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Anti-apoptotic activity of *Bak Foong Pills* and its ingredients on 6-hydroxydopamine-induced neurotoxicity in PC12 cells

Rui Rui Jia [a,b], Yu Lin Gou [a], Lok Sze Ho [a], Chuen-Pei Ng [a], Ning Hua Tan [b], Hsiao Chang Chan [a,*]

[a] *Epithelial Cell Biology Research Center, Department of Physiology, The Chinese University of Hong Kong, Shatin, Hong Kong, China*
[b] *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan, China*

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Abstract

Bak Foong Pills (BFP), a traditional Chinese medicine used for centuries for the enhancement of women's health, was shown to display neuro-protective activity in the 1-methyl-4-phenyl-1,2,4,6,-tetrahydro-pyridine (MPTP)-induced mouse model in a previous study. In order to elucidate its mechanism of action, we investigated the anti-apoptotic properties of Bak Foong Pills and its main ingredients, including *Panax ginseng*, *Angelica sinensis*, *Glycyrrhiza uralensis*, and *Ligusticum chuanxiong*, in the 6-hydroxydopamine (6-OHDA)-treated PC12 cell model. The addition of the neurotoxin could cause significant cell death and reduction of cell proliferation, as shown in the results determined by MTT assay, nitric oxide (NO) measurement and flow cytometric propidium iodine (PI) staining analysis, while pre-treatment of PC12 cell with either BFP or its main ingredients prevented the toxicity to some degree. In addition, the neurotoxin caused an elevated activation of caspase-3, the key enzyme for activation of the cellular apoptotic cascade, whereas BFP or its main ingredients inhibited the activation of caspase-3. These results strongly indicate that BFP and its main ingredients may provide a useful therapeutic strategy for the treatment of neurodegenerative diseases, such as Parkinson's disease.
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1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by rigidity, bradykinesia, resting tremor and gait disturbance. A major pathological hallmark of Parkinson's disease is the degeneration of nigrostriatal dopaminergic neurons, resulting in the reduced release of dopamine into the striatum (Dunnett and Bjorklund, 1999; Olanow and Tatton, 1999; Forno, 1996; Hardy and Gwinn-Hardy, 1998; Gibb and Lees, 1988). Even though the cause of Parkinson's disease remains largely unknown, the onset and progression of the disease is thought to involve many biochemical pathways induced by oxidative stress, including apoptosis (reviewed by Halliwell (1992)).

Recently, advances have been made towards understanding the mechanisms of cell death. Apoptosis is a genetically controlled cell 'suicide' program, inherent to every eukaryotic cell. Normally, apoptosis plays a major role in tissue development and homeostasis. However, 'inappropriate' activation of apoptosis is also fundamental to the pathogenesis of tissue damage, both in acute and chronic disease states (Mochizuki et al., 1997). Indeed, apoptosis is now recognized to be a major factor in the pathogenesis of numerous neurological diseases, ranging from acute cerebral stroke to Parkinson's

* Corresponding author. Tel.: +852 2609 6839; fax: +852 2603 5022.
E-mail address: hsiaocchan@cuhk.edu.hk (H.C. Chan).

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and Alzheimer's diseases (Marks and Berg, 1999). The 6-OHDA-induced cytotoxicity model has been the standard model system for studying oxidative stress-induced neurodegeneration because the neurotoxin 6-OHDA can cause similar features of neuronal damage and neuron cell death in the brain (Soto-Otero et al., 2000; He et al., 2000).

Bak Foong Pill (BFP, also known as Bai Feng Wan), an over-the-counter traditional Chinese medicine with 26 herbal ingredients, has a long history in treating gynecological disorders, such as dysmenorrhea, irregular menstrual cycle and irregular bleeding. Our previous results have demonstrated the protective effect of BFP in the MPTP-induced animal model of PD (Liu et al., 2004). In the present study, using the rat pheochromocytoma cell line (PC12), the standard model for neuronal functional studies, we further examined the protective activity of BFP and its main ingredients on the neuronal apoptosis induced by 6-OHDA. In order to explore whether the drugs are indeed involved in protective effects against apoptosis, we also analyzed the level of caspase-3 activation, a key enzyme essential for the execution of apoptosis. The results indicate that, similar to the results shown in our previous animal model study (Liu et al., 2004), BFP gave protection against 6-OHDA-induced neuronal cell death, which is at least partly associated with inhibition of the central apoptotic cascade involving caspase-3.

2. Materials and methods

2.1. Materials

The PC12 cell line was obtained from ATCC. 6-OHDA and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Griess reagent system was purchased from Promega (Madison, WI, USA). The antibodies against caspase-3 (the inactive pro-form) and cleaved caspase-3 (the active enzymatic form) and secondary antibodies were purchased from Cell Signaling (Beverly, MA, USA). Other common chemicals were of reagent grade and were from commercial sources in the USA.

2.2. Extraction of BFP and ingredients of BFP

The ingredients in the herbal medicine BFP used in this study are listed as follows (Latin, Chinese): *Panax ginseng*, Renshen; *Angelica sinensis*, Danggui; *Glycyrrhiza uralensis*, Gancao; and *Ligusticum chuanxiong*, Chuanxiong. Five hundred grams of BFP (obtained from Eu Yan Sang Company, Hong Kong) was ground and passed through 80 μm meshes. The organically soluble fraction of BFP was successively extracted three times using 70% aqueous ethanol at 1 l:200 g powder ratio. The filtrate was concentrated to 200 ml at 60 °C and dried using a freeze dryer to yield the "BFP-E-ext" fraction. The water soluble fraction was extracted three times at 60 °C with distilled water at 1 ml:1 g powder ratio and dried by a freeze dryer to yield the "BFP-W-ext" fraction. The individual herbal ingredients in BFP were supplied by Eu Yan Sang Co. (Hong Kong). Again, each single powdered ingredient (200 g) was extracted three times using 70% ethanol. The filtrate was concentrated to 200 ml at 60 °C and dried using a freeze dryer to yield the individual ingredient extracts. All samples were stored at 4 °C.

2.3. Cell culture

The PC12 cells retain dopaminergic characteristics and have been widely used for neurobiological and neurochemical studies in Parkinson's disease. The cells were maintained in DMEM supplemented with 10% horse serum, 5% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were cultured in a humidified incubator aerated with 95% air and 5% CO_2 at 37 °C. All experiments were carried out 24–48 h after the cells were seeded. The cells were routinely passaged when approaching the sub-confluent stage and they were usually plated in 25 cm^2 culture flasks, with seeding culture at 1:5 ratio.

2.4. Cell viability assay

For the measurement of cell viability, MTT reagent is absorbed into the living PC12 cells and converted into formazan. The chemical conversion is facilitated by succinate dehydrogenase in the mitochondria of living cells. The amount of accumulated formazan indicates the activity of succinate dehydrogenase, and thus reflects the cell viability (Yamamoto et al., 2000). For our purpose, cells were seeded in 96-well plates at a density of 1×10^4 cells per well. The cultures were grown at 37 °C for 24 h, then the media were changed to those containing varying concentrations of BFP and their ingredients (1000, 500, 250 or 125 μg/ml) and 100 μM 6-OHDA. After incubation for up to 48 h, MTT solution (5 mg/ml in PBS) was added to the 96-well plates and the cells were allowed to incubate for 4 h at 37 °C. After the medium had been removed, the cells and dye crystals (formazan) were dissolved by adding 100 μl dimethylsulfoxide (DMSO) and the light absorbance was measured at 570 nm using a model 550 microplate reader (Bio-Rad, Hercules, CA, USA).

2.5. Nitrite content measurement (Griess reagent system)

One of the means to detect nitric oxide (NO) formation is to measure nitrite (NO_2^-), one of the

breakdown products of NO. The level of nitrite can be quantified by the Griess reaction (Stuehr and Marletta, 1985). In brief, 50 μl of culture supernatants following treatment with BFP and its components were collected and placed into a new 96-well microplate. Then, 50 μl sulfanilamide (2 mg/ml) solution was added to each well and incubated at room temperature for 10 min, followed by the addition of 50 μl NED solution (1 mg/ml *N*-(1-naphthyl)-ethylenediamine). After incubation for 30 min, absorbance was measured with a spectrophotometer at a wavelength of 535 nm. The absorbance of a sample was compared with that of standard sodium nitrite solution.

2.6. Flow cytometric analysis

To quantify the level of apoptosis, we chose a flow cytometric propidium iodide-based staining method to detect cellular events that have undergone DNA fragmentation, a late apoptotic event. The BFP-treated PC12 cells were harvested and washed twice with 1× cold PBS and permeabilized with 80% ethanol. The cells were centrifuged for 10 min and resuspended in 500 μl TE buffer containing 40 μg/ml propidium iodide (PI) and 40 μg/ml RNase A and incubated at 37 °C for 30 min. The PI-stained cells at different fluorescence intensities were measured with an FACScan flow cytometer (Coulter, Fullerton, CA, USA).

2.7. Western blotting analysis

The cleaved and non-cleaved forms of the enzyme caspase-3 were observed with Western blot analysis. Briefly, 5×10^6 cells were washed twice in cold PBS and incubated for 10 min on ice in a lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% deoxycholic acid, 1% Nonidet P40, 0.1% sodium dodecyl sulfate (SDS), 1 mM PMSF, and 100 mg/ml leupeptin. Cell lysates were clarified by centrifugation at 15,000 rpm for 20 min at 4 °C; the clear supernatants were saved for Western blotting. To ensure equal loading of the protein samples, protein concentrations of the cell lysates were determined by Bio-Rad DC protein assay kit (Hercules, CA, USA). An equal amount (total of 20 μg) of protein was separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in 1× Tris-buffered saline containing 0.05% Tween-20 (TBST) for 1 h. After blocking, the membrane was incubated with 2% skim milk in TBST, containing either the primary mouse monoclonal antibody against caspase-3 (1:500, Cell Signaling, Beverly, MA, USA), or the monoclonal antibody against cleaved caspase-3 (1:500, Cell Signaling, Beverly, MA, USA) for another hour. The membranes were then washed with 1× TBST three times, followed by an additional incubation with 2% skim milk in TBST, containing an HRP-conjugated anti-rabbit antibody as the secondary antibody (1:10,000). The detection of protein bands was performed using the enhanced chemiluminescence (ECL) detection system. The results of several western blots were quantified with an image analyzer.

2.8. Statistical analysis

Data are expressed as mean ± standard error of the mean (S.E.M.) where applicable, and n is the number of tests. Significance testing used was Student's t-test and one-way analysis of variance (ANOVA). The significance level was set at $p < 0.05$.

3. Results

3.1. Growth kinetics and cell viability of PC12 cells following exposure to 6-OHDA

In order to evaluate the viability of PC12 cells after exposure to oxidative injury, the cells were treated with different concentrations of 6-OHDA (0: control group, 1, 10, 100 and 1000 μM). After incubation for 24 h, cell viability was measured by the MTT assay. There was significantly less production of formazan, indicating progressively lower cell count, in higher concentrations of 6-OHDA. One hundred micromolars of 6-OHDA, which led to 50% cell viability, was selected for subsequent experiments (Fig. 1A).

To investigate the neuro-protective effects of BFP and its ingredients, PC12 cells were pre-treated with the medium containing BFP for 24 h. PC12 cells were then washed and exposed to 100 μM 6-OHDA for 24 h. Pre-treatment with either BFP or its ingredients at 1000 μg/ml significantly increased cell viability (Fig. 1B).

3.2. Decreased NO production by BFP and its ingredients in 6-OHDA-induced cell death

Since nitric oxide (NO) can trigger cell death in the CNS (Dawson and Dawson, 1996), we measured the level of NO indirectly by determining nitrite formation in the PC12 cultures that were treated with 100 μM 6-OHDA for 24 h. The nitrite generation induced by 6-OHDA, measured by the Griess reagent system, was upregulated approximately 75% over the untreated control and this increase was reduced by pre-treatment with 1000 μg/ml BFP and its ingredients (Fig. 2).

3.3. The protective effect of BFP and its ingredients against 6-OHDA-induced DNA fragmentation

In order to quantify apoptosis based on late apoptotic nuclear events such as DNA fragmentation,

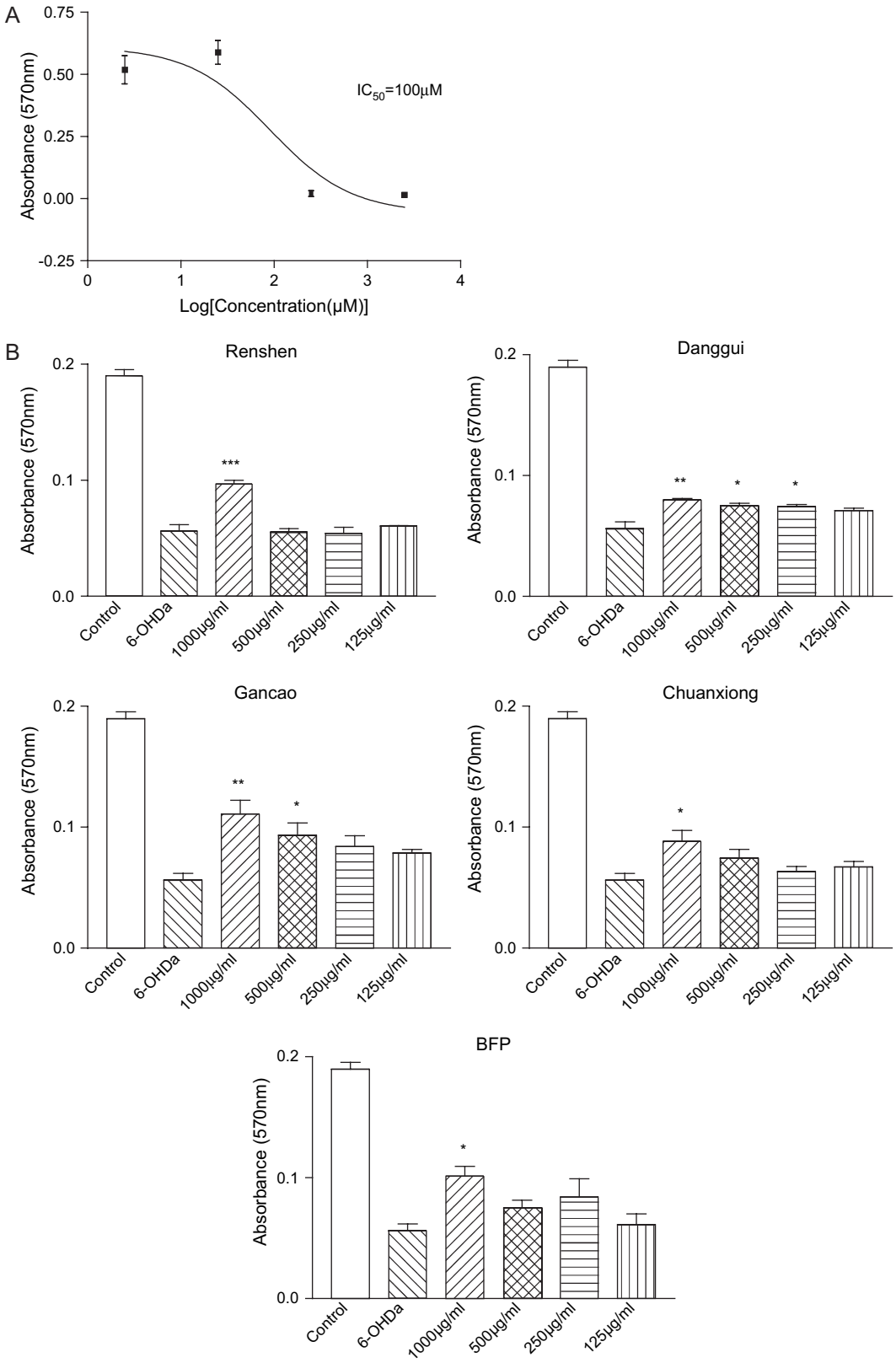

Fig. 1. (A) 6-OHDA-induced neurotoxicity in PC12 cells. PC12 cells were treated with different concentrations of 6-OHDA (1000, 100, 10, and 1 μM). Cell viability was measured after 24 h by the MTT assay and IC50 values calculated. Data points are the means ± SEM of three independent experiments and considered statistically significant if $p < 0.05$. (B) Protection by BFP and its ingredients against 6-OHDA-induced neurotoxicity in PC12 cells. PC12 cells were treated with 6-OHDA (100 μM) at different concentrations in herbal medicine. The cell viability was measured after 48 h by the MTT assay and IC50 values calculated. Data points are the means ± SEM of three independent experiments and considered statistically significant if $p < 0.05$ compared with 6-OHDA-treated cells.

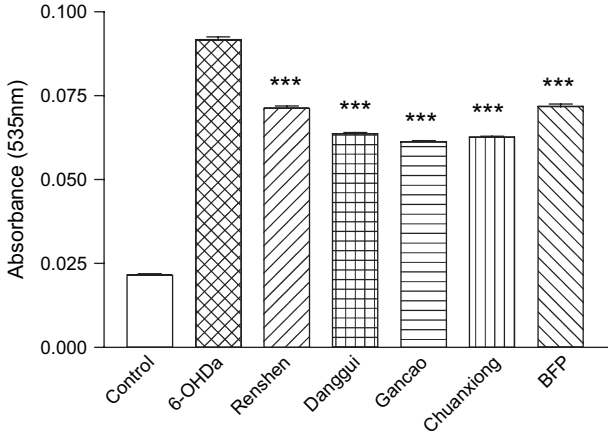

Fig. 2. BFP and its individual ingredients suppressed generation of NO in 6-OHDA-treated cells. PC12 cells were treated with 6-OHDA (100 μM), following pre-treatment with BFP and its ingredients (1000 μg/ml). Nitrite formation was measured after 24 h by the Griess reagent and calculated as % of control. Data are means ± SEM from three independent cultures, each performed in triplicate with comparable results. The data are statistically significant ($p < 0.05$) compared with the 6-OHDA-treated cells.

a propidium iodide-based flow cytometric analysis following ethanol permeabilization was used to analyze the protective effect of BFP and its ingredients against 6-OHDA-induced cell apoptosis. The PI stain used in the flow cytometric analysis specifically targets DNA and detects the integrity of the DNA content based on varying fluorescence intensity. In a cell cycle/DNA content analysis histogram, the cell population with lower fluorescence intensity (the sub-G0/G1 population marked as "apoptosis" in the cell cycle profile) indicates the apoptotic cells undergoing DNA fragmentation (Fig. 3). In untreated cells, the apoptotic sub-G0/G1 fraction was minimal (8.0% of total counted 10,000 events) (Fig. 3A). After 24 h exposure to 100 μM 6-OHDA, the percentage of cells with DNA fragmentation increased to 51.5% (Fig. 3B). When BFP and its different ingredients were added to the cells, the fraction of cells in the sub-G0/G1 phase decreased to 14.9%, 26%, 13.7%, 15.3%, and 26.9% following 24 h pre-treatment with Renshen, Danggui, Gancao, Chuanxiong, and BFP, respectively (Fig. 3C–G).

3.4. The protective effect of BFP and its ingredients against 6-OHDA-induced caspase-3 activation

To ascertain whether BFP affects downstream apoptotic signaling, Western blotting of PC12 cell homogenates examining caspase-3 activation was performed. The Western blotting employed a specific antibody against the active form of caspase-3 (17 kDa) to determine the level of caspase-3 activation. In PC12 cells pre-treated with either BFP or its ingredients (Fig. 4, lanes C–G), the level of activated caspase-3 (17 kDa)/inactive pro-caspase-3 (35 kDa) decreased dramatically following 6-OHDA treatment, whereas PC12 cells treated with only 100 μM 6-OHDA resulted in significant activation of caspase-3 (Fig. 4, lane B). These findings suggest that BFP and its ingredients inhibit downstream apoptotic signaling, including the activation of caspase-3 (Fig. 4).

4. Discussion

Neurons are post-mitotic cells that cannot divide or regenerate, except under a few special circumstances. Accumulating evidence shows that neuronal apoptosis or cell death can be induced by a wide range of insults, such as oxidative stress (Coyle and Puttfarcken, 1993; Gotz et al., 1994), insufficiency of trophic factors, excitatory amino acids, and many neurotoxins, such as MPTP (Przedborski and Jackson-Lewis, 1998) and 6-OHDA (Glinka and Youdim, 1995). Excessive neuronal apoptosis then may lead to massive neuronal tissue damage, as seen in some human neurodegenerative diseases, like Parkinson's and Alzheimer's diseases. Thus, the use of anti-apoptotic agents as a way of neuroprotection could be a potential therapy to slow or ameliorate the progression of neurodegenerative diseases.

In this study, we examined the neuro-protective effects of BFP and its ingredients against 6-OHDA-induced apoptosis. We observed that the viability of the PC12 neuronal cells was significantly enhanced by pre-treatment with BFP and its main ingredients (*P. ginseng*, *A. sinensis*, *G. uralensis*, and *L. chuanxiong*) compared with the cells treated with only 6-OHDA. *P. ginseng* and *G. uralensis* appeared to exert a stronger anti-apoptotic effect than BFP and other components (Fig. 1). Using a flow cytometric analysis that detects DNA fragmentation, a large sub-G0/G1 population (cells undergoing DNA fragmentation) was observed in the 6-OHDA-treated group. This sub-G0/G1 cell fraction was reduced by pre-treatment with BFP or its ingredients, particularly with *P. ginseng*, *G. uralensis*, and *L. chuanxiong*, to a level comparable to that of the control group (Fig. 3). Together, these results indicate that BFP and its ingredients exert their protective effects by inhibition of apoptosis.

Recent reports have indicated that caspases play an essential role in neuronal cell death during development as well as after neuronal injury (Green, 1998). The caspase family is a class of cysteine proteases that is involved in a cascade of proteolytic cleavages leading to the eventual fragmentation of DNA in mammalian cells (Li et al., 1998). Of particular interest is caspase-3, the most widely studied member of the caspase family and the most important executioner of apoptosis, since it is responsible for the direct proteolytic cleavage of various cellular target proteins (Du et al., 1997). Thus, caspase-3

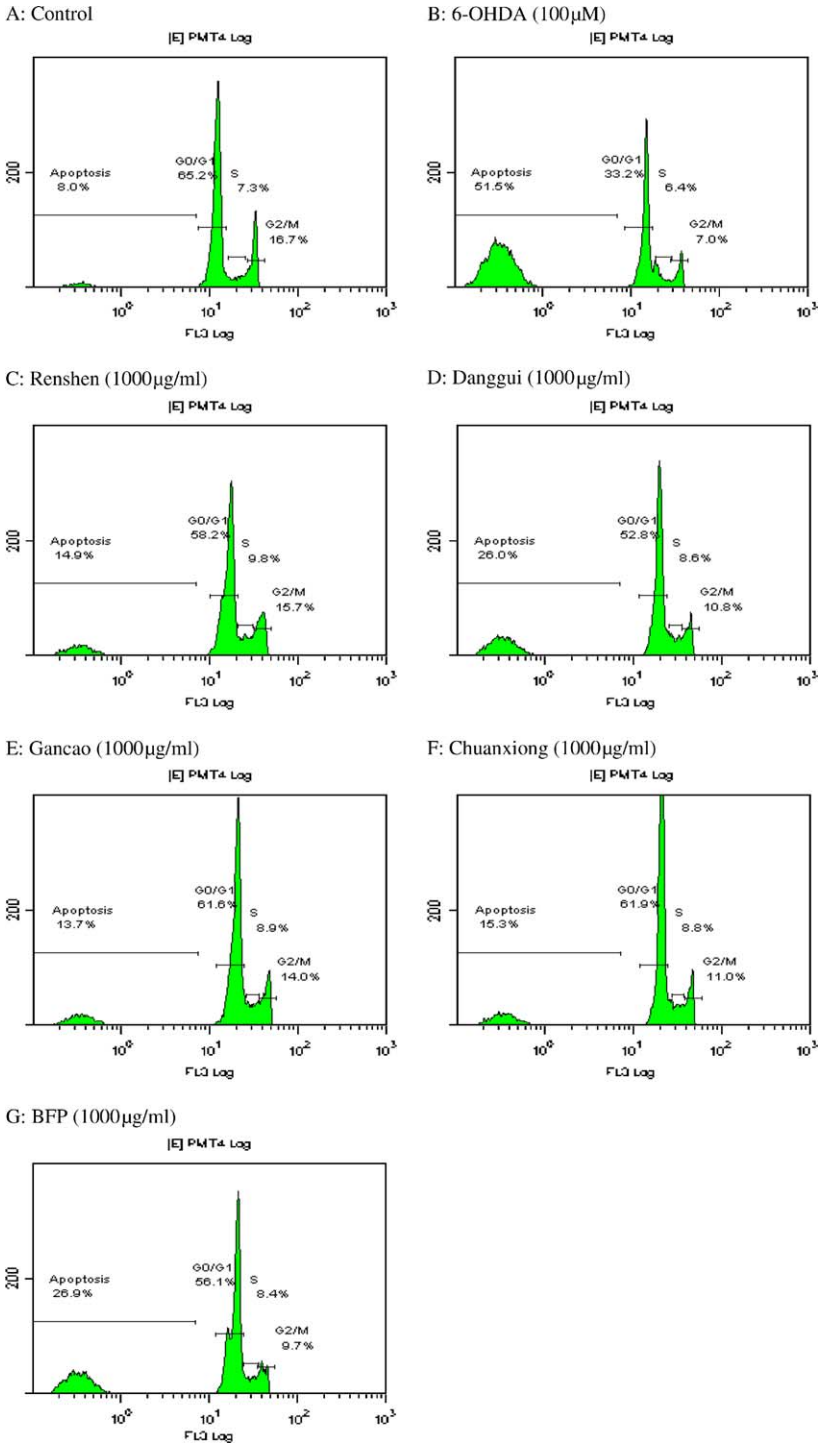

Fig. 3. Flow cytometric histograms of control PC12 cells and PC12 cells treated with 100 μM 6-OHDA alone or pre-treated with BFP and its ingredients (1000 μg/ml) for 24 h. After incubation, the cells were harvested and labeled with propidium iodide. (A) Control; (B) 100 μM 6-OHDA treated alone; (C) 1000 μg/ml Renshen + 100 μM 6-OHDA; (D) 1000 μg/ml Danggui + 100 μM 6-OHDA; (E) 1000 μg/ml Gancao + 100 μM 6-OHDA; (F) 1000 μg/ml Chuanxiong + 100 μM 6-OHDA; (G) 1000 μg/ml BFP + 100 μM 6-OHDA.

is often used as a marker for detection of apoptosis (Chen et al., 1998). Activation of caspase-3 is an essential step in the execution of apoptosis and its inhibition blocks apoptotic cell death. As demonstrated in our experiment, 6-OHDA-induced apoptotic signaling leading to the activation of caspase-3, yet pre-treatment of PC12 cells with BFP and its main ingredients apparently inhibited the cleavage and activation of caspase-3. *P. ginseng* and *A. sinensis* displayed the most obvious inhibitory effect (Fig. 4).

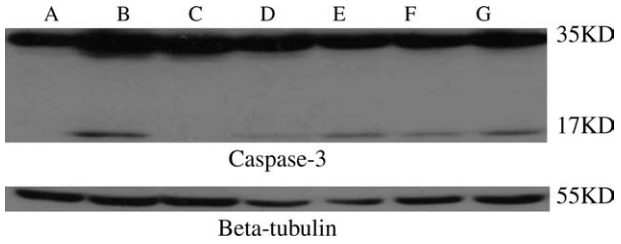

Fig. 4. Activation of caspase-3 in PC12 cells treated with 100 μM 6-OHDA alone or pre-treated with BFP and its ingredients (1000 μg/ml) for 24 h. The positions of standard molecular weight markers in kDa are indicated on the left side of the blots (pro-caspase-3 (35 kDa) and activated caspase-3 (17 kDa)). Lanes: (A) Control; (B) 100 μM 6-OHDA treated alone; (C) 1000 μg/ml Renshen + 100 μM 6-OHDA; (D) 1000 μg/ml Danggui + 100 μM 6-OHDA; (E) 1000 μg/ml Gancao + 100 μM 6-OHDA; (F) 1000 μg/ml Chuanxiong + 100 μM 6-OHDA; (G) 1000 μg/ml BFP + 100 μM 6-OHDA.

This result indicates that BFP and its main ingredients exert anti-apoptotic activity partly by preventing the apoptotic signaling that leads to the activation of caspase-3.

Nitric oxide (NO) has also been thought to be one of the important endogenous mediators for neuronal cell death (Dawson and Dawson, 1996). Excess NO is normally generated by inducible NOS (I-NOS) to induce neuronal apoptosis, when the neuronal cells are challenged by environmental stress (Dawson et al., 1991; Bredt and Snyder, 1992; Zhang et al., 1994; Heneka et al., 1998). Our experimental data showed that BFP and their ingredients exhibit some inhibitory activities against 6-OHDA-induced generation of NO (Fig. 2), which may partly contribute to their anti-apoptotic actions.

At present, the underlying cellular and molecular neuro-protective mechanisms of BFP are not fully understood. However, our results demonstrate that several mechanisms, separately or in association, may be involved in the neuro-protective effects of BFP. The individual ingredients, such as *P. ginseng*, demonstrated a higher anti-apoptotic activity than BFP and its other main ingredients, suggesting that *P. ginseng* is a more potent agent guarding against neuronal apoptosis. This observation is in agreement with the results reported by Kim et al. (2003), a study also involving PC12 neuronal cells. In summary, anti-apoptotic effects are possible mechanisms for the Chinese medicine-mediated neuro-protection, and the results of this study demonstrate that neuro-protective effects of BFP may be partly attributed to its anti-apoptotic signaling, which prevents the activation of caspase-3 and production of NO. Moreover, *P. ginseng* could play a much more important role in the anti-apoptosis of neurons, and could be the primary component responsible for the anti-apoptotic activity of BFP.

Acknowledgements

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