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The effects of acupuncture and traditional Chinese medicines on apoptosis of brain tissue in a rat intracerebral hemorrhage model

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HIGHLIGHTS

- Chinese herbs and acupuncture may improve neural impairment and reduce apoptosis.
- Significant differences in TUNEL assay were found between treatment groups.
- Differences of BCL-2, BAX, and caspase-3 were observed between treatment groups.

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ABSTRACT

Objective: This study aimed to evaluate the effects of Chinese herbs and acupuncture on the neuronal apoptosis and the expression of apoptosis-related genes in the brain tissue of rats following intracerebral hemorrhage (ICH).
Methods: Collagenase VII was injected into the caudate nucleus of Sprague–Dawley rats to induce ICH. Chinese herbs (musk, borneol and tetrandrine) were intragastrically administered, and acupuncture was performed using Dazhui, Fengfu and Shuigou acupoints. Each group was further subdivided into 4 subgroups based on treatment duration (6-hour, 24-hour, 72-hour, and 1-week). Neurological impairment score, TUNEL assay and apoptotic markers, BCL-2, BAX, and caspase-3 were used to evaluate the apoptosis status after ICH and subsequent treatment.
Results: Chinese herbal therapy and acupuncture improved neurological impairment compared with no therapy and sham-operated animals. Significant differences in TUNEL positive cells were found between treatment groups ($p < 0.001$) and over time ($p < 0.001$). Differential expression of BCL-2, BAX, and caspase-3 was observed between treatment groups ($p = 0.014$ for BAX and <0.05 for BCL-2 and caspase-3) and treatment duration groups ($p = 0.006$ for BAX and <0.05 BCL-2 and caspase-3).
Conclusions: Results indicate that Chinese herbs and acupuncture may improve neural impairment and reduce apoptosis, although there was no difference between therapies in a rat model of ICH. Additional experiments are needed to further clarify the role of these therapies following ICH.

1. Introduction

Intracerebral hemorrhage (ICH) refers to primary non-traumatic parenchymal hemorrhage in the brain and is one of the most severe types of stroke [1]. ICH is the second most common subtype of stroke after ischemic stroke and accounts for approximately 10% to 20% of all strokes [2]. The most common cause of ICH is hypertension [3]. As compared to ischemic stroke, ICH is associated with a poor prognosis, neurological impairments, and a high mortality rate. The mortality rate for patients with ICH patients has been reported to range from 35% to 52% (within 30 days of ICH), and greater than 50% of patients have been reported to die within 2 days [4]. Cerebral edema, increased intracranial pressure and herniation are the major causes of death in ICH patients.

Brain cell death after ICH may be mediated in part by apoptotic mechanisms [5]. Apoptosis, or programmed cell death, functions to eliminate dying cells across the cell cycle and contributes to normal development and tissue homeostasis [6]. However, unchecked apoptosis has been associated with neurodegenerative disorders, including stroke [5,7]. Following ICH, apoptosis has been reported to be a significant contributor to neural death [8]. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells and activation of caspase-3 have also been reported following intracerebral hemorrhage [9], both of which are markers of apoptosis. TUNEL staining is a widely used method for the detection of DNA fragmentation, a characteristic of apoptotic cell death [10].

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Chinese herbs and acupuncture have been used as a clinical treatment for various diseases in Oriental medicine, including stroke. Clinical studies have revealed that Chinese herbs and acupuncture may improve the symptoms of stroke, attenuate neurological dysfunction and promote the absorption of hematoma in the acute phase and remission phase of ICH [11–14].

We currently report the effect of Chinese herbs (musk, borneol, and tetrandrine) and acupuncture (Dazhui, Fengfu and Shuigou acupoints) on neuronal apoptosis in rats examined via TUNEL assay and immunohistochemistry for caspase-3. It was hypothesized that early intervention by adjuvant therapy may reduce apoptosis and assist recovery following ICH.

2. Materials and methods

2.1. Animals

A total of 160 Sprague–Dawley rats were divided into 6 groups: the control group (n = 8), the sham operated group (n = 32), the intracerebral hemorrhage (ICH) group (n = 56), the ICH and acupuncture treatment (acupuncture) group (n = 32), the ICH and traditional Chinese medicine (medicine) group (n = 32), and the ICH and combination of both acupuncture and the traditional Chinese medicine (combination) group (n = 32). Except for the control and ICH groups, each group was further subdivided into 4 time (treatment duration) groups (i.e., 6-hour, 24-hour, 72-hour, and 1-week).

Rats were male (specific pathogen free), aged 3 months, and weighed 250–300 g (purchased from the Experimental Animal Center of Guangzhou University of Chinese Medicine). Experiments were conducted in the Experimental Animal Center of Guangzhou University of Chinese Medicine. Animals were housed in a controlled environment with 12 h:12 h light:dark cycle at 23 ± 2 °C at a humidity of 40–70%. Animals were provided water and food ad libitum.

2.2. Intracranial hemorrhage model

Animals were intraperitoneally anesthetized with 10% chloral hydrate at 300 mg/kg. Rats were then placed in a prone position and fixed in a stereotaxic instrument (Stoelting, USA). Hair was removed from the head and scalp and skin was sterilized. An approximately 0.8 cm incision was made at the midline of the scalp exposing the skull. A hole was drilled at the site 0.2 mm posterior and 2.9 mm right to the midline of bregma. A microinjector (Zhenhai Glass Instrument Factory; 0.7-mm needle, Ningbo, China) was then inserted vertically into the brain to a depth of 6.0 mm. Collagenase VII (Sigma, Germany; 0.6 U; 0.2 U/μl) was injected into the right caudate nucleus at a rate of 1 μl/min. After injection, the needle remained in the injection site for 5 min, and was then withdrawn. The wound was closed and sterilized. In the sham surgery experimental group, the same protocol was adhered to, however normal saline was injected (3 μl) into the brain in place of Collagenase VII. A non-surgical control group did not receive any surgical procedures. All procedures were performed using aseptic conditions.

After surgery, neurological function was evaluated using the NDS system described by Gerriets et al [15]. A neurological score of 1–5 was indicative of establishment of ICH. Rats with score of "0" and those that died during or after surgery were excluded from the study.

2.3. Acupuncture therapy

Acupuncture was performed on 1 h after the establishment of ICH. Acupuncture was performed once daily thereafter. Rats were sacrificed 1 h after the last acupuncture treatment. A 30-gauge filiform needle (1 in.; Suzhou Tianyi Acupuncture Instruments Ltd., Suzhou, China) was used for all acupuncture. Briefly, acupuncture was administered at the following acupoints: Dazhui (2 mm straight stab), Fengfu (1 mm posterior oblique stab downward), and Shuigou (1 mm straight or upward stab). Acupuncture of the Dazhui and Fengfu acupoints was performed using the reinforcing–reducing method once every 10 min (acupuncturing for 30 s and retaining the needle for 30 min). The Shuigou acupoint was acupunctured for 30 s without retaining the needle.

2.4. Chinese herbal treatment

The intragastric injection of Chinese herbs was performed 1 h after the establishment of ICH and thereafter once every 12 h. In the combined therapy group, rats received treatment with Chinese herbs and acupuncture. The administration of acupuncture and Chinese herbs was aligned for those animals being treated in combination. Rats were sacrificed 1 h after the last intragastric injection of Chinese herbs.

Musk (Yunnan Tengchong Pharmaceutical, Yunnan, China), Borneol (Guangdong Health America Pharmaceutical Co. Ltd., Guangdong, China), and Tetrandrine (Guangzhou Tai Zong Trading Co., Ltd., Guangzhou, China) were mixed at a ratio of 1:3:4. The dose (amount per kg) of this mixture used in the current study for rats was calculated according to the dose for humans (N [rat] = 5.75 M [human]).

The routine dose of tetrandrine for adults is 400 mg, twice daily; thus, the dose for rats was (800 mg/60 kg) × 5.75 = 76.67 mg/kg (7.667 mg/100 g/d). Rats were intragastrically injected with tetrandrine at 3.83 mg/100 g [5 mg/ml]. The routine dose of musk for humans is 100 mg, twice daily; thus, the dose for rats was (200 mg/60 kg) × 5.75 = 19.17 mg/kg (1.917 mg/100 g/d). Rats were intragastrically injected with musk at 0.95 mg/100 g [1.586 mg/ml]. The routine dose of borneol for humans is 300 mg, twice daily; thus, the dose for rats was (600 mg/60 kg) × 5.75 = 57.50 mg/kg (5.75 mg/100 g/d). Rats were intragastrically injected with borneol at 2.875 mg/100 g [3.753 mg/ml].

For those animals that did not receive administration of Chinese herbs, the volume of normal saline was calculated as follows: 7.667 mg/100 g/d × body weight (g) ÷ 5 mg/ml. Injection protocols were aligned with all other groups.

2.5. Detection of neuronal apoptosis

Upon removal, brain tissues were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and cut into 4-μm sections. TUNEL staining was performed using the TUNET-AP kit (Boehringer Mannheim) and BCIP-NBT kit (Beijing Zhongshan Company, Beijing, China). Stained sections were observed at a magnification of ×200 (Olympus BX50), and 4 fields were randomly selected from each section and captured for further quantitative analysis with an image analysis system (Guangzhou Yiming Image analysis software). Apoptotic cells associated with the hematoma were quantified for all sections. The apoptotic bodies were visualized as black or violet-blue granules in the nucleus without background.

2.6. Immunohistochemistry for BCL-2/BAX and caspase-3

The brain tissues were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and cut into 4-μm sections which were then incubated with rabbit anti-rat primary antibodies (1:100; BCL-2, BAX and caspase-3; Wuhan Boster, Wuhan, China) at 37 °C for 1 h and then with goat anti-rabbit biotinylated secondary antibody (Wuhan Boster) at 37 °C for 20 min. Cells were visualized with DAB and counterstained with hematoxylin. Sections were then observed under a light microscope at a magnification of ×200, and 4 fields were randomly selected from each section and captured for further quantitative analysis of BCL-2/BAX and caspase-3. BCL-2/BAX and caspase-3-positive cells were visualized as yellow-brown granules in the cytoplasm or on the cell membrane.