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Original Article

Effects of Buyang Huanwu decoction combined with edaravone on mitochondrial apoptotic pathway of nerve cells in mice with cerebral ischemia-reperfusion injury

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Abstract: Objective: To study the neuroprotective effect and underlying mechanism of Buyang Huanwu Decoction (BUHWD) combined with edaravone in mice with cerebral ischemia-reperfusion injury. Method: Ischemia-reperfusion injury in middle cerebral artery was induced by modified occlusion method. Ninety mice were randomly divided into 5 groups (n=18): sham operation group, model group, BYHWD group (10 ml/kg BYHWD by irrigation), edaravone (ED) group (3 mg/kg ED by injection via the tail vein), and BYHWD+ED group (3 mg/kg of the mixture by injection via the tail vein). The above drugs were given once daily for 7 days consecutively. Neurologic impairment scores were recorded for each group at 1, 3 and 7 d postoperatively. Infarct volume of the brain was assessed by TTC staining at 7 d postoperatively. Neuronal apoptosis was assessed by TUNEL assay. Expression levels of Bcl-2, Bax and Caspase-3 in the ischemic cortex were detected by Western Blot. The results were compared between the groups using one-way ANOVA. Pairwise comparison was done by LSD t-test. Results: After ischemia-reperfusion, neurologic impairment scores decreased over time. For the same time point, the score of the model group was the highest and that of the combined treatment group was the lowest ($P<0.01$). At 7 d after surgery, the ischemia area of BYHWD+ED group was the smallest ($P<0.01$). After treatments, all groups showed a decline in apoptotic cells ($P<0.05$). Upregulation of Bcl-2 with downregulation of Bax and Caspase-3 was the most significant in BYHWD+ED group ($P<0.05$). Conclusion: The combined treatment had a synergistic neuroprotective effect in mice following cerebral ischemia-reperfusion injury. The working mechanism was related to reduction in cell apoptosis and regulation of proteins in mitochondrial apoptotic pathway.

Keywords: Buyang Huanwu decoction, edaravone, cerebral ischemia-reperfusion injury, apoptosis

Introduction

Cerebral apoplexy is the second most prevalent fatal disease and the leading cause of disability. Ischemic stroke accounts for over 80% of all cases with cerebral apoplexy, with middle cerebral artery occlusion (MCAO) being the most common condition [1]. Ischemic brain injury involves multiples steps and responses, where mitochondrial apoptotic pathway is the primary pathway for neurons following cerebral ischemia [2-4]. Cell apoptosis is regulated by Bcl-2 and Bax which act on the mitochondrial permeability. Bcl-2 is inhibitory of cell apoptosis, while Bax is pro-apoptotic. The two proteins interact in the programmed cell death [5, 6]. Caspase-3 is one of the most important downstream effectors of several apoptotic pathways and a key enzyme in triggering apoptosis mediated by different factors. Caspase-3 is involved in cerebral ischemia-reperfusion injury by regulating cell apoptosis [7]. Bcl-2 and Caspase families play a synergistic role in regulating the mitochondrial apoptotic pathway, and intervening mitochondrial apoptotic pathway has a neuroprotective effect in cerebral ischemia-reperfusion injury.

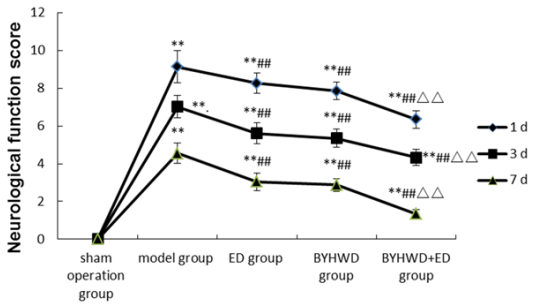

Figure 1. Neurologic impairment scores of each group. Note: compared with the sham operation group, **P<0.01; compared with the model group, ##P<0.01; compared with ED or BYHWD group, ΔΔP<0.01.

Buyang Huanwu Decoction (BYHWD) is a classical prescription for benefiting qi, activating blood circulation and regulating the meridians. Edavarone (ED) can resist oxygen free radicals. The effects of BYHWD combined with ED on cerebral ischemia-reperfusion injury in mice and on mitochondrial apoptotic pathway of neurons were discussed. By analyzing the mechanism of neuroprotective effects, we hope to provide experimental data to the combination of Chinese and western medicine in ischemic cerebrovascular diseases.

Materials and methods

Animals

Ninety C57BL/6 mice aged about 8 months and weighing 45-80 g (45 males and 45 females) were provided by Shanghai Sippr-bk Laboratory Animals Co., Ltd. (laboratory animal license: SCXK (Shanghai) 2008-0016).

Drugs and reagents

Edavarone injection (Simcere Pharmaceutical Group, license No.: 080825), Buyang Huanwu Decoction (prepared by preparation room of xxx hospital). The decoction was prepared by boiling 120 g *Astragalus membranaceus*, 6 g *Angelica sinensis*, 3 g *Ligusticum wallichii*, 3 g earthworm, 5 g red peony root, 3 g *Carthamus* tinctorius and 3 g peach seeds. The raw decoction was concentrated to 2 g/ml. Other reagents were: riphenyltetrazolium chloride (TTC) (Sinopharm Chemical Reagent Co., Ltd., license No. 20040328), TUNEL assay kit (Roche, Swiss, license No. 11684817910), immunohistochemistry staining kit for Bcl-2 (license No. 20070223), Bax (license No. 20070223 and Caspase-3 (license No. 20070227) (Wuhan Boster Biological Technology Ltd.).

Modeling

MCAO was induced in mice by modified occlusion method. Thread was removed 90 min after induction of ischemia and reperfusion was performed. The model was successfully built according to neurologic impairment score 2 h postoperatively. For the sham operation group, the right common carotid artery was separated and the right external carotid artery was severed without occlusion.

Grouping and treatments

Before formal experiment, mice were acclimatized for 3 d. The mice were randomly divided into 5 groups (n=18) and treated with different drugs at 2 h postoperatively: BYHWD group (10 ml/kg BYHWD by irrigation), edaravone (ED) group (3 mg/kg ED by injection via the tail vein), BYHWD+ED group (3 mg/kg of the drug mixture by injection via the tail vein), model group and sham operation group (gastric irrigation and injection via the tail vein of equal amount of normal saline, respectively). The above drugs were given once daily for 7 days consecutively.

Neurologic impairment scores

Neurologic impairment scores were measured at 1 d, 3 d and 7 d postoperatively in accordance with the literature [9]. The full score was 10. Modeling was considered successful for score ≥2, and mice which had score <2 or died were eliminated. The higher the score, the more severe the neurologic impairment was.

Determination of infarct volume of brain

Mice were sacrificed at 7 d postoperatively to harvest the brain tissues. From 2 m behind the bregma, coronal slices were made at an interval of 2 mm and 5 slices were obtained. The brain slices were dyed in freshly prepared 1% TTC solution in the dark for 30 min and fixed in 4% paraformaldehyde. The tissues were photographed and the infarct area was delineated using Photoshop software. Infarct area and the

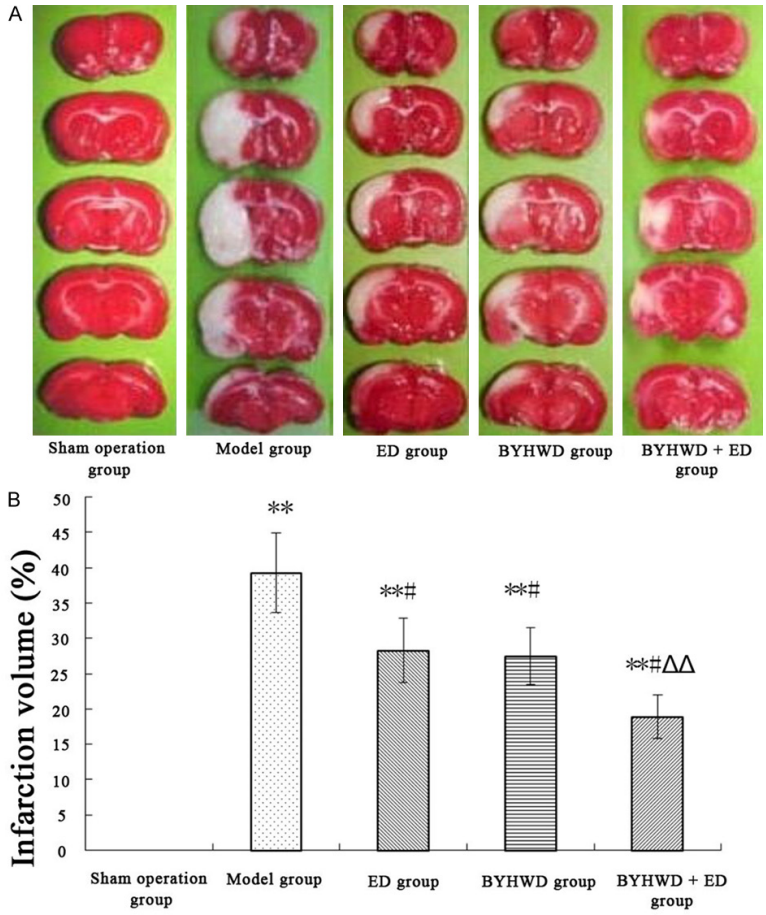

Figure 2. Comparison of infarct volume by TTC staining. Note: A. Representative TTC stained coronal sections showing infarct area in the ischemic cerebral hemisphere as distinct pale-stained area at 7 d after surgery. B. Percentage of infarct volume of the brain in each group at 7 d after surgery; compared with the sham operation group, **P<0.01; compared with the model group, #P<0.05; compared with ED or BYHWD group, ΔΔP<0.01.

area of left and right brain hemispheres were measured. Infarct volume was calculated by multiplying total area by slice thickness (mm^3). Percentage of infarct volume (%)= volume of pale region of infarction/(volume of pale region of infarction + volume of non-pale region of infarction) × 100%.

TUNEL assay

Brain tissues were fixed in 4% paraformaldehyde and the medium was replaced 24 h later by 30% sucrose every 2-3 days. The tissues were embedded in OCT and cut into coronal slices of 6 μm thickness using a freezing microtome. TUNEL assay was conducted according to user's instruction manual.

Western blot detection of Bcl-2, Bax and Caspase-3 in ischemic cortex

At 7 d after modeling, brain cortex around the ischemic region was collected from 6 mice in each group. The tissues were added with a proper amount of protease inhibitor cock-tail and homogenated on ice. Centrifugation was performed at 13000 r/min and 4°C for 20 min and the supernatant was collected. Protein concentration of the brain tissues was determined using BCA assay kit. After the addition of 5 × SDS-PAGE loading buffer, the proteins were denatured by heating at 99°C and separated by 10% SDS-PAGE. The proteins were blotted to PVDF membranes using wet transfer method, which were sealed at room temperature on a shaker for 2 h. Then Bcl-2 (1:500), Bax (1:500) and Caspase-3 (1:800) antibodies and internal reference gene a-Tubulin were added to incubate the cells at 4°C overnight. The membranes were further incubated at room temperature on a shaker for 2 h and washed with TBST for three times, 10 min each time. With the addition of HRP-labeled secondary antibodies, the membranes were incubated at room temperature on a shaker for 2 h and washed with TBST for three times, 10 min each time. Finally, the protein levels were assayed using an ECL Western Blotting kit.

Statistical analysis

SPSS 17.0 software was used for statistical analysis. Data were reported as $\bar{x} \pm s$. One-way ANOVA was adopted for multiple comparisons and LSD t-test for pairwise comparisons. P<0.05 was considered significant difference.

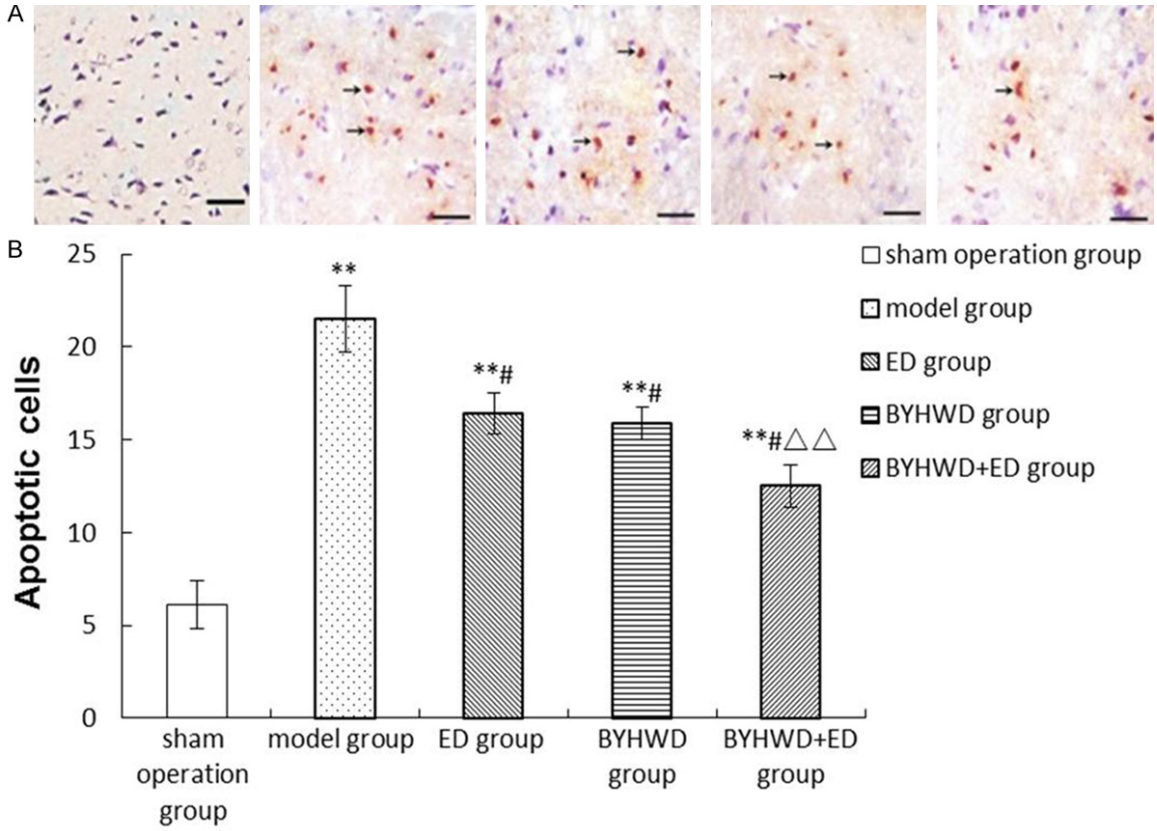

Figure 3. Effects of drug treatments on cell apoptosis following cerebral ischemia-reperfusion injury. Note: A. Representative photomicrographs of TUNEL staining in the ischemic penumbra 7 day after MCAO. The arrows mean apoptotic cells (TUNEL-positive cells). Magnification: 400 ×, Scale bar =20 um. A-sham operation group; B-model group; C-ED group; D-BYHWD group; E-BYHWD+ED group; B. Quantitative analysis of the number of apoptotic cells .Data are expressed as the mean ± SD (n=6). **P<0.01, as compared with the sham operation group; #P<0.05, as compared with the model group; ΔΔP<0.01, as compared with ED group or BYHWD group.

Results

Effects of different treatments on neurologic impairment scores

As shown in **Figure 1**, neurologic impairment scores at 1 d, 3 d and 7 d after cerebral ischemia-reperfusion injury decreased over time. At 7 d, the model group and all treatment groups showed a significant reduction in neurologic impairment score as compared with that at 1 d and 3 d (P<0.05). In contrast, neurologic deficit was not observed in the sham operation group. At the same time point, neurologic impairment scores were the highest in the model group, followed by single drug treatments. The neurologic impairment scores were the lowest in BYHWD+ED group and the difference compared with any other groups reached a significant level (P<0.01).

Comparison of infarct volume between the groups

As shown in **Figure 2A**, by TTC staining, the white region was the infarct region and the red region was the normal brain tissue at 7 d after cerebral ischemia-reperfusion injury. In **Figure 2B**, the infarct volume was 0 for the sham operation group and that of the model group increased significantly (P<0.01). As compared with the model group, the infarct volume of each drug treatment group decreased considerably (P<0.05). The reduction was most significant in BYHWD+ED group (P<0.01).

Effects of different treatments on cell apoptosis after cerebral ischemia-reperfusion injury

TUNEL assay was performed to detect apoptotic cells following cerebral ischemia-reperfu-

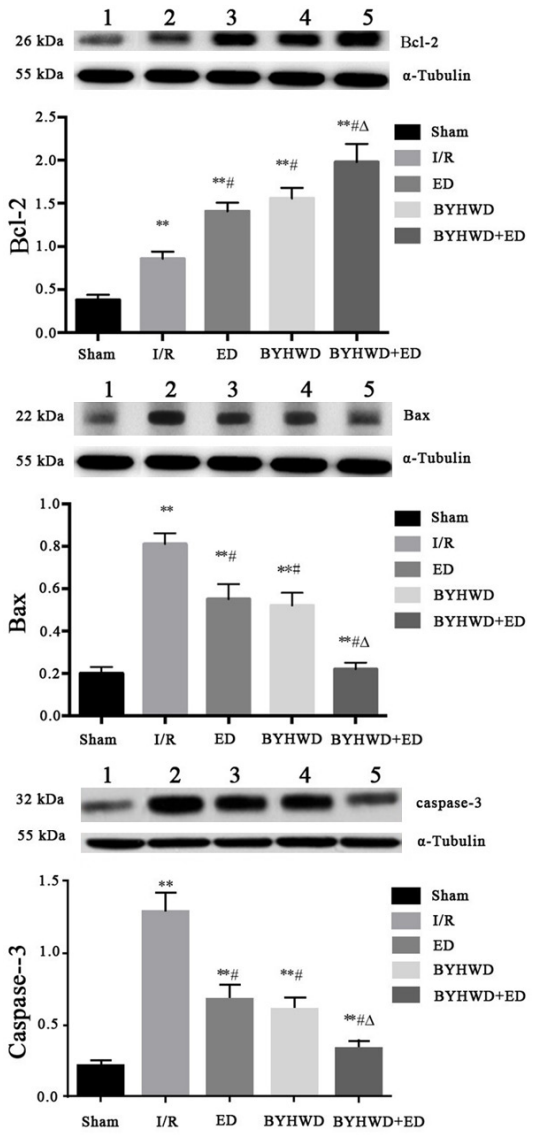

Figure 4. Western Blot detection of Bcl-2, Bax and Caspase-3 in each group after cerebral ischemia-reperfusion injury. Note: 1: Sham operation group; 2. I/R group; 3: ED group; 4: BYHWD group; 5: BYHWD+ED group. compared with the sham operation group: **P<0.01; compared with the model group, #P<0.05; compared with ED or BYHWD group, ΔP<0.05.

sion injury. Under the light microscope, the nuclei of apoptotic cells were brown, as indicated by the arrow in **Figure 3A**. As to the number of apoptotic cells, it increased significantly in the model group as compared with the sham operation group (P<0.01). The number of apoptotic cells decreased significantly in each drug treatment group as compared with the model group (P<0.05), and the decrease was the greatest in BYHWD+ED group (P<0.01). However, BYHWD and ED groups did not differ significantly from each other (P>0.05) (**Figure 3**).

Effects of drug treatments on cortical expressions of Bcl-2, Bax and Caspase-3 in mitochondrial apoptotic pathway

Western Blot was conducted to determine the expressions of Bcl-2, Bax and Caspase-3 in the ischemic cortex at 7 d after cerebral ischemia-reperfusion injury. In the sham operation group, Bcl-2, Bax and Caspase-3 were lowly expressed. As compared with the sham operation group, Bcl-2, Bax and Casepase-3 were upregulated significantly in the model group and each drug treatment group (P<0.01). As compared with the model group, Bcl-2 in each drug treatment was upregulated considerably (P<0.05), while Bax and Caspase-3 were downregulated (P< 0.05). Changes in the above proteins were greater in BYHWD+ED group as compared with BYHWD group and ED group (P<0.05). However, the difference between BYHWD group and ED group did not reach a significant level (P>0.05) (**Figure 4**).

Discussion

Nerve cell damage caused by cerebral ischemia-reperfusion injury consists of necrosis and apoptosis. Cells in the center of the infarct region become necrotic quickly, while the cells in the peripheral semi-dark ischemic region show delayed death, which is predominantly apoptosis [10]. Reducing necrosis of nerve cells in the periphery of the infarct region is crucial for treating ischemic brain diseases. Mitochondrial apoptotic pathway is one important pathway triggered by brain ischemia, which leads to changes of mitochondrial morphology. For example, increased membrane permeability can promote the release of cytochrome C, resulting in protease cascade and cell apoptosis [3, 4]. Mitochondrial apoptosis is mainly regulated by Bcl-2 family and involved in the formation of channels in outer mitochondrial membrane. Bcl-2 and Bax are the most representative genes involved in cell apoptosis. The former inhibits cell apoptosis, while the latter promotes it. Bcl-2 binds to Bax and overexpression of Bax will facilitate cell apoptosis and counteract the anti-apoptotic effect of Bcl-2. Bcl-2/Bax ratio is related to the survival and death of cells. The higher the ratio, the lower the survival, and the lower the ratio, the higher the apoptosis rate will be [11-13]. Caspase-3

acts as the key executor of protease cascade and activate programmed death mediated by different factors [7]. Inhibiting Caspase-3 expression can help prevent deterioration of brain damage and improve nerve function.

BYHWD was described in Correction on Errors in Medical Classics by Wang Qing in Qing Dynasty. Generally used as a classical prescription for benefiting qi, activating blood circulation and regulating the meridians, BYHWD exerts neuroprotective effects by acting on multiple targets and pathways [14, 15]. ED can clear free oxygen radicals, inhibit lipid peroxidation and promote the secretion of neurotrophic factors [16, 17]. We applied BYHWD combined with ED to MCAO models in mice and the results showed that BYHWD+ED group had a more significant reduction in neurologic impairment scores as compared with the model group and single drug treatment groups. This means the combined drug use had a synergistic effect in reducing neurologic deficit caused by cerebral ischemia-reperfusion injury in mice. Moreover, the combined drug treatment outperformed any single drug treatments. At 1 d after modeling, the neurologic impairment scores reached the highest for all groups and decreased significantly at 7 d. Neurologic impairment scores were gradually improved over time, though the change was very slight at an early stage. This fact was indicative of the length of time window appropriate for treatment after ischemia-reperfusion injury. The degree of nerve damage was measured by infarct volume of the brain and the results showed that the combined treatment group was superior to either the model group or single drug groups in this indicator.

TUNEL assay indicated that there were more apoptotic cells in the model group as compared with all drug treatment groups. Moreover, the combined treatment group was more effective in inhibiting cell apoptosis than any single drug treatment group. Although TUNEL assay is most commonly used for detecting cell apoptosis [18], it may have some difficulty in discriminating between apoptotic and necrotic cells. To avoid false positive result, pro- and anti- apoptotic genes were further detected. According to Western Blot, Bcl-2, Bax and Caspase-3 were upregulated significantly in the semi-dark ischemic region at 7 d after cerebral ischemia-reperfusion injury. This indicated that the mitochondrial apoptotic mechanism was activated by cerebral ischemia-reperfusion injury. After treatments, Bcl-2 expression increased ($P<0.05$), while the expressions of Bax ($P<0.05$) and Caspase-3 ($P<0.05$) decreased as compared with the model group. These changes were most salient in the combined treatment group ($P<0.05$). It was inferred that BYHWD and ED inhibited nerve cell apoptosis, facilitated nerve function recovery and exerted the neuroprotective effect by upregulating Bcl-2 and downregulating Bax and Caspase-3.

To conclude, BYHWD combined with ED effectively alleviated nerve cell damage and infarct area of the brain in mice following cerebral ischemia-reperfusion injury. The two drugs can act synergistically by changing the expressions of Bcl-2, Bax and Caspase-3 in the mitochondrial apoptotic pathway. The above results demonstrated the benefits of combined Chinese and western medicine in acute ischemic cerebrovascular disease, and more clinical trials and practice are required to confirm this.

Disclosure of conflict of interest

None.

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