (P<0.05).

*BAEP*BAEP is a sensitive, non-invasive and easily reproducible method to evaluate the vertebrobasilar failure. BAEP changes, including peak latencies (PL) of wave I, III and V, interpeak latencies (IPL) of wave I-III, III-V and I-V, were used to measure the neuronal dysfunction of PCIV before and after pharmacological intervening. As to PL I (fig. 4A), there were no significant latencies within five groups before modeling (P>0.05), however, the latencies of MC, LXYD, HXYD and YXQN groups were significantly increased after modeling (P<0.05); after pharmacological intervening, the latencies were still significantly increased in the MC and YXQN group (P<0.05), but there were no significant latencies in the LXYD and HXYD group (P>0.05). As to PL III (fig. 4B), there were no significant latencies within five groups before modeling (P>0.05), however, the latencies of MC, LXYD, HXYD and YXQN groups were significantly increased after modeling (P<0.05); after pharmacological intervening, the latencies were still significantly increased in the MC and YXQN group (P<0.05), but there were no significant latencies in the LXYD and HXYD group (P>0.05). As to PL V (fig. 4C), there were no significant latencies within five groups before modeling (P>0.05), however, the latencies of MC, LXYD, HXYD and YXQN groups were significantly increased after modeling (P<0.05); after pharmacological intervening, the latencies of MC, LXYD and YXQN group were significantly higher than that of NC group (P<0.05) and the latencies of LXYD and HXYD group were significantly lower than that of MC group (P<0.05). As to IPL I-III (fig. 4D), there were no significant latencies within five groups before modeling (P>0.05), however, the latency of LXYD group was significantly increased after modeling (P<0.05); after pharmacological intervening, there were no significant latencies within five groups (P>0.05). As to IPL III-V (fig. 4E), there were no significant latencies within five groups before modeling (P>0.05), however, the latencies of HXYD and YXQN groups were significantly increased after modeling (P<0.05); after pharmacological intervening, the latency of MC group was still significantly higher than that of NC group (P<0.05) and the latencies of LXYD, HXYD and YXQN group were significantly lower than that of MC group (P<0.05). As to IPL I-V (fig. 4F), there were no significant latencies within five groups before modeling (P>0.05), however, the latencies of HXYD and YXQN groups were significantly increased after modeling (P<0.05); after pharmacological intervening, the latency of MC group was still significantly higher than that of NC group (P<0.05) and the latencies of LXYD, HXYD and YXQN group were significantly lower than that of MC group (P<0.05).

Hemorheology test

The hemorheology test was performed to evaluated the effect of XYD and YXQN on rabbits with PCIV. As shown in fig. 5A and B, there were no significant differences of the whole blood viscosity (WBV) at high and low shear rate within five groups before modeling (P>0.05). After modeling, the WBV at high and low shear rate was significantly increased (P<0.05). After pharmacological intervening, the WBV at high and low shear rate of MC and LXYD group were significantly higher than those of NC group (P<0.05) and the WBV at high and low shear rate of HXYD and YXQN group were significantly lower than those of MC group (P<0.05). As shown in fig. 5C, there were no significant differences of the plasma viscosity (PV) within five groups before modeling. After modeling, the PV was significantly increased (P<0.05). After pharmacological intervening, the PV of MC and LXYD group were significantly higher than those of NC group (P<0.05) and the PV of HXYD group was significantly lower than those of MC group (P<0.05).

Blood lipids test

As shown in fig. 6A, there were no significant differences of total cholesterol (TC) within five groups before modeling (P>0.05) and the levels of TC were significantly increased after modeling (P<0.05). After pharmacological intervening, the level of TC in the HXYD group was significantly lower than those of MC, LXYD and YXQN group (P<0.05). As shown in fig. 6B, there were no significant differences of triglyceride (TG) within five groups before modeling (P>0.05) and the levels of TG were significantly increased after modeling (P<0.05). After pharmacological intervening, the levels of TG in the LXYD and HXYD group were significantly lower than that of MC group (P<0.05). As shown in fig. 6C, there were no significant differences of low-density lipoprotein (LDL) within five groups before modeling (P>0.05) and the levels of LDL were significantly increased after modeling (P<0.05). After pharmacological intervening, the levels of LDL in the LXYD and HXYD group were significantly lower than that of MC group (P<0.05) and the level of LDL in the HXYD group was significantly lower than that of YXQN group (P<0.05).

*The effect of XYD and YXQN on the levels of ET-1 and CGRP*As shown in fig. 7A, there were no significant differences of ET-1 within five groups before modeling (P>0.05) and the levels of ET-1 were significantly increased after modeling (P<0.05). After pharmacological intervening, the levels of ET-1 in the LXYD, HXYD and YXQN group were significantly lower than that of MC group (P<0.05); the level of ET-1 in the HXYD group was significantly lower than that of YXQN group (P<0.05). As shown in fig. 7B, there were no significant differences of CGRP within five groups before modeling (P>0.05) and the levels of CGRP were significantly decreased after modeling (P<0.05). After pharmacological intervening, the levels of CGRP in the LXYD, HXYD and YXQN group were significantly higher than that of MC group (P<0.05); the level of CGRP in the HXYD group was significantly higher than that of YXQN group (P<0.05).

Detection of puerarin concentration in the plasma by LC-MS

The extracted ion chromatograms obtained from the puerarin sample, blank plasma, blank plasma with puerarin (20 ng/mL) and plasma sample from YXD-treated rabbits were presented in fig. 8A-D. The retention time of puerarin was found to be approximately 3.6 min. The calibration curve of puerarin was y = 12832x + 28876 (R^2=0.9979) and the concentration range of puerarin was 2-100 ng/mL (fig. 8E). As shown in fig. 8F, there were no significant differences of puerarin concentrations in the plasma between LXYD and HXYD group before pharmacological intervening (P>0.05). After treating with XYD, the concentrations of puerarin in the LXYD and HXYD group were significantly increased after pharmacological intervening (P<0.05) and the concentration of puerarin in the HXYD group was significantly higher than that of LXYD group (P<0.05).

Histopathological examination

The morphological characteristics of vertebral artery after pharmacological intervening were observed by H&E staining. Normal arterial lumen, smooth arterial wall, continuous endarterium and normal morphology of smooth muscle cells were observed in the fig. 9A. As shown in fig. 9B, the vertebral artery of MC group exhibited significant stenosis of arterial lumen, serious fibroplastic proliferation of arterial wall, wrinkled and discontinuous endarterium, hyperplasia and hypertrophy of smooth muscle cells. After pharmacological intervening, the vertebral artery showed the reduced stenosis of arterial lumen and less proliferation of fibrous tissue in the arterial wall (fig. 9C-E).

The morphological characteristics of nuclei vestibularis after pharmacological intervening was observed by TEM test. Mitochondria and nerve fibers with normal morphological characteristics were observed in the neural cells of nuclei vestibularis in the NC group (fig. 10A-C); mitochondrial edema, degenerated nerve fibers, cytoplasmic shrinkage, swelling, nuclear pyknosis, islocation and necrosis of neural cells of nuclei vestibularis in the MC group (fig. 10D-F); After pharmacological intervening, the degeneration of mitochondria, nerve fibers and neural cells was reduced in the LXYD, HXYD and YXQN group (fig. 10G-O).

DISCUSSION

Based on the TCM theory, vertigo is caused by a loss of nourishment in the upper orifices resulting from pathogenic wind, heat, phlegm, deficiency, or stasis and PCIV is a kind of vertigo characterized by Qi-deficiency and blood-stasis syndrome. In this study, we established a rabbit model of PCIV based on by feeding high-fat diet companied with paravertebral sclerotherapy and rotation exercise After modeling, the body weight of rabbits significantly decreased and the symptoms of Qi-deficiency and blood-stasis, including lackluster, sparse fur, less activities, and decreasing amount of feces, were observed. Moreover, the rabbits with PCIV exhibited decreased CBF, prolonged step-down time, abnormal BAEP and morphological changes of vertebral artery and neural cells in the nuclei vestibularis, which proved the success of PCIV modeling. The treatment of PCIV by Chinese herbal medicine, such as *Buyang Huanwu* decoction and *Yang-Xue-Qin-Nao* granules, has shown satisfactory efficacy in the clinical practice (Dzieciolowska-Baran and Gawlikowska-Sroka, 2015; Park *et al*., 2014). Based on our clinical experiences of treating PCIV by using Chinese herbs, we have created a new TCM prescription *Xuan-Yun-Ding* (XYD) which has been functionally characterized by Qi supplement and improving blood circulation. The main components of XYD were *Huangqi* (Radix Astragali seu Hedysari), *Gegen* (Radix Puerariae lobatae) and *Danggui* (Radix Angelica sinensis). Cao *et al*. reported that *Huangqi* could significantly increase activities of antioxidants and decrease the level of reactive oxygen species, which indicated its neuroprotective function and ameliorating effect on blood stasis (Cao *et al*., 2014). Liu *et al*. suggested that the anti-inflammatory effect and neuronal apoptosis inhibition of *Gegen* might be beneficial to protect cerebral hypoxia-ischemia injury and improve cerebral circulation (Liu *et al*., 2013). Kim *et al*. indicated that *Danggui* could ameliorate the neurological dysfunction of cerebral ischemia *via* improving antioxidant defense system and exhibited the neuroprotective effects (Kim *et al*., 2016).

In the present study, we found that XYD could ameliorate symptoms of Qi-deficiency and blood-stasis in the PCIV rabbits. Furthermore, the therapeutic effects of XYD on PCIV were investigated based on step-down test, REG, BAEP, blood tests of hemorheology, lipids, ET-1 and CGRP, histopathological examination. The decreased

step-down time after treating with XYD indicated that XYD could improve the equilibrium function and limb coordination. According to the results of REG, we found that the CBF of high-dose XYD treated rabbits significantly increased, indicating the positive effects of XYD on improving cerebral circulation. Similarly, Wu *et al*. reported that *Gegen* (Radix Puerariae lobatae) could increase the cerebral blood perfusion and improve the cerebral microcirculation (Wu *et al*., 2014). Subsequently, the BAEP results showed that the PL and IPL of XYD treated PCIV rabbits significantly decreased, proving that XYD could ameliorate the dysfunction of brainstem auditory pathway in PCIV rabbits. Qu *et al*. suggested that 200 mg/kg puerarin treatment significantly ameliorated the thresholds of auditory brainstem response of mice (Qu *et al*., 2015). Moreover, the results of blood tests further showed that XYD could decrease the blood viscosity, reduce the levels of TC, TG and LDL, decrease the level of ET-1 and increase the level of CGRP. Similarly, the therapeutic effect of puerarin on blood-stasis in rats was investigated and the results of hemorheology tests showed that puerarin could significantly reduce the blood viscosity and inhibit blood stasis by regulating metabolism (Zou *et al*., 2015). Bao *et al*. found that the levels of TC, TG and LDL-C were significantly lower in high-/middle-dose puerarin-treated groups than those in the model group feeding with high-fat diet (Bao *et al*., 2015). The amount and activity of vasoactive substances, ET-1 (vaso-excitor peptide) and CGRP (vaso-dilator peptide), maintained stability in the physiological state, however, at the ischemic state, the level of ET-1 expression significantly increased and the synthesis of CGRP which could simulate vasodilation and protect vascular endothelium would decrease (Homma *et al*., 2014). Zhao *et al*. reported that the puerarin not only increased the level of prostacyclin expression in cerebral tissue, but also reduced the activity of plasma plasminogen activator inhibitor and decreased the expression level of ET-1 in the cerebral tissue, indicating that puerarin possessed protective effects on the cerebral ischemia-reperfusion injury (Zhao *et al*., 2007). Furthermore, the histopathological examinations of vertebral artery proved that the atherosclerotic changes of PCIV rabbits in the XYD-treated groups significantly alleviated. The abnormal ultrastructure of neuronal cells in the nuclei vestibularis, including mitochondrial edema, degenerated nerve fibers, cytoplasmic shrinkage, swelling, nuclear pyknosis, islocation and necrosis, significantly reduced after treating with XYD, proving the neuroprotective effects of XYD on nuclei vestibularis. Finally, puerarin, the most abundant ingredient of *Gegen*, which was one of the main effective components of XYD, was investigated for detecting its blood concentration. The results showed that the blood concentrations of puerarin in the LXYD and HXYD group were 10.9±3.785 and 20.98±3.127 ng/mL, respectively and we suggested that puerarin might be the main effective component of XYD. However, further researches on the mechanism of protective effects of XYD on neural cells and vascular endothelial cell should be explored via *in vitro* experiments.

CONCLUSION

In summary, we suggested that XYD could ameliorate the symptoms of vertigo, Qi-deficiency and blood stasis in PCIV rabbits via effectively regulating the levels of blood lipids and vasoactive substances, decreasing blood viscosity, increasing CBF and protecting vestibular function. Moreover, puerarin might be the main effective component of XYD.

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