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Effect of Total Phenolic Acid on the Repeated Cerebral Ischemia-Reperfusion Model in Mice

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ABSTRACT

This study aim to investigate the effects of total phenolic acid on the neuronal enolase and brain biochemical factors in the cerebral ischemia-reperfusion mice model and to assess the protection mechanism of cerebral ischemia-reperfusion injury. The repeated cerebral ischemia-reperfusion mice model was used where bilateral common carotid artery was targeted to block blood flow, reperfusion and then re-blocking the arteries. Brain tissues were examined 24 hours post-drug administration to determine the level of biochemical indicators such as serum NSE in the brain tissue. To investigate the impact of total phenolic acid on the biochemical parameters in the repeated cerebral ischemia-reperfusion mice, the mouse model of cerebral ischemia repeated reperfusion was successfully replicated. The doses of total phenolic acid of D. canes have significantly reduced the levels of IL-6 and TNF-α in brain tissue and decreased the level of NSE in the serum. Collectively, it was observed that the total phenolic acid can protect the brain tissue by inhibiting the expression of inflammatory factors, repairing damaged nerve cells and improving the pathological changes of cerebral ischemia, proposing its therapeutic potential in treating such anomalies in future.

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Authors' Contribution
MM designed the study, performed experimental work and analyzed the data. XF and YM helped in microscopic examinations. HZ and JJ wrote the article.

Key words
Succulent total phenolic acid, Cerebral ischemia-reperfusion, Animal model

INTRODUCTION

The big blood vine is the dried cane of *Sargentodoxa cuneata (Olive.)* Rehd.er Wils., bitter, slightly sweet, non-toxic, mild, flat, liver, pericardium, stomach, large intestine Zhu Jing (Zhao *et al*., 2014) Transient blood supply in the brain is a common acute cerebrovascular disease. The pathological changes at the beginning of the disease are mainly of inflammatory natures. The adhesion of leukocytes to vascular endothelial cells, leukocyte migration and infiltration in the blood are landmark changes of inflammation. The current study found that repeated cerebral ischemia-reperfusion is associated with immune inflammatory responses, mainly through the pro-inflammatory factors (such as TNF-α) and its pro-inflammatory mediators. In the process of the disease development, the inflammatory responses also proceed, and the anti-inflammatory factor such as IL-6 in the cytokine balance system is correspondingly increased. Additionally, the nerve cells begin to be impaired due to disease development (Naderi *et al*., 2018) resulting in the pathological anomalies.

Traditional Chinese medicine theory advocates that repeated cerebral ischemia and reperfusion are related to various factors such as "qi-stagnation" and "qi-deficiency". Qi deficiency, blood stasis and heat toxicity are important factors influencing the occurrence of diseases. Among them, heat toxicity is regarded as affecting model of repeated cerebral ischemia reperfusion. Therefore, based on the previous research, this experiment used the drug (with Qingrejiedu Huoxuetongluo), and applied the model of repeated cerebral ischemia-reperfusion in mice. They observed its effects on biochemical indicators and pathological parameters of the mice. The effect of tested drugs on repeated cerebral ischemia-reperfusion models was obvious (Cheng *et al*., 2012).

In this study, we explored the therapeutic effects of Qingrejiedu Huoxuetongluo on the repeated cerebral ischemia-reperfusion model, and provided new ideas for prevention and treatment of cerebral ischemia by Chinese medicine. Additionally, explored the mechanism of repeated cerebral ischemia-reperfusion in mice treated with Qingrejiedu Huoxuetongluo. It was revealed that total phenolic acid could improve the levels of IL-6 and TNF-α in blood and brain homogenate. We also observed the effect of total phenolic acid on the pathological tissues of repeated cerebral ischemia-reperfusion. These finding propose a new treatment of clinical cerebral ischemia, which can be exploited to alleviate the impact of this disease in future.

MATERIALS AND METHODS

Experimental animals

Kunming mice (n=98 males) of specific-pathogen free (SPF) grade were purchased from Shandong Lukang Pharmaceutical Co., Ltd., each weighting from 19-22g. All

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experiments were performed according to standard animal ethics and welfare guidelines under the certificate number: 37005400000002, provided by the animal experiment centre license number SYXK (Yu) 2015-0005.

Drugs and reagents

Total phenolic acid of S. sinensis was provided by the Chemistry Department of Henan University of Traditional Chinese Medicine, and was determined by HPLC method 70.04%. The Nimodipine (Yabao Pharmaceutical Group Co., Ltd., batch number 140861); Naoluotong Capsule (Jilin Jinbao Pharmaceutical Co., Ltd., batch number 150401); Neuron-specific enolase ELISA assay kit (R&D, batch number 20150601A); Interleukin-6 ELISA Kit, (R&D, Lot 20150601A); Tumor necrosis factor alpha ELISA kit (R&D, batch 20150601A) were used in this study.

Experimental instruments

Bench-top low-speed automatic balancing centrifuge from Changsha Xiangzhi Centrifuge Instrument Co., Ltd. (TDL-40B) and UV, Shanghai Tianmei Scientific Instrument Co., Ltd. (UV1000); microplate reader, BIO-RAD (BIORAD-680) were used.

Modeling and administration

Healthy male SPF mice with a body weight of 19-22g were randomly divided into 7 groups according to their body weight. Each group consisted of 14 rats; sham operation group, model group, nimodipine group, brain collateral group, large, medium and small doses of the total phenolic acid group. Nimodipine group was treated with 30mg/kg, clinical dose 15 times, Naoluotong group was treated with 750mg/kg, clinical dose 15 times; large, medium and small doses of blood vines total phenolic acid group was treated with 450mg/kg, 225mg/ Kg, 112.5mg/ kg doses, respectively. The sham operation group and the model group were given the same volume of normal saline (0.2ml/10g).

These groups were given dose once a day for one week as described by Zhang *et al*. (2016). The water was withdrawn in batches at 8:00 pm on the 6th day, and the drug was weighed in batches after 12 hours. After 1 hour, these were anesthetized by intra-peritoneal injection of 10% chloral hydrate (Ogren *et al*., 2018). Precisely, the elbow was excised from the peripheral nerves of the bilateral common carotid arteries (CCA). The CCA was then isolated. The bilateral common carotid arteries were clamped with a micro-arterial clamp. After 10 min of ischemia, the arteriolar clip was removed and the normal perfusion of the vessels was restored for 10 min Han *et al*., 2018). Then, the ischemic state was maintained for another 10 min, and the blood supply to the blood vessels was resumed, and the model of repeated cerebral ischemia-reperfusion in mice was prepared. The wound was sutured and penicillin was applied at the wound. In the sham operation group, the blood flow was not blocked and was only exposed the bilateral common carotid arteries. The ambient temperature was maintained at 25 to 26 °C during the operation.

Test indicators

All mouse models were successfully operated and after 24 hours of reperfusion of blood flow in the blood vessels, the blood was taken from the eyeballs and centrifuged at 3500 r/min for 10 min (Li *et al*., 2018). Serum was taken and the serum NSE contents were measured (Zong and Zhang, 2017). The brain was quickly removed after the cervical vertebrae were sacrificed. Half of the sagittal cuts were placed in 10% formalin solution for one week, embedded in paraffin for HE staining and the other half was washed with physiological saline. In a large beaker containing ice cubes, a 10% brain homogenate was prepared with a glass homogenizer, centrifuged at 4 °C, 3000 r / min for 10 min, and the supernatant was stored at a temperature below -20 °C to determine IL-6 and TNF-α concentrations in the brain homogenate as reported before (Singh *et al*., 2018).

Data analysis

Data analysis was performed using SPSS 19.0 statistical software package for statistical processing of data. The measurement data were expressed as mean ± standard deviation (± s). One-way ANOVA was used for comparison between groups, and the least significant difference was used for variance test. The LSD method uses the Games-howell test for variance and the Radit test for grade data.

RESULTS

Effects on serum NSE levels in mice with repeated cerebral ischemia-reperfusion

It was observed that the mortality of the model group was the highest (Table I), and the mortality of the mice in the drug-administered group was decreased. These finding directly indicate that the administration group can reduce the mortality of the mice with repeated cerebral ischemia-reperfusion models to varying degrees (Table I). Reduced brain tissue damage and protection of the brain tissue was observed. Additionally, it was noticed that compared to the sham operation group, the NSE levels in the model group was significantly increased (Table I), indicating that the modelling is successful. Compared to the model group, the NSE levels of the positive drug group, the large and medium

Table I. Effect on mortality and serum NSE levels in mice with repeated cerebral ischemia-reperfusion (±s).

Group	Number of mice started	Number of surviving mice	Dose (mg/kg)	Mortality rate(%)	NSE(ng/ml)
Mock surgical group	14	14	--	0	1.58±0.30**
Model group	14	9	--	35.71	2.52±0.50
Nimodipine group	14	12	30	14.29	1.52±0.26**
Naoluotong group	14	12	750	14.29	1.63±0.29**
Large dose of Big blood vine total phenolic acid group	14	12	450	14.29	1.59±0.26**
Medium dose of Big blood vine total phenolic acid group	14	12	225	14.29	1.70±0.25**
Small dose of Big blood vine total phenolic acid group	14	11	112.5	21.43	1.90±0.29*

Note: Compared with the model group, $^{**}P<0.01$, $^{*}P<0.05$ (the same below)

Table II. Effects on the levels of IL-6 and TNF-α in brain tissue of mice with repeated cerebral ischemia-reperfusion (±s).

Group	Number of animals	Dose(mg/kg)	IL-6(pg/ml)	TNF-α(pg/ml)
Mock surgical group	14	--	9.94±1.99**	33.54±4.74**
Model group	9	--	14.56±1.53	48.49±6.20
Nimodipine group	12	30	10.53±2.68**	36.07±5.78**
Naoluotong group	12	750	10.59±2.36**	36.04±6.10**
Large dose Spatholobus Grandiflprum group	12	450	10.92±2.20**	37.44±7.91**
Medium dose of Spatholobus Grandiflprum group	12	225	10.98±2.19**	39.13±6.56*
Small dose of Spatholobus Grandiflprum group	11	112.5	11.70±1.98*	39.51±6.94*

dose groups was significantly reduced. The NSE levels in the low-dose group of the tested drug was significantly reduced, indicating that each drug-administered group can reduce the level of serum NSE with a different degree, and has the effect of protecting neurons in the model of repeated cerebral ischemia-reperfusion.

Effects on the levels of IL-6 and TNF-α in brain tissue of mice with repeated cerebral ischemia-reperfusion

Compared to the sham operation group, the levels of IL-6 and TNF-α in the brain tissue of the model group were increased significantly (Table II), indicating that the model was successful. Additionally, compared to the model group, in the positive drug group the tested drug was large and medium. In the dose group, the level of IL-6 in the brain tissue of the mice was significantly decreased, and the level of IL-6 in the brain of the mice in the low dose group was significantly decreased. In contrast, the levels of TNF-α in the brain of the positive, middle and middle dose groups were significantly affected. The level of TNF-α in the brain tissue of mice in the low-dose group was significantly decreased. These finding indicate that the administration in each group had decreased levels of IL-6 and TNF-α in the brain of mice with repeated cerebral ischemia-reperfusion and reduced inflammatory responses.

Effects of pathological changes in the cortex of brain tissue in mice with repeated cerebral ischemia-reperfusion

Ridit test showed that compared to the sham operation group, the model group had statistically significance ($P<0.01$) impacts. The model group had significant pathological changes in the cortical area of the brain tissue, and highlight the success of the model. Compared with the model group, the nimodipine group carried statistically significant differences between the naoluotong group and the large and medium doses of the total phenolic acid group ($P<0.01$). The small doses of the total phenolic acid group were statistically significant ($P<0.05$), indicating the drug-administered group can protect brain tissue to different degrees and alleviate the pathological damage of brain cortex in mice with repeated cerebral ischemia-reperfusion (Figs. 1 and 2).

Effects of pathological changes in hippocampus of mice with repeated cerebral ischemia-reperfusion

Compared with the sham operation group, the Ridit 4x5test showed significant statistical significance ($P<0.01$),

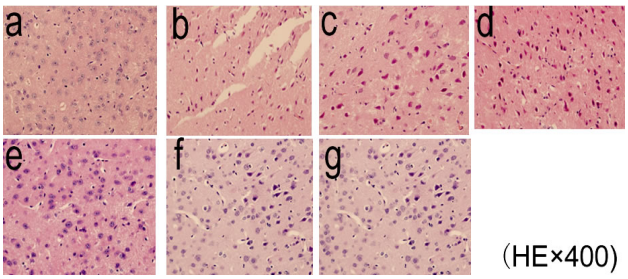

Fig. 1. Effect of pathological changes in brain cortex of mice with repeated cerebral ischemia-reperfusion (HE×400). a. Blank Group; b. Model Group; c. Nimodipine Group; d. Naoluotong Group; e. High-dose Spatholobus Grandiflprum Group; f. Mid-dose Spatholobus Grandiflprum Group; g. Low-dose Spatholobus Grandiflprum Group.

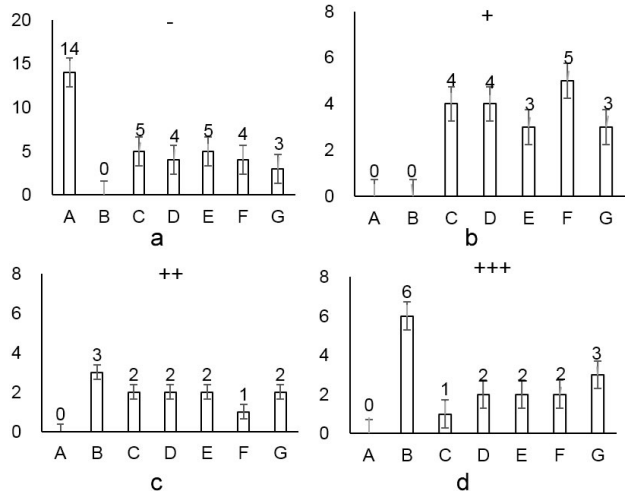

Fig. 2. Effect of pathological changes in the cortex of brain tissue of mice with repeated cerebral ischemia-reperfusion. a: Cerebral cortical nerve cells are normal; b: Cerebral cortical individual nerve cell edema, individual neuron degeneration, cytoplasm light staining, fuzzy structure, individual neuron necrosis; c: Cerebral cortex a small number of nerve cell edema, scattered, a small number of neuronal degeneration The cytoplasm is lightly stained and the structure is fuzzy; d: Cerebral cortical nerve cells edema, most of the neurons are necrotic; A: Blank Group; B: Model Group; C: Nimodipine Group; D: Naoluotong Group; E: High-dose Spatholobus Grandiflprum Group; F: Mid-dose Spatholobus Grandiflprum Group; G: Low-dose Spatholobus Grandiflprum Group; -: Cerebral cortical nerve cells are normal; +: Cerebral cortical individual nerve cell edema, individual neuron degeneration, cytoplasm light staining, fuzzy structure, individual neuron necrosis; ++: Cerebral cortex a small number of nerve cell edema, scattered, a small number of neuronal degeneration The cytoplasm is lightly stained and the structure is fuzzy; +++: Cerebral cortical nerve cells edema, most of the neurons are necrotic.

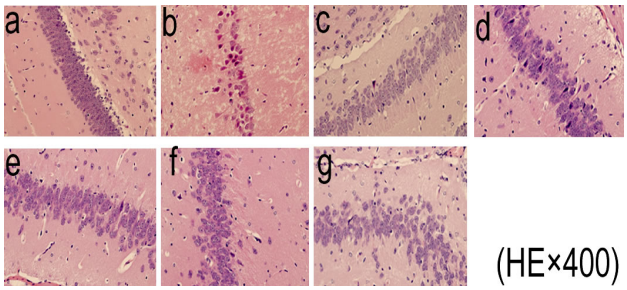

Fig. 3. Effect of pathological changes in hippocampus of mice with repeated cerebral ischemia-reperfusion model (HE×400)

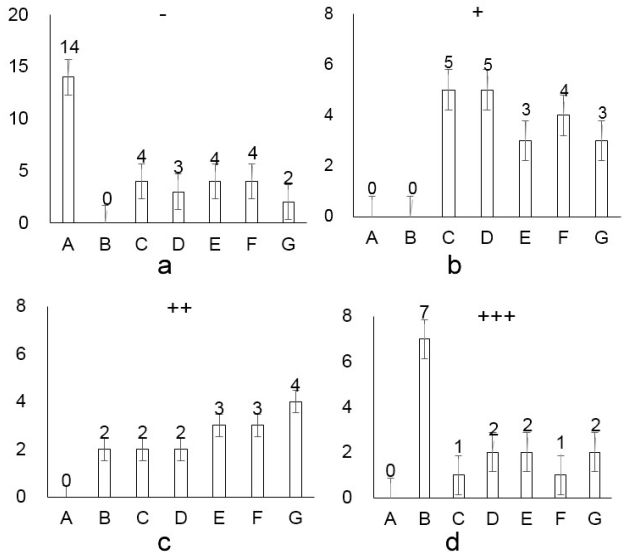

Fig. 4. Effect of pathological changes in hippocampus of mice with repeated cerebral ischemia-reperfusion model; A: Blank Group; B: Model Group; C: Nimodipine Group; D: Naoluotong Group; E: High-dose Spatholobus Grandiflprum Group; F: Mid-dose Spatholobus Grandiflprum Group; G: Low-dose Spatholobus Grandiflprum Group; -: Brain cells in the hippocampus are normal; +: brain hippocampus, individual nerve cells edema, scattered, individual neuron degeneration, cytoplasm lightly stained, fuzzy structure; ++: a few hippocampal edema in the hippocampus of the brain, a few neuron degeneration, cytoplasm Light staining, fuzzy structure; ++: cerebral hippocampal neuronal edema, most of the neuron necrosis.

indicating that the model group had significant pathological changes in the hippocampus of the mice brain tissue. There were statistically significant differences between the Diping group, the Naoluotong group, and the large and medium doses of the total phenolic acid group ($P<0.01$). Additionally, the small doses of the total phenolic acid

group were statistically significant ($P<0.05$). These results indicate that each drug-administered group could protect brain tissue to different degrees and alleviate the pathological damage of the hippocampus in mice with repeated cerebral ischemia-reperfusion (Figs. 3 and 4).

DISCUSSION

Cerebral ischemia is a common condition in both middle-aged and elderly population, and proposing novel treatments is key to prevent and treat these conditions (Fu *et al*., 2016). Cerebral ischemia-reperfusion injury refers to the recovery of blood supply after a certain period of brain ischemia, but the brain function has not recovered (Zhang *et al*., 2018), and it has caused more serious dysfunction (Wahul *et al*., 2018). According to the traditional Chinese medicine therapeutics, the incidence of cerebral ischemia is related to qi stagnation, qi deficiency, and similar other factors. Qi deficiency, blood stasis and heat toxicity are important factors influencing the occurrence of disease (Xu *et al*., 2018). The relationship between the syndrome components of the combination analysis found that the cerebral ischemic factor was the most common syndrome of qi deficiency and turbidity (Valente *et al*., 2018). Western medicine believes that the pathogenesis of cerebral ischemic diseases is complex, including inflammation, cell damage amongst others (Zou *et al*., 2017). Prevention and treatment of ischemic brain injury are few of the important research topics in modern medicine (Miao *et al*., 2017).. Traditional Chinese medicine carries multi-target effects (Xu *et al*., 2017), which are effective in treating ischemic cerebrovascular diseases, with negligible side effects and costs but broader application prospects (Miao *et al*., 2018).

There are many inflammatory mediators involved in the pathological process of cerebral ischemia-reperfusion injury (Miao *et al*., 2013), and their expression is up-regulated, and promoting or inhibiting the progression of inflammatory responses (Kang *et al*., 2017). In this study, by detecting the changes of IL-6 and TNF-α in the brain of mice ((Kang *et al*., 2017), it was revealed that Daphnia sinensis inhibited the expression of pro-inflammatory cytokines by regulating the inflammatory factors after cerebral ischemia, thereby exerting protective neurons. To prevent and treat cerebral ischemic damage, cerebral ischemia is accompanied by nerve cell damage, and neuron-specific enolase (NES) which are specific markers of the central nervous system (Xiong *et al*., 2018; Nishikawa and Suzuk, 2017) and are closely related to brain damage (Kang *et al*., 2018). The NES is present in neurons and secretory cells (Miao *et al*., 2017) and when the nerve cells are damaged, NES enters the cerebrospinal fluid and systemic circulation (Cruz *et al*., 2018). Therefore, the level of NES can be used to assess the damage of the nervous system (Le *et al*., 2017).

By observing the effects of total phenolic acid on the mortality, brain IL-6, TNF-α level and serum NSE level in mice, it is suggested that the total phenolic acid can inhibit inflammation (Song and Du, 2019). These also reduce the damage of nerve cells, improve the pathological changes of brain tissue, and achieve the effect of reducing animal mortality and treating cerebral ischemia-reperfusion model (Diaz and Yepes, 2018). The results of this study can provide guidance for the clinical application of total phenolic acid, and provide theoretical and practical guidance for the clinical application of Huoxue Tongluo and Baidu Sanyu in the therapeutics of cerebral ischemia (Cao *et al*., 2018).

CONCLUSION

Total phenolic acid can improve the levels of inflammatory factors in animal models of repeated cerebral ischemia-reperfusion, regulate central neurons, and protect the cranial nerves. At the same time, the drug can significantly improve the pathological changes in the cortex and hippocampus of the mice. Finding presented in this study show that the total phenolic acid could improve the immune response and central nervous system in cerebral ischemia by regulating inflammatory factors and central nervous system-specific markers. These finding clearly attribute to the therapeutic impacts and treating cerebral ischemia. Taken together, the total phenolic acid treatment of repeated cerebral ischemia-reperfusion has a dose-dependent impact and specifically we have determined the optimal dose of 450mg/kg in repeated cerebral ischemia-reperfusion mice model.

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Statement of conflict of interest

There is no conflict of interests regarding the publication of the manuscript.

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