Original Article

Investigation on the Mode of Action of the Traditional Chinese Medical Prescription-Yiqihuoxue Formula, an Effective Extravasation Treatment for Cerebral Vascular Microemboli in ApoE-/- mice

Chao Jiang[a,b], Ting Wang[c], Zhong-Ju Xu[d], Xu Chao[e,f], Pierre Duez[g]

[a]Department of Neurology, The Second Affiliated Hospital of Xi'an Medical University, [c]Department of Management Science and Engineering, School of Economics and Management, Xidian University, [f]Department of Biochemistry, The College of Basic Medicine Sciences, Shanxi University of Chinese Medicine, Xi'an, [b]Department of Emergency, Longhua Hospital Shanghai University of Traditional Chinese Medicine, [d]Department of Preventive Treatment, Shanghai Guanghua Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai, [e]Department of Scientific Research, The Second Affiliated Hospital of Shanxi University of Chinese Medicine, Xianyang, China, [g]Unit of Therapeutic Chemistry and Pharmacognosy, University of Mons, Mons, Belgium

Abstract

Objective: The objective of this study was to investigate the mechanisms underlying anti-embolism and extravasational effects of traditional Chinese medical prescription YiqiHuoxue (YQHX) formula in ApoE-/-mice with cerebral vascular microemboli. **Materials and Methods:** An ApoE-/-mice model with microemboli was developed by infusing fluorescently labeled heterologous fibrin-rich microparticles into the internal carotid artery of ApoE-/-gene knockout male mice through the common carotid artery. Before microemboli injection, the animals were randomly divided into four groups of 10 animals, treated daily for 6 weeks by intragastric administration: The ApoE-/-control group (physiological saline, 0.2 mL/10 g/d), YQHX group (0.2 ml/10 g/d), clopidogrel group (3 mg/kg/d), and atorvastatin group (3 mg/kg/d); a further group was constituted of normal male C57BL/6J mice (with the same genetic background as ApoE-/-mice; normal control group; no treatment; microemboli injection). The mice in each microemboli group were divided into three subgroups, the 2-h, 24-h, and 72-h subgroups, corresponding to the time after microemboli injection. Two hours (or 24 h or 72 h) after microemboli injection, the changes in aortic intima and brain tissue were analyzed by histopathology, the amounts of fluorescent emboli being measured by fluorescence microscopy image analysis. Comparison points included the microemboli induced loss of aorta functions and pathological changes, atherosclerotic plaque, brain ultrastructure and functions, and embolus extravasation. **Results:** Loss of aorta functions and adverse pathological changes, atherosclerotic plaque, serious damage in brain ultrastructure and functions, and reduced thrombus elimination were obviously serious in microemboli injected ApoE-/-mice. These symptoms were significantly relieved by the YQHX pretreatment: (i) the ratio of thrombus accumulation was increased with a significant decrease in thrombus extravasation in ApoE-/-mice, while YQHX induced an increased thrombus extravasation; (ii) the degree of aortic intimal thickening and brain tissue structural disorders were significantly increased in ApoE-/-mice, but overtly inhibited in the YQHX group; (iii) YQHX restored cell viability and homeostasis in the brain; (iv) YQHX regulated the expression of pro- and anti-inflammatory cytokines in the aorta; and (v) YQHX reduced cortical nerve nuclei pyknosis, edema, liquefaction, and necrosis induced by brain hypoxia, especially in the 24 h and 72 h groups. **Conclusions:** These findings indicate that the protective effects of YQHX on the brain against microemboli-induced injury may be attributed to the activation of extravasation mechanisms, which are involved in the cerebrovascular injury pathway and constitutively important in the progression of ischemic stroke.

Keywords: Angelicae sinensis radix, Astragali radix, Carthami flos, Chuanxiong rhizome, Cinnamomi ramulus, extravasation, ischemic stroke, *Lumbricus*, microemboli, Paeoniae radix rubra, Persicae semen, *Salviae miltiorrhizae* radix et rhizome, Spatholobi caulis, Yiqihuoxue decoction

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Address for correspondence: Prof. Zhong-Ju Xu, Shanghai Guanghua Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai, 200052, China.
E-mail: xuzhongju531@163.com
Prof. Xu Chao,
The Second Affiliated Hospital of Shaanxi University of Chinese Medicine, Xianyang, 712000, China.
The College of Basic Medicine Sciences, Shaanxi University of Chinese Medicine, Xi'an, 712046, China.
E-mail: chaoxu2004@126.com

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Introduction

Ischemic stroke (IS) is a leading cause of long-term disability and death worldwide.[1-3] Because of the growth of the older global population, the incidence of IS has increased in recent decades; as this pathology and its consequences impose a considerable economic and family burden,[4] IS is very worthy of the attention of the whole society. IS contributes to a loss of brain function, mainly due to a reduction in cerebral blood flow. In a given therapeutic window, an overwhelming number of studies and clinical trials confirm the efficacy of thrombolytic therapy in improving the clinical outcome and recovery of patients with acute ischemic stroke (AIS). It seems particularly important to develop measures to prevent IS as early as possible. Microembolic signals (MES), detectable by transcranial Doppler monitoring, are associated with increased risk of first or recurrent IS. MES seem to detect a key factor in the pathogenesis and early recurrence of IS and their detection has been applied to the investigation of stroke etiology and eventual prophylactic treatments. Contrast enhancement (e.g., with sulfur hexafluoride, intravenous [IV] administration) is not necessary for MES detection, but it may be required for vascular examinations that are relevant in stroke diagnosis; such agents, however, contain microbubbles that could be confused with stroke-induced microemboli.[5] In 2010, the discovery of a new mechanism of cerebral microvascular recanalization and cerebrovascular clearance by embolus extravasation was rated by the American Heart Association/American Stroke Association as one of the ten major research advances in the field of stroke.[6] The modulation of such extravasation mechanisms is expected to provide clues for new primary or secondary prevention drugs in patients at risk of IS.

An overwhelming number of studies and clinical trials indicate that some Chinese herbs can act on complex diseases through multiple targets and levels, thanks to their multiple active ingredients.[7-9] In particular, Traditional Chinese Medicines (TCM) prescription drugs have historically been developed to significantly enhance synergies and broaden their application scopes; such complex drugs are increasingly investigated, yielding clues to original mechanisms of action.[10] In the prevention of IS, it is of great significance to improve hemorheology and to regulate coagulation, platelet function and fibrinolysis, thus inhibiting thrombosis and preventing ischemia-reperfusion injuries; some TCM compounds have been shown to play an important role in this field. In addition, an in-depth study of TCM intended for IS prevention/treatment would be interesting to research compounds promoting extravasation of cerebral vascular microemboli.

The Yiqihuoxue (YQHX) Decoction was designed[11] based on the well-known TCM formula Buyang Huanwu decoction (BYHWD). BYHWD, consisting of Astragali radix, *Lumbricus*, Chuanxiong rhizoma, Angelicae Sinensis radix, Paeoniae radix rubra, Carthami flos and Persicae semen, first described in 1830 A.D. by Qing-Ren Wang in Yilingaicuo, has been used for hundreds of years in China to *"nourish Qi"* and *"enrich Blood,"*, to prevent and treat or improve the recovery of neurological functions in stroke-induced disabilities.

Cerebrovascular microemboli seem to correspond to tangible (micro) particles that linger in blood vessels through cardiogenic or atherosclerotic plaque shedding, eventually leading to the formation of arterio-arterial emboli. Our previous clinical studies have found that YQHX can significantly improve the neurological function and living ability of IS patients positive for MES.[11] As IS is treated by the TCM method of *"invigorating qi and blood, transforming proclivity, and dredging collaterals"* with drugs known to alleviate so-called *"blood stasis"* and *"phlegm pool,"* we hypothesize that these TCM concepts may, at least in part, correspond to a clearance effect of these particles from cerebral vessels, improving cerebral blood circulation. Other effects, however, cannot be excluded, notably a reduction in intima injuries and so, whether the neuroprotective effect of YQHX on IS is related to regulation of cerebral vascular microemboli extravasation remains unclear. Therefore, we aimed to investigate the effects and mechanisms of YQHX on the extravasation of microemboli and its neuroprotective effects *in vivo*. This study will provide further insight into the mechanisms of complex TCMs in the prevention and treatment of IS.

Materials and Methods

Composition and preparation of Yiqihuoxue formula

The Yiqihuoxue (YQHX) Decoction was obtained from the Second Affiliated Hospital of Xi'an Medical University and the full components are Astragali radix (60 g), *Lumbricus* (20 g), Chuanxiong rhizome (12 g), Angelicae sinensis radix (15 g), Paeoniae radix rubra (12 g), Carthami flos (6 g), Persicae semen (6 g), Salviae *miltiorrhizae* radix et rhizome (12 g), Cinnamomi ramulus (6 g), Spatholobi caulis (12 g); in this formula, Astragali radix, and Angelicae sinensis radix are two major components. YQHX was boiled for 0.5 h with 500 mL ultrapure water and filtered on cellulose. In *in vivo* studies, 250 μl YQHX were fed to mice twice a day; this is equivalent to 0.25 g/10 g/d of the crude drug.

Construction of microemboli injected mice model

The preparation of fluorescence-labeled microemboli and subsequent surgical procedures were designed by referring to and improving on described animal model methods,[12] injecting allogeneic microparticles produced *in vitro*. To prepare autologous thrombi, blood was collected from the left ventricle of rats and completely dried in a drying box at 80°C. The dried blood clots were refined and made into emboli through 200 μm sieve holes. A suspension of 12,000 microemboli/mL in saline solution was mixed (30:1, v/v) with a 10 mg/mL stock solution of Texas Red®-X (succinimidyl ester), prepared in anhydrous dimethyl sulfoxide, and oscillated, for 4 h at room temperature, away from light. The mice, fasted for 12 h before the operation, could drink water *ad libitum*. They were anesthetized with 10% chloral hydrate intraperitoneally (350 mg/kg); in the supine position, an incision was made in the neck at the midline and

the left common carotid artery (CCA), internal carotid, and external carotid were exposed. The proximal end of the CCA was ligated to separate the external carotid artery (ECA) and the internal carotid artery (ICA). A disposable IV infusion needle No. 4.5 was inserted into the CCA between the two lines of the ICA, and 200 μL of the suspension containing the fluorescent microemboli were injected (injection time is more than 2 min); the distal end was ligated, the needle withdrawn and the ECA proximal ligation line loosened. The wound was sutured and slathered with erythromycin eye ointment.

Animals and grouping

Forty healthy 8-week-old male ApoE-/-mice weighing 18–20 g and 10 C57BL/6J mice with the same genetic background as ApoE-/-were obtained from the Laboratory Animal Center of Xi'an Jiaotong University, Xi'an, China. Animals were housed at room temperature, 22°C ± 1°C with a 12-h light-dark cycle, and fed standard diet and tap water *ad libitum*. Animals were randomly divided into four groups of 10 animals, treated daily for 6 weeks by intragastric administration: The ApoE-/-model group (physiological saline, 0.2 mL/10 g/d), YQHX group (0.2 mL/10 g/d), clopidogrel group (3 mg/kg/d), and atorvastatin group (3 mg/kg/d); a further group was constituted of normal male C57BL/6J mice (normal control group; no treatment; microemboli injection). Each group was again divided into three subgroups: 2 h, 24 h, and 72 h. The experimental design and drug administration plan are depicted in Figure 1. At specified time points, mice were killed by cervical dislocation for subsequent analyses.

Evaluation of aortic pathology

After 6 weeks pretreatment, one mice in each group was perfused with normal saline and fixational fluids (4% paraformaldehyde) through the heart; the whole aorta was stripped from the base of heart, fixed with 4% paraformaldehyde, embedded into paraffin, sliced using a microtome at a thickness of 5 μm and stained with hematoxylin and eosin. The intima, adventitia, and plaque changes were observed under light microscope and quantitatively analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc. 1700 Rockville Pike, Suite 240 Rockville, MD 20852 USA).

Evaluation of brain tissue

Mouse brain tissues were isolated over ice and fixed in 4% paraformaldehyde for 48 h. Serial coronal sections (4 μm in thickness) at the level of the ischemic penumbra were prepared for hematoxylin and eosin staining and further histological and immunohistochemical analysis. Cells with high degrees of eosinophilia and pyknotic nuclei were considered as necrotic neurons. A necrotic index was calculated as necrotic neuron numbers per 100 neurons under a ×40. Immunohistochemistry staining was performed according to the manufacturer's instruction with primary and secondary antibodies antibodies (Texas Red®-X, succinimidyl ester, Life Technologies, T6134); Lectin from *Lycopersicon esculentum* (FITC conjugate, Sigma-Aldrich, L0401).

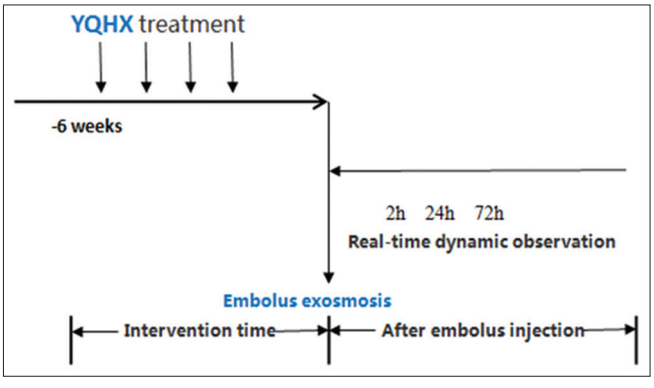

Figure 1: Schematic diagram of experimental design

Quantitative analysis of microemboli extravasation

Microemboli extravasation was determined at ultra-early, acute, and subacute stages. Two, 24, and 72 h after 200 μL fluorescence-labeled fibrin thrombus were injected into the brain through the ICA, and the brain tissue was fixed with 4% paraformaldehyde for 12 h, precipitated in 30% sucrose solution, frozen, sliced at a thickness of 50 μm, and observed by fluorescence microscopy with excitation wavelength at 596 nm. (Multi-function enzyme labeling instrument [Tecan, M1000pro], laser confocal microscope [Zeiss, LM710]). Five representative sections were sampled from roughly the same brain part of three animals from each group; for each slice, three nonoverlapping visual fields were randomly selected and on the same area, the number of fluorescent particles was counted.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Differences between control and experimental groups were analyzed using one- or two-way analysis of variance followed by Levene's test, depending on homoscedasticity, Fisher's Least Significant Difference test (homogeneous variances), or Games-Howell test (nonhomogeneous variances) were applied. $P < 0.05$ was accepted as statistically significant. All the calculations were performed using the SPSS 20.0 (IBM, 1 New Orchard Road, Armonk, New York, United States).

Results

Observation of fluorescence-labeled microemboli

Before injecting in mice, we observed and quantified the fluorescent microemboli, following the same procedure as for the analysis of extravasation. This allowed to standardize the injected microemboli, an important experimental parameter [Figure 2]. A suspension corresponding to 12,000 microemboli/mL, size ranging from 8 μm to 20 μm, was used for 200 μL injections.

Observation on the general situation after microemboli injection

After injection, there was no significant difference in body weight among the five groups [ANOVA, $n \leq 10$, $F \leq 0.623$, $P > 0.05$, Figure 3]. One mouse in the model control group died in the 2nd week of feeding for unknown reasons. In the

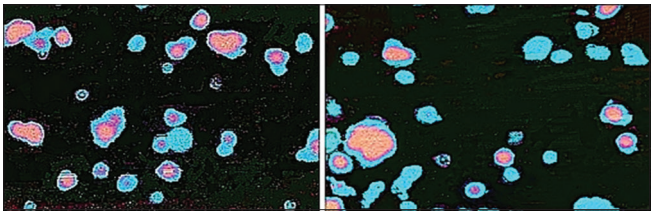

Figure 2: Observation of labeled emboli under fluorescence microscope (excitation wavelength 596 nm, ×100 times)

YQHX group, one mouse developed mania symptoms and died. The successful rate of operation was 97.9%. Three mice (2 in the atorvastatin group and 1 in the YQHX group) showed a decrease in active diet after the operation. One mouse had the Horner's sign of the eye in the blank group, and a contralateral anterior limb flexion when lifted by the tail. Because of the small number of subjects, it is unclear whether pretreatments or operation parameters are at cause.

Histological analysis of aorta and brain

As shown in Figure 4a, 6 weeks pretreatment resulted in the mice of the ApoE-/-control group into a high number of plaque tissue, foam cells gathered, thickened aortic intima, incomplete endothelium, unclear intimal structure, compared to the normal control group (blank group). By contrast, in ApoE-/-mice pretreated with YQHX, clopidogrel, and atorvastatin, the aortic intima presented significantly reduced foam cells, plaque tissue, and intracellular fat vacuoles, suggesting effective preservation of the intima; interestingly, the YQHX pretreatment had the most significant protective effect [Table 1, $P < 0.05$].

Regarding the structure of brain tissue, the mice of the ApoE-/-control group showed significantly higher pyknosis of neurons in hippocampal regions 1 and 4 and dentate gyrus, compared with the normal control group, and accompanied by moderate cortical edema [Figure 4a]. The 6 weeks of atorvastatin, clopidogrel, and YQHX pretreatments could improve the pyknosis and degeneration of hippocampal neurons in areas 1 and 4; here also, the YQHX pretreatment was more efficient ($P < 0.05$) Figure 4b. These results suggest that a YQHX pretreatment may play a neuroprotective role toward the integrity of periventricular and hippocampal tissues.

Quantitative analysis of microemboli extravasation after ischemic brain injury

As shown in Table 2 and Figure 4, there was a slight amount of microemboli extravasation 2 h after injection; interestingly, extravasation significantly increased between 24 and 72 h, comparatively with the increase observed between 2 and 24 h [Figure 5]. Curiously, microemboli extravasation was the highest in the normal control group (ApoE+/+) and the lowest in the ApoE-/-/control group; there is no obvious explanation to this finding, but this is probably due to the difference in ApoE expression.

The rate of microemboli extravasation increases with time among the five different groups [Figures 6 and 7], and there

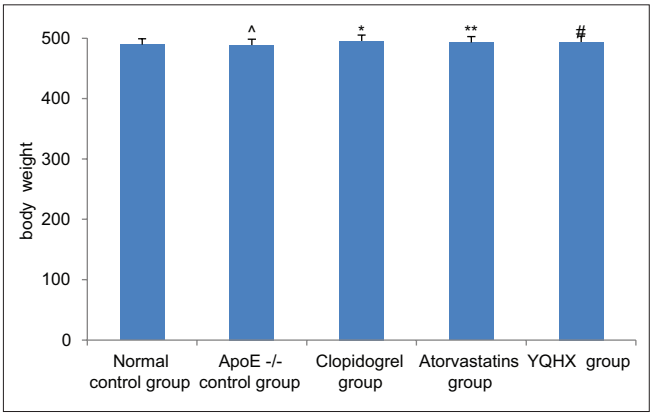

Figure 3: Comparisons of the body weights in all groups after microemboli injection. Data are expressed as (mean ± standard deviation; $n = 9$). $^{\wedge}P > 0.05$, $^{*}P > 0.05$, $^{**}P > 0.05$ versus control; $^{\#}P > 0.05$ versus normal

Table 1: Comparison of the relative area of aortic plaques according to mice pre-treatment ($\bar{x}$±standard deviation)

Group	*n*	Patch area	*P*
ApoE -/- control	10	36.2±5.3*	
Atorvastatin group	10	21.5±4.3*	0.046
Clopidogrel group	10	20.1±6.4*	0.043
YQHX group	45	14.3±4.6*,#	0.031

Compared with the ApoE-/-control group, the degree of aortic plaque in all groups was significantly reduced (*P<0.05), but the plaque area was significantly improved after YQHX intervention (#P<0.05). YQHX: YiqiHuoxue formula

Table 2: The number of microemboli extravasated at different time points (2 h, 24 h, 72 h) after injection

Group	Number of visual fields	2 h	24 h	72 h
Normal control	45	11.2±3.6	24.0±6.5	51.9±6.2
ApoE -/- control	45	6.3±1.6*	12.3±4.1	34.8±5.1#
Atorvastatin	45	7.1±2.3*	17.0±4.6**	40.0±5.2#
Clopidogrel	45	7.1±3.1*	23.4±5.3**	41.4±6.0#
YQHX	45	6.5±3.2*	20.4±6.2**	41.9±6.5#

Data were expressed as mean±SD (n=3). *P<0.05 versus normal control; **P<0.05 versus ApoE -/- control group, #P<0.05 versus YQHX group. SD: Standard deviation, YQHX: YiqiHuoxue formula

was a significant difference in embolic extravasation at 24h compared to 2h (*P<0.05), and there was a significant difference in embolic extravasation at 72h compared to 24h (**P<0.05), and the amount of YQHX group extravasation was more obvious compared with other groups (#P<0.05).

Discussion

IS is characterized by the reduction of blood supply to the brain, leading to a series of events such as neuronal damage and reperfusion injury, all of which lead to often severe neurological deficit. Therefore, the research for effective IS preventive and therapeutic options are intense. During normal physiological

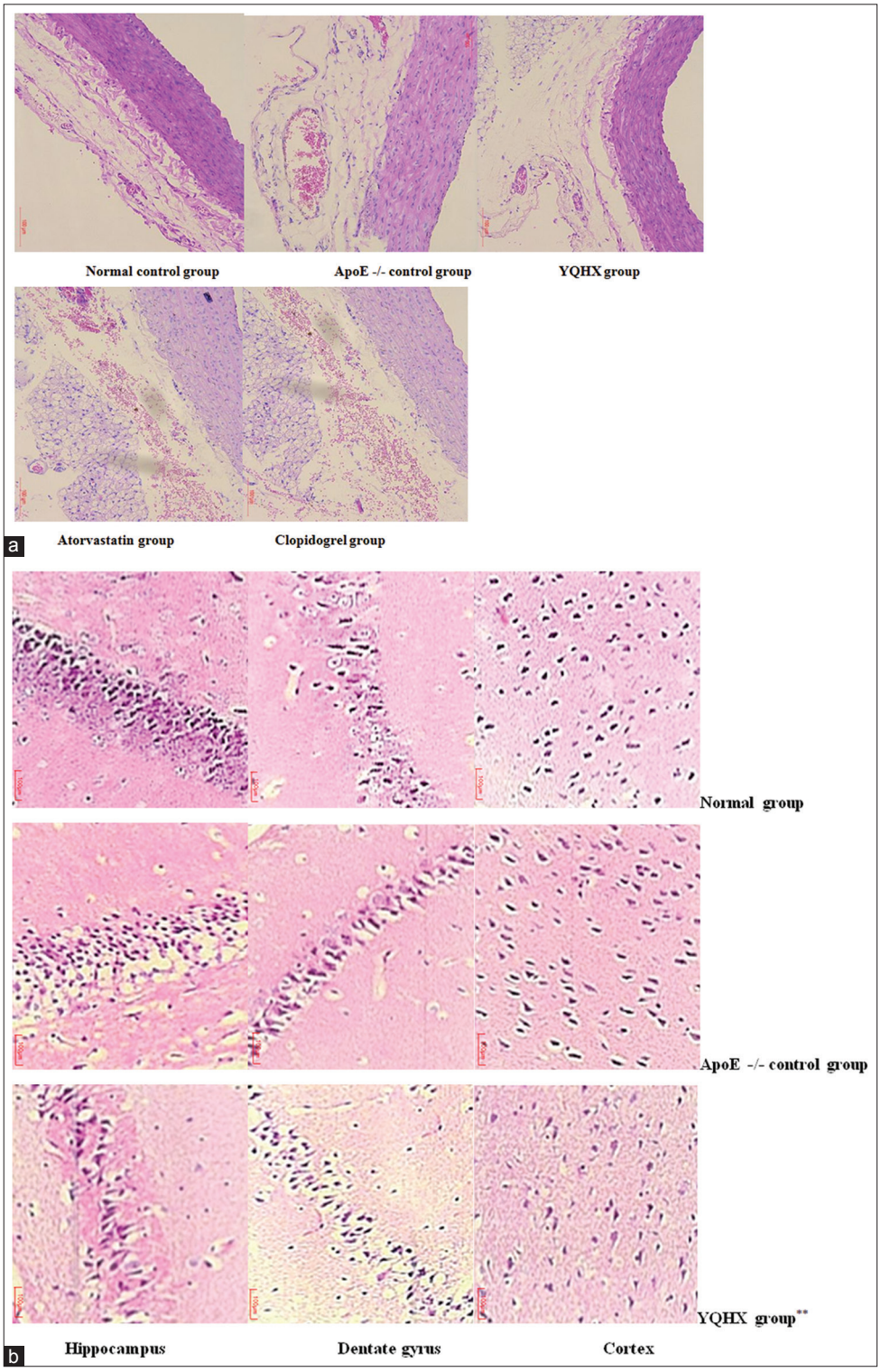

Figure 4: YiqiHuoxue improved aortic intima and brain tissue ultrastructures. Scale bars = 100 μm. (a) Effect of atorvastatin, clopidogrel and YiqiHuoxue pretreatments on aortic intima ultrastructures. (b) Effect of YiqiHuoxue pretreatment on the brain tissue ultrastructures. **indicates the YQHX pretreatment was more efficient compared to the positive control group and the normal group ($P < 0.05$).

conditions, cerebral functions and viability are dependent on uninterrupted blood flow through the microvasculature, yielding adequate oxygen, and glucose delivery.[11] Robust mechanisms must have evolved to ensure microvascular patency. Many types of animal models of focal cerebral ischemia, in which thromboembolic model may be a good simulation of the whole process of human cerebral ischemia, have been proposed to evaluate the efficacy of potential therapeutic agents, notably

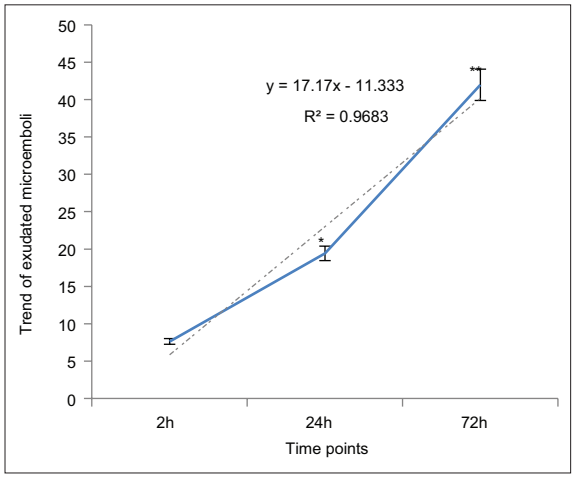

Figure 5: The changing trend of microemboli extravasation with time. Note: Data were expressed as mean ± standard deviation. *$P < 0.05$ versus 2 h; **$P < 0.05$ versus 24 h

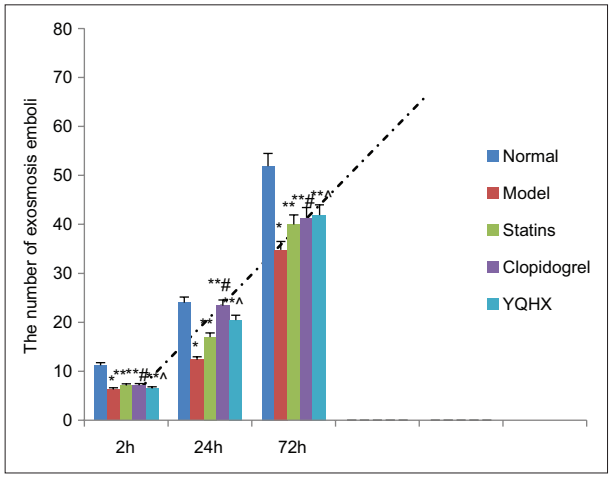

Figure 6: Comparison of microemboli extravasation between different groups according to postinjection time

thrombolytics. Microemboli, microparticles that contain lipids, fibrins and various cellular components, most often caused by cardiogenic or atherosclerotic plaque shedding, are trapped in the blood vessels. As these microemboli cause mild and transient damage, the brain seems to have evolved effective extravasation mechanisms that clear these microsized particles from the vessels; quite recently, it has been confirmed that microemboli extravasation may be a very important mechanism for cerebral microvascular recanalization.[12,13] The search for therapies or compounds that could enhance extravasation is an attractive prospect to develop strategies that protect the neuronal tissue and functions. During ischemia, microvascular embolization determines to a large extent the degree of local ischemia after macrovascular occlusion, with a series of damages, including oxidative stress and aggravated neural necrosis, often leading to severe brain lesions. The combination in an animal model of focal cerebral ischemia and microparticles appears as an interesting set-up for in-depth study of microemboli clearance, which could be of great significance for the treatment and prevention of IS.

The concepts of focal cerebral ischemia and microemboli are not specifically described in TCMs, but some of their clinical manifestations can be ascribed to TCM syndromes, notably *"blood turbidity," "stasis syndrome," "chest arthralgia," "stroke," "vertigo," "withered."* Similarly, the TCM *"apoplexy,"* which is located in the brain, and caused by aging, *"deficiency of Qi," "blood stasis,"* and *"obstruction of the choroid,"* is consistent with the pathological process of "cerebral atherosclerosis-microemboli formation" recognized by Western medicine at present. The TCM theory also holds that IS is a disease caused by *"blood stasis"* and *"deficiency of Qi,"* characterized by *"poor operation of Qi and blood"* and *"loss of nourishment of muscles and veins due to internal emotional injury," "loss of coordination between work and escape,"* improper diet, climate change and so on. TCM treatment principles will aim at *"invigorating Qi," "dredging collaterals," "activating blood circulation"* and *"removing blood stasis."*

The TCM formula BYHWD, representative of prescriptions *"invigorating Qi and activating blood circulation,"* has been preconized in China for hundreds of years to improve the recovery of neurological function in stroke-induced disabilities.[14] The neuroprotective mechanisms underlying this classic TCM formula include the stimulation of neural proliferation, the modulation of AKT1 mRNA and JNK1/2 expression levels, alterations in Ca^{2+} levels, and a selective decrease of some metabolic glutamic acid receptor-1 RNA.[15,16] Many studies have reported that BYHWD was neuroprotective in cerebral ischemic models; notably, intragastric administration of BYHWD in Wistar rats, after cerebral ischemia-reperfusion, reduces the infarct volume of brain tissue, inhibiting local malondialdehyde formation and tumor necrosis factor-a, interleukin (IL)-1 β, IL-6 secretion.[17] BYHWD seems clinically well tolerated[18] and in a Chinese clinical trial,[17] its safety has been confirmed on patients with acute cerebral ischemia. Nevertheless, despite its clinical and laboratory effectiveness, it is difficult to draw a definite conclusion on BYHWD safety.

Adhering to the knowledge of ancients rather than sticking to their viewpoints, our group proposes a new compound prescription, YQHX; highlighting the role of *"tonifying Qi and activating blood circulation,"* S. *miltiorrhizae* radix et rhizoma, Cinnamomi ramulus, and Spatholobi caulis were added to BYHWD, taking into account the following reasons:

1. According to the TCM theory, the roots of Salvia miltiorrhiza have the function of "promoting blood circulation and removing blood stasis," "dredging menstruation and relieving pain," "clearing the heart and removing annoyance," "cooling blood and eliminating carbuncle." Zhang Xuewen,[19] a master of Chinese medicine, preconized a series of combinations of *Salvia miltiorrhiza* (i) with *Cinnamomum cassia*, for "running blood and dredging

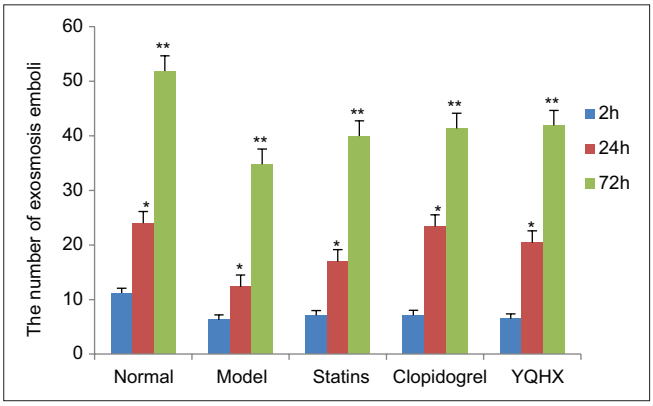

Figure 7: Changes of microemboli extravasation at different time points according to experimental group. Note: *$P < 0.05$ versus the number of extravasated microemboli at 2 h; **$P < 0.05$ versus the number of extravasated microemboli at 24 h

yang;" (ii) with *Astragalus membranaceus* for "invigorating Qi and rescuing emergency;" (iii) with *Angelica sinensis* for" activating blood, not hurting blood, tonifying blood, removing blood stasis"

2. The stems of *Spatholobus suberectus* are indicated for *"activating blood circulation and soothing tendons,"* and *"regulating menstruation."* TCM master Ban Xiuwen[18] preconized that its main therapeutic application is to *"replenish blood and treat deficiency and skillfully treat blood stasis"*
3. The twigs of *C. cassia "induce sweating and relieve muscle, warming meridians, aiding Yang and removing Qi."* The Huangqi Guizhi Wuwu decoction[20,21] has been shown to significantly improve a series of clinical symptoms, notably in diabetic peripheral neuropathy, cerebrovascular and cardiovascular diseases
4. Modern pharmacological studies have shown that the above three drugs also have positive effects on vascular endothelium repair, platelet aggregation inhibition, atherosclerosis, coronary artery dilatation, microcirculation, inflammation and on blood lipid levels, oxidation and metabolic imbalance.[22-25]

In developing this new compound formula, our group assumes that YQHX, the combination of these three drugs with BYHWD, may significantly *"promote blood circulation and remove blood stasis,"* in accordance with the multiple beneficial effects of *"invigorating Qi and blood"* known from clinical and experimental studies of IS. Our previous studies have shown that YQHX is clinically effective on IS patients with positive MES, significantly reducing the number of intracranial MES in patients with AIS, improving the clinical symptoms and prognosis of patients.

In the present study, we investigate for the first time the effects of YQHX on the extravasation of microemboli from ischemic brains.

In our ApoE-/-mice model, histological parameters affected by brain infarction were improved by YQHX and other

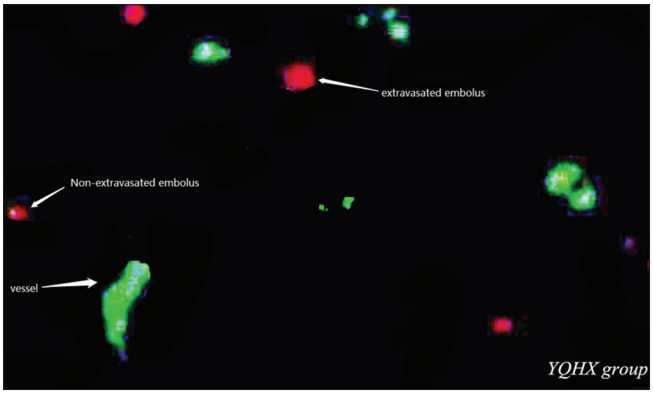

Figure 8: YiqiHuoxue promotes extravasation of microemboli

tested treatments [Figure 4b]. *In vivo*, using fluorescence double-labeling technique (simultaneous labeled microemboli particles and cerebral vessels) and two-photon transmission electron microscopy [Figure 8], we carried out dynamic observation of microemboli extravasation, indicating that the degree of neurological damage in mice is inversely related to the number of particles extravasation, this process being faster along postinfarction time [Figures 5-7].

The intima hyperplasia, endothelial cell structure disorder, foam cell aggregation and atherosclerotic plaque formation in ApoE-/-model mice were affected by pretreatments with atorvastatin, clopidogrel and YQHX, correlating with enhanced extravasation and clearance of microemboli. The same was observed for the pathological changes in hippocampus, dentate gyrus, and cortex [Figure 4].

Although only a few animals (2.1%) were adversely impacted by pretreatment and/or surgery, the exact reason remains uncertain. Whether the removal ability of emboli is different due to different methods of operation or intervention remains to be verified by further experiments. Given that the success rate of the mouse ICA fluorescence microprobe injection model is high, with a low occurrence of animal injury and death, and that the instruments needed for the experiment are easy to obtain, this ApoE-/-microemboli model is a simple model to study the clearance effect of cerebral vessels on microemboli.

The positive effects of YQHX on microemboli clearance are obvious. The reasons may be that the compound prescription is reasonable, the composition is complex, and the effective parts could have synergetic effects, with many targets of action. Nevertheless, the exact mechanism of microemboli extravasation needs to be further studied to elucidate the exact role of YQHX components, chemical compounds, eventual synergies, and therapeutic targets.

Conclusions

The present findings provide some preliminary evidence that microemboli can be cleared in brain ischemic injury, which should undoubtedly be beneficial to the recovery of brain function. Firstly, we confirmed that cerebral microemboli

extravasation is a process affected by many factors, resulting in limited emboli clearance, unblockage of cerebral vessels, and a series of behavioral changes. Second, the complex composition of YQHX comprises constituents inferred or shown to be beneficial for the treatment of ischemic cerebrovascular diseases. YQHX treatment exerts cerebral vessel protective effects in ischemic conditions both *in vivo* and vitro. YQHX treatment promotes the microemboli extravasation in IS mice and improves vascular endothelial function. *In vitro*, YQHX has a pharmacological potential in the treatment of brain ischemic injury by improving the damage of cerebral neurons induced by ischemia and hypoxia. These findings correlate with our previous study on the regulation of MES in patients treated by YQHX.[11] Therefore, the protective mechanism of YQHX on the brain may be due to the regulation of microemboli extravasation, a mechanism that is worthy of further studies.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

Authors' contributions

All authors contributed to this study. CJ, CX and ZJ X designed the study. CJ and ZJ X carried out the *in vitro* study and its corresponding molecular studies. CJ and CX carried out the animal study and its corresponding molecular studies, as well as histopathological examinations. TW and DP contributed to the interpretation and statistical analysis of the data; JC, TW and DP wrote and revised the manuscript. All authors read and approved the final manuscript.

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Conflicts of interest

There are no conflicts of interest.

References

1. GBD 2017 Disease and Injury Incidence and Prevalence Collaborators. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: A systematic analysis for the Global Burden of Disease Study 2017. Lancet 2018;392:1789-858.
2. Sun S, Stewart JD, Eliot MN, Yanosky JD, Liao D, Tinker LF, *et al*. Short-term exposure to air pollution and incidence of stroke in the Women's Health Initiative. Environ Int 2019;132:105065.
3. Alkhouli M, Alqahtani F, Tarabishy A, Sandhu G, Rihal CS. Incidence, predictors, and outcomes of acute ischemic stroke following percutaneous coronary intervention. JACC Cardiovasc Interv 2019;12:1497-506.
4. Fekadu G, Chelkeba L, Kebede A. Risk factors, clinical presentations and predictors of stroke among adult patients admitted to stroke unit of Jimma University Medical Center, South West Ethiopia: Prospective observational study. BMC Neurol 2019;19:187.
5. Aarli SJ, Novotny V, Thomassen L, Kvistad CE, Logallo N, Fromm A. Persistent microembolic signals in the cerebral circulation on transcranial Doppler after intravenous sulfur hexafluoride microbubble infusion. J Neuroimaging 2019;00:1-4.
6. Lam CK, Yoo T, Hiner B, Liu Z, Grutzendler J. Embolus extravasation is an alternative mechanism for cerebral microvascular recanalization. Nature 2010;465:478-82.
7. Liao CC, Li JM, Chen CH, Lin CL, Hsieh CL. Effect of *Paeonia lactiflora*, a traditional Chinese herb, on migraines based on clinical application and animal behavior analyses. Biomed Pharmacother 2019;118:109276.
8. Pu ZH, Peng C, Xie XF, Luo M, Zhu H, Feng R, *et al*. Alkaloids from the rhizomes of *Ligusticum striatum* exert antimigraine effects through regulating 55-HT receptor and c-Jun. J Ethnopharmacol 2019;237:39-46.
9. Shan CS, Xu QQ, Shi YH, Wang Y, He ZX, Zheng GQ. Chuanxiong formulae for migraine: A systematic review and meta-analysis of high-quality randomized controlled trials. Front Pharmacol 2018;9:589.
10. Xu Q, Bauer R, Hendry BM, Fan TP, Zhao Z, Duez P, *et al*. The quest for modernisation of traditional Chinese medicine. BMC Complement Altern Med 2013;13:132.
11. Jiang C, Wang T, Hou Q, Li HP, Sang CN, Yin Z, *et al*. Clinical observation of Yiqi Huoxue granule in intervention of microemboli positive patients with acute ischemic stroke[J]. J Cardiovasc Cerebrovasc Dis Integr Tradit Chin West Med 2019;17:2588-90.
12. Wu Y, Bian L, Ni X, Ning M, Zhao Y, Ling R. A mechanistic investigation into non-infarcted brain injury induced by cerebral artery microemboli. Mol Biol Rep 2013;40:1283-90.
13. van der Wijk AE, Lachkar N, de Vos J, Grootemaat AE, van der Wel NN, Hordijk PL, *et al*. Extravasation of microspheres in a rat model of silent brain infarcts. Stroke 2019;50:1590-4.
14. Grutzendler J, Murikinati S, Hiner B, Ji L, Lam CK, Yoo T, *et al*. Angiophagy prevents early embolus washout but recanalizes microvessels through embolus extravasation. Sci Transl Med 2014;6:226ra31.
15. van Veluw SJ, Shih AY, Smith EE, Chen C, Schneider JA, Wardlaw JM, *et al*. Detection, risk factors, and functional consequences of cerebral microinfarcts. Lancet Neurol 2017;16:730-40.
16. She Y, Shao L, Zhang Y, Hao Y, Cai Y, Cheng Z, *et al*. Neuroprotective effect of glycosides in Buyang Huanwu Decoction on pyroptosis following cerebral ischemia-reperfusion injury in rats. J Ethnopharmacol 2019;242:112051.
17. Li P, Tang T, Liu T, Zhou J, Cui H, He Z, *et al*. Systematic analysis of tRNA-derived small RNAs reveals novel potential therapeutic targets of Traditional Chinese Medicine (Buyang-Huanwu-Decoction) on intracerebral hemorrhage. Int J Biol Sci 2019;15:895-908.
18. Yang P, Chen A, Qin Y, Yin J, Cai X, Fan YJ, *et al*. Buyang Huanwu Decoction combined with BMSCs transplantation promotes recovery after spinal cord injury by rescuing axotomized red nucleus neurons. J Ethnopharmacol 2019;228:123-31.
19. Li JH, Liu AJ, Li HQ, Wang Y, Shang HC, Zheng GQ. Buyang Huanwu Decoction for healthcare: Evidence-based theoretical interpretations of treating different diseases with the same method and target of vascularity. Evid Based Complement Alternat Med 2014;2014:506783.
20. Hao CZ, Wu F, Shen J, Lu L, Fu DL, Liao WJ, *et al*. Clinical efficacy and safety of Buyang Huanwu Decoction for acute ischemic stroke: A systematic review and meta-analysis of 19 randomized controlled trials. Evid Based Complement Alternat Med 2012;2012:630124.
21. Guo J. Summary of Zhang Xuewen's experience in using Salvia miltiorrhiza. Shandong J Tradit Chin Med 2010;29:712-3.
22. Zhang WF, Dai M, Zhang LL, Liu QX, Lin CZ, Yu LL, *et al*. Ban

Xiuwen, a master of Chinese medicine, used Tripterygium wilfordii to treat gynecological diseases. Glob Tradit Chin Med 2018;5:784-6.
23. Han ZY, Tian M, Liu Z, Lu JQ, Ning Y. Present situation of pharmacological research on Huangqi Guizhi Wuwu decoction. Heilongjiang Med 2013;26:777-9.
24. Li Y, Song X, Zhang G. Effect of Huangqi Guizhi Wuwu decoction combined with acupuncture on deficiency of mental meridians and activity of daily living in patients with acute ischemic stroke. J Integr Tradit Chin West Med Cardiovasc Cerebrovasc Dis 2019;17:43-6.
25. Guan J, Feng X, Liu J. Advances in pharmacological effects of *Tripterygium wilfordii* on prevention and treatment of arteriosclerosis. New Drugs Tradit Chin Med Clin Pharmacol 2019;3:385-9.