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Danhong injection

A modulator for Golgi structural stability after cerebral ischemia-reperfusion injury

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Research Highlights

(1) Transforming growth factor-β1 expression increased, while expression of Golgi matrix protein GM130 expression decreased following cerebral ischemia-reperfusion injury.
(2) *Danhong* injection, a traditional Chinese medicine, can improve the expression of transforming growth factor beta 1 and GM130, and maintain the stability of neuronal Golgi morphology and structure after cerebral ischemia-reperfusion, thus exerting a neuroprotective effect.

Abstract

The cerebral ischemia-reperfusion model was established using the suture occlusion method, and rats were intraperitoneally given 8 mL/kg *Danhong* injection once a day prior to model establishment. Rat brain tissues were harvested at 6, 24, 48, 72 hours after reperfusion. Immunohistochemical staining showed that transforming growth factor-β1 expression increased, while Golgi matrix protein GM130 expression decreased after cerebral ischemia-reperfusion. *Danhong* injection was shown to significantly up-regulate the expression of transforming growth factor-β1 and GM130, and expression levels peaked at 7 days after reperfusion. At 7 days after cerebral ischemia-reperfusion, Golgi morphology was damaged in untreated rats, while Golgi morphology breakage was not observed after intervention with *Danhong* injection. These experimental findings indicate that *Danhong* injection can up-regulate the expression of transforming growth factor-β1 and GM130, and maintain Golgi stability, thus playing a neuroprotective role in rats after cerebral ischemia-reperfusion.

Key Words

neural regeneration; traditional Chinese medicine; *Danhong* injection; cerebral ischemia-reperfusion; transforming growth factor-β1; GM130; Golgi; matrix protein; brain injury; neuroprotection; grants-supported paper; neuroregeneration

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Conflicts of interest: None declared.

INTRODUCTION

Ischemia-reperfusion injury is the key driving-force in ischemic brain damage, and may further aggravate cerebral infarction[1]. Transforming growth factor-β1 is a universal multifunctional polypeptide cytokine, which has been shown to have a protective effect against nerve cell damage[2]. Golgi morphology may be damaged after cerebral ischemia-reperfusion[3]. Transforming growth factor-β1 exists in the Golgi apparatus of nerve cells, and further protects the Golgi apparatus[3]. GM130 is an important matrix protein appearing on the Golgi surface[3], however, variation of GM130 expression during cerebral ischemia-reperfusion injury has not been studied yet.

Ethical approval: This study received permission from the Animal Ethics Committee of Central South University, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

Danhong injection, a Chinese patented drug, has been widely used in the treatment of ischemic cerebrovascular diseases[4-5]. *Danhong* injection has been shown to induce a positive effect against cerebral infarction through its anti-oxidant, anti-platelet aggregation, anti-thrombotic, and circulation improving effects[4-5]. However, the neuroprotective mechanisms involved require further investigation. Whether *Danhong* injection can protect the Golgi apparatus from damage after cerebral ischemia-reperfusion remains unclear.

The present study aimed to explore the neuroprotective effect of *Danhong* injection through observing Golgi morphology, and transforming growth factor-β1 and GM130 expression in a rat model of cerebral ischemia-reperfusion injury.

RESULTS

Quantitative analysis of experimental animals

A total of 96 Sprague-Dawley rats were randomly divided into four groups: normal group (n = 8), sham operation group (n = 8), model group (ischemia-reperfusion model; n = 40), and *Danhong* injection group (ischemia-reperfusion model + *Danhong* injection; n = 40). The model and *Danhong* injection groups were sub-divided into five subgroups (n = 8), which included 6, 24, 48, 72 hours and 7 days after ischemia-reperfusion groups. All 96 rats were all involved in the final analysis.

Danhong injection alleviated hippocampal changes in rats after cerebral ischemia-reperfusion

Hematoxylin-eosin staining showed that nerve cells in the hippocampal CA1 region were arranged in neat rows and were morphologically normal in the normal and sham operation groups. In the model group, cells became sparse, vacuolar changes were observed, and increases in gaps between cells and blurry membrane boundaries were evident. In addition, nerve cells were denatured and necrotic to varying degrees. A large area of nerve cell degeneration and necrosis was visible at 7 days after reperfusion, as well as cell shrinkage, nuclear condensation, and disappearance of nucleoli. After *Danhong* injection, nerve cell degeneration and necrosis were significantly weakened when compared with the model group, and nerve cell damage in the 7-day *Danhong* injection subgroup was lighter than the remaining subgroups (Figure 1).

Danhong injection up-regulated transforming growth factor-β1 and GM130 expression in the rat hippocampal CA3 region after cerebral ischemia-reperfusion

Immunohistochemical staining showed that transforming growth factor-β1 expression was rare in normal and sham operation groups, and significantly increased in the model and *Danhong* injection groups when compared with the normal and sham operation groups (P < 0.05). Transforming growth factor-β1 expression in the *Danhong* injection group was significantly higher than that in the model group (P < 0.05), with the exception of the 6-hour time point, at which a significant difference was not observed between the two groups (P > 0.05). In the model group, transforming growth factor-β1 expression increased gradually, and peaked at 48 hours after reperfusion, which was significantly higher than the other time points (P < 0.05). In the *Danhong* injection group, transforming growth factor-β1 expression also increased, and peaked at 7 days after reperfusion, which was significantly higher than the other time points (P < 0.05; Figure 2, Table 1).

GM130 was positively expressed in the forebrain cortex of rats in the normal and sham operation groups, which was significantly higher than that in the model and *Danhong* injection groups (P < 0.05). However, there was no significant difference when compared with the *Danhong* injection 7-day subgroup (P > 0.05). At 6 hours after reperfusion, a significant difference in GM130 expression was not observed between the *Danhong* injection group and model group (P > 0.05); at other time points,

GM130 expression in the *Danhong* injection group was significantly higher than that in the model group ($P < 0.05$). In the model group, GM130 expression decreased gradually and the levels were the lowest at 7 days, with significant differences when compared with other time points ($P < 0.05$). In the *Danhong* injection group, GM130 expression increased and peaked at 7 days, which was significantly higher than the other time points ($P < 0.05$; Table 2, Figure 3).

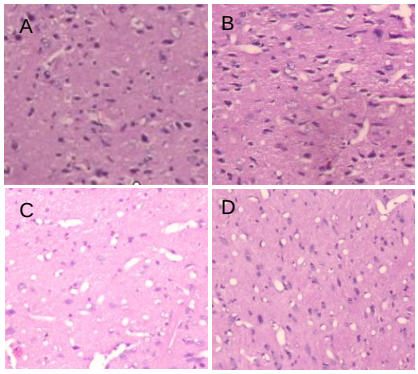

Figure 1 Effect of *Danhong* injection on morphological changes of brain tissue in the rat hippocampal CA1 region after cerebral ischemia-reperfusion (hematoxylin-eosin staining, light microscope, × 200).

(A) Normal group; (B) sham operation group; (C) model group (ischemia-reperfusion 7 days); (D) *Danhong* injection group (injection 7 days).

The number of nerve cells was significantly reduced in the model group, while increased in the *Danhong* injection group.

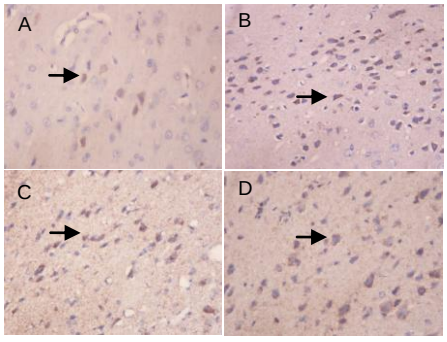

Figure 2 Effect of *Danhong* injection on transforming growth factor-β1 expression in the rat hippocampal CA3 region after cerebral ischemia-reperfusion (hematoxylin-eosin staining, × 200).

(A)Sham operation group; (B) model group (ischemia-reperfusion 48 hours); (C) model group (ischemia-reperfusion 7 days); (D) *Danhong* injection group (injection 7 days).

Transforming growth factor-β1 expression increased in the model group, peaked at 48 hours, and then decreased. Transforming growth factor-β1 expression further increased in the *Danhong* injection group. Arrows indicate transforming growth factor-β1 positive cells.

Danhong injection improved Golgi structure in the forebrain cortex in rats with cerebral ischemia-reperfusion injury

GM130, a specific marker of the Golgi apparatus, was used to immunohistochemically stain the Golgi[3]. Under the light microscope, we found that Golgi network structure was normal in all groups, except in the 7-day model subgroup. Some Golgi apparatus structures stained lightly, meanwhile granules appeared to decrease or disappear, and some parts appeared broken in the 7-day model subgroup (Figure 4).

DISCUSSION

Transforming growth factor-β1, constituted by dimeric peptides, can mediate the cerebral ischemia-reperfusion process, execute an important cerebral protective role, and prevent ischemia-caused damage of nerve cells. Thus, it is considered as a protective index of nerve injury[2]. Astrocytes and microglia have the function of responding to the ischemic signal and releasing transforming growth factor-β1, which can protect neurons against cerebral ischemia and hypoxia through various mechanisms[6]. This study found that transforming growth factor-β1 expression increased after ischemia-reperfusion, and peaked at 48 hours. This evidence proves that transforming growth factor-β1 was released to withstand cerebral ischemic injury in nerve cells, inducing a strong neuroprotective effect[7-8]. The decline of transforming growth factor-β1 expression may be associated with the severity of brain injury. When nerve cells cannot tolerate ischemia, necrosis increases, leading to a decrease in transforming growth factor-β1. Subsequently, this reduction may aggravate neurological dysfunction and nerve damage changes from reversible to irreversible. Lesné *et al* [9] found that transforming growth factor-β1 was not detected in the cerebral ischemia irreversible damage period, which supports the theory that transforming growth factor-β1 expression only increases in the reversible period, thus playing a protective role. Therefore, transforming growth factor-β1 can be regarded as a sign of neuronal survival.

After cerebral ischemia-reperfusion occurs, various mechanisms of injury can change the morphology and function of multiple subcellular ultrastructures with a series of subcellular stress reactions[10]. Damage to Golgi structure directly affects functions such as protein processing, modification as well as transportation with cell physiological dysfunction, and eventually leads to disease development[11].

Table 1 *Danhong* injection up-regulated transforming growth factor-β1 expression (*n*/200-fold visual field) in the rat hippocampal CA3 region after cerebral ischemia-reperfusion

Group	Time after reperfusion				
	6 hours	24 hours	48 hours	72 hours	7 days
Normal	53.43±4.45				
Sham operation	54.48±4.51				
Model	84.85±7.48[a]	103.37±8.10[a]	122.03±7.87[ac]	102.48±7.06[a]	99.32±5.42[a]
Danhong injection	85.06±7.99[ac]	133.90±5.48[abc]	135.26±6.24[abc]	138.00±8.29[abc]	161.06±5.29[ab]

Data are expressed as mean ± SD; *n* = eight rats in each group at each time point. [a]*P* < 0.05, *vs*. normal group and sham operation group; [b]*P* < 0.05, *vs*. model group at the same time point; [c]*P* < 0.05, *vs*. 7 days subgroup (one-way analysis of variance followed by least significant difference *t*-test).

Table 2 *Danhong* injection up-regulated GM130 expression (*n*/200-fold visual field) in the rat hippocampal CA3 region after cerebral ischemia-reperfusion

Group	Time after reperfusion				
	6 hours	24 hours	48 hours	72 hours	7 days
Normal	163.72±6.24				
Sham operation	162.82±5.98				
Model	117.11±6.89[ac]	116.41±6.53[ac]	113.47±5.56[ac]	102.48±7.06[ac]	99.32±5.42[a]
Danhong injection	120.09±7.66[ac]	126.18±8.97[abc]	130.89±8.01[abc]	147.92±6.99[abc]	158.72±5.71[b]

Data are expressed as mean ± SD; *n* = eight rats in each group at each time point. [a]*P* < 0.05, *vs*. normal group and sham operation group; [b]*P* < 0.05, *vs*. model group at the same time point; [c]*P* < 0.05, *vs*. 7 days subgroup (one-way analysis of variance followed by least significant difference *t*-test).

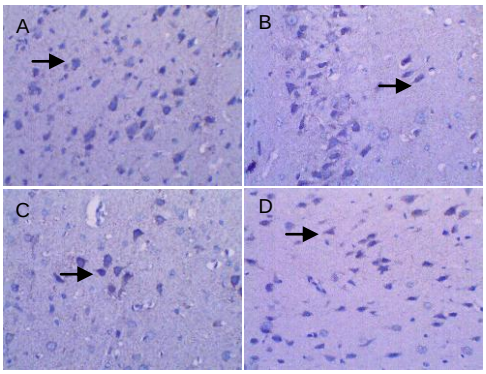

Figure 3 Effect of *Danhong* injection on GM130 expression in the rat hippocampal CA3 region after cerebral ischemia-reperfusion (hematoxylin-eosin staining, × 200).

(A) Sham operation group; (B) model group (ischemia-reperfusion 48 hours); (C) model group (ischemia-reperfusion 7 days); (D) *Danhong* injection group (injection 7 days).

GM130 expression decreased in the model group and expression levels were lowest at 7 days. GM130 expression increased in the *Danhong* injection group. Arrows indicate GM130-positive cells.

The metabolic status of nerve cells can be reflected by changes to the Golgi apparatus. Golgi changes in some neurological diseases, for example, prion protein disease, Parkinson's disease, amyotrophic lateral sclerosis and Alzheimer's disease, and it participates in the pathophysiology of these diseases[12-15]. Nakagomi *et al* [16] insisted that once cells were stimulated a lot, the Golgi may sense and identify these signals with emerging stress, and then change their structural elements.

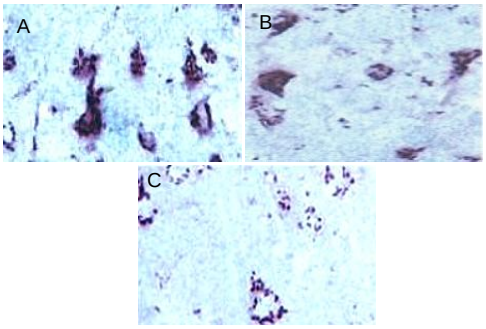

Figure 4 Effect of *Danhong* injection on Golgi structure in the forebrain cortex in rats with cerebral ischemia-reperfusion injury (immunohistochemical staining, × 400).

(A) Sham operation group; (B) *Danhong* injection group (injection 7 days); (C) model group (ischemia-reperfusion 7 days).

The Golgi apparatus stained lightly only in the 7-day model group, and granules decreased or disappeared, and some appeared broken.

In this study, the morphology of the Golgi apparatus was normal within the first 72 hours after ischemia-reperfusion, due to the existence of some protective mechan-

isms in the body. At 7 days, a variety of injury mechanisms (oxygen free radicals, calcium overload, inflammatory cytokines, and excitotoxic amino acids) culminated in a cascade of effects. Thus, the body cannot withstand these damaging processes and Golgi morphology is damaged. Damage to Golgi morphology and function may be aggravated as time after cerebral ischemia-reperfusion progresses.

GM130, a peripheral membrane protein of the Golgi, is involved in processing and modification, vesicle transportation, cell migration, material exchange, mitosis, microtubule generation, maintaining structural integrity of the smooth surface mesh, and GM130 is found in the Golgi zone and Golgi stack with GRASP65[17-23]. Liu[24] found that once HeLa cells were infected by CVB3, GM130 expression reduced; when the Golgi was completely broken, GM130 expression disappeared. Zhao[25] found that after PC12 cells were exposed to rotenone, GM130 expression decreased, and Golgi structure became damaged. When GM130 was silently expressed using gene technology, the morphology of the Golgi apparatus exhibited a distinct change, which directly affected their function[26]. If cells died, GM130 may destroy[27], and the Golgi apparatus disintegrates[28]. This study showed that GM130 expression gradually decreased after ischemia-reperfusion, and that expression was significantly different when compared with the normal group at 7 days, while Golgi morphology was destroyed. This is evidence that down-regulation of GM130 expression can reflect destruction of the Golgi apparatus accurately.

Danhong injection contains two pharmacodynamic ingredients (*Salvia* and *Safflower*), and its main ingredients are safflower yellow pigment, tanshinone and Salvia phenol. *Danhong* injection has achieved positive effects on the treatment of cerebral infarction where it acts as a vasodilator, anti-oxidant, anti-platelet aggregator, anti-thrombotic, improves immune stress function, improved circulation, and eliminates inflammation of the blood vessel wall[4-5, 29-32]. The increase in transforming growth factor-β1 expression had a protective effect on cerebral ischemia-reperfusion injury and regulated the structure and function of the Golgi apparatus[3]. This study found that survival of nerve cells increased in rats with cerebral ischemia reperfusion after *Danhong* injection intervention, while necrosis reduced and pathological damage of nerve cells in the *Danhong* injection 7-day subgroup was significantly reduced than that in other time point groups. Meanwhile, transforming growth factor-β1 and GM130 expression in brain tissue increased, and the morphology of the Golgi apparatus appeared normal. Therefore, we have shown that *Danhong* injection may play a neuroprotective role in protecting the Golgi apparatus, thus promoting neuronal survival and inhibiting cell apoptosis.

In summary, after cerebral ischemia-reperfusion, transforming growth factor-β1 expression increased, GM130 expression decreased, and Golgi stability was damaged. *Danhong* injection may increase the expression of transforming growth factor-β1 and GM130, and involves in the pathophysiological process of Golgi stability, which may be one of the protective mechanisms underlying nerve injury.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

Experiments were performed in the Laboratory Animal Center of Central South University, China from May 2010 to April 2011.

Materials

Animals

A total of 96 healthy, male Sprague-Dawley rats, aged 5 months, weighing 250–300 g, were provided by the Laboratory Animal Center of Central South University, China (license No. SCXK (Xiang) 2009-0012). All experiments were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[33].

Drugs

Danhong injection (batch No. 101166, 10 mL per ampulla) comprised of 750 g *Salvia*, 250 g *safflower* and 7 g *sodium chloride*, was purchased from Heze Step Length Pharmaceutical Co., Ltd. (Heze, Shandong Province, China; National Medicine Permit No. Z20026866).

Methods

Establishment of middle cerebral artery occlusion model

The right middle cerebral artery occlusion model was established using the Longa suture method[34]. Experimental animals were fasted for 12 hours, and deprived of water for 6 hours prior to the operation. Rats were anesthetized with 10% (v/v) chloral hydrate (0.3 mL/100 g). The carotid

artery, internal carotid artery and external carotid artery were separated, and an incision was made on the carotid artery using ophthalmic scissors. A nylon cord (0.26 mm diameter) was inserted into the internal carotid artery from the incision of the carotid artery, with the length of the line being 18–20 mm. Resistance implied that the line had reached the beginning of the right middle cerebral artery, thus blocking blood flow of the vessel. Reperfusion was given after 2 hours of cerebral ischemia, after which the skin wound was sutured. The sham operation group only underwent blood vessel separation, and received no other operation.

Drug intervention

Danhong was injected intraperitoneally 1 day before the experiment (8 mL/kg, once a day)[4] and injections were terminated until tissue harvesting. Rats were euthanized at the indicated time points. The model and sham operation groups were injected with the same dose of normal saline.

Preparation of tissue samples

After rats were decapitated, the forebrain (cortex, hippocampus) was removed and fixed in 4% (w/v) paraformaldehyde. After processing, including conventional dehydration, waxing, and paraffin embedding, sections were sliced into 5 μm slices. Slices were attached to poly-L-lysine treated slides for hematoxylin-eosin and immunohistochemical staining.

Immunohistochemical staining for the expression of transforming growth factor-β1 and GM130

The brain tissue of rats was formalin-fixed and embedded into paraffin sections. Slices were dewaxed by alcohol and hydrated in distilled water. Slices were immersed in citrate, and boiled for 20 minutes in an electric furnace. After natural cooling, antigen repair solutions were allowed to reach room temperature for 10 minutes. After normal goat serum was added, sections were placed in a wet box at 37°C for 30 minutes, and the serum was discarded without washing. Primary antibodies (rabbit anti-rat transforming growth factor-β1 monoclonal antibody, 1:50; rabbit anti-rat GM130 monoclonal antibody, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were applied and incubated at 4°C overnight. Negative controls were treated with PBS or serum. Secondary antibody (goat anti-rabbit IgG, 1:200; Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) and horseradish peroxidase-labeled chain Avidin (Zhongshan Golden Bridge Biotechnology Co., Ltd.) were respectively added at 37°C for 30 minutes each. After slices were developed with 3,3'-diaminobenzidine (Zhongshan Golden Bridge Biotechnology Co., Ltd.) at room temperature, the staining reaction was observed under the microscope, and sections were washed with distilled water to terminate the reaction. Subsequently, slices were stained with hematoxylin for 5 minutes, dehydrated, cleared in xylene, mounted, and coverslipped. All slices were observed on the same bio-optical microscope (Olympus, Tokyo, Japan), and images were analyzed using the PAS9000 pathology image analysis system (Peking Beiruida Medical Technology Co., Ltd., Beijing, China). Five slices were randomly selected from each rat for observation under 200 × magnification. The quantity of transforming growth factor-β1- and GM130-positive cells in the forebrain cortex were counted in five visual fields, and the average was calculated as the number of positive cells at each time point.

Statistical analysis

Data were expressed as mean ± SD and analyzed using SPSS 18.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance was used to compare data among multiple groups. The least significant difference *t*-test method was used for pairwise mean comparison. A value of $P < 0.05$ was considered statistically significant.

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