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Original Article

Protective effects of traditional Chinese herbal decoction Baihe Dihuang Tang on serum-deprived PC12 cells

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Abstract: Objective: This study aimed to explore the neuroprotective effect of Baihe Dihuang Tang (BDT), a traditional Chinese herbal decoction used for nervous-mental system diseases, on serum-deprived PC12 cell. Methods: BDT treatment time and concentration were determined through 4,5-dimethylthiazol-2-yl,2,5-diphenyl tetrazolium (MTT) assay. PC12 cells were randomly divided into three groups: control (cells cultured in serum-containing medium), model (cells cultured in serum-deprived medium), and BDT (cells cultured in serum-deprived medium and treated with 3 mg/mL BDT for 24 h) groups. Cell morphology was observed under an inverted phase contrast microscope. The ultrastructure of cells was studied under a transmission electron microscopy. Membrane potential was determined using a laser scanning confocal microscope. Total protein and ATP levels were analyzed by the bicinchoninic acid method and firefly luciferase method, respectively. Results: Compared with the model group, PC12 cells in the BDT group exhibited extensive morphology and adhered to the culture plate. The ultrastructure was modified and possessed a smooth membrane and nuclear surface, increased microvilli-like membrane protrusions, homogeneously distributed organelles, rare pinocytosis and phagocytosis, abundant ribosomes, rare vacuoles, and normal-shaped nucleus. BDT also enhanced the total cell protein content, ATP level, and membrane hyperpolarization. Conclusion: BDT could reduce cell death and neural excitability and regulate energy metabolism caused by serum deprivation. We suggest that BDT could modify the hypometabolic state and neural overactivity in chronic fatigue syndrome (CFS). This work may provide new insights into the possibility of using BDT as a therapeutic agent for CFS.

Keywords: Baihe Dihuang Tang, PC12 cell, ultrastructure, cellular membrane potential, chronic fatigue syndrome

Introduction

Baihe Dihuang Tang (BDT) was first recorded in "Synopsis of Prescriptions of the Golden Chamber" (*Jinkui Yaolue*) by Zhongjing Zhang, a prestigious specialist in traditional Chinese medicine (TCM) during 210 A.D. [1]. BDT was initially used to cure *Baihe Bing*, also called Lily disease. According to Zhang, "*Baihe Bing* is characterized by general malaise and inability to eat, talk, lie down, or walk. A patient with this disease often appears quiescent and may sometimes have an appetite, while other times not. The patient feels cold but has no chills or feels hot but has no fever. A bitter taste disturbs his/her mouth, and his/her urine becomes red [1]". BDT consists of *Lilii Bulbus* (*Baihe*) and *Rehmanniae Radix* (*Dihuang*). Integrative pharmacology studies predicted that the action of BDT may be related to regulating neurotrophic factors, disorder of amino acids, and purine metabolism and catabolism [2]. At present, BDT is a widely used recipe in TCM to cure nervous-mental illnesses [3].

Chronic fatigue syndrome (CFS)/Myalgic Encephalomyelitis (ME) is a generalized disease that persists for more than 6 months. It has unknown etiology, and manifests as a heterogeneous multisystem disorder, including neurological problems, immune dysfunction, hormonal imbalance, gastrointestinal symptoms,

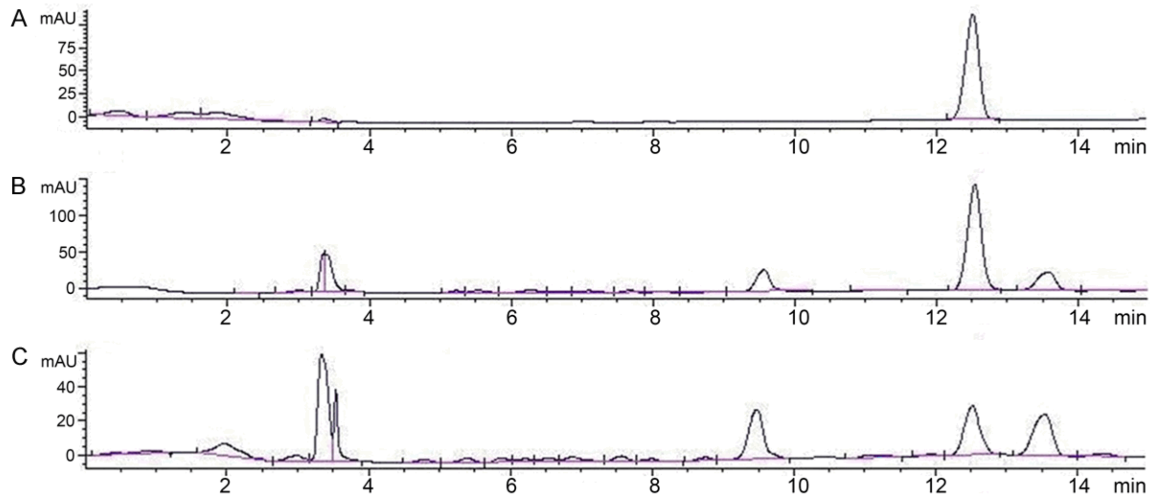

Figure 1. High-performance liquid chromatography chromatography. A. Catalpol standard solution. B. Rehmanniae Radix. C. Baihe Dihuang Tang. mAU: milli-absorbance units.

and joint and muscle pain [4, 5]. CFS occurs in approximately 0.1% to 5% of the world population [6, 7]. Despite the high social burden and heavy economic burden caused by CFS, no specific therapy is available. Western medicine offers potent cures for diseases with a single cause and definite pathophysiology. However, the etiology of CFS remains unidentified [8]. Thus, only graded exercise therapy and cognitive behavioral therapy have been shown to be effective for CFS [9]. TCM herbal formulas have multiple targets on a disease with network connection [10, 11]. A new TCM recipe for CFS treatment must be developed. CFS has several similarities to *Baihe Bing* in terms of symptoms, etiology, pathology, and treatment [12, 13]. In this regard, we speculated that BDT could be a potential therapy for CFS.

BDT can regulate neurological dysfunction, such as depression and psychological suboptimal health state [14, 15]; however, few works have examined its effects on CFS. Rat pheochromocytoma PC12 cells are similar to sympathetic neurons in terms of morphology and function and are widely used for neuronal disease research, such as in in vitro cell models [16]. Patients with CFS are usually under a hypometabolic survival state [17], so we used serum deprivation to simulate a similar living condition. The effect of BDT on modifying neuronal metabolism was evaluated by the analysis of cell morphology, ultrastructure, total protein content, and ATP level. The effect of BDT on neural activity was determined by measuring membrane potential. The present results may possibly reinforce the application of BDT for CFS.

Materials and methods

Preparation of BDT

BDT used in this study is composed of Lilii Bulbus (Baihe, the dried bulbs of Lilium lancifolium Thunb., 80 g) and Rehmanniae Radix (Dihuang, the dried root of Rehmannia glutinosa Libosch., 60 g) at a ratio of 4:3 [18]. The crude drugs were bought from Guo Yi Tang Chinese Herbal Medicine Store (Fuzhou, Fujian, China). The herbs were boiled in 1200 mL of water for 30 min, and the decoction was filtered. The residues were added with 900 mL of water, boiled, and filtered again. The extracts were combined, dried under vacuum, and stored at -20°C.

The catalpol in Rehmanniae Radix was proved to be neuroprotective [19]. Thus, the catalpol was used for quantitative analysis of BDT. It was detected by Agilent 1200 high-performance liquid chromatography system (Agilent, santa Clara, CA, USA) with a C-18 column (4.6×250 mm, 5 μm). The mobile phase consisted of acetonitrile-0.1% phosphoric aq. (1:99) at a flow rate of 1 mL/min. The column temperature was 30°C and the detection wavelength was 210 nm. The content of catalpol in BDT was found to be 0.22% (**Figure 1**).

Cell culture

Rat PC12 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were incubated at 37°C in an incubator with 90% humidified atmosphere (Thermo Fisher Scientific, Waltham, MA, USA) containing 5% CO_2. The cells were then cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. RPMI 1640 medium, FBS, penicillin, and streptomycin were acquired from Hyclone (Logan, UT, USA).

Cell treatment condition selection

BDT treatment condition was selected with MTT assay. For selection of BDT treatment time, PC12 cells were incubated in serum-deprived medium for 0, 12, 24, and 48 h. Cell viability was observed under an inverted phase contrast microscope (IX70, Olympus, Tokyo, Japan).

For selection of BDT treatment concentration, PC12 cells were seeded in 96-well plates at a concentration of $2×10^5$ cells/mL, with 100 μL of the cells per well. The cells were then pretreated with BDT at various concentrations (0, 1.5, 3, 6, 12, 24, and 48 mg/mL) for 4 h. Each well was added with 4,5-dimethylthiazol-2-yl, 2,5-diphenyl tetrazolium (MTT, Sigma Aldrich, St Louis, MO, USA) at 37°C for 4 h. The purple-blue MTT formazan precipitate was dissolved using dimethylsulfoxide (Sigma Aldrich). Absorbance was determined using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 570 nm.

Cell grouping and treatment

After the proper BDT treatment condition was selected, PC12 cells were randomly divided into three groups. Cells in the control group were cultured in serum-containing medium. Cells in the model group were cultured in serum-deprived medium. Cells in the BDT group were cultured in serum-deprived medium and treated with BDT.

Cell morphology observation

PC12 cells were seeded into six-well plates at a density of $2×10^5$ cells/mL. Cell morphology was observed and photographed by an inverted phase contrast microscope at a magnification of 200×.

Cell ultrastructure observation

Cells were collected and fixed by glutaraldehyde and osmium tetroxide, dehydrated with alcohol and acetone, and embedded in epoxy resin. The samples were ultrathin sectioned and stained with uranylacetate and lead citrate. The cell ultrastructure was observed and photographed using a transmission electron microscope (H-7650, Hitachi, Japan).

Cellular total protein content and ATP level determination

Cellular total protein and ATP level analyses were conducted using our previously reported method [20]. Protein content was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). ATP level was measured using a firefly luciferase-based ATP assay kit (Beyotime Institute of Biotechnology). PC12 cells were plated in a 24-well plate. After intervention, the cells were rinsed with phosphate-buffered saline (pH 7.4, Gibco Life Technologies), disrupted in lysis buffer (Sigma Aldrich), and centrifuged. The supernatant was collected and mixed with BCA protein/ATP detection working solution. Absorbance was recorded at 562 nm. Standard curve of protein or ATP was generated using the standard. ATP level was expressed as normalized luminance (NRLU, in nmol/mg protein) [21].

Cell membrane potential assay

Cells were seeded into a six-well plate, added with bis-(1,3-dibarbituric acid)-trimethine oxanol [diBAC4(3), Sigma Aldrich], and incubated at 37°C for 30 min. Cell membrane potential was examined under a laser confocal scanning microscope (LSM710, Carl Zeiss Meditec, Jena, Germany) with laser light at 488 nm.

Statistical analysis

SPSS 23.0 (SPSS, Chicago, Illinois, USA) was used for statistical analyses. Quantitative data are expressed as mean ± standard deviation ($\bar{x}$ ± sd) of at least triplicate experiments. Variance among groups were analyzed using Student's *t*-test and one-way analysis of vari-

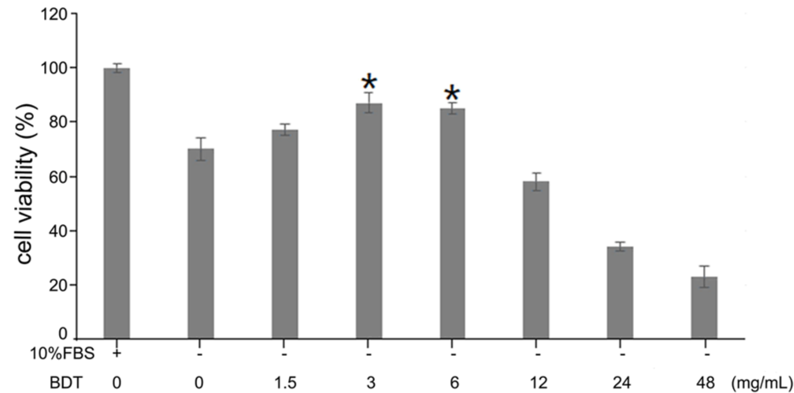

Figure 2. Changes in the viability of serum-deprived PC12 cells determined through MTT assay. PC12 cells were pretreated with BDT (0, 1.5, 3, 6, 12, 24, and 48 mg/mL) for 24 h. Cell survival rate was analyzed with MTT assay. Cell viability was expressed as the percentage (%) of cells cultured in medium containing 10% fetal bovine serum. Data are presented as mean ± SD (*n* = 3). *P<0.05 is considered statistically significant versus the model group.

ance (ANOVA) followed by least significant difference test (LSD-t). *P* value<0.05 was considered statistically significant.

Results

BDT treatment condition selected

The viability of cells treated with different times or concentrations of BDT was investigated to select the proper treatment condition. Based on the MTT assay results, the viability of PC12 cells was decreased by serum deprivation in a time-dependent manner. Compared with cells under regular culture condition, serum-deprived PC12 cells shrunk and were extensively detached from the culture plate with prolonged culture time. Hence, 24 h of culture time was selected for this study.

The viability of PC12 cells changed with increasing BDT dose (**Figure 2**). Cell viability was notably increased in PC12 cells treated with low concentrations of BDT (1.5, 3, and 6 mg/mL). Meanwhile, the cells died when treated with high concentrations of BDT (12, 24, and 48 mg/mL). Treatment with 3 mg/mL BDT exhibited significant protective effect relative to the model group with the minimal dose (P<0.05). We selected 3 mg/mL BDT for our experiment.

Cell morphology

Cell morphology was observed to assess the growth status of PC12 cells. After exposure to the serum-deprived medium, cells in the model group exhibited a degenerated, shrunk, and round morphology and were detached from the culture plate (**Figure 3B**). After BDT treatment, the cell adherence rate to the plate and the cell volume were increased. This finding indicated the improved cellular morphology of cells in the BDT group (**Figure 3C**). Compared with cells in the model group, the morphology of PC12 cells cultured in medium containing 10% fetal bovine serum and treated with 3 mg/mL BDT for 24 h was modified (**Figure 3D**), indicated that the present BDT treatment condition was cytoprotective.

Cell ultrastructure

Cellular ultrastructure was investigated to detect the material basis of changes in cellular functions. Compared with cells in the control group (**Figure 4A**, **4B**), cells in the model group exhibited increased pinocytosis vesicles and phagosomes and opened cellular boundaries, indicating cell nutrition deficiency. The loss of rough endoplasmic indicated abnormal protein synthesis. The absence of mitochondria reflected abnormal energy metabolism. Irregularly shaped nucleus and condensed chromosomes indicated DNA injuries (**Figure 4C**, **4D**).

After BDT treatment, the cell surface became smoother and the number of microvilli-like membrane protrusions was increased. The organelles were homogeneously distributed in the cytoplasm. The cells had rare vacuoles, abundant ribosomes and mitochondria, and improved Golgi complex. The nucleus exhibited a normal shape. These findings suggested that BDT attenuated serum deprivation-induced injuries in PC12 cells (**Figure 4E**, **4F**).

Cellular total protein content and ATP levels

Total protein content was determined to detect the effect of BDT on cell nutrition (**Figure 5A**). Compared with that in the control group, the

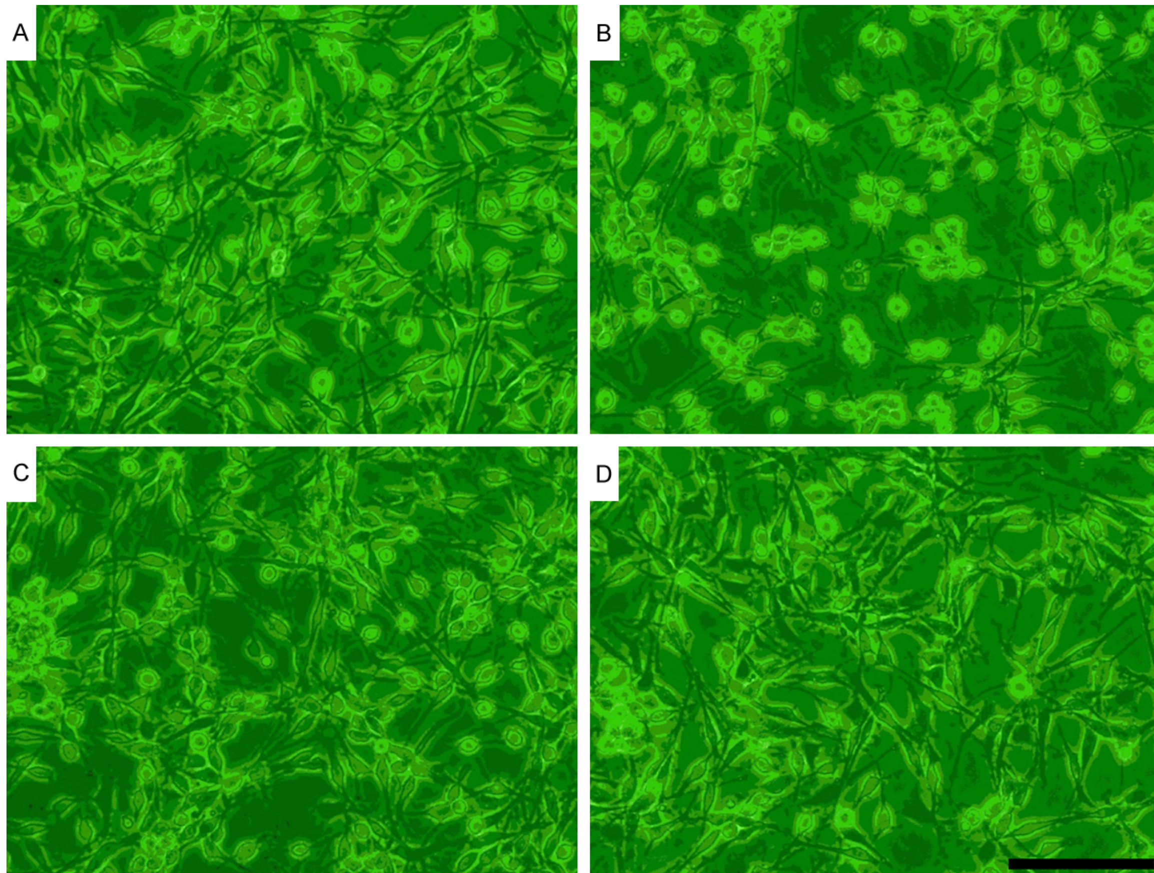

Figure 3. Morphological changes in PC12 cells observed under an inverted phase contrast microscope at magnification of 200×. A. Cells in the control group were cultured in serum-containing medium. The cells exhibited neurite structure and were attached to the plate. B. Cells in the model group were cultured in serum-deprived medium. The cells exhibited degenerated, shrunk, and round morphology and were detached from the culture plate. C. Cells in the BDT group were cultured in serum-deprived medium and treated with 3 mg/mL BDT for 24 h. The cells adhered to the plate, and the cellular volume was increased. D. PC12 cells cultured in medium containing 10% fetal bovine serum and treated with 3 mg/mL BDT for 24 h. The morphology of these cells was similar to that of cells in the control group. Bar = 10 μm.

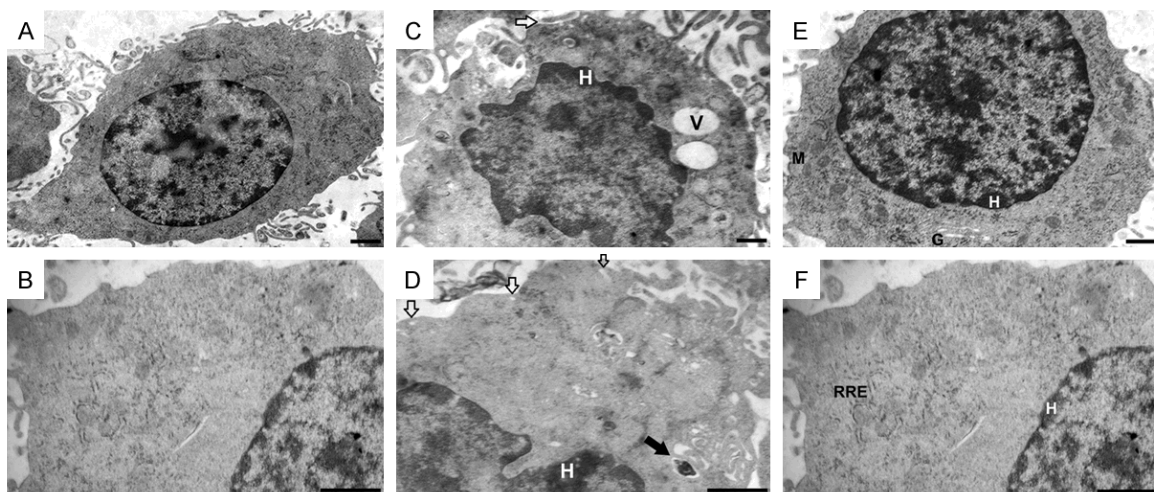

Figure 4. Ultrastructural changes in PC12 cells determined through transmission electron microscopy. Magnification 15,000× and 30,000×. A, B. Cells in the control group were cultured in serum-containing medium. Abundant

microvilli-like membrane protrusions and ribosomes and scattered distribution of heterochromatin were observed. C, D. Cells in the model group were cultured in serum-deprived medium. The number of microvilli-like membrane protrusions was decreased, and the incidence rates of membrane traffic events including pinocytosis and phagocytosis were increased. The ribosomes were dissociated, and their numbers decreased. The number of vacuoles was increased. Partly swollen mitochondria and partly dilatant endoplasmic reticulum were observed. Heterochromatin was condensed and clung to the nuclear envelope, and irregular nuclear borders were observed. E, F. Cells in the BDT group were cultured in serum-deprived medium and treated with 3 mg/mL BDT for 24 h. The occurrence of pinocytosis and phagocytosis was decreased. The ribosomes and mitochondria were abundant. Cell nucleus was spherical, and heterochromatin had scattered distribution. White arrows: Pinocytosis vesicles; Black arrows phagosome; V: vacuoles; RRE: ribosomes in the rough endoplasmic reticulum; H: heterochromatin; M: mitochondrion; G: Golgi complex. Bar = 1 μm.

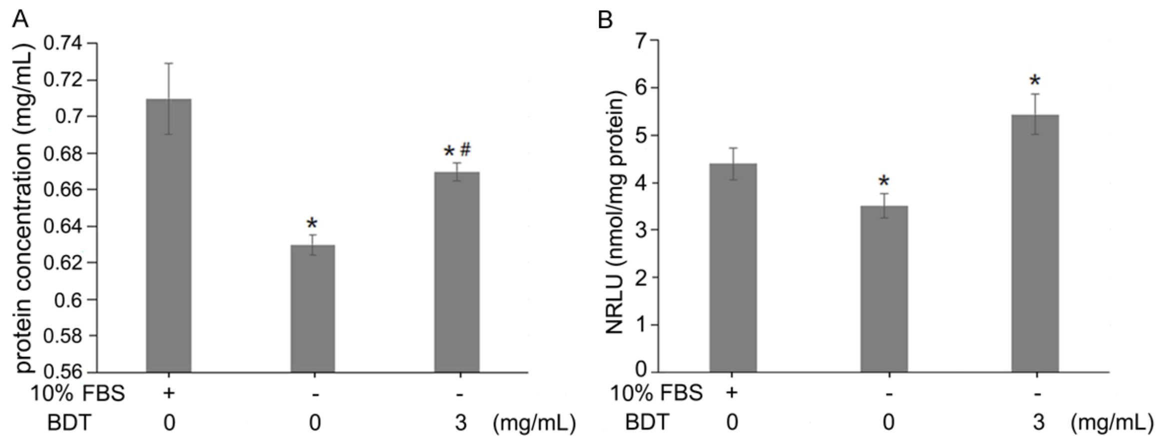

Figure 5. A. Total protein changes in PC12 cells determined through BCA method. B. Cellular ATP levels in the three groups determined through firefly luciferase-based ATP assay. ATP level was expressed as the normalized luminance (NRLU) of firefly luciferase (nmol/mg protein). Data are presented as mean ± SD (*n* = 3). *P<0.05 versus the control group. #P<0.05 versus the model group.

total protein level was significantly decreased in the model group (P<0.05) but increased after the BDT treatment (P<0.05). Hence, BDT could improve the total protein content in serum-deprived PC12 cells. However, the protein level did not recover to the normal control under the present intervention.

Cellular ATP levels were investigated to examine the effect of BDT on cell energy metabolism (**Figure 5B**). The ATP level in the model group was significantly decreased compared with that in the control group (P<0.05), indicating that serum deprivation decreased the energy metabolism. After 24 h of BDT treatment, the ATP level in the BDT group was significantly increased compared with that in the control group (P<0.05), suggesting that BDT enhanced the energy metabolism.

Cell membrane potential

Cell membrane potential was measured to detect the effect of BDT on cell activity. Compared with that in the control group, the fluorescence intensity in the model group was increased after being incubated in serum-deprived medium (P<0.05, **Figure 6A**, **6B**, **6D**), indicating reduced resting potential. After BDT treatment, the fluorescence intensity in the BDT group decreased compared with that in the model group (P<0.05, **Figure 6B-D**), indicating improved membrane hyperpolarization. The fluorescence intensity in the BDT group was significantly higher than that in the control group, suggesting that BDT treatment still did not restore the membrane potential of serum-deprived PC12 cells to the control level.

Discussion

In several aspects, *Baihe Bing* is similar to CFS. In terms of etiopathogenesis, both are induced by infectious agents (“exogenous disease” in TCM), immune dysfunction (“vital qi deficiency”), sleep disorders (“kidney essence deficiency”), endocrine-metabolic dysfunction (“kidney essence deficiency”), and neuropsychiatric fac-

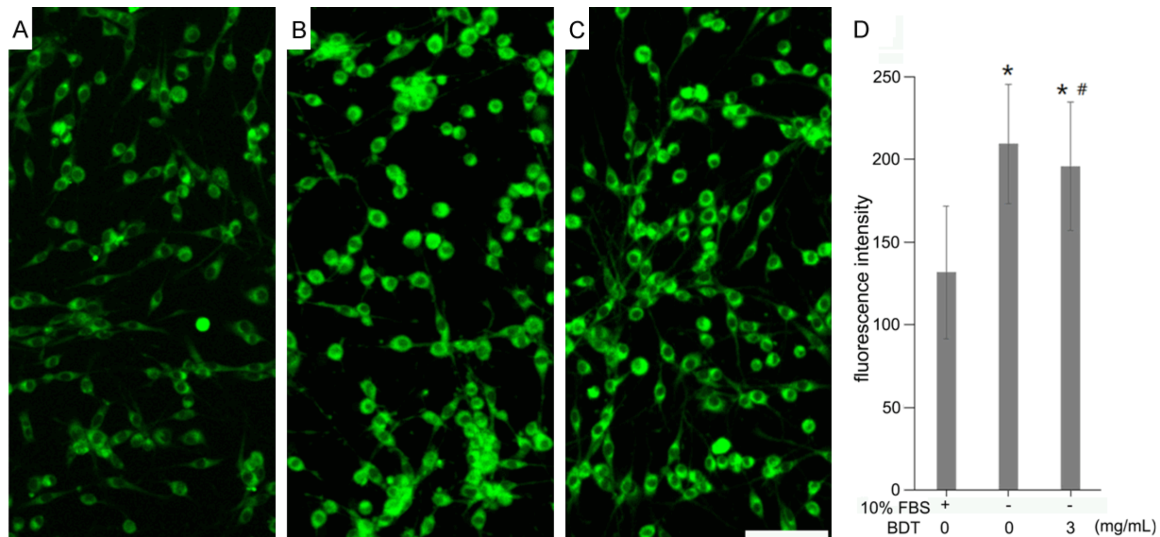

Figure 6. Membrane potential changes in PC12 cells. A-C. PC12 cells were stained green by diBAC4 (3) under a laser scanning confocal microscope. A. Cells in the control group were cultured in serum-containing medium. B. Cells in the model group were cultured in serum-deprived medium. C. Cells in the BDT group were cultured in serum-deprived medium and treated with 3 mg/mL BDT for 24 h. D. Histograms represent the fluorescence intensity comparison among the three groups. Fluorescence intensity values were obtained through a laser scanning confocal microscope. Data are presented as mean ± SD (n = 3). *P<0.05 versus the control group. #P<0.05 versus the model group. Bar = 50 μm.

tors ("stagnation syndrome"). In terms of symptoms, both are manifested by abnormal actions, emotions, sleep patterns, and diet. In therapy, both need physical exercise, psychotherapy, antidepressants, immunomodulatory agents, and antiviral agents [9, 12, 13, 22]. BDT is mainly used to cure *Baihe Bing*. Thus, we hypothesized that BDT may be a remedy for CFS. We detected its possible effect on serum-deprived PC12 cells.

Studies have shown that patients with CFS are under a hypometabolic survival state, but at the cost ofserious long-term disability, pain, and suffering [17]. Neural activation in patients with CFS should be enhanced to satisfy the demands of daily reaction and movement and compensate for function loss [23]. Hence, we explored the effect of BDT on protein synthesis, ATP production, and cell excitability.

In the present research, the total protein level was increased in serum-deprived PC12 cells after BDT intervention. The ultrastructures of organelles involved in protein synthesis, such as ribosomes, rough endoplasmic reticulum, and Golgi complex, were regulated. These results indicated improved protein synthesis after BDT intervention. The number of mitochondria and the ATP level were increased, indicating improved ATP production in the BDT group. On the basis of the total protein and ATP results, we inferred that the abnormal metabolic state of patients with CFS could be modulated with BDT treatment.

The major symptoms of CFS remain to be fatigability and post-exertion, which are also implicated in energy metabolism [24]. After exertion, patients with CFS manifest reduced ATP synthesis and ability to restore high-energy phosphate storage [25]. Patients with CFS also display decreased ATP production compared with healthy controls, and these abnormalities worsen on repeated exercise tests [26]. We infer that BDT may ameliorate the physical ability of patients with CFS by improving ATP production. CFS induces emotional distress, including autonomic nervous system disorders and hormonal imbalance, which may be the possible cause of irregular energy metabolism [27]. Emotional changes can be regulated through neural activity. Thus, the effect of BDT on neural excitement was investigated by observing cell membrane potential changes with/without intervention.

Neuronal excitability in resting neurons can be modulated through cell membrane polarization and be decreased by membrane hyperpolari-

zation [28, 29]. In the present study, BDT improved the hyperpolarization of serum-deprived PC12 cells, thereby attenuating neural excitability. Membrane hyperpolarization may occur when the number of N-methyl-D-aspartate (NMDA) receptors decreased [30]. The overactivation of NMDA receptors causes a series of reactions that may be the mechanism of neuroinflammation [31]. Furthermore, neuroinflammation is a possible pathophysiology of CFS [32]. PC12 cells are similar to sympathetic neurons and are generally used as cell models to study nervous system diseases. Enhanced sympathetic nervous activity may be the pathogeny of adolescent CFS [33]. The neuroprotective effect of BDT on CFS may be associated with inhibiting neuroinflammation through NMDA receptors and the overactivity of sympathetic nerves.

In conclusion, our study presented evidence for the protective effect of BDT on serum-deprived PC12 cells through analyses of cell viability, protein synthesis, ATP production, and cell excitement. BDT may regulate the hypometabolic state and neural overactivity, which are related to the main symptoms and the possible pathophysiology of CFS. Previous studies have showed that serum-deprived PC12 cells could be protected through Ras/MEK/ERK signaling pathway [34]. In-depth studies must be conducted to verify the molecular mechanism of BDT neuroprotective function. Further work is necessary to confirm our results.

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Disclosure of conflict of interest

None.

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