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Shuyusan-containing serum protects SH-SY5Y cells against corticosterone-induced impairment

Liping Chen¹, Zhigao Sun², Fawei Wang¹, Chengyong Xu², Miao Geng³, Hongyan Chen³, Dongmei Duan¹

1 Department of Traditional Chinese Medicine, Hainan Branch of Chinese PLA General Hospital, Sanya 572004, Hainan Province, China

2 Department of Internal Medicine, Hainan Branch of Chinese PLA General Hospital, Sanya 572004, Hainan Province, China

3 Institute of Geriatrics, General Hospital of Chinese PLA, Beijing 100853, China

Research Highlights

- (1) *Shuyusan*-containing serum increased the survival rate of corticosterone-injured SH-SY5Y cells, and improved cell morphology.
- (2) High-dose *Shuyusan*-containing serum decreased Bax expression and elevated Bcl-2 expression in corticosterone-injured SH-SY5Y cells.
- (3) *Shuyusan*-containing serum increased brain-derived neurotrophic factor mRNA expression in corticosterone-injured SH-SY5Y cells, and blocked cell apoptosis.

Abstract

The Chinese herb *Shuyusan*, whose main constituent is jasminoidin, has been shown to protect SH-SY5Y cells against corticosterone-induced damage. SH-SY5Y cells injured by 400 $\mu\text{mol/L}$ corticosterone were treated with 5 and 30 $\mu\text{g/mL}$ *Shuyusan*-containing serum. Results revealed that *Shuyusan*-containing serum elevated the survival rate of SH-SY5Y cells, reduced Bax expression, increased Bcl-2 expression, markedly elevated brain-derived neurotrophic factor mRNA expression, and blocked cell apoptosis. Moreover, the effect of high-dose (30 $\mu\text{g/mL}$) *Shuyusan*-containing serum was more remarkable. Therefore, *Shuyusan*-containing serum appears to protect SH-SY5Y cells against corticosterone-induced impairment by adjusting the expression of apoptosis-associated proteins and brain-derived neurotrophic factor. Moreover, high-dose *Shuyusan*-containing serum has a protective effect on high-dose corticosterone-induced impairment.

Key Words

neural regeneration; traditional Chinese medicine; *Shuyusan*-containing serum; SH-SY5Y cells; corticosterone; Bcl-2; Bax; apoptosis; brain-derived neurotrophic factor; grants-supported paper; neuroregeneration

INTRODUCTION

Because of our fast-paced and competitive society, psychological pressures and the incidence of depression has gradually increased^[1]. The occurrence of depression has been shown to be strongly associated with pressure^[2] or unexpected events^[3]. Long-term chronic stress is an important

factor for inducing depression^[4]. Recurrent stress to the cerebral cortex activates the hypothalamic-pituitary-adrenal axis^[5], which causes hypothalamic-pituitary-adrenal axis hyperfunction, increases adrenal cortical hormone levels, and induces hippocampal neuronal injury^[6-7].

Synthetic antidepressants present a narrow spectrum and side effects following long-

Corresponding author:
Liping Chen, M.D., Chief physician, Master's supervisor, Department of Traditional Chinese Medicine, Hainan Branch of Chinese PLA General Hospital, Sanya 572004, Hainan Province, China, lipingschen@yahoo.com.cn.

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Conflicts of interest: None declared.

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term application. Therefore, natural drugs that are effective and have fewer side effects are popular with some medical workers and patients. Recently, medical practitioners have shown interest in the use of Chinese medicines for the treatment of diseases and in the adjustment of the human response to stress^[8]. Depression is known as “depressed syndrome” in Chinese medicine. The main reasons for depression are liver-*Qi* stagnation and heart-spirit denutrition. Recently, numerous studies addressing the use of Chinese medicines for depression have emerged, including the use of Chinese medicinal compounds^[9] and the combination of Western and Chinese medicines^[10]. In addition, studies have investigated the mechanism of action of Chinese medicines for depression^[11]. *Shuyusan*, which is composed of semen ziziphi spinosae, turmeric root tuber and cape jasmine fruit, has been shown to regulate the heart and liver, disperse depressed liver-energy, regulate vital energy, nourish the heart and relieve depression, and have a good effect on treating depression and post-stroke depression^[12].

Our pilot data reveals that *Shuyusan* antagonizes depression, improves clinical symptoms of depression in patients, improves the behavior of a rat model of chronic unforeseeable stress-induced depression, and increases neurotransmitter and neuropeptide content in the brains of rats with chronic stress-induced depression, resulting in an antidepressant effect^[13]. Moreover, jasminoidin, a major ingredient of *Shuyusan*, protects SH-SY5Y cells against high-dose corticosterone-induced injury^[14]. However, whether *Shuyusan*-containing serum protects neural cells and its mechanism of action remain unclear.

Cells from the SH-SY5Y human neuroblastoma cell line are characterized by their low differentiation, pyramidal shape, and obvious axons. In addition, SH-SY5Y cells have been extensively used in the study of nervous system disease onset and mechanisms of drug action^[15-16]. SH-SY5Y cells share some physiological functions with normal neurons^[15-16]. For example, Bcl-2 and Bax ex-

pression are associated with the regulation of cell apoptosis^[17-18]. This study explored the protective mechanisms of *Shuyusan*-containing serum on SH-SY5Y cells against high-dose corticosterone-induced impairment by analyzing the cell growth cycle, apoptosis, Bcl-2 and Bax protein expression, and brain-derived neurotrophic factor mRNA expression.

RESULTS

***Shuyusan*-containing serum increased the survival rate of SH-SY5Y cells injured by corticosterone**

The 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and cell counting kit-8 results revealed that corticosterone significantly reduced the survival rate of SH-SY5Y cells ($P < 0.05$), showing obvious injury. *Shuyusan*-containing serum increased the survival rate of SH-SY5Y cells injured by corticosterone ($P < 0.05$, $P < 0.01$). The high dose of corticosterone (30 $\mu\text{mol/L}$) showed a more potent effect. However, there was no significant difference in the effect of *Shuyusan* on the survival rate of these SH-SY5Y cells ($P > 0.05$; Table 1).

***Shuyusan*-containing serum improved the morphology of corticosterone-injured SH-SY5Y cells**

Microscopy revealed that cells in the control group grew well and were spindle-shaped or triangular, with a high transmittance. Corticosterone-injured cells showed a decreased density, loose arrangement, became long, thin, and wrinkled, and had a large intercellular space and low transmittance. After intervention of *Shuyusan*-containing serum, cell injury visibly reduced, and cells exhibited a clear boundary, increased density, uniform growth and an increased transmittance. Moreover, the effects of high-dose *Shuyusan*-containing serum were better (Figure 1).

***Shuyusan*-containing serum reduced apoptosis of corticosterone-injured SH-SY5Y cells**

Flow cytometry revealed that the apoptotic

rate of SH-SY5Y cells was less than 3% in the control group, while the apoptotic rate of corticosterone-injured SH-SY5Y cells reached 29%.

Table 1 Effect of *Shuyusan*-containing serum on the survival rate of corticosterone-injured SH-SY5Y cells

Group	Absorbance at 570 nm (MTT)	Survival rate (%) (cell counting kit-8)
Control	2.07±0.08	100
Model	1.13±0.16 ^a	49.21±3.14
Low-dose <i>Shuyusan</i> -containing serum	1.50±0.13 ^b	72.37±4.51
High-dose <i>Shuyusan</i> -containing serum	1.72±0.17 ^c	81.49±3.63

The doses of *Shuyusan*-containing serum in the low- and high-dose *Shuyusan*-containing serum groups were 5 µg/mL and 30 µg/mL, respectively.

The MTT assay showed that high absorbance values represented high survival rate of cells at 570 nm. For the cell counting kit-8, cell survival rate (%) = absorbance value in the experimental group/absorbance value in the control group × 100%.

All data are expressed as mean ± SD. Experiments were repeated three times. Intergroup comparisons were performed using the *F* test. ^a*P* < 0.05, vs. control group; ^b*P* < 0.05, ^c*P* < 0.01, vs. model group. MTT: 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

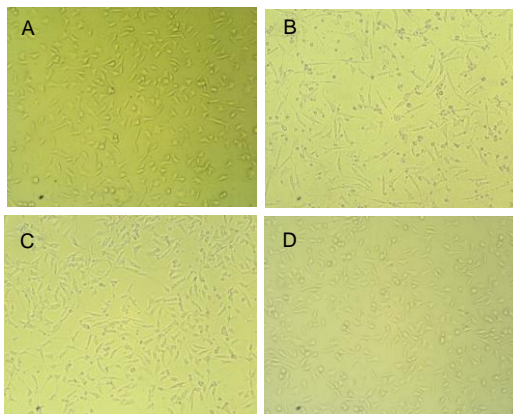


Figure 1 Morphology of corticosterone-injured SH-SY5Y cells after intervention with *Shuyusan*-containing serum (fluorescence microscope, × 200).

SH-SY5Y cells stained with Hoechst 33258 appeared blue color. The doses of *Shuyusan*-containing serum in the low- and high-dose *Shuyusan*-containing serum groups were 5 µg/mL and 30 µg/mL, respectively.

(A) Control group: Cells were spindle-shaped or triangular.

(B) Model group: Cells became long, thin and wrinkled, with a large intercellular space.

(C, D) Low- and high-dose *Shuyusan*-containing serum groups: clear cell boundaries, reduced shrinkage, and increased cell density.

The apoptotic rate of corticosterone-injured SH-SY5Y cells decreased (23%) in the low-dose *Shuyusan*-containing serum group, while the apoptotic rate of corticosterone-injured SH-SY5Y cells significantly reduced (14%) in the high-dose *Shuyusan*-containing serum group (Figure 2).

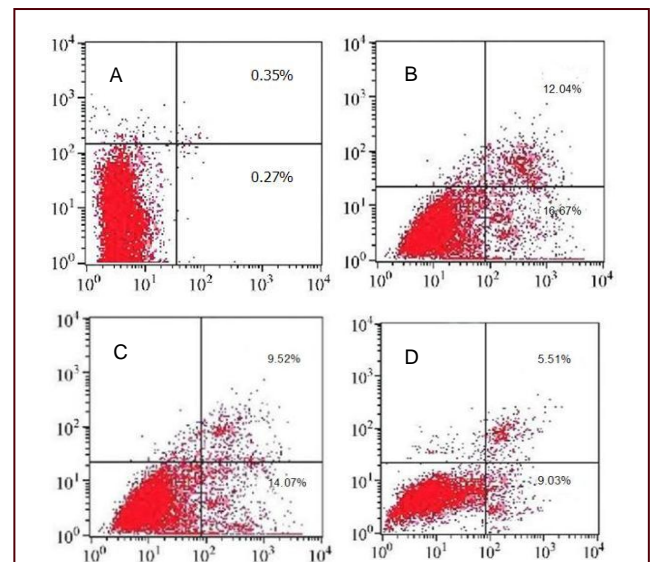


Figure 2 Effect of *Shuyusan*-containing serum on corticosterone-induced apoptosis in SH-SY5Y cells (flow cytometry).

Left lower quadrant: Normal cells; right lower quadrant: early apoptotic cells; right upper quadrant: late apoptotic cells; left upper quadrant: necrotic cells. The doses of *Shuyusan*-containing serum in the low- and high-dose *Shuyusan*-containing serum groups were 5 µg/mL and 30 µg/mL, respectively.

(A) Control group: Apoptotic rate was less than 3%.

(B) Model group: Apoptotic rate was 29%.

(C) Low-dose *Shuyusan*-containing serum group: Apoptotic rate was 23%.

(D) High-dose *Shuyusan*-containing serum group: Apoptotic rate was 14%.

***Shuyusan*-containing serum affected Bax and Bcl-2 expression in corticosterone-injured SH-SY5Y cells**

Immunofluorescence staining results revealed that compared with the control group, Bax expression significantly increased, and Bcl-2 expression significantly decreased in corticosterone-injured SH-SY5Y cells (*P* < 0.05 and *P* < 0.01, respectively). No significant changes in Bax and Bcl-2 expression were visible in the low-dose *Shuyusan*-containing serum group. However, Bax expression significantly diminished and Bcl-2 expression significantly elevated in the high-dose *Shuyusan*-containing serum group (*P* < 0.05 and *P* < 0.01, respectively). Compared with the low-dose *Shuyusan*-containing serum group, Bax expression was lower and Bcl-2 expression was higher in the high-dose *Shuyusan*-containing serum group (*P* < 0.05; Figure 3, Table 2).

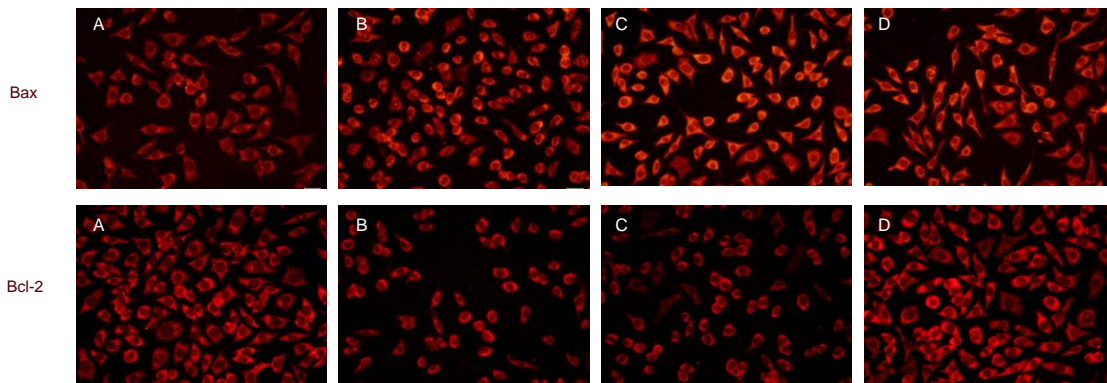


Figure 3 Effect of *Shuyusan*-containing serum on Bax and Bcl-2 expression in corticosterone-injured SH-SY5Y cells (immunofluorescence staining, $\times 400$).

Cy3-positive cells appeared red. The doses of *Shuyusan*-containing serum in the low- and high-dose *Shuyusan*-containing serum groups were 5 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$, respectively.

(A) Control group: Less Bax expression and more Bcl-2 expression in normal cells.

(B) Model group: Bax expression increased, but Bcl-2 expression was inhibited in the corticosterone-injured SH-SY5Y cells.

(C) Low-dose *Shuyusan*-containing serum group: Low-dose *Shuyusan*-containing serum non-significantly reduced Bax expression and increased Bcl-2 expression in the corticosterone-injured SH-SY5Y cells.

(D) High-dose *Shuyusan*-containing serum group: High-dose *Shuyusan*-containing serum obviously inhibited Bax expression and increased Bcl-2 expression in corticosterone-injured SH-SY5Y cells.

Table 2 Effects of *Shuyusan*-containing serum on Bax and Bcl-2 expression in corticosterone-injured SH-SY5Y cells

Group	Average absorbance		Number of positive cells (/400-fold field)	
	Bcl-2	Bax	Bcl-2	Bax
Control	1.904 \pm 0.072	0.478 \pm 0.021	27.902 \pm 4.104	5.809 \pm 0.706
Model	0.602 \pm 0.003 ^b	1.742 \pm 0.049 ^b	11.070 \pm 2.030 ^a	20.170 \pm 3.052 ^b
Low-dose <i>Shuyusan</i> -containing serum	1.083 \pm 0.039	1.362 \pm 0.205 ^c	18.726 \pm 3.917 ^c	13.350 \pm 1.170
High-dose <i>Shuyusan</i> -containing serum	1.407 \pm 0.061 ^c	0.693 \pm 0.085 ^{de}	24.305 \pm 2.151 ^d	9.705 \pm 0.506 ^c

The doses of *Shuyusan*-containing serum in the low- and high-dose *Shuyusan*-containing serum groups were 5 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$, respectively. All data are expressed as mean \pm SD. Experiments were repeated three times. Intergroup comparisons were performed using the *F* test. ^a $P < 0.05$, ^b $P < 0.01$, vs. control group; ^c $P < 0.05$, ^d $P < 0.01$, vs. model group; ^e $P < 0.05$, vs. low-dose *Shuyusan*-containing serum group.

***Shuyusan*-containing serum increased brain-derived neurotrophic factor mRNA expression in corticosterone-injured SH-SY5Y cells**

Reverse transcription-PCR results revealed that compared with the control group, brain-derived neurotrophic factor mRNA expression significantly decreased in corticosterone-injured SH-SY5Y cells ($P < 0.01$). Brain-derived neurotrophic factor mRNA expression significantly increased in the corticosterone-injured SH-SY5Y cells of the high- and low-dose *Shuyusan*-containing serum groups ($P < 0.01$ and $P < 0.05$, respectively). The effect of high-dose *Shuyusan*-containing serum on increasing brain-derived neurotrophic factor mRNA expression was better than that of low-dose *Shuyusan*-containing serum ($P < 0.05$; Figure 4).

DISCUSSION

The pathogenesis of depression is complicated, and is associated with environment, outside forces, psychological factors, neuroendocrine disturbances, and monoamine transmitter disturbances^[19-20]. Previous studies have mainly addressed the effects of monoamine transmitter disturbances on the pathogenesis of depression, but recent studies have focused on the effects of the hypothalamic-pituitary-adrenal axis stress reaction on the pathogenesis of depression^[21]. Stress induces a persistent increase in corticosterone levels in the body, and causes over-activation of the hypothalamic-pituitary-adrenal axis^[22], which leads to damage of hippocampal neurons^[23].

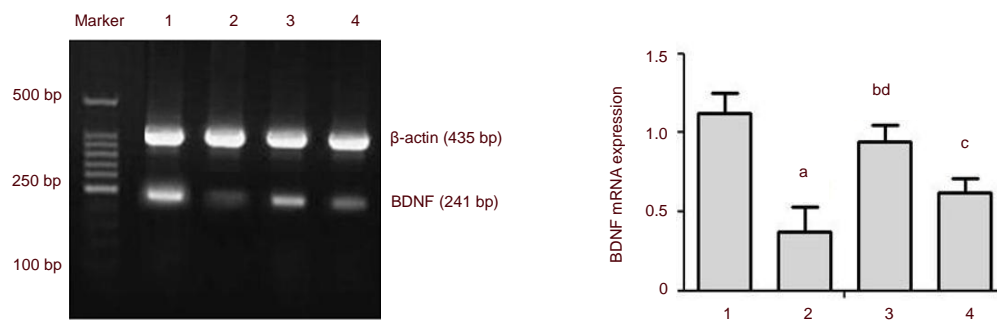


Figure 4 Effects of *Shuyusan*-containing serum on brain-derived neurotrophic factor (BDNF) mRNA expression in corticosterone-injured SH-SY5Y cells.

The doses of *Shuyusan*-containing serum in the low- and high-dose *Shuyusan*-containing serum groups were 5 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$, respectively. BDNF mRNA expression was expressed as a ratio of the absorbance of BDNF mRNA to β -actin mRNA. β -actin served as the internal reference, and BDNF as the target gene.

All data are expressed as mean \pm SD. Experiments were repeated three times. Intergroup comparisons were performed using the *F* test. ^a $P < 0.01$, vs. control group; ^b $P < 0.01$, ^c $P < 0.05$, vs. model group; ^d $P < 0.05$, vs. low-dose *Shuyusan*-containing serum group. 1: Control group; 2: model group; 3: high-dose *Shuyusan*-containing serum group; 4: high-dose *Shuyusan*-containing serum group.

High concentrations of corticosterone levels lead to a decrease in brain-derived neurotrophic factor mRNA expression in the hippocampi^[24], which reduces regeneration in hippocampal neurons and results in depression^[25]. Results demonstrated that the survival rate of high-dose corticosterone-injured SH-SY5Y cells was reduced, and that apoptotic cell death markedly increased. High-dose *Shuyusan*-containing serum visibly elevated the survival rate and reduced apoptosis of corticosterone-injured SH-SY5Y cells. Fluorescence staining demonstrated that some SH-SY5Y cells with a reduced body surface area were visible at 48 hours following intervention with high-dose corticosterone. Normal appearing SH-SY5Y cells with obviously increased nuclei were detected following treatment with high-dose *Shuyusan*-dose serum, suggesting that *Shuyusan*-containing serum can antagonize high-dose corticosterone-induced injury to SH-SY5Y cells and that it has a strong inhibitory effect on SH-SY5Y cell apoptosis. The above-mentioned inhibitory effect was also associated with a reduction in Bcl-2 protein expression and the promotion of Bax protein expression. Bcl-2 protein expression was lower, but Bax protein levels were higher in the model group than those in the control group, indicating that corticosterone probably exerted its effects on inducing SH-SY5Y cell apoptosis by reducing Bcl-2 expression and contributing to Bax expression. *Shuyusan*-containing serum diminished Bax expression, increased Bcl-2 expression and suppressed cell apoptosis. Bcl-2, an antiapoptotic gene, can prevent various factor-induced cell apoptosis^[26], while Bax is a proapoptotic gene. The ratio of Bcl-2 to Bax is a key regulatory factor

for cell apoptosis^[27]. The inhibitory effect of *Shuyusan* on SH-SY5Y cell apoptosis is associated with elevating Bcl-2 protein expression and reducing Bax protein expression, resulting in the inhibition of apoptosis^[28].

Brain-derived neurotrophic factor is an endogenous protein that widely exists in the human brain and peripheral nervous system^[29], and a reduction in brain-derived neurotrophic factor levels can contribute to the onset of depression^[30]. Increased brain-derived neurotrophic factor expression has been shown to promote neuronal survival, growth and differentiation and to upregulate hippocampal synaptic plasticity, which has been considered a common mechanism of action of various antidepressants^[31-32]. Brain-derived neurotrophic factor levels significantly reduced in the hippocampi and cortex of the prefrontal lobe of depression patients^[33-34]. Decreased brain-derived neurotrophic factor mRNA expression is associated with neuronal injury^[35]. Results from this study demonstrated that compared with the control group, brain-derived neurotrophic factor mRNA expression significantly decreased in the model group, but high-dose *Shuyusan*-containing serum significantly increased brain-derived neurotrophic factor mRNA expression in SH-SY5Y cells, indicating that *Shuyusan*-containing serum acts by elevating brain-derived neurotrophic factor mRNA expression in the corticosterone-injured cells. Various traditional Chinese therapies have proven effective against depression, such as syndrome-type based therapies, single ingredient recipes, or proven recipes that target the cause of depression, such as mental factors and emotional regulation^[11]. Some studies have addressed the mechanisms of action of

Chinese herbs for depression from the aspect of brain neurotransmitter levels^[36], hormone levels^[8] and animal behavior^[37], and the use of Chinese medicine monomers for the treatment of hippocampal neuronal injury^[9]. However, few studies have focused on the protective effects of traditional Chinese drug-containing serum on SH-SY5Y cells against high-dose corticosterone-induced injury. The use of Chinese medicines involves syndrome differentiation, treatment and compound application. Thus, it is important to use living cells to study the antidepressive mechanisms of traditional Chinese drug-containing serums. Thus, the present study investigated the protective mechanisms of *Shuyusan*-containing serum in high-dose corticosterone-injured SH-SY5Y cells.

In summary, *Shuyusan*-containing serum elevated the survival rate of high-dose corticosterone-injured SH-SY5Y cells, reduced Bax expression, increased Bcl-2 expression, markedly elevated brain-derived neurotrophic factor mRNA expression, and blocked cell apoptosis, indicating that *Shuyusan*-containing serum protects SH-SY5Y cells against high-dose corticosterone-induced injury.

MATERIALS AND METHODS

Design

A traditional Chinese drug-containing serum, living cell biology study.

Time and setting

Experiments were performed at the Institute of Geriatrics, General Hospital of Chinese PLA, China from July 2011 to March 2012.

Materials

Cells

The human neuroblastoma cell line SH-SY5Y was a gift from the Six Institute, Academy of Military Medical Sciences of Chinese PLA, China.

Animals

A total of 15 clean male Sprague-Dawley rats aged 2 months old and weighing 200 ± 10 g were provided by the Experimental Animal Center, General Hospital of Chinese PLA, China (license No. SLXK (Jing) 2009-0007). All rats were housed at the Rodents Animal Laboratory (specific pathogen free), Experimental Animal Center, General Hospital of Chinese PLA, China at $22 \pm 2^\circ\text{C}$, a humidity of 40–70%, in 12-hour light/dark cycles. All protocols were conducted in accordance with the

Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China^[38].

Drugs

Shuyusan was prepared by the Manufacturing Laboratory, General Hospital of Chinese PLA, China, and was composed of *cape jasmine fruit* 10 g, *turmeric root tuber* 15 g, *semen ziziphi spinosae* 15 g, *Chinese magnolivine fruit* 6 g and *honey-fried herba ephedrae* 10 g. *Shuyusan* was supplied and identified by the Dispensary of Traditional Chinese Medicine, General Hospital of Chinese PLA, China. Crude drugs (560 g) were immersed in 5 000 mL water for 0.5 hours, boiled, decocted for 0.5 hours, and then cooled for filtration. The drugs were condensed in a thermostatic water bath at 80°C to obtain 5 g/mL and stored at 4°C .

Methods

Preparation of *Shuyusan*-containing serum

Rats were gastrically administered 0.375 g/mL *Shuyusan* 2 mL, twice a day, for 3 consecutive days (based on the clinical oral dose^[39]). One hour after oral administration of *Shuyusan* (entire dose, once) on day 4, blood was obtained from the abdominal aorta, and centrifuged at 3 000 r/min for 20 minutes. Subsequently, *Shuyusan*-containing serum was inactivated at 56°C for 30 minutes, filtered with a 0.22 μm micropore filter, and stored at -70°C .

SH-SY5Y cell culture

5×10^6 SH-SY5Y cells were collected, digested with 0.25% (w/v) trypsin (Gibco, Carlsbad, CA, USA) and 0.02% (w/v) ethylenediamine tetraacetic acid for 3 minutes, and incubated with Dulbecco's modified eagle medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/mL penicillin and 100 U/mL streptomycin in 5% (v/v) CO_2 at 37°C . The medium was replaced twice every week. When cells reached 90% confluence, cells were digested with 0.25% (w/v) trypsin for passage. Following adjustment with the cell counter (Nanjing Yu'an Instrument Co., Ltd., Nanjing, Jiangsu Province, China), 1×10^5 cells/mL were incubated in a 96-well plate (80 μL /well) for 12 hours.

Establishment of high-dose corticosterone-induced impairment model

In accordance with a previous study^[14], 1×10^5 cells/mL were treated with 10 μL of 400 $\mu\text{mol/L}$ corticosterone (Sigma, San Jose, CA, USA) for 48 hours to establish a model of high-dose corticosterone-induced impairment.

Intervention with Shuyusan-containing serum

At 12 hours before model establishment, SH-SY5Y cells in the high- and low-dose *Shuyusan*-containing serum groups were respectively treated with 30 µg/mL and 5 µg/mL *Shuyusan*-containing serum.

Measurement of cell survival rate

Cell concentration was adjusted to 1×10^4 cells/mL. 5 g/L 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT; Sigma; 20 µL) was added to each well at 37°C in a 5% (v/v) CO₂ incubator (Shellab, Cornelius, NC, USA) for 4 hours. The supernatant was removed when hepatic crystals formed. Dimethyl sulfoxide (Sigma; 150 µL) was added to each well and shaken for 10 minutes to thoroughly dissolve the hepatic crystals. Absorbance values were measured at 570 nm using a microplate reader (Polar star Galaxy; BMG, Offenburg, Germany). An average value of the six wells was calculated using the following formula: cell survival rate = absorbance value in the experimental group/absorbance value in the control group × 100%. Experiments were repeated three times.

Cell morphology observed by Hoechst 33258 fluorescence staining

SH-SY5Y cells (1×10^4 /mL) in each group were fixed in fresh 4% (w/v) paraformaldehyde (pH 7.14) at 37°C for 30 minutes, washed twice with PBS, and stained with 5 mg/L Hoechst 33258 (Sigma) for 30 minutes. After a wash with PBS, cells were mounted with a mounting fluid (pH 5.15) containing 20 mmol/L citric acid, 50 mmol/L disodium hydrogen phosphate, 50% (v/v) glycerol, and then observed under a fluorescence microscope (Olympus, Hatagaya, Japan).

Immunofluorescence staining for Bcl-2 and Bax expression

After three washes with PBS, SH-SY5Y cells (1×10^6 cells/mL) in each group were fixed in 4% (w/v) paraformaldehyde for 20 minutes, washed three times with PBS, blocked in normal goat serum at 37°C for 20 minutes, and incubated with rabbit anti-Bcl-2 or Bax antibodies (1: 200; Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) at 4°C overnight. Cells were rewarmed for 10 minutes, washed three times with PBS, each for 5 minutes, and incubated with Cy3-labeled goat anti-rabbit IgG (1:20–1:100; Beijing Biosynthesis Biotechnology) at 37°C for 90 minutes. Following three washes in PBS (each for 5 minutes), cells were mounted with glycerol buffer solution and observed using the fluorescence microscope. Bax- and Bcl-2-positive cells were quantified and the average absorbance value was

determined using the Image-Pro plus 5.1 image analysis system.

Reverse transcription-PCR for brain-derived neurotrophic factor mRNA expression in cells

Cells (10^6 – 10^7) in each group were collected, and total RNA (Omega R6934, New York, NY, USA) was extracted. Nucleic acid concentrations were measured using an ultraviolet spectrophotometer (Beckman, Brea, CA, USA). Total RNA (0.5 µg) was treated with DNase I (Fermentas, Ottawa, Canada), and reverse transcribed into cDNA. cDNA (1 µL) was added to 8.2 µL double distilled water. Brain-derived neurotrophic factor and β-actin were amplified on a quantitative PCR device (ABI790, Milwaukee, WI, USA). Primers were prepared in accordance with a previous study^[40].

Primer sequences are as follows:

Primer	Sequence	Product length (bp)
Brain-derived neurotrophic factor	Upstream: 5'-TGG CTG ACA CTT TTT AGC AC-3'	131
	Downstream: 5'-CAA AGG CAC TTG ACT GCT GA-3'	
β-actin	Upstream: 5'-GCT ACA GCT TCA CCA CCA CA-3'	84
	Downstream: 5'-GCC ATC TCT TGC TCG AAG T-3'	

The cycle profile was as follows: predenaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 45 seconds and elongation at 72°C for 20 seconds, and a final incubation at 72°C for 10 minutes. PCR products were electrophoresed on a 2% (w/v) agarose gel, followed by imaging using an image acquisition and analysis system (Tanon Science & Technology Co., Ltd., Shanghai, China). Brain-derived neurotrophic factor mRNA expression was expressed as the ratio of absorbance of brain-derived neurotrophic factor mRNA and β-actin mRNA.

Propidium iodide staining and flow cytometry

Cells were centrifuged at 1 000 r/min for 10 minutes. After removal of supernatant and two washes with PBS, cells were fixed in 70% (v/v) ice alcohol at –20°C for 24 hours, and centrifuged at 1 000 r/min for 10 minutes. After removal of supernatant and two washes with PBS, cells were adjusted to 1×10^6 cells/mL, treated with RNase (Sigma) to a final concentration of 100 µg/mL in a 37°C water bath for 30 minutes, and stained with propidium iodide (Sigma) at 4°C in the dark for 30 minutes to a final concentration of 50 µg/mL. Cell aggregates were

discarded by filtering with a 350-mesh nylon filter membrane. Cellular DNA content in different phases was detected by flow cytometry (Epics XL; Coulter, Fullerton, CA, USA) at an excitation wavelength of 488 nm and an emission wavelength of 610 nm. Experiments were repeated three times.

Statistical analysis

Data were expressed as mean \pm SD, and analyzed using SPSS 13.5 software (SPSS, Chicago, IL, USA), followed by one-way analysis of variance. Multiple comparisons among groups were performed using the Student-Newman-Keuls test ($\alpha = 0.05$).

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