Protection of Effective Component Group from Xiaoshuan Tongluo on Brain Injury after Chronic Hypoperfusion in Rats

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Abstract: **Objective** To investigate the protective effects of purified effective component group in extract from Xiaoshuan Tongluo (CGXT) formula on chronic brain ischemia in rats. **Methods** CGXT 75, 150, and 300 mg/kg or vehicle were ig administered daily for four weeks to rats with bilateral common carotid arteries ligation (BCCAL). From the day 24 to 28 after BCCAL, Morris water maze was performed to assess the learning and memory impairment of rats. Four weeks after BCCAL, brain gray and white matter damage were assessed. **Results** In Morris test, the mean escape latency of rats in the CGXT (150 and 300 mg/kg) groups was significantly shorter than that in the vehicle group. CGXT also attenuated the neuronal damage in hippocampus and cortex and reduced the pathological damage in the optic tract and corpus callosum. **Conclusion** CGXT could improve learning and memory impairment resulted from BCCAL in rats. These results provide the experimental basis for the clinical use of CGXT in stroke treatment and may help in investigation of multimodal therapy strategies in ischemic cerebrovascular diseases including stroke.

Key words: effective component group in extract from Xiaoshuan Tongluo formula; learning and memory; Morris water maze; stroke; white matter damage

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Introduction

Over the last decade, accumulating studies demonstrated that cerebral white matter (WM) was vulnerable to ischemic injury, the neurological consequences of ischemic insult reflected the influence of stroke on both gray matter (GM) and WM (Alix, 2006; Pantoni, 2006), and that stroke was not only a degeneration of neurons but also a cerebral disease (Young *et al*, 2007). The concept that not only neurons, but also WM including astrocytes, oligodendrocytes, microglia, and all entities affected by cerebral ischemia should be considered in the development of innovative strategies of stroke therapy and have attracted more and more attention (Young *et al*, 2007). Therefore, the strategies of whole brain protection are proposed and multimodal drugs targeting multi-events are required (Green and Shuaib, 2006). In addition, traditional herbal medicines will be important candidates for their integrated concept of treatment.

In China, there has been accumulated fruitful clinical experience on traditional Chinese medicine (TCM) in treating stroke over the past thousands of years, and it is believed that TCM achieves their sufficient effects through possible mechanisms known as multiple components directing to multiple patho-physiological events (Gong and Sucher, 1999). Xiaoshuan Tongluo (XSTL) is a famous traditional Chinese herbal formula which has been widely used in China for stroke treatment and included in the *Chinese Pharmacopoeia* since 1990. It consists of 11 crude herbs and has many effects such as antithrombosis, antiplatelet aggregation, and anti-oxidation. In this herbal formula, some bioactive constituents have been previously identified. These include quercitin, ginsenoside

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Rb_1, ginsenoside Rg_1, and natoginsenoside R_1. And recently, effective component group from Xiaoshuan Tongluo (CGXT) formula was obtained using advanced pharmaceutical extraction and purification technologies (Zhang *et al*, 2006).

In the present work, we investigated the effects of CGXT on learning and memory impairment after chronic hypoperfusion, and then the protective effects of CGXT on GM and WM damage induced by chronic hypoperfusion in rats.

Materials and methods

Preparation of CGXT from herbal formula

CGXT was prepared as described before (Zhang *et al*, 2006) and its chemical consistency was verified with the HPLC fingerprint analysis (Fig. 1).

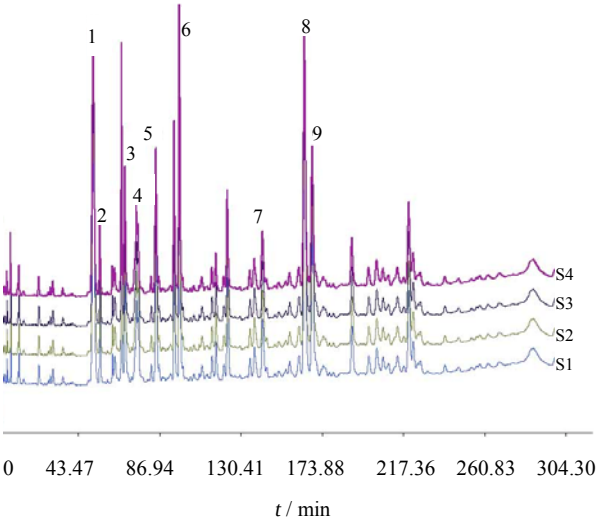

Fig. 1 HPLC fingerprints for four batches of CGXT

3: rutin 4: salvianolic acid B 7: ligustilide 8: dihydrotanshinone I 9: isoalantolactone

The HPLC fingerprint analysis was conducted with an Alltech C_{18} (150 mm × 4.6 mm, 5 μm) on an Agilent HP 1200 series LC system consisting of a G1310A pump, a G1322A Degasser, a G1315D Diode-Array Detector, and an SPD CTO—10As^{VP} column oven using acetonitrile (A) and solvent (B) (0.05 mol/L dibasic sodium phosphate, adjusted with phosphoric acid to pH 3.5) as mobile phase in a gradient elution matter (percentage of A: 0－15 min 5%, 15－280 min 5%－70%, 280－300 min 70 %) at a flow rate of 1.0 mL/min. Detection was performed at 210 nm at 30 ℃.

Experimental protocol

Animal care and treatment procedures conformed to *Guidelines of the Laboratory Animal Center of the Chinese Academy of Medical Sciences* (Beijing, China). Adult male Sprague-Dawley rats (280－320 g) were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and maintained on a 12 h light-dark cycle with free access to food and water. Rats were randomly allocated into five groups (12 animals per group) after acclimating for one week: CGXT treatment groups with ig administration of 75, 150, and 300 mg/(kg·d) CGXT after bilateral common carotid arteries ligation (BCCAL); the vehicle group: rats of this group underwent BCCAL but were daily ig administrated with identical volume vehicle (1% Tween-80); the Sham group: the rats underwent same surgical protocol except for BCCAL. For BCCAL, animals were anesthetized with pentobarbital-sodium (50 mg/kg, ip) and the bilateral common carotid arteries were carefully separated from the cervical sympathetic and vagal nerves and ligated permanently through a midline incision. Spatial learning and memory were assessed in all rats on consecutive 5 d from the day 24 after BCCAL. On the day 28 after BCCAL, all rats were euthanized and the brain was dissected out immediately and sectioned for subsequent analyses.

Morris water maze (MWM) test

The MWM test was performed to evaluate BCCAL-related learning and memory deficits using the method described previously with minor modification (Vorhees and Williams, 2006). Briefly, in a 150 cm diameter circular pool filled with 30 cm deep water, a circular transparent acrylic platform was prepared, the top surface of it was 3 cm below water. Rats were released facing the wall, and the time from start to the hidden platform was recorded as the escape latency. Tests were performed on consecutive 4 d from day 24 after surgery to day 27. And a probe test, in which the hidden platform was removed, was conducted on the day 28 after BCCAL. The rats were released into water from the opposite quadrant with respect to the training one. The rats were allowed to swim for 60 s in the pool before they were removed from water by hand.

Histological analysis

After the behavioral experiments, four rats per group were deeply anesthetized with ip pentobarbital-sodium (60 mg/kg), and perfused transcardially with saline followed by 4% polyoxymethylene in 0.1 mol/L phosphate-buffer saline (PBS, pH 7.4). Brains were

removed, post-fixed in 4% paraformaldehyde for 4 h and then cryoprotected in 30% sucrose in PBS. Brain tissues were then cut into 5 μm thick coronal sections on a sliding microtome. Brain tissue sections were stained with hematoxylin and eosin (H & E) or Klüver-Barrera for pathological examination. The severity of the WM lesions was graded as normal (grade 0), a disarrangement of the nerve fibers (grade 1), the formation of marked vacuoles (grade 2), and the disappearance of myelinated fibers (grade 3) (Wakita *et al*, 2003).

Statistical analysis

All values are expressed as $\bar{x} \pm s$. Performance in the learning trials of MWM was assessed by mean escape latency (time to reach platform) and the data were analyzed using repeated measures and multi-variate analysis of variance (ANOVA) process of the general linear models (GLM) in SPSS 13.0 and giving comparison among different groups. One-way ANOVA was used to analyze the probe trial data (target quadrant spent time). $P < 0.05$ was considered significant.

Results

Improvement of learning and memory

In the MWM test, almost all of the rats could not find the hidden platform within 60 s in the first day of learning trials (Fig. 2A). It suggested that the groups did not start the test at different performance levels. During the four-day learning trials, the latency became more and more short and the latency of the rats in the vehicle-treatment group was significantly longer than that in the Sham-operated rats (Fig. 2B). Administration of 150 and 300 mg/kg, but not 75 mg/kg, of CGXT significantly improved spatial learning in the rats with BCCAL for four weeks. As shown in Fig. 2C, the escape latency of animals treated with 150 or 300 mg/kg CGXT was significantly shorter than that of vehicle-treatment animals on the learning days 3 and 4 ($P < 0.05$). The latency of 75 mg/kg CGXT-treatment

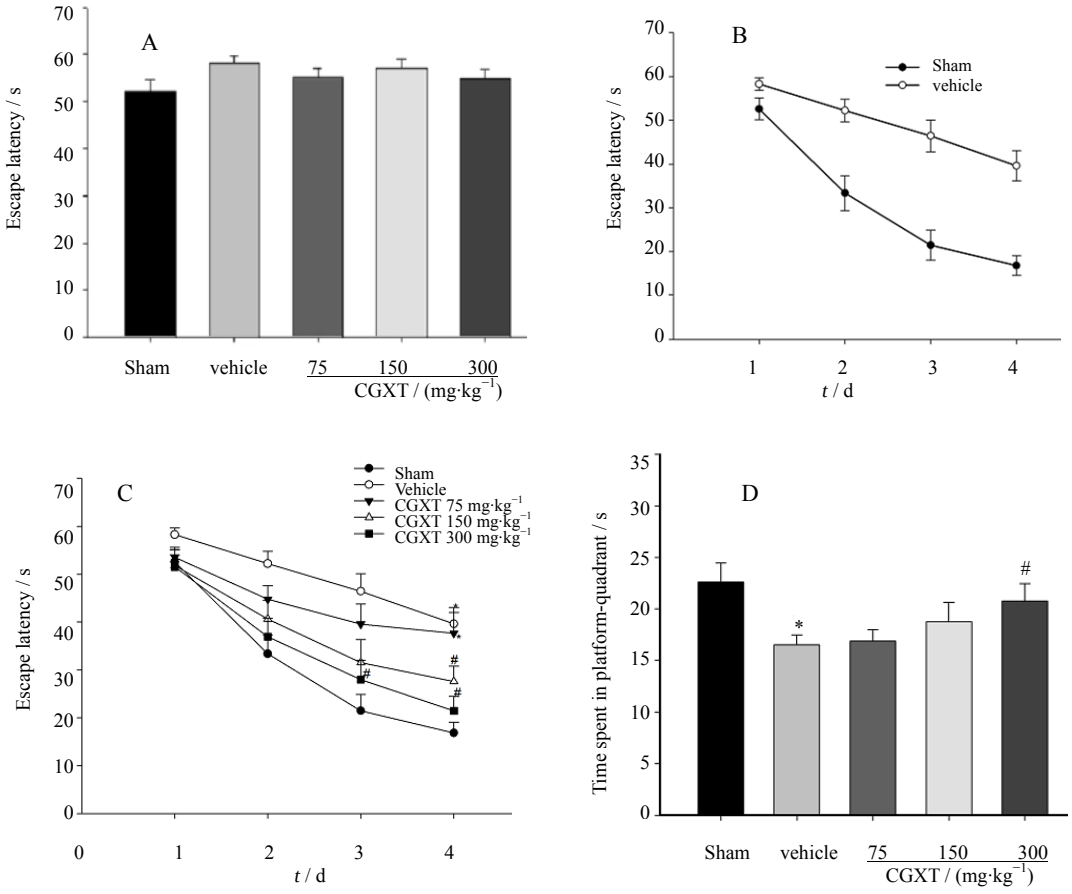

Fig. 2 Effects of CGXT on learning and memory of hypoperfused rats in MWM test

A: Latency in the first day of learning trials

B: Vehicle group spent more time to find the hidden platform in the training phase than did in Sham group

C: Results of escape latency in hidden-platform test

D: Time spent in target quadrant in the probe test. MWM was performed from days 24 to 28 after BCCAL

$^{*}P < 0.05$ *vs* Sham group; $^{\#}P < 0.05$ *vs* vehicle group ($\bar{x} \pm s$, $n = 12$)

rats showed no significant difference as compared with vehicle-treatment rats on training days 3 and 4.

After training for 4 d, probe tests were conducted on the day 5 to assess the effects of CGXT on the memory deficits induced by BCCAL. As shown in Fig. 2D, we plotted the performance of different treatment groups during the probe trials by analyzing the time in the target quadrant where the hidden platform located. Compared with vehicle, 150 or 300 mg/kg of CGXT increased the time in the target quadrant. It suggested that CGXT could improve the memory deficits induced by BCCAL.

Neuronal protective effects of CGXT

In rats with BCCAL for four weeks, the characteristic neuropathological changes of ischemic damage such as shrinkage and triangulation of the nucleus and cytoplasm could increase eosinophilia of the cytoplasm, and neuronal loss was observed in the hippocampus and cerebral cortex (Figs. 3B and 3E) associated with inflammation characterized by lymphocyte and macrophage infiltration. CGXT treatment markedly attenuated these pathologic changes and alleviated the chronic hypoperfusion-induced neuronal damage (Figs. 3C and 3F).

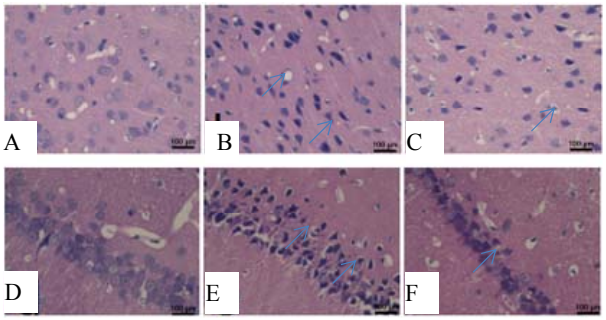

Fig. 3 Photographs of H & E staining in hippocampus CA1 subfield (A – C) and cortex (D – F) after BCCAL

WM damage

In the vehicle-treated rats subjected to chronic cerebral hypoperfusion for four weeks, significant WM lesions were observed in the optic tract and corpus callosum characterized as disarrangement of the nerve fibers, vacuole formation, and marked rarefaction (Fig. 4). And the WM injury was improved by the CGXT treatment. In the 300 mg/(kg·d) CGXT-treated animals, the grading score significantly decreased in the optic tract and corpus callosum ($P < 0.05$), and a moderate reduction was observed in the 150 mg/(kg·d) CGXT-treated group compared with the vehicle group.

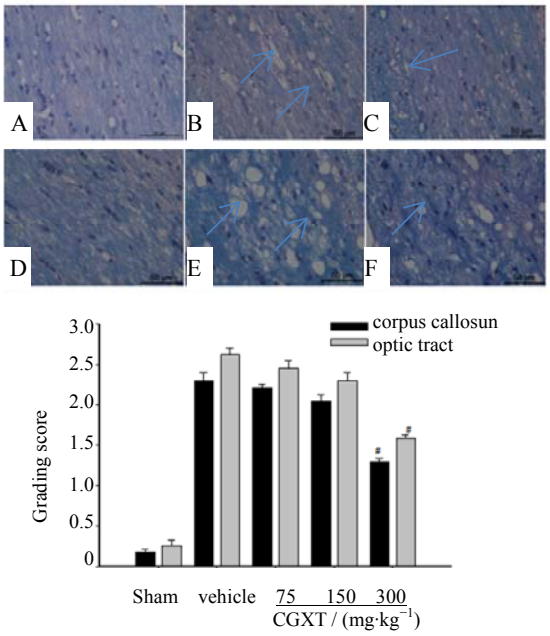

Fig. 4 Photographs of Klüver-Barrera staining in corpus callosum (A – C) and medial part of optic tract (D – F)

Ten fields were viewed in each slice and four rats used for Klüver-Barrera staining. The animal received vehicle (B and E) or 300 mg·kg^{-1} of CGXT (C, and F) for 28 d. In the CGXT-treated rats, the extent of WM rarefaction was reduced

$^{\#}P < 0.05$ *vs* vehicle group ($\bar{x} \pm s$, $n = 4$)

Discussion

In the present study, we investigated the effects of CGXT, an extract from a famous Chinese herbal formula XSTL, on the impairment of learning and memory, the neuronal damage, and WM lesions induced by chronic hypoperfusion. The major findings were that CGXT significantly improved learning and memory deficits, and attenuated both GM and WM damages induced by chronic cerebral hypoperfusion in rats.

Chinese herbal medicines have been widely used in China, Japan, and South Korea for treatment of various complicated diseases including stroke. Traditionally, herbs are decocted for drinking as the medicine. Thus, the actual medicine consumed contains unknown thousands of compounds, and the non-standard decocting procedure and unstable extracts might influence the desirable therapeutic effects in clinic. Preparing Chinese herbal medicines with standardized pharmaceutical extraction processes could probably avoid this problem. In our study, CGXT was prepared with an optimized standard process which mainly focused on maximizing the contents of known bioactive chemical compounds or clusters in the formula as well as preserving the major

pharmacological activities of the formula. The chemical consistency of CGXT was controlled by standard process and proved by the HPLC fingerprint analysis in which the fingerprints of the four batches of CGXT were almost identical (Fig. 1). The fingerprint analysis also revealed that CGXT contained the known bioactive compounds from these 11 Chinese herbs, including quercetin, rutin, curcumin, ginsenoside Rb_1, ginsenoside Rg_1, salvianolic acid B, notoginsenoside R_1, isoalantolactone, and astragaloside IV (Fig. 1).

BCCAL could induce learning and memory deficits and be highly suitable for the research of pathophysiology of chronic hypoperfusion and the testing of potentially neuroprotective drugs (Farkas, Luiten, and Bari, 2007). Our present study revealed that rats after BCCAL for four weeks showed significant learning and memory impairment, which could be ameliorated by CGXT. It is consistent with previous traditional clinical practices in China.

It has been demonstrated that many complex pathophysiological cascade events were involved in ischemic cerebral injury and recovery. And the failure of over 286 clinical trials suggested that unimodal targeting of key events in stroke pathophysiology was not effective in providing long-term benefits (Young *et al*, 2007). Otherwise, the TCM, characterized as multimodal therapeutic strategies according to integral and systematic theory of TCM, has accumulated fruitful clinical experience in treating stroke over the past thousand years (Kim, 2005). CGXT, the extract from famous medicinal Chinese formula XSTL may gain its therapeutic benefits through simultaneously targeting multiple events involved in ischemic cerebral injury. The flavonoids, such as quercetin, rutin, and curcumin, have anti-oxidative and anti-inflammatory effects; Saponins, such as ginsenoside Rb_1, ginsenoside Rg_1, and notoginsenoside R_1, protect nerve cells from excitotoxic, oxidative, and inflammatory injury, improve the microcirculatory disturbance, and attenuate neurodegeneration in ischemic cerebrovascular diseases. It can be speculated that the multimodal therapeutic effects of Chinese medicine, as in the case of CGXT, could provide the benefits for the treatment and recovery of ischemic cerebral injury.

One of the reasons for the failure of neuro-protection in stroke may be the lack of WM protection. The present study suggested that CGXT could protect not only neuronal injury, but WM lesions after hypoperfusion in the rats. Both GM and WM protective actions might contribute to its clinical benefits for stroke. And the protective effects of CGXT on the WM lesions might be gained from its multiple components, such as anti-oxidation of flavonoids and axonal regeneration stimulatory effects of *Astragalus mongholicus* Bunge (Tohda *et al*, 2006). But the exact mechanisms are still under further investigation.

In summary, the current study demonstrates that CGXT could protect brain from both GM and WM injury, and improve learning and memory deficits induced by chronic cerebral ischemia. Thus, these results provide an experimental basis for the clinical practical use of CGXT in stroke treatment, and may help in investigation of multimodal therapeutical strategies of ischemic cerebrovascular diseases including stroke.

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