Efficacy of Bunao Fuyuan decoction on cerebral ischemia and reperfusion injury in vitro

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Abstract

OBJECTIVE: To investigate the protective efficacy of Bunao Fuyuan decoction (BNFY) on cerebral Ischemia/reperfusion (I/R) injury.

METHODS: The mouse PC12 cells were chosen, and the oxidative-glucose deprivation/re-oxygenation (OGD/R) injury model were established to simulate cerebral I/R injury. Atorvastatin was selected as a positive drug, and a gradient dose of BNFY was given for 6, 12 and 24 h. 3-(4,5)-dimethylthiahi-azo (-z-y)-3,5-di- phenyterazoliumromide (MTT) assay were used to detect cell viability at each time point. Cell apoptosis was measured by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining. enzyme linked immunosorbornt assay were used to detect the expression of key regulators (toll-like receptor 4 (TLR4), nuclear factor kappa-B (NF-κB), p-p38 mitogen-activated protein kinase (MAPK) and p-Akt (also known as protein kinase B, PKB)) of cell survival and inflammatory response.

RESULTS: The results of MTT assay and TUNEL staining assay revealed that BNFY significantly increased cell viability and inhibited cell apoptosis of PC12 cells following OGD/R, respectively. Furthermore, the expression of TNF-α, IL-6, IL-1β and PAF were decreased after BNFY treatment. And the results of Western blot assay showed that BNFY downregulated TLR4, NF-κB, p-p38 MAPK expression and upregulated p-Akt expression.

CONCLUSION: Our findings suggest that BNFY may play a role in protecting OGD/R injured PC12 cells through inhibiting the inflammatory response and cell apoptosis.

Keywords: Inflammatory response; Ischemic stroke; PC12; Oxidative-glucose deprivation/re-oxygenation; Bunao Fuyuan decoction

INTRODUCTION

Ischemic stroke is a clinically common cerebrovascular disease with high rates of morbidity and is one of the major causes of severe disability and death worldwide.1,2 Restoring blood and oxygen, the primary concern of clinical treatment for ischemic stroke, causes cerebral apoptosis and necrosis, which is termed ischemia/reperfusion (I/R) injury.3 Increasing evidence has demonstrated that inflammatory reaction plays a pivotal role in the pathogenesis of cerebral I/R injury.4 I/R triggers acutely activate the immune system and the subsequent inflammatory cascade, resulting in irreversible
neuronal damage and deterioration of brain injury. Anti-inflammatory therapy could be an efficacious therapeutic strategy to attenuate the progression of cerebral I/R injury and prevent brain function. Therefore, research and development of neuroprotective drugs to reduce apoptosis and inhibit inflammatory response is clinically imperative for the treatment of ischemic stroke.

Traditional Chinese Medicine and classic prescriptions, such as astragulus, ginseng, and angelica, is widely used in China for the treatment of I/R injury. Bunao Fuyuan (BNFY) decoction is developed from Buyang Huanwu decoction, which has long been used for the treatment of ischemic stroke. The separate component of Buyang Huanwu decoction decreases the level of inflammatory factor. Therefore, we presume that BNYF has potential therapeutic effect on cerebral I/R.

In the present study, we aimed to investigate the efficacy of BNYF on cerebral I/R injury, and elucidate the possible molecular mechanism underlying its action.

**MATERIALS AND METHODS**

**Cell and culture**

PC12 (pheochromocytoma) were purchased from American Type Cell Culture Collection (ATCC; Manassas, VA, USA) and cultured with Dulbecco modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 mg/mL streptomycin in a humidified incubator with an atmosphere of 5% CO₂ at 37 °C.

**BNFY preparation**

The decoction was prepared with Huangqi (Radix Astragali Mongolici), Danshen (Radix Angelicae Sinensis), Taoren (Semen Pericae), Chishao (Radix Paeoniae Rubra), Danshen (Radix Salviae Miltiorrhizae), Chuanxiong (Rhizoma Chuanxiong), Shuizhi (Hirudo), Shichangpu (Paeoniae Radix Alba), Chuanxiao (Radix Glycyrrhizae), and Chuanniu (Radix Cyathulae).

**OGD/R and drug treatment**

To mimic ischemia/reperfusion conditions in vitro, PC12 cells were incubated with glucose-free DMEM (Gibco, Grand Island, NY, USA) without foetal bovine serum (FBS) in an anaerobic chamber containing a gas mixture composed of 5% CO₂ and 95% N₂ at 37 °C for 6 h. Then, the glucose-free DMEM was replaced with complete medium and the plates were removed from the anoxia incubator and cultured under normal conditions for 6, 12, and 24 h. For the drug treatment groups, different dose (0.2, 0.5 and 1.0 mg/mL) of BNFY or atorvastatin (10 μM) was given with the complete medium after OGD. Cells that were not exposed to OGD and incubated under normal conditions continuously were used as control.

**3-(4,5)-dimethylthiahiazolo -(2-y1)-3,5-di-phenyterazoliumromide (MTT) assay**

Cell viability was determined using the MTT assay. Briefly, PC12 cells were seeded into 96-well plates at a density of 5 × 10⁴ cells/well for 24 h. At 6, 12, and 24 h after BNFY treatment, MTT (Promega, Madison, WI, USA) with a final concentration of 0.5 mg/mL was added to each experimental well for incubation at 37 °C for 4 h. Then, to dissolve the formazan crystals, the MTT solution was replaced with 100 μL dimethyl sulfoxide (DMSO). The absorbance of each well was read at 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Each experiment was repeated in quadruplicate, and independent experiments were performed for three times.

**Terminal dUTP nick-end Labeling (TUNEL) assay**

The TUNEL assay was used to assess cell apoptosis. After BNFY treatment, PC12 cells were fixed with 4% paraformaldehyde. TUNEL positive cells displayed green staining within the nucleus and 5 fields for each section were randomly selected for the calculation of positive cells.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)**

Total RNA from PC12 cells was extracted and purified using Trizol reagent (15596026, Invitrogen, Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) according to the manufacturer’s protocol. The concentration of RNA was measured using NanoDrop 2000c (Thermo Fisher Scientific, Inc., Carlsbad, CA, USA). And the TaqMan Reverse Transcription Kit (Takara Bio Inc., Dalian, China) was used to obtain cDNA followed the manufacturer’s instructions. RT-qPCR was performed using a Perfect Real Time SYBR Premix Ex Taq Kit (Takara Bio Inc., Dalian, China) with an ABI 7500 thermocycler (Thermo Fisher Scientific, Inc., Carlsbad, CA, USA). The reaction conditions for PCR were as follows: Pre-degeneration at 95 °C for 5 min and following 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The relative expressions of mRNAs were analyzed by the 2⁻ΔΔCt method. Independent experiments were repeated three times. The primers used were as follows: Bcl-2, Forward 5‘-CCGAAACCTTGTGTGACCCGAGG-3’ and Reverse 5‘-GGTGTACCCCGTCGGTAAGA-3’. Caspase-3, Forward 5‘-ACAGTTCTTCAGCCGCTGACTG-3’ and Reverse 5‘-GGACATCAATACGGGATCCTG-3’. β-actin, Forward 5‘-GGGCCCTATACACACCCAGCCG-3’ and Reverse 5‘-GCATCCCTCGCTGGAATGCTG-3’.

**Enzyme linked immunosorbent assay (ELISA)**

Following the drug treatment, cell culture supernatant was collected. The levels of interleukin (IL)-1β, interleukin (IL)-6, tumor necrosis factor (TNF)-α and Platelet activating factor (PAF) were measured by ELISA kits IL-1β (#56169T, Cell Signaling Technology;
BNFY suppression of OGD/R induced apoptosis in PC12 cells

To examine the anti-apoptotic effect of BNFY, TUNEL assay was performed to test the typical DNA laddering pattern of PC12 cells after I/R. And the results suggested that, the number of TUNEL-positive cells in the PC12 cells exposed to OGD/R was significantly increased compared with control group (Figure 3), while BNFY significantly alleviated cell apoptosis induced by OGD/R. Furthermore, RT-qPCR was used to assess the levels of caspase-3 and Bcl-2. As indicated in Figure 4A, BNFY significantly decreased OGD/R induced caspase-3 and Bcl-2 expression.

BNFY inhibited OGD/R induced pro-inflammatory factors expression

Inflammatory response has been implicated in the pathogenesis of cerebral I/R injury. Therefore, pro-inflammatory cytokines TNF-α, IL-6, IL-1β and platelet-activating factor (PAF) expression were detected using ELISA assay. The levels of TNF-α, IL-6, IL-1β and PAF were significantly increased in PC-12 cells subjected to OGD/R, compared with the control group (Figure 4B). And this increase was significantly reduced by pretreatment with BNFY treatment (Figure 4B).

BNFY decreased NF-κB p65 and p-p38 MAPK expression and increased p-AKT expression in PC12 cells exposed to OGD/R

To elucidate the molecular mechanism underlying BNFY mediated neuroprotection, we investigated the efficacy of BNFY on the several key regulators of cell survival and inflammatory response in OGD/R treated PC12 cells. The results of Western blot showed that, the OGD/R treatment significantly increased TLR4, NF-κB p65 and p-p38 MAPK expression in PC-12 cells, compared with the control group (Figure 5). While BNFY significantly decreased OGD/R, and induced TLR4, NF-κB p65 and p-p38 MAPK expression (Figure 5). Moreover, OGD/R significantly repressed the activation of the phosphorylated AKT. BNFY significantly increased the level of p-AKT. Meanwhile, there were no significant differences in the AKT and MAPK levels among those different treatment groups.

DISCUSSION

In the study, we investigate the efficacy of BNFY on I/R injury. OGD/R injury model was established to simulate cerebral I/R injury. Our results demonstrated...
that BNFY significantly lowered the susceptibility of PC12 cells to OGD/R injury in vitro, indicating that BNFY may have potential neuroprotective efficacy on cerebral I/R injury. BNFY significantly reduced the OGD/R induced production of pro-inflammatory cytokines TNF-α, IL-6, IL-1β and PAF. Moreover, the results of TUNEL indicated that BNFY significantly inhibited apoptosis in PC12 cells exposed to OGD/R. Those data suggested that BNFY may prevent OGD/R induced neuronal damage via inhibiting inflammatory response and cell apoptosis to exert neuroprotective efficacy.

There exist numerous survival and inflammation-related signaling pathways that have been implicated in cerebral I/R injury, such as TLR4/NF-κB, p38 MAPK and AKT. For example, NF-κB, as a nuclear transcrip-
Figure 2 BNFY promoted cell viability in OGD/R-treated PC12 cells
Different dose (0.2, 0.5 and 1.0 mg/mL) of Bunao Fuyuan decoction (BNFY) or atorvastatin (10 μM) was given for 6, 12 and 24 h after oxidative-glucose deprivation (OGD). MTT assay was used to evaluate cell viability. Con: PC12 cells were incubated with normal medium. Model: PC12 cells were incubated with medium, which mimic ischemia/reperfusion conditions. Positive: PC12 cells were underwent OGD/re-oxygenation (OGD/R) and treated with 10 μM atorvastatin. BNFY 0.2 mg/mL: PC12 cells were underwent OGD/R and treated with 0.2 mg/mL BNFY. BNFY 0.5 mg/mL: PC12 cells were underwent OGD/R and treated with 0.5 mg/mL BNFY. BNFY 1.0 mg/mL: PC12 cells were underwent OGD/R and treated with 1.0 mg/mL BNFY. *P < 0.001 vs Con, †P < 0.01 vs model.

Figure 3 BNFY inhibited OGD/R induced cell apoptosis in PC12 cells
Cells were treated with Bunao Fuyuan decoction (BNFY) (0.5 mg/mL) or atorvastatin (10 μM) for 12 h after oxidative-glucose deprivation (OGD). TUNEL assay was used to assess cell apoptosis. A1-A2: con; PC12 cells were incubated with normal medium. B1-B3: model; PC12 cells were incubated with medium, which mimic ischemia/reperfusion conditions. C1-C3: positive; PC12 cells were underwent OGD/re-oxygenation (OGD/R) and treated with 10 μM atorvastatin. D1-D3: BNFY 0.5 mg/mL: PC12 cells were underwent OGD/R and treated with 0.5 mg/mL BNFY. E: apoptosis cells of groups. *P < 0.001 vs Con, †P < 0.01 vs model.
In conclusion, our study demonstrated that BNFY could reduce I/R injury via inhibiting inflammatory responses and cell apoptosis, and was involved in several signaling pathways including TLR4/NF-κB, p38 MAPK and AKT, which is still required to be further investigated. Unfortunately, the use of a single PC12 cell line is a limitation of our study. Comprehensive analysis of animal model should be performed in future studies.

REFERENCES

BNFY decreased NF-kB p65 and p-p38 MAPK expression and increased p-AKT expression in PC12 cells exposed to OGD/R. A: cells were incubated with BUNao Fuyu decoction (BNFY) (0.5 mg/mL) or atorvastatin (10 μM) for 12 h after oxidative-glucose deprivation (OGD). B1-B6: Western blot assay was performed to detect TLR4, NF-kB p65, p-p38 MAPK, p38 MAPK, p-AKT and AKT expression. 1: con: PC12 cells were incubated with normal medium. 2: model: PC12 cells were incubated with medium, which mimic ischemia/reperfusion conditions. 3: positive: PC12 cells were exposed to OGD/re-oxygenation (OGD/R) and treated with 10 μM atorvastatin. 4: BNFY 0.5 mg/mL: PC12 cells were exposed to OGD/R and treated with 0.5 mg/mL BNFY. NF-kB nuclear factor kappa-B; MAPK: p-p38 mitogen-activated protein kinase; p-Akt: protein kinase B. *p < 0.01 vs Con, †p < 0.05 vs model.

8 Huang XP, Tan H, Chen BY, Deng CQ. Combination of total Astragalus extract and total Panax notoginseng saponins strengthened the protective effects on brain damage through improving energy metabolism and inhibiting apoptosis after cerebral ischemia-reperfusion in mice. Chin J Integr Med 2017; 23(6): 445-452.
10 Han JY, Li Q, Ma ZZ, Fan JY. Effects and mechanisms of compound Chinese medicine and major ingredients on microcirculatory dysfunction and organ injury induced by ischemia/reperfusion. Pharmacol Ther 2017; 177(10): 146-173.
14 Ming YC, Chao HC, Chu SM, Luo CC. Heparin-binding epidermal growth factor-like growth factor (HB-EGF) protected intestinal ischemia-reperfusion injury through...


