Total Flavone of Hawthorn Leaf inhibits neuronal apoptosis in brain tissue of rat models of chronic cerebral ischemia

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Abstract

BACKGROUND: Cerebrovascular disease often causes dysfunction of the brain nerve, and nerve cell apoptosis is the important factor of cerebral nerve dysfunction. The excessive expression of c-fos can block the transduction of intracellular signal so that producing some apoptosis-promoting factors, which involve in nerve cell apoptosis process after ischemia injury of brain. Bcl-2 is an inhibited factor. It might to be the key to treat ischemic cerebrovascular disease by inhibiting or reducing the apoptosis of nerve cells after ischemia injury.

OBJECTIVE: To investigate the therapeutic effect and mechanism of the Total Flavone of Hawthorn Leaf on chronic cerebral ischemia rats.

METHODS: A total of 72 healthy male Sprague-Dawley rats were randomly divided into sham surgery group, model group, Total Flavone of Hawthorn Leaf group and ginkgo leaf group. Permanent bilateral carotid artery ligation was used to prepare chronic cerebral ischemia model in the model group, Total Flavone of Hawthorn Leaf group and ginkgo leaf group. Total Flavone of Hawthorn Leaf group and ginkgo leaf group respectively received 140 mg/kg Total Flavone of Hawthorn Leaf and 12.3 mg/kg ginkgo leaf intragastrically for 36 days from 36 days after model induction. Model group and sham surgery group received 3.5 mL/kg physiological saline intragastrically.

RESULTS AND CONCLUSION: Compared with the model group, the expression of c-fos protein significantly deceased in the Total Flavone of Hawthorn Leaf group ($P < 0.01$), Bcl-2 expression levels significantly increased ($P < 0.01$), and Ca^{2+} content decreased ($P < 0.05$). Moreover, no significant difference in above indexes was detected between Total Flavone of Hawthorn Leaf group and ginkgo leaf group ($P > 0.05$). These data indicated that the protective effect of Total Flavone of Hawthorn Leaf on chronic cerebral ischemia was associated with its inhibition of neuronal apoptosis. Its mechanism of anti-apoptosis might be associated with up-regulating expression of Bcl-2, down-regulating expression of c-fos and decreasing Ca^{2+} content in brain.

Subject headings: brain ischemia; flavones; crataegus; ginkgo biloba; apoptosis

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INTRODUCTION

Cerebral ischemia is one of the central nervous system diseases characterized by decrease in cerebral circulation blood flow, which is common in clinic. According to the World Health Organization data[1], in 57 countries, 40 countries ranked the cerebrovascular disease mortality for the first three, and first in China, seriously affecting the quality of human life and survival[2]. There is extremely close relationship between apoptosis and ischemic damage of brain. On one hand, after cerebral ischemia, the excessive expression of c-fos can block the transduction of intracellular signal so that producing some apoptosis-promoting factors[3], which involve in nerve cell apoptosis process after ischemia injury of brain. On the other hand, Bcl-2 which related to cell apoptosis closely is an inhibited factor. Obviously, it is the key to treat ischemic cerebrovascular disease by inhibiting or reducing the apoptosis of nerve cell after ischemia injury[4]. Ginkgo leaf is recognized having significant effect on cerebral ischemia disease, but its price is more expensive. Total Flavone of Hawthorn Leaf is a flavonoid extracted from hawthorn leaves, can lower lipid, improve antioxidation and have other functions[5]. However, it has not been clear whether Total Flavone of Hawthorn Leaf has any influence on the expression of c-fos

and Bcl-2 yet. This research adopted a method that ligaturing bilateral carotid artery of the rats permanently to build the model of chronic cerebral ischemia, to observe Total Flavone of Hawthorn Leaf's influence on the expression of c-fos and Bcl-2 in cerebral ischemia rats.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment.

Time and setting

This study was performed in the Institute of Basic Medical, Chengde Medical College in China from September 2012 to November 2013.

Materials

Animals

A total of 72 adult, clean, male, Sprague-Dawley rats, weighting 250–300 g and aged 12–14 weeks, were provided by Beijing Tonglihua Experimental Animal Technical Co., Ltd. (case number: SCXK (Jing) 2012- 0001). All rats lived in the same room, were allowed free access to water and ventilated. All experimental procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China.

Drugs

Total Flavone of Hawthorn Leaf was gathered from Chengde local hawthorn leaves from July to August. Total Flavone of Hawthorn Leaf was extracted by water frying alcohol sinking method, with a purity of 65.2%.

Ginkgo leaf was provided by China Yantai Rongchang as long as the Cable Co., Ltd.

Main reagents are listed as follows:

Reagent	Source
c-fos and Bcl-2 polyclonal antibodies	Wuhan Boster Biotechnology Co., Ltd., China
3,3'-Diaminobenzidine chromogenic reagent kit	Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., China

Methods

Experimental groups

All 72 rats were randomly divided into sham surgery group, model group, Total Flavone of Hawthorn Leaf group and ginkgo leaf group.

Establishment of rat models of chronic cerebral ischemia by ligating bilateral common carotid artery

Using the method called permanent bilateral carotid artery ligation[6], chronic cerebral ischemia model was prepared. The animals were anesthetized with 30 mg/kg sodium pentobarbital by enterocoelia injection. A median sagittal incision was made on the neck, followed by blunt dissection of the subcutaneous tissue and fascia, freed bilateral carotid artery at the carotid triangle, double ligation both sides of carotid arteries, and cut from the middle by electric knife, and then the incision was sutured. Bilateral carotid artery was only separated, without ligating in the sham surgery group. After natural awakening, in accordance with Longa's standard[7], there was 5 levels 4 scores, 1 to 3 scores in the experimental groups.

Drug intervention

At 36 days postoperation, rats in the Total Flavone of Hawthorn Leaf and ginkgo leaf groups were respectively given Total Flavone of Hawthorn Leaf 140 mg/kg per day (equivalent to twice the clinical dose) and gingko leaf 12.3 mg/kg per day (equivalent to one time the clinical dose) by gavage for 36 days. Sham surgery group and model group were given the saline 3.5 mL/kg per day .

Immunohistochemical detection of c-fos expression in the rat brain

At 1 hour after the last drug given, aniamls were anesthetized with 4% chloral hydrate, followed by heart perfusion and fixation with 10% formaldehyde. Brain tissue was taken out which was 2 mm to 8 mm behind optic chiasma, conventionally paraffin embed, and sliced into 5 μm coronal slices. Slices were dewaxed with the dimethy lbenzene, dehydrated by graded alcohol, incubated with 0.3% H_2O_2 for 30 minutes, and blocked by goat serum for 30 minutes. Subsequently, c-fos antibody (1:100) 40 μL was added in 4 ℃ refrigerator for a night. Then sections were incubated with biotin-labeled goat anti-rabbit IgG 50 μL for 30 minutes, visualized for 5 minutes with 3,3'-diaminobenzidine, counterstained by hematoxylin, dewatered in gradient alcohol, permeabilized, and mounted with neutral gum, and then observed under the optical microscope (400 ×; Olympus, Tokyo, Japan). Absorbance of the product was analyzed which was positive expression by MINT image analysis system (Xingwan Electronics Factory, Dongguan, Guangdong Province, China).

Western blot assay of Bcl-2 expression in the rat brain

At 1 hour after the last drug given, animals were decapitated, leptomeninges was peeled. Brain tissue was taken out which was 2 to 8 mm behind optic chiasma. The tissue was washed with 0.01 mol/L PBS. Lysate and 10 μL phenylmethyl sulfonylfluoride (10 mg/mL) were added, followed by 4 ℃ homogenate, centrifugation at 10 000 r/min for 30 minutes. The supernatant was taken for protein quantization by BCA protein assay kit. After sodium dodecyl sulfate polyacrylamide gel electropheresis, samples were transferred to membrane, which were blocked with 10% skim milk powder, and then treated with Bcl-2 primary antibody (1:150), overnight at 4 ℃, followed by incubation with horseradish peroxidase-labeled secondary antibody (1:1 000) at room temperature for 2.5 hours. Super ECL Plus ultra-sensitive luminescent liquid was used. Samples were exposed in X-ray film cassette and developed by D72 developer. The Quantity One software (Discovery Series, USA) was utilized to analyze the grey value of X-ray film.

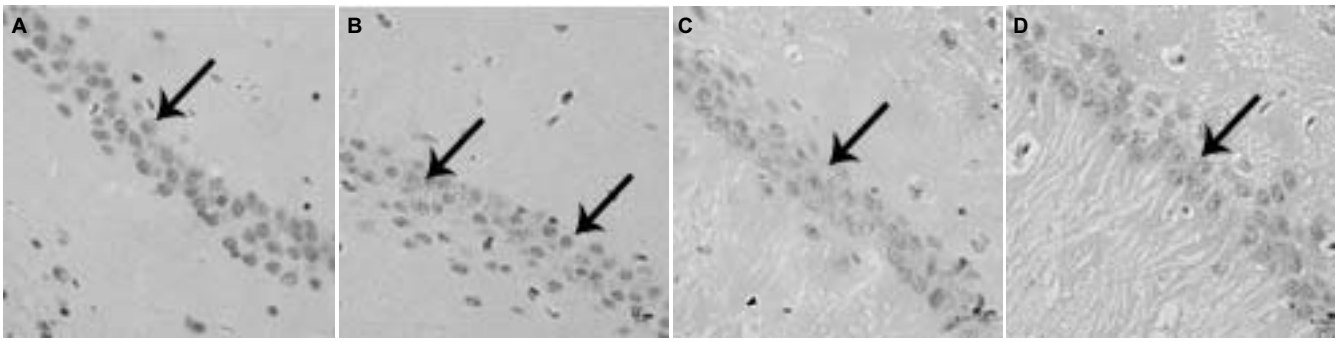

Figure 1 Effects of Total Flavone of Hawthorn Leaf on c-fos expression in brain tissues of rats with chronic cerebral ischemia (immunohistochemical staining, ×400)
Note: A: Sham surgery group; B: model group; C: ginkgo leaf group; D: Total Flavone of Hawthorn Leaf group. Arrows: positive expression.

Table 1 Effects of Total Flavone of Hawthorn Leaf on c-fos quantitative expression in brain tissues of rats with chronic cerebral ischemia ($\bar{x}\pm s$, n=6, absorbance value)

Group	c-fos expression
Sham surgery	0.010 9±0.000 3
Model	0.046 3±0.005 8[a]
Ginkgo leaf	0.016 8±0.000 5[b]
Total Flavone of Hawthorn Leaf	0.017 6±0.000 6[b]

Note: [a]P < 0.01, *vs.* sham surgery group; [b]P < 0.01, *vs.* model group.

Table 2 Effects of Total Flavone of Hawthorn Leaf on Bcl-2 quantitative expression in brain tissues of rats with chronic cerebral ischemia ($\bar{x}\pm s$, n=6, mg/L)

Group	Bcl-2 expression
Sham surgery	0.28±0.02
Model	0.33±0.06[a]
Ginkgo leaf	0.70±0.08[b]
Total Flavone of Hawthorn Leaf	0.68±0.09[b]

Note: [a]P < 0.01, *vs.* sham surgery group; [b]P < 0.01, *vs.* model group.

Spectrophotometry of the content of Ca^{2+} in the rat brain

At 1 hour after the last drug given, the rats were decollated. Brain tissue was gotten out and leptomeninges was peeled. The tissue was rinsed with ultrapure water to remove residual blood, baked in oven at 60 ℃ for 48 hours, and then weighed by analytical balance. Concentrated nitric acid was added to digest the tissue into solution gradually. Samples were diluted with 1% $CaCl_2$. In the end, spectrophotometry was used to detect the content of Ca^{2+} in the sample.

Main outcome measures

There were expressions of c-fos and Bcl-2, as well as the content of Ca^{2+} in the rat brain.

Statistical analysis

Data were expressed as $\bar{x}\pm s$, and analyzed using SPSS16.0 statistical software (SPSS, Chicago, IL, USA). One-way analysis of variance was utilized to analyze the comparison between groups. A value of P < 0.05 was considered statistically significant.

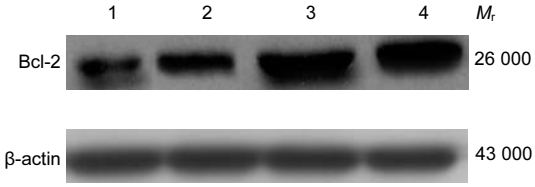

Figure 2 Effects of Total Flavone of Hawthorn Leaf on Bcl-2 expression in brain tissues of rats with chronic cerebral ischemia
Note: 1: Sham surgery group; 2: model group; 3: ginkgo leaf group; 4: Total Flavone of Hawthorn Leaf group.

Table 3 Effects of Total Flavone of Hawthorn Leaf on Ca^{2+} content in brain tissues of rats with chronic cerebral ischemia ($\bar{x}\pm s$, n=6, μg/g)

Group	Ca^{2+} content
Sham surgery	106.59±6.87
Model	231.51±11.47[a]
Ginkgo leaf	142.32±5.17[b]
Total Flavone of Hawthorn Leaf	145.91±5.78[b]

Note: [a]P < 0.01, *vs.* sham surgery group; [b]P < 0.05, *vs.* model group.

RESULTS

Quantitative analysis of experimental animals

A total of 72 rats were included in the final analysis

Total Flavone of Hawthorn Leaf inhibited c-fos protein expression in the brain of rats with chronic cerebral ischemia

Compared with sham surgery group, c-fos protein expression in the rat brain was elevated by (324.78±50.37)% in the model group (P < 0.01). Compared with the model group, c-fos protein expression in the rat brain was reduced by (63.71±11.42)% and (61.98 ±11.27)% in the ginkgo leaf and Total Flavone of Hawthorn Leaf groups (P < 0.01). There was no difference between ginkgo leaf and Total Flavone of Hawthorn Leaf groups (P > 0.05; **Figure 1**, **Table 1**).

Total Flavone of Hawthorn Leaf promoted Bcl-2 protein expression in rats of each group

There was little expression of Bcl-2 protein in the rat brain in sham surgery group. Compared with sham surgery group, Bcl-2 protein in the rat brain was

increased by (17.89±3.19)% in the model group ($P < 0.05$). Compared with model group, Bcl-2 protein expression in the rat brain was increased greatly by (112.12±6.14)% and (106.01±5.81)% in the ginkgo leaf and Total Flavone of Hawthorn Leaf groups ($P < 0.01$). There was no difference between ginkgo leaf and Total Flavone of Hawthorn Leaf groups ($P > 0.05$; **Figure 2**, **Table 2**).

Total Flavone of Hawthorn Leaf decreased Ca^{2+} content in rats of each group

The Ca^{2+} content in the rat brain rats was significantly increased (117.20±5.18)% in the model group compared with the sham surgery group ($P < 0.01$). Compared with the model group, Ca^{2+} content in the rat brain reduced by (38.53±2.13)% and (36.97±2.01)% in the ginkgo leaf and Total Flavone of Hawthorn Leaf groups, with significant differences ($P < 0.05$). There was no difference between Ginkgo leaf and Total Flavone of Hawthorn Leaf groups ($P > 0.05$; **Table 3**).

DISCUSSION

Cerebrovascular disease is a group of clinical disease caused by various reasons which lead to cerebral injury, in which ischemic cerebrovascular disease is the most common type[8]. Its incidence, morbidity and mortality are high[9]. This disease seriously influences the patients' quality of life, often accompanies with learning and cognitive dysfunction[10]. The number of seniors over the age of 65 who suffer from the disease in China has exceeded 200 million. With the aging of population, the incidence of cerebrovascular disease increases year by year. Cerebrovascular disease often causes dysfunction of the brain nerve, and nerve cell apoptosis is the important factor of cerebral nerve dysfunction[11]. It is the key to the treatment of ischemia to inhibit or reduce the apoptosis of damaged nerve cells[3]. *c-fos* gene which is a member of mys family, can encode nuclear DNA binding protein, and regulate gene transcription[12]. Under normal physiological condition, *c-fos* gene exists in the central nervous system, and is in a low expression or stationary state, and not easy to detect[13]. However, after cerebral ischemia injury, as an immediate and early response gene, *c-fos* gene can make reaction to express quickly[14], under the stimulation of growth factors. The expression products called fos protein can promote cell proliferation. However, excessive expression of *c-fos* will increase cell apoptosis[15]. Ischemia can also make a lot of Ca^{2+} flow in, causing Ca^{2+} in nerve cells overload[16]. This causes injury or death of nerve cells, even nerve dysfunction. This study shows that the Ca^{2+} content and c-fos protein expression in model group were significantly higher than sham surgery group ($P < 0.01$), which proved that cerebral ischemia could induce apoptosis of nerve cells. Ca^{2+} content and c-fos protein expression in Total Flavone of Hawthorn Leaf group reduced a lot compared with model group ($P < 0.05$), suggesting that Total Flavone of Hawthorn Leaf could inhibit the excessive expression of c-fos protein, reduce the overload of Ca^{2+}, and lighten damage of nerve cell.

Bcl-2 is a protein which is recognized to have an inhibitory effect on cell apoptosis[17], could inhibit the Bax transfer from cytoplasm to the mitochondrial membrane, protect potential gradient of mitochondrion and regulate intracellular Ca^{2+} steady state and redox state, thereby, inhibit the intrinsic apoptotic pathway[18]. As a result, the expression of Bcl-2 protein determines the tropism of nerve cell survival or apoptosis directly. The results of this study showed: because of brain tissue ischemia and hypoxia, expression of Bcl-2 protein was restrained, and the c-fos excessively expressed, Ca^{2+} overload, thus accelerated the neural cell apoptosis. Application of Total Flavone of Hawthorn Leaf could obviously increase the Bcl-2 protein expression in ischemic brain tissue, thus inhibit or reverse nerve cell apoptosis. As a result, the mechanism of Total Flavone of Hawthorn Leaf may be related to its inhibition of Ca^{2+} overload, promoting the expression of antiapoptotic protein Bcl-2 and reducing the expression of promoting apoptosis protein c-fos. However, further research is needed to determine the specific mechanism that the Total Flavone of Hawthorn Leaf reduces neural cell apoptosis caused by cerebral ischemia.

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山楂叶总黄酮抑制慢性脑缺血模型大鼠脑组织神经元的凋亡

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文章亮点：

实验特点在于通过采用永久性双侧颈总动脉结扎法制备脑缺血模型，证实山楂叶总黄酮对慢性脑缺血的保护作用与抑制脑神经细胞凋亡有关，其抗凋亡的机制可能与上调 Bcl-2 表达，下调 c-fos 表达，降低脑组织 Ca^{2+}含量有关。

关键词：
实验动物；组织工程；慢性脑缺血；山楂叶总黄酮；银杏叶片；c-fos；Bcl-2；Ca^{2+}；细胞凋亡
主题词：
脑缺血；黄酮类；山楂属；银杏；细胞凋亡
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摘要

背景：脑血管疾病常引起大脑神经功能缺失，而神经细胞凋亡是造成脑神经功能缺失的重要因素，c-fos 的过度表达能阻断细胞内信号的转导而产生一些促调亡因子，参与脑缺血损伤后的神经细胞凋亡过程，Bcl-2 是抑制细胞凋亡因子，抑制或减少损伤后神经细胞凋亡可能成为缺血性脑血管病治疗的关键。

目的：探索山楂叶总黄酮对慢性脑缺血模型大鼠的治疗作用及机制。

方法：72 只健康雄性 SD 大鼠随机等分为假手术组、模型组、山楂叶总黄酮组和银杏叶片组。后 3 组大鼠采用永久性双侧颈总动脉结扎法制备脑缺血模型。山楂叶总黄酮组和银杏叶片组大鼠分别于造模后 36 d 起连续 36 d 灌胃 140 mg/kg 山楂叶总黄酮和 12.3 mg/kg 银杏叶片，而模型组和假手术组大鼠灌胃 3.5 mL/kg 生理盐水。

结果与结论：与模型组比较，山楂叶总黄酮组模型大鼠缺血脑组织中 c-fos 表达水平显著降低($P < 0.01$)，Bcl-2 表达水平显著增加($P < 0.01$)，Ca^{2+}含量降低($P < 0.05$)，且上述指标与银杏叶片组比较差异无显著性意义($P > 0.05$)。提示山楂叶总黄酮对慢性脑缺血的保护作用与抑制脑神经细胞凋亡有关，其抗凋亡的机制可能与上调 Bcl-2 表达，下调 c-fos 表达，降低脑组织 Ca^{2+}含量有关。

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利益冲突：文章及内容不涉及相关利益冲突。

伦理要求：实验经过承德动物伦理委员会批准。

学术术语：山楂叶总黄酮-中药山楂提取物中黄酮类化合物的总称，主要成分为芦丁、牡荆素、牡荆素葡萄糖苷、牡荆素鼠李糖苷、槲皮素和金丝桃苷等。

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