Effects of Buyang Huanwu Decoction on Neurite Outgrowth and Differentiation of Neuroepithelial Stem Cells

Jin-Hao Sun[1, 2], Ying-Mao Gao[1], Lin Yang[1], Xian Wang[1], Li-Hua Bao[1], Wen-Jing Liu[1], and David Yew[3]

[1]*Institute of Anatomy and Histology & Embryology*
School of Medicine, Shandong University
Jinan 250012, Shandong, P.R. China
[2]*Department of Neurobiology*
Yale University School of Medicine
New Haven, CT 06520, USA
and
[3]*Department of Anatomy*
The Chinese University of Hong Kong
Shantin, N.T. Hong Kong

Abstract

To determine the effects of Buyang Huanwu Decoction (BYHWD), a traditional Chinese medicine, on neurite outgrowth and differentiation of neuroepithelial stem cells (NEPs), NEPs were isolated from embryonic neural tube and cultured in medium with rat serum containing BYHWD, which was prepared from rats administrated orally with BYHWD. The average neurite length of NEPs grew significantly longer in rat serum containing BYHWD than in control serum without BYHWD. More neurofilament (NF) positive cells and glial fibrillary acidic protein (GFAP) positive cells were detected in NEPs cultured in the presence of BYHWD. Besides, when cultured NEPs were loaded with Fluo-3-AM, the fluorescence intensity obtained from NEPs cultured in serum with BYHWD was significantly lower than that from NEPs cultured in control serum without BYHWD. Our results indicate that BYHWD could exert a promotion effect on neurite outgrowth and differentiation of NEPs.

Key Words: buyang Huanwu decoction, neuroepithelium, neurite, differentiation

Introduction

Buyang Huanwu Decoction (BYHWD) is a popular traditional Chinese medicine that is widely used in China to invigorate the body, promote blood circulation and activate meridians (energetic channels). In clinical applications, BYHWD has proven effective in treating cerebrovascular diseases for hundreds of years (10, 14). Previous studies have shown that BYHWD could exert neuroprotective effects against neuronal injury (6, 11) and could also promote peripheral nerve regeneration *in vivo* (4). However, the mechanisms by which BYHWD protected the nervous system *in vivo* were not clear. In recent studies, BYHWD demonstrated a protective effect against hypoxia on cortical neurons in culture (13). Similarly, we recently showed that BYHWD played a demonstratable protective role in oxidative injured Schwann cells and could down-regulate intracellular Ca^{2+} level of Schwann cells (15). Ca^{2+} channels are closely associated with cellular differentiation and neurite outgrowth (3). Based on these data, we hypothesized that BYHWD may stimulate growth and differentiation of neuroepithelial stem cells (NEPs). To date, there have been very limited studies of this phenomenon.

Corresponding author: Dr. Ying-Mao Gao, Institute of Anatomy and Histology & Embryology, School of Medicine, Shandong University, Jinan 250012, Shandong, P.R. China. Tel: +86-531-88382047, Fax: +86-531-88382047, E-mail: sunjinhao@sdu.edu.cn
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NEPs originated from the neural plate and its successor, the neural tube, during embryonic development. These primitive neural stem cells were often used as a candidate in drug experiments for their characteristics in neuronal development and potential clinical application (12). In this study, we investigated the effects of BYHWD on these NEPs. To test our hypothesis, a serum pharmacological testing method, which has proven effective in pharmacological research on traditional Chinese medicine, was employed (1).

Materials and Methods

Cell Culture

Neural tubes were dissected from Wistar rats of embryonic day 11 (E11, day of gestation) and were put into cold, sterile D-Hank's buffer. Tissues surrounding the NEPs were removed under a microscope. For histologic evaluation of the embryonic neural tube, the classical hematoxylin & eosin (HE) staining method was used. NEPs suspensions were prepared through a series of D-Hank's rinses, digested with 0.125% trypsin for 10 min at 37°C, then washed in D-Hank's buffer containing 0.004% DNase to disperse the cells into solution. The resulted NEPs suspensions were used to examine the effects of BYHWD. To characterize the neural tube-derived NEPs, the dissociated cells were suspended in N2 medium, a defined DMEM/F12-based medium (Life Technologies, Paisley, UK) supplemented with B27 (Gibco, Carlsbad, CA, USA), human recombinant EGF (20 ng/ml; Sigma, St. Louise, MO, USA), human recombinant basic FGF (20 ng/ml; Sigma). Five ml of the cell suspension at a density of 3×10^4 cells/ml were plated into each well of uncoated six-well plates and incubated at 37°C in a humidified incubator with 5% CO_2. The cells grew as free-floating neurospheres *in vitro* for 7 days, and then were plated onto poly-L-lysine coated coverslips and cultured for 2 h in DMEM/F12 containing 10% newborn calf serum. The cells were examined with nestin staining, a marker of neural stem cells.

Preparation of BYHWD

Huangqi, danggui, chishao, chuanxiong, honghua, taoren and dilong were purchased from Jianlian Herb companies in Jinan (Shandong, China), and were identified by botanists Dr. Jingde Wu and Dr. Fengqi Lu in School of Pharmacy, Shandong University. BYHWD was extracted as previously described (16). Briefly, powdered huangqi, danggui (Guiwei), chishao, chuanxiong, honghua, taoren and dilong were mixed at a ratio of 120:6:4.5:3:3:3:3 according to "Yilin Gaicuo" (Correction on Errors in Medical Classics). The mixture was boiled for 30 min three times in water at a volume of 10, 8, and 6 folds of the dry weight of the herb mixture, respectively. The resulted BYHWD solution was concentrated and lyophilized, and the dried BYHWD powder was stored at 4°C until use.

Preparation of BYHWD-Containing Serum

Adult male Wistar rats (180-220 g) aged 8-10 weeks were obtained from the Laboratory Animal Center of Shandong University. All procedures were performed in accordance with the China Animals (Scientific) Procedures Act. A modified method was used as previously reported (16). Briefly, rats were randomly distributed into two groups. In first group, each animal was orally administrated BYHWD solution at a concentration of 1 g/ml (dry BYHWD power) for 6 ml/kg twice daily (7:00 a.m. and 7:00 p.m.), and for a total of seven doses. Blood was obtained from each animal by cardiac puncture 1 h after the last administration and the serum from each animal was pooled together, termed as BYHWD-containing serum (BYHWD-CS). In second group, rats were orally administrated normal saline in the same protocol; their serum was used as control serum. Both the BYHWD-CS and control serum were inactivated by heating at 56°C for 30 min, filtered through a 0.22 μm filter, and stored at –20°C until use.

Measurement of Neurite Length

NEPs suspensions were plated onto 24-well plates and cultured in DMEM/F12 supplemented with 10% BYHWD-CS or equivalent control serum at a final concentration of 3×10^5 cells/ml. So, the cultured cells were divided into BYHWD-CS group and control serum group. The length of a neurite was measured under a microscope at 24 h and 48 h *in vitro* culture, respectively. Both in BYHWD-CS group and the control serum group, three wells were examined. In each well, five fields were photographed through the microscope at a magnification of 200×. The first field was selected in the center of the well. The other fields were selected in the four directions next to the first field. The longest neurite from each cell was selected for measurement. Neurite length was defined as the distance between the cell body and the farthest tip of the neurite.

Immunocytochemical Staining

At day 7 in culture, the differentiation of NEPs was examined by NF and GFAP immunocytochemical

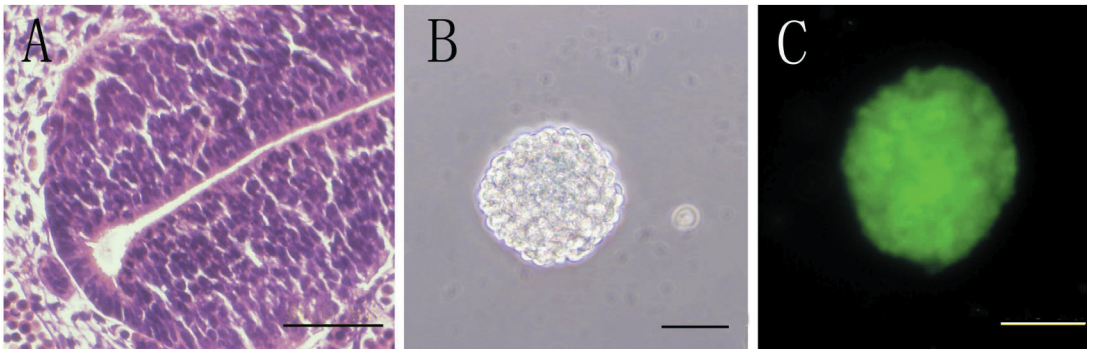

Fig. 1. Neural tube of embryonic day 11 with HE staining (A). Neurospheres were generated from embryonic neural tube at day 7 *in vitro* culture (B) and stained with the antibody against nestin (C). Scale bars: 50 μm.

staining. Neurospheres were examined by nestin staining when cultured for 2 h in medium containing newborn calf serum. Cells and spheres attached to coverslips were fixed in 4% paraformaldehyde for 10 min and incubated in 50% methanol and 3% hydrogen peroxide for 20 min, then subsequently blocked with 10% normal goat serum for 60 min and incubated overnight with one of the following primary antibodies: rabbit anti-neurofilament medium (NF), rabbit anti-glial fibrillary acidic protein (GFAP), (both diluted 1:200, Sigma), mouse anti-nestin (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) diluted 1:500. After three washes with phosphate-buffered saline (PBS), spheres were treated with fluorescein isothiocyanate (FITC) labeled anti-mouse IgG for 30 min at 37°C and then examined under a fluorescence microscope. Cells were incubated in biotinylated secondary (goat-anti-rabbit both 1:200, Sigma), and then incubated in avidin-biotin complex (vectastain ABC kit, Vector Lab, Burlinhame, CA, USA). After rinsing with PBS and Tris-buffered saline, cells were developed for 5-30 min in 0.04% hydrogen peroxide and 0.05% 3, 3-diaminobenzidine (Sigma). Controls with omission of the primary antibody were performed on selected spheres and cells to verify the specificity of staining. Morphometric quantification of NF-positive (NF+) and GFAP-positive (GFAP+) cells was performed in 30 randomly selected high power fields (400×) in both BYHWD-CS group and control serum group. Moreover, the ratio of NF+ cells to GFAP+ cells in these two groups was examined.

Calcium Measurement

Intracellular calcium ($[Ca^{2+}]_i$) concentration was measured to determine the mechanisms of BYHWD-CS on the outgrowth and differentiation of NEPs. NEPs suspensions were seeded onto poly-L-lysine coated 24-well plates and cultured for 48 h in DMEM/F12 medium containing 10% BYHWD-CS (six wells) or equivalent control serum (six wells) to a final concentration of 3×10^5 cells/ml. Fluo-3-AM stock solution was prepared in DMSO (Sigma), and then diluted into culture medium. The final concentration of Fluo-3-AM was 5 μM (0.1% DMSO). The cells were incubated with Fluo-3-AM for additional 45 min at room temperature and rinsed with D-Hank's buffer before analysis (9). Three fields in each well were selected. $[Ca^{2+}]_i$ concentration in the cultured NEPs was measured using laser scanning confocal microscopy (LSCM, Leica, Bensheim, Germany).

Statistical Analysis

The length of neuritis, amount of NF+, and GFAP+ cells, the average intensity of fluorescence and the radio of NF+ cells to GFAP+ cells were presented as mean ± SD. SPSS 10.0 software was used in experiment. A Student's *t*-test was used for statistical analysis and significance was set at $P < 0.05$.

Results

Isolation and Identification of NEPs

NEPs resided within the epithelium of the neural tube. With HE staining, these pseudostratified columnar epitheliums showed different nuclear layers (Fig. 1A). In our experiment, NEPs were isolated and cultured in serum-free medium. At day 7 *in vitro* culture, neurospheres were clearly evident (Fig. 1B). Undifferentiated spheres were all immuno-positive for the immature cell marker nestin (Fig. 1C).

Effects of BYHWD-CS on Neurite Growth of NEPs

We observed the effects of BYHWD-CS on neurite extending. At 24 h after seeding, more differentiated cells were seen in BYHWD-CS treated cultures (Fig.

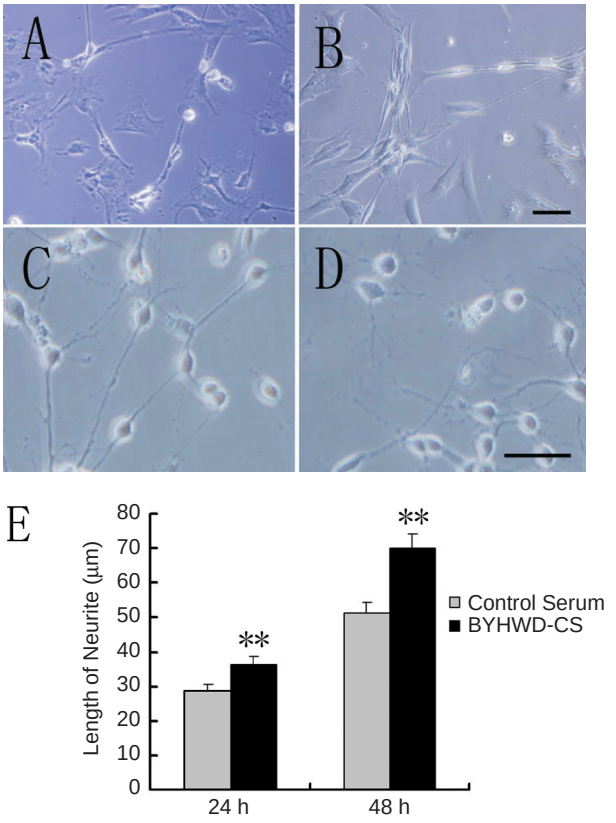

Fig. 2. NEPs extended neurites at 24 h (A) and 48 h (C) *in vitro* culture with BYHWD-CS. In control serum group, NEPs were cultured at 24 h (B) and 48 h (D) *in vitro*, respectively. $^{**}P < 0.01$, difference compared with control serum group (E). Scale bars: 50 μm.

2A), and the average length of the extended neurites was 36.4 μm. In the control serum group, neurite extension was not evident (Fig. 2B); the average length of the neurites was 28.8 μm, which was significantly shorter than that in BYHWD-CS culture ($t = 6.06$, $P < 0.01$, Fig. 2E). Moreover, at 48 h in culture, neurites connected with one another was presented in both BYHWD-CS group (Fig. 2C) and control serum group (Fig. 2D). The average lengths of neurites in BYHWD-CS group and control serum group were 70.2 μm and 51.4 μm, respectively ($t = 8.72$, $P < 0.01$, Fig. 2E).

Effects of BYHWD-CS on the Differentiation of NEPs

At day 7 *in vitro* culture, immunocytochemical staining showed that the number of NF+ cells detected in BYHWD-CS group was significantly higher than that detected in control serum group ($t = 22.12$, $P < 0.01$, Fig. 3, A and B). In BYHWD-CS group more neurites extending from cell bodies were observed. Similarly, the number of GFAP+ cells detected in BYHWD-CS group was also significantly higher than that detected in control serum group ($t = 13.45$, $P < 0.01$, Fig. 3, C and D), but the ratio of NF+ cells to GFAP+ cells in BYHWD-CS group was higher than that in control serum group ($t = 10.13$, $P < 0.01$, Fig. 3F).

Effects of BYHWD-CS on Neuronal $[Ca^{2+}]_i$

After Fluo-3-AM loading, NEPs in the two groups showed bright fluorescence compared with the dark background of the medium. The cells in BYHWD-CS group showed less fluorescence than that in control serum group (Fig. 4, A and B). And, the average fluorescence intensity obtained from the cultured neurons in BYHWD-CS was significantly lower than that in control serum ($t = 9.63$, $P < 0.01$, Fig. 4C).

Discussion

We studied the effects of Buyang huanwu Decoction on neurite outgrowth and differentiation of NEPs. The results showed that BYHWD-CS had significant neurite extending effects on these cells. Also, BYHWD-CS promoted NEPs' differentiation into neurons and astrocytes. Our studies provided direct evidence that BYHWD-CS could affect the outgrowth and differentiation of NEPs. Furthermore, the radio of neurons to astrocytes was higher in BYHWD-CS group. Probably, BYHWD demonstrated a specific role in neurons. Our results were consistent with *in vivo* studies, in which Li and his colleagues reported that more neurons could be detected in hippocampus treated with BYHWD after ischemia (11).

To further address the effects of BYHWD on NEPs, we measured the intracellular calcium. These results showed that $[Ca^{2+}]_i$ of NEPs in BYHWD-CS was significantly lower than in control serum. It indicated that BYHWD could reduce Ca^{2+} overloading of NEPs. As an important intracellular secondary messenger, Ca^{2+} was closely associated with cellular differentiation and neurite outgrowth (3). Previous studies indicated that calcium overload could cause cell death during injury, and suppressing $[Ca^{2+}]_i$ by photorelease of a Ca^{2+} chelator could accelerate axon extension (5, 7). During the process of isolation, digestion and culture, NEPs were probably injured by the *in vitro* microenvironment (2), and the down-regulating $[Ca^{2+}]_i$ by BYHWD might contribute to its effect on neurite growth and differentiation of NEPs.

In this study, a serum pharmacological testing method was employed. In recent years, serum pharmacology has been proved to be effective in pharmacological research on traditional Chinese medicine (1, 8). BYHWD contained many chemical constituents, such as flavonoids, alkaloids and

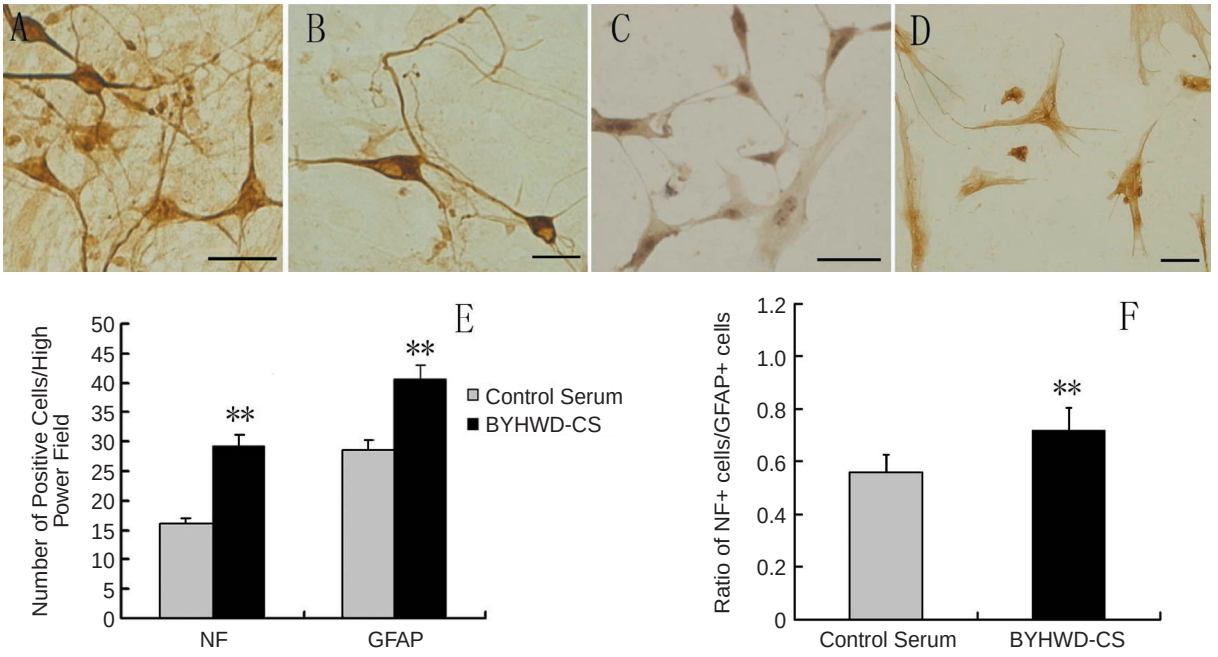

Fig. 3. At day 7 in culture, NF positive cells were detected in BYHWD-CS group (A) and control serum group (B); GFAP positive cells in BYHWD-CS group (C) and control serum group (D). $^{**}P < 0.01$, difference compared with control serum group (E, F). Scale bars: 50 μm.

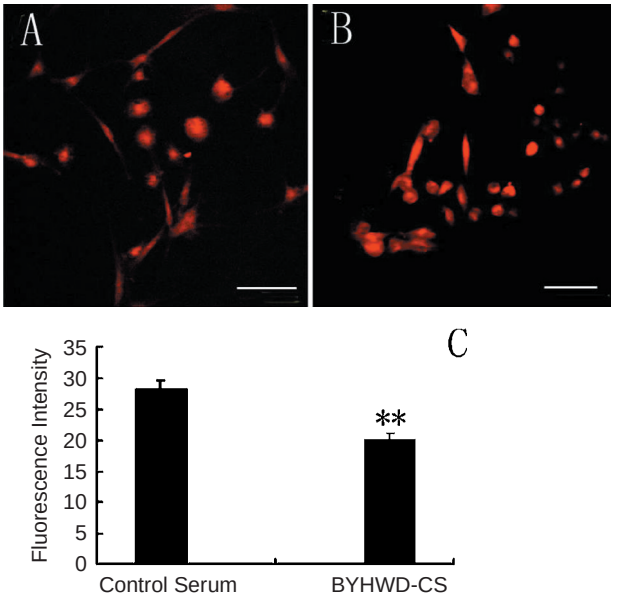

Fig. 4. NEPs were loaded with fluo-3-AM, fluorescence distributions were showed in BYHWD-CS group (A) and control serum group (B). $^{**}P < 0.01$, difference compared with control serum group (C). Scale bars: 50 μm.

saponins. Recently, the constituents of BYHWD in pig serum were identified using combined high-performance liquid chromatography (HPLC), photodiodearray detection and mass spectrometry techniques (16). Forty-five characteristic HPLC peaks containing drug were detected in serum from pigs administrated BYHWD orally. Nine chemical structures were metabolites of the constituents of BYHWD *in vivo*, including calycosin, formononetin and glucoside. Thus, it is therefore worth investigating the active constituent of BYHWD-CS in the future.

In conclusion, using a serum pharmacological testing method, we showed that BYHWD could promote the neurite growth and differentiation of NEPs. Down-regulation of intracellular Ca^{2+} might be one of the mechanisms underlying this process.

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