Chinese Herbal Complex ‘Bu Shen Jie Du Fang’ (BSJDF) Modulated Autophagy in an MPP⁺-Induced Cell Model of Parkinson’s Disease

Cuifang Liu,1 Xiaobo Huang,1 Shengxiang Qiu,2 Wenqiang Chen,1 Weihong Li,3 Haiyan Zhang,3 Tao Wang,4 Xue Wang,5 and Xiling Wu1

1Department of Chinese Medicine, Xuanwu Hospital, Capital Medical University, Beijing, China
2Key Laboratory of Plant Resources Conservation and Sustainable Utilization, Guangdong Provincial Key Laboratory of Applied Botany, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, China
3Department of Cell Biology, Capital Medical University, Beijing, China
4Department of Neurosurgery, Xuanwu Hospital, Capital Medical University, Beijing, China
5Department of library, Xuanwu Hospital, Capital Medical University, Beijing, China

Correspondence should be addressed to Xiaobo Huang; ccmu_pdr@163.com

Received 13 October 2018; Revised 17 January 2019; Accepted 6 February 2019; Published 13 March 2019

Academic Editor: Carmen Mannucci

Copyright © 2019 Cuifang Liu et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Autophagy plays an important role in the development of Parkinson disease (PD). Previous studies showed that autophagy could protect cells from α-synuclein toxicity and promote functional coupling of mitochondria. But it is still a question whether modulating autophagy can be used to treat PD. In traditional Chinese medicine, a specific Chinese herbal complex called Bu Shen Jie Du Fang (BSJDF) has a long history of treating motor impairments similar to Parkinson disease, while its mechanism is still unclear. As a pilot study, we aimed to evaluate the efficacy and its mechanism of Bu Shen Jie Du Fang in an MPP⁺-induced cell model of Parkinson’s disease. And the phase contrast microscope (PCM) revealed that the BSJDF group had the greatest surviving cell counts compared with all other treated cell groups except the normal group. And Cell Counting Kit 8 (CCK8) assays showed a similar result. In BSJDF group, 3.7 × 10⁷ cells/dish was identified by hemocytometer counts, which was significantly higher than other groups except the normal cells (p<0.05). In the BSJDF group, autophagy can be observed by transmission electron microscopy (TEM). Protein expression of Atg12 and LC3 in the BSJDF group was upregulated compared to the PD model group (p<0.05). Atg12 mRNA expression was also upregulated in the BSJDF group (p<0.05). In conclusion, our study indicated that the therapeutic mechanisms of BSJDF may be mediated by stimulating autophagy, and modulating autophagy can be used to treat PD.

1. Introduction

Parkinson’s disease (PD) is a progressive, degenerative neurologic disease characterized by a tremor that is maximal at rest, slowness of movement, rigidity, and postural instability [1]. Its incidence is 8 to 18 per 100,000 person-years based on prospective population-based studies with either record-based or in-person case finding [2–4]. Currently, the therapy is limited, only capable of slowing down the progression of this disabling neurodegenerative disorder [5]. Drug therapy and surgery mainly target impairments related to dopaminergic lesions [6]. L-DOPA, used as the pharmacological replacement of dopamine (DA), is the major pharmaceutical drug for symptom control, but most patients still experience motor fluctuations as the disease progresses [5, 7]. Moreover, drug therapy becomes less effective or causes complications like disabling dyskinesias in later stages of the disease [6].

The etiology and pathology of the disease remain largely unknown. Many studies have addressed the issue of autophagy in PD. Previous studies suggested that dysregulation of autophagy is implicated in the pathogenesis of idiopathic and familial PD [8, 9]. Ravikumar and colleagues [10] demonstrated in vivo that enhancing autophagy provided protection in neurodegeneration. Whether “excessive” autophagy mediates “autophagic cell death” is perhaps one of the most controversial areas in autophagy research [11].
Autophagy plays a complex role in PD, and it may play a fundamental role in the development of disease, thus its regulation may be important to find a new therapy [8, 11]. In the autophagy signaling, Beclin-1, LC3, and Atg12 play essential roles in the initiation step of autophagy. Beclin-1 serves as a critical regulator at the beginning of autophagic vesicle nucleation [12]. Beclin1-P13KC3 is an essential complex for autophagosome formation, which plays a role in nucleation and initial phagophore membrane formation [13]. LC3 (the mammalian homolog of Atg8), which have two cellular forms, LC3-I (a cytoplasmic form of LC3) and LC3-II (a cleaved form) [14], was considered as a specific marker for autophagy [14]. Except the Atg8/LC3 lipidation system, the Atg5-Atg12 conjugation system also plays essential roles in autophagosome initiation and expansion [13]. Atg12 is also involved in autophagosome formation [15]. So Beclin-1, LC3, and Atg12 are useful representative proteins for investigating autophagy.

Traditional Chinese medicine, including a specific Chinese herbal complex called Bu Shen Jie Du Fang (BSJDF), has a long history of treating motor impairments similar to Parkinson disease. Clinical observations showed that BSJDF enhances functional capacity in PD patients, without leading to motor fluctuations. Bu Shen Jie Du Fang (BSJDF) is composed of Rehmannia glutinosa, Cistanche deserticola, Paonia lactiflora Pall, Radix Angelica sinensis, Puerariae Radix, Rhizoma Coptidis, Radix Scutellariae, Antelope Horn Powder, and Glycyrrhizae Radix in a weight ratio of 5:5:4:4:5:4:4:1:2. Earlier reports revealed that Radix Scutellariae induced autophagic cell death in SMMC-7721 cells [16, 17]; Rhizoma Coptidis stimulated autophagy and suppressed the proinflammatory phenotype of macrophages [18]. Furthermore, Glycyrrhizae Radix has been shown to induce autophagic cell death in cervical and breast cancer, as well as androgen-sensitive prostate adenocarcinomas and adenoid cystic carcinoma cancer cells [19]. The present study was designed to explore the therapeutic mechanisms of BSJDF: whether it is mediated by stimulating autophagy and whether stimulating autophagy can be used to treat PD. We investigated the effect of BSJDF on pheochromocytoma 12 (PC12) cells treated with the neurotoxin MPP+ (a metabolite of MPTP (1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine) to induce PD in vivo [3, 20–22].

2. Results

2.1. Cell Survival Was Increased in the BSJDF Group. We assessed the effect of cotreating PC12 cells with different drugs by cell survival counts at 48 h. The PCM (phase contrast microscope) demonstrated that the highest number of survival cells in the BSJDF group rather than the others, except the normal group (Figure 1(d)). We counted the survival cells by hemocytometer and then calculated and analyzed the results. And the statistics also showed that BSJDF group has the maximum surviving cells compared with other groups except the normal group (Figure 2 D). Cell Counting Kit 8 (CCK-8) revealed the correlation between the counts and OD (Optical Density, which is absorbance values) (Figure 3). The result proved that BSJDF group had the largest OD than all other groups except the normal group (Figure 4 D). And BSJDF group had the greatest surviving cell counts compared with all other groups except the normal group.

2.2. The Autophagy Was Clearly Observed by Transmission Electron Microscopy (TEM) in Rapamycin Group and BSJDF Group. To investigate whether BSJDF induces autophagy, PC12 cells were assigned to groups and treated for 48 h, as described above, before TEM examination. As we know, autophagy was indicated as double-membrane enclosed autophagosomes by TEM, and we can observe autophagy in the rapamycin group (Figure 5(b); red arrow) and the BSJDF group (Figure 5(d); red arrow). TEM result also showed the cells in PD model group and 3-MA group exhibited impaired autophagy (Figures 5(c) and 5(e)). NH4Cl markedly increased the number of these intracellular vacuoles (Figure 5(f)).

2.3. Protein Expression of Atg12 and LC3 Was Increased by BSJDF. To investigate whether cell survival following BSJDF treatment was mediated by autophagy, we analyzed Beclin-1, Atg12, and LC3 expression by western blotting. And the result showed that protein expression of Atg12 and LC3 was upregulated in the BSJDF group. The remarkable increased Atg12 expression had a statistically significant difference (p<0.05) compared with other groups except the rapamycin group (Figure 6(a)). The expression of Atg12 between rapamycin group and BSJDF group did not show a statistically significant difference (p>0.05). The upregulated LC3 expression also had a statistically significant difference (p<0.05) (Figure 6(c)). Also, we can find that Atg12 expression was significantly higher (p<0.05) in rapamycin group except BSJDF group (Figure 6(a)).

2.4. mRNA Expression of LC3 Was Upregulated by BSJDF. RT-qPCR revealed a significantly higher mRNA expression of Atg12 in the BSJDF group than other groups except the rapamycin group (p<0.05) (Figure 7(a)). In the rapamycin group, we observed a higher mRNA expression of Atg12, which had a statistically significant difference (p<0.01) compared with other groups, except the BSJDF group (Figure 7(a)). Not only the Atg12 mRNA but also Beclin-1 showed higher expression which had a statistically significant difference (p<0.05), as did LC3 mRNA expression (p<0.01), in the rapamycin group compared with the others (Figure 7(c)).

3. Discussion

In the present study, we explored the underlying modulated mechanism of BSJDF on the principal aspect autophagy relating mRNA and proteins. We examined the neuroprotective effects of BSJDF by PCM, hemocytometer, and CCK-8. And autophagic process was visualized using TEM. To elucidate the possible mechanisms of how BSJDF regulates autophagy, we performed western blotting and RT-qPCR. Our results showed that BSJDF can protect PC12 cells (Figures 1, 2, and 4 D) which was based a higher cervical cells counts than other groups except the normal condition in BSJDF group by PCM, hemocytometer, and CCK-8. And BSJDF.
Figure 1: The cells were reviewed under PCM enlarged 100× and 1000×. (a) The normal group which is untreated PC12 cells; (b) the rapamycin group treated with MPP⁺ for 24 h and then rapamycin for 24 h; (c) the PD model group treated with MPP⁺ for 48 h; (d) the BSJDF group treated with MPP⁺ for 24 h and then BSJDF serum for 24 h; (e) the 3-MA group treated with MPP⁺ for 24 h and then 3-MA for 24 h; (f) the NH₄Cl group treated with MPP⁺ for 24 h and then NH₄Cl for 24 h. The cell number in the BSJDF group is larger than the others except the normal group.

could activate autophagy (Figure 5(d)) by TEM. BSJDF also increased protein expression of Atg12 and LC3 (Figures 6(a) and 6(c)) and upregulated Atg12 mRNA expression (Figure 7(a)). Collectively, these findings indicate that BSJDF improves cell survival by inducing autophagy in the PC12 cell PD model. Autophagy plays an important role in cell fate and maintaining cellular metabolic balance [9]. Anglade et al. (1997) identified apoptosis and autophagy as a possible mechanism when they discovered that nigrostriatal neurons were lost in PD patients [23]. Since then, many studies have reported that autophagy exerted an important protective effect on neurons [15, 24, 25]. Autophagy-mediated clearance of aggresomes has been implicated in many neurodegenerative diseases, and autophagy regulation is considered as a potential method in treatment of PD [26–29]. Indeed, autophagy is a key step in the development of PD. In our research, we established an in vitro PD model in MPP⁺-induced PC12 cells, which provided a stable and reliable assay for estimating the effects of anti-PD drugs [28].

We did not observe an increase in the protein or mRNA expression of Beclin-1 (Figures 6(b) and 7(b)), Beclin-1 overexpression via lentivirus delivery is beneficial for PD [30] in the setting of BSJDF treatment. But BSJDF can increase Atg12 and LC3 protein expression (Figures 6(a) and 6(c)) and upregulate Atg12 mRNA expression (Figure 7(a)). Interestingly, Atg12 protein overexpression inhibits autophagosome formation in HEK-293 cells [31], and changing Atg12 protein levels contributes to the development of sporadic PD [15]. Meanwhile, upregulation of LC3 protein is sufficient to enhance autophagic activity and reduce the accumulation
Evidence-Based Complementary and Alternative Medicine

Figure 2: The survival cells counted by hemocytometer. A, the normal group, the survival cells were $7.3 \times 10^7$; B, the rapamycin group, the survival cells were $2.8 \times 10^7$; C, the PD model group, the survival cells were $1.8 \times 10^7$; D, the BSJDF group, the survival cells were $3.4 \times 10^7$; E, the 3-MA group, the survival cells were $1.6 \times 10^7$; F, the NH$_4$CL group, the survival cells were $1.6 \times 10^7$. The number of cells surviving in the BSJDF group was larger than other groups except the normal group ($p<0.05$), *$p<0.05$ and **$p<0.01$.

Figure 3: The OD results in different groups by CCK8. A, the normal group; B, the rapamycin group; C, the PD model group; D, the BSJDF group; E, the 3-MA group; F, the NH$_4$CL group. The number of cells surviving in the BSJDF group has more cell counts than the others, except the normal group ($p<0.05$), *$p<0.05$ and **$p<0.01$.

4. Materials and Methods

4.1. The Preparation of Materials and BSJDF Serum

4.1.1. Medications. The component substances used to make BSJDF were as follows: Rehmannia glutinosa, Cistanche deserticola, Paeonia lactiflora Pall, Radix Angelica Sinensis, Puerariae Radix, Coptidis Rhizoma, Scutellariae Radix, Antelope Horn Powder, and Glycyrrhiza uralensis. These herbs were mixed based on a dry weight ratio of 5:5:4:4:5:4:4:1:2, respectively. All of the plants were extracted by standard methods according to the Chinese Pharmacopoeia. The manufacturing process of BSJDF begins with decoction. The decocted solutions were mixed together, and the Antelope horn powder was added to produce BSJDF, which was stored in the refrigerator for later use at 4°C. The final concentration of the solution was 0.255 g/mL (equivalent to the dry weight of raw materials in 400 ml liquid).

4.1.2. BSJDF Serum Preparation. Ten Sprague–Dawley rats (5 males, 5 females) were purchased from the Laboratory Animal Center of Xuanwu Hospital, Beijing, China. All animal experiments were approved by the Institutional Animal Care and Use Committee of Xuanwu Hospital, Capital Medical University, China, and conducted according to guidelines laid out by the National Institutes of Health. Animals initially...
Figure 5: The formation of autophagosomes in treated cells was evaluated by TEM. (a) the normal group; (b) the rapamycin group; (c) the PD model group; (d) the BSJDF group; (e) the 3-MA group; (f) the NH$_4$CL group. The arrowheads autophagy (scale bar: 2.0 µm). "Red arrow" indicates autophagosomes.

Table 1: Component herbs of BSJDF.

| Botanical plant name               | family                  | Part used       | Ratio of composition |
|-----------------------------------|-------------------------|-----------------|----------------------|
| Rehmannia glutinosa              | Scrophulariaceae        | root and rhizome| 5                    |
| Cistanche deserticola            | Orobanchaceae           | Fleshy stem     | 5                    |
| Paeonia lactiflora Pall          | Paeoniaceae             | Radix           | 4                    |
| Radix Angelica Sinensis          | Apiaceae                | Radix           | 4                    |
| Puerariae Radix                  | Fabaceae                | Radix           | 5                    |
| Coptidis Rhizoma                 | Ranunculaceae           | Rhizome         | 4                    |
| Scutellariae Radix               | Labiatae                | Radix           | 4                    |
| Cornu Bubali                     | Cornu Bubali            | Horn            | 1                    |
| Glycyrrhizae radix               | Leguminosae             | Radix           | 2                    |

weighed 200-250 g and were housed individually at 21°C with a 12:12 h light/dark cycle. Rats had free access to standard food and drinking water.

Animals were treated with BSJDF (5.1 g/kg body weight per day) for 3 d, by oral administration. Blood was collected from the abdominal aorta 2 h after the final administration and then centrifuged at 3000 rpm for 20 min to obtain serum. The serum was heated in a 56°C water bath for 30 min and then stored at −20°C before further analysis [32].

4.1.3. Reagents and Antibodies. Cell culture medium (Dulbecco’s minimum essential medium, DMEM) RIMPI640, fetal bovine serum (FBS), heat-inactivated horse serum, and penicillin-streptomycin liquid were obtained from Gibco (Grand Island, NY, USA). Rabbit anti-Beclin-1 antibody, rabbit anti-Atg12 antibody, rabbit anti-LC3A/B antibody, rabbit anti-GAPDH antibody, and goat anti-rabbit IgG H&L were obtained from Abcam (Cambridge, UK). We purchased MPP+ iodide and NH$_4$CL from Sigma Co. (St. Louis, MO, USA). All other materials were purchased from Sigma Co., except where indicated, and were of analytical grade. ExpressPlus PAGE Gels and Tris-MOPS-SDS Running Buffer Powder were purchased from GenScript (Nanjing, China). The RNPrep pure cell kit, FastKing RT Kit (with gDNase), and Talent qPCR PreMix (SYBR Green) were purchased from TIANGEN (Beijing, China).

4.2. Cell Culture and Cell Counts. Rat PC12 cells (Cat. 311C0001CCC000024; National Infrastructure of Cell Line Resource, China) were cultured in DMEM supplemented
Figure 6: 1, the normal group; 2, the rapamycin group; 3, the PD model group; 4, the BSJDF group; 5, the 3-MA group; 6, the NH₄CL group. GAPDH was used as a loading control. (a) Atg12 western blot densitometry in ST. (b) Beclin-1 western blot densitometry in ST. (c) LC3 western blot densitometry in ST. ∗p<0.05 and ∗∗p<0.01.
with 5% FBS and 10% heat-inactivated horse serum, before adding 100 μg/mL penicillin-streptomycin liquid to the medium. The cells were cultured at 37°C in a humidified mix of 5% CO₂ and 95% air. Cells were split 1:4 every 3 d; experiments were performed on cells at passages 4 to 8 when they were at 80–90% confluence. All experiments were carried out 24–48 h after the cells were seeded [33].

We divided cells into six treatment groups: (1) the normal group were cultured with 5% FBS and 10% heat-inactivated horse serum; (2) the PD model control group treated with 200 mM MPP⁺ in 5% FBS and 10% heat-inactivated horse serum for 48 h; (3) the rapamycin group, which can induce autophagy in a variety of cell types [34] via its methoxy group, were treated with 200 mM MPP⁺ in 5% FBS and 10% heat-inactivated horse serum for 24 h, before being cocultured with 100 nM rapamycin (Cell Signaling Technology, Beverly, MA, USA) for 24 h; (4) the BSJDF group were treated with 200 mM MPP⁺ in 5% FBS and 10% heat-inactivated horse serum for 24 h, before being cocultured with 10% BSJDF serum for 24 h; (5) the 3-methyladenine (3-MA) group, which as a PI3K and autophagy inhibitor [35] is the most commonly used

Figure 7: 1, the normal group; 2, the rapamycin group; 3, the PD model group; 4, the BSJDF group; 5, the 3-MA group; 6, the NH₄Cl group. GAPDH was used as a loading control. (a) Atg12 mRNA expression level in RT-qPCR. (b) Beclin-1 mRNA expression level in RT-qPCR. (c) LC3 mRNA expression level in RT-qPCR. ∗ p < 0.05 and ∗∗ p < 0.01.
specific inhibitor of autophagic removal [36], were treated with 200 mM MPP\(^+\) in 5% FBS and 10% heat-inactivated horse serum for 24 h and then cocultured with 15 mM 3-MA (Sigma) for 24 h; and (6) the NH\(_4\)Cl (ammonium chloride) group, a specific and rapid inhibitor of induced autophagy-mediated proteolysis and inhibits autolysosome formation [37], were treated with 200 mM MPP\(^+\) in 5% FBS and 10% heat-inactivated horse serum for 24 h and then cocultured with 10 mM NH\(_4\)Cl (Cell Signaling Technology) for 24 h. After 48 h, all cells were examined using a phase contrast microscope (PCM; Axio Observer 3m; Carl Zeiss, Jena, Germany) under 1000\(\times\) and 10000\(\times\) magnification and the survival cells counted by hemocytometer.

Cell counts were assessed by Cell Counting Kit 8 (CCK-8; Solarbio, China) assays according to the manufacturer's instructions. First, we divided PC12 cells into three groups and labeled. In the first group we placed six wells of 96-well plate at a density of 1 \(\times\) 10\(^4\) cells per well, in the second group 5 \(\times\) 10\(^4\) cells per well were plated into 96-well plate in six wells, and in the third group 2.5 \(\times\) 10\(^4\) cells per well were plated into 96-well plate in six wells. Cells were cultured in complete medium (DMEM including 10% fetal bovine serum (FBS) and 5% heat-inactivated horse serum) at 37°C in 5% CO\(_2\). 10 \(\mu\)L CCK8 was added for 2 h. The spectrometric absorbance was measured by microplate reader (Thermo Scientific, Massachusetts, US) at 450 nm and then analyzed the correlation between the counts and absorbance values and marked a standard curve.

PC12 cells of six difference treatment groups were plated into 96-well plate with 1 \(\times\) 10\(^4\) cells per well in six wells and cultured in corresponding medium at 37°C in 5% CO\(_2\). Then 10 \(\mu\)L CCK8 solution was added for 24 h and absorbance values were determined at 450 nm. and spectrometric absorbance was measured by microplate reader at a wavelength of 450 nm. Depending on the standard curve, the system calculates the number of different groups in the sample.

**4.3. Observing Autophagy by Transmission Electron Microscopy (TEM).** Autophagosome morphology was examined with TEM. After 48 h, PC12 cells were fixed in ice-cold 2.5% glutaraldehyde in 0.1 mol/L phosphate-buffered saline (PBS) at 4°C for 1.5-2 h. Then cells were postfixed for 1 h in 1% osmium tetroxide in the same buffer, dehydrated in graded alcohols and acetones, and embedded in Epon 812 at the laboratory of Capital Medical University (Beijing, China). Samples were sectioned with an LKB-1 ultramicrotome in 50 to 60 nm thick slices. Then the sections were stained with 3% uranyl acetate and lead citrate and examined with a transmission electron microscope (JEM-1400plus, JEOL, Tokyo, Japan).

**4.4. Protein and mRNA Expression.** We measured the protein and mRNA expression of Beclin-1, Atg12, LC3. Protein was extracted as follows: cell culture solutions were centrifuged at 800 rpm for 5 min, the supernatant was discarded, cells were collected, radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China) in phenylmethanesulfonyl fluoride was added, and the resultant solutions were placed on ice for 30 min. The supernatants were collected after centrifugation at 12,000 g at 4°C for 20 min. Protein concentration was determined using a BCA Protein Assay Kit (Beyotime, Shanghai, China), and whole lysates were mixed with 5x loading buffer at a ratio of 1:5. Samples were heated at 95°C for 15 min and were separated on SDS–polyacrylamide gels. The separated proteins were then transferred to polyvinylidene difluoride membranes. The blots were first probed with a primary antibody. After incubation with horseradish peroxidase- (HRP-) conjugated secondary antibody, autoradiograms were prepared using an enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. The primary antibodies were rabbit anti-LC3, anti-Beclin-1, anti-ATG12, and antiglyceraldehyde 3-phosphate dehydrogenase (GAPDH). The secondary antibody was goat anti-rabbit. We used GAPDH as a protein loading control. Protein levels were first normalized to GAPDH and then normalized to the experimental control. Images shown in the figures represent data from five animals. Western blot densitometry was performed with AlphaView software.

Total RNA was extracted from PC12 cells using the RNAprep pure cell kit; then the RNA was reverse transcribed to cDNA using the FastKing RT Kit (with gDNase). We conducted cDNA synthesis in a reaction mixture containing 2 \(\mu\)l 5x gDNA buffer, 2 \(\mu\)l total RNA, and 6 \(\mu\)l RNase-free ddH\(_2\)O. The total reaction volume of 10 ml was mixed, centrifuged for 30 s, and stained at 42°C for 3 min. Then we added another reaction mixture containing 2 \(\mu\)l 10x King RT Buffer, 2 \(\mu\)l FastKing RT Enzyme Mix, 2 \(\mu\)l FQ-RT Primer Mix, and 5 \(\mu\)l RNase-free ddH\(_2\)O. The total reaction volume was 20 ml. Reverse transcription was performed at 42°C for 30 min and then 99°C for 5 min and stored at 20°C for RT-qPCR. The details of all oligonucleotide primer sequences, predicted product lengths, and GenBank accession numbers for sequences amplified by RT-PCR are listed in Table 2. The RT-PCR reaction mixture using the Talent qPCR Premix (SYBR Green) consisted of 0.6 \(\mu\)l forward primer, 0.6 \(\mu\)l reverse primer, 1 \(\mu\)l cDNA, and 78 \(\mu\)l RNase-free ddH\(_2\)O. The total reaction volume was 20 ml. The reaction was performed under the following conditions: pre-denaturation at 95°C for 5 min, denaturation at 95°C for 10 s, annealing at 60°C for 10 s, extension at 72°C for 20 s for a total of 45 cycles and then denaturation at 95°C for 5 s, annealing at 65°C for 1 min, denaturation at 97°C, continuous for a total of 1 cycles; then a final extension step was performed at 40°C for 30 s. An RT-PCR assay was carried out with a Roche LightCycler480 (Basel, Switzerland).

**4.5. Statistical Analysis.** All statistical analyses were carried out using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Data were analyzed using SPSS (SPSS 22.0 for Windows, IBM Corp., Armonk, NY) and expressed as mean ± SD of the indicated number of independent experiments. Statistical significance among groups was evaluated by a one-way analysis of variance (ANOVA) followed by Bonferroni-corrected comparison tests. A value of p < 0.05 was considered statistically significant for all tests.
Table 2: Sequences of primers used for RT-PCR.

| Name    | Oligo      | Primer sequence | Predicted size(bp) | Genebank accession |
|---------|------------|-----------------|--------------------|--------------------|
| Atg12   | Forward    | aaacgaagaaatgggctgtg  | 148                | NM_001038495.1    |
|         | Reverse    | gtcccaaccttcttgcttg | 148                |                    |
| LC3     | Forward    | gctgtccttgaaaaagcacc | 121                | NM_012823.2        |
|         | Reverse    | gttcaccagcaggaagaag | 121                |                    |
| Beclin-1| Forward    | ggccaataagatgggtctga | 182                | NM_001034117.1     |
|         | Reverse    | gttcaccagcaggaagaag | 182                |                    |
| β-actin | Forward    | gctgacaggatgcagaagga | 124                | NM_031144          |
|         | Reverse    | tggacagtgaggccagaga | 124                |                    |
| GAPDH   | Forward    | cctgcaccaccaactgtctta | 120                | NM_0170008         |
|         | Reverse    | ggccatcaccagtccttga | 120                |                    |

5. Conclusions

In conclusion, this study is the first to investigate the possible mechanism of autophagy signaling, through which BSJDF improved survival in the PC12 cell model of PD. Our findings indicated that BSJDF improves MPP⁺-induced injury. We found that BSJDF protected PC12 cells by inducing autophagy. However, its effect was not solely attributed to autophagy induction because a greater number of cells survived following treatment with BSJDF than rapamycin, an autophagy inducer (Figures 1, 2, and 4 B, D). We therefore hypothesize that BSJDF regulates the balance of autophagy, but the specific underlying mechanism remains to be elucidated. Our research provides a new way, which is worth going into additional research, for the development of PD medicine in the future.

Data Availability

The data used to support the findings of this study are included within the article.

Ethical Approval

All animal experiments were approved by the Institutional Animal Care and Use Committee of Xuanwu Hospital, Capital Medical University, China, and conducted according to guidelines laid out by the National Institutes of Health.

Conflicts of Interest

We declare that we have no conflicts of interest.

Authors’ Contributions

Cuifang Liu worked on all the experiments and wrote and modified the modification. Tao Wang provided some important guidance and advices in the process of writing and modification. Shengxiang Qiu analyzed the main results and provided guidance and advices in writing the paper. Weihong Li provided guidance and advices in all experiments and helped in finishing the research. Haiyan Zhang provided guidance and advices in all experiments and helped finishing the research. Xue Wang provided some interesting guidance and advices in the process of writing and modification. Wenqiang Chen provided some guidance and advices in the process of writing and modification. Xiaobo Huang designed the research work. Xiling Wu provided some help in the experiments.

Funding

The National Science Foundation [grant number 81574036]

Acknowledgments

We thank Jing Ann in the department of cell biology, Xuanwu Hospital, Capital Medical University, for providing experimental technical help. This work is supported by the National Science Foundation [Grant no. 81574036].

References

[1] H. H. Fernandez, “2015 Update on Parkinson disease,” Cleveland Clinic Journal of Medicine, vol. 82, no. 9, pp. 563–568, 2015.
[2] A. Elbaz, L. Carcaillon, S. Kab, and F. Moisan, “Epidemiology of Parkinson’s disease,” Rev Neurol (Paris), vol. 172, no. 1, pp. 14–26, 2016.
[3] P. Michel, E. Hirsch, and S. Hunot, “Understanding dopaminergic cell death pathways in parkinson disease,” Neuron, vol. 90, no. 4, pp. 675–691, 2016.
[4] A. Lee and R. M. Gilbert, “Epidemiology of Parkinson disease,” Neurologic Clinics, vol. 34, no. 4, pp. 955–965, 2016.
[5] J. Obergasteiger, G. Frapporti, P. P. Pramstaller, A. A. Hicks, and M. Volta, “A new hypothesis for Parkinson’s disease pathogenesis: GTPase-p38 MAPK signaling and autophagy as convergence points of etiology and genomics,” Molecular Neurodegeneration, vol. 13, no. 1, 2018.
[6] D. L. M. Radder, I. H. Sturkenboom, M. van Nimwegen, S. H. Keus, B. R. Bloem, and N. M. de Vries, “Physical therapy and occupational therapy in Parkinson’s disease,” International Journal of Neuroscience, vol. 127, no. 10, pp. 930–943, 2017.
[7] R. Katzenschlager, W. Poeoe, O. Rascol et al., “Apomorphine subcutaneous infusion in patients with Parkinson’s disease with persistent motor fluctuations (TOLEDO): a multicentre, double-blind, randomised, placebo-controlled trial,” The Lancet Neurology, vol. 17, no. 9, pp. 749–759, 2018.
[8] Y. Miki, S. Shimoyama, T. Kon et al., “Alteration of autophagy-related proteins in peripheral blood mononuclear cells of patients with Parkinson’s disease,” Neurobiology of Aging, vol. 63, pp. 33–43, 2018.

[9] S. Ghavami, S. Shojaei, and B. Yeganef, “Autophagy and apoptosis dysfunction in neurodegenerative disorders,” Prog Neurobiol, vol. 112, pp. 24–49, 2014.

[10] B. Ravikumar, C. Vacher, Z. Berger et al., “Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease,” Nature Genetics, vol. 36, no. 6, pp. 585–595, 2004.

[11] S. J. Cherra III and C. T. Chu, “Autophagy in neuroprotection and neurodegeneration: a question of balance,” Future Neurology, vol. 3, no. 3, pp. 309–323, 2008.

[12] H. Shi, H. Shi, F. Ren, D. Chen, Y. Chen, and Z. Duan, “Naringin in Ganshuang Granule suppresses activation of hepatic stellate cells for anti-fibrosis effect by inhibition of mammalian target of rapamycin,” Journal of Cellular and Molecular Medicine, vol. 21, no. 3, pp. 500–509, 2017.

[13] S.-F. Wang, M.-Y. Wu, C.-Z. Cai, M. Li, and J.-H. Lu, “Autophagy modulators from traditional Chinese medicine: Mechanisms and therapeutic potentials for cancer and neurodegenerative diseases,” Journal of Ethnopharmacology, vol. 194, pp. 861–876, 2016.

[14] N. Bae, T. Ahn, S. Chung et al., “The neuroprotective effect of modified Yeoldahanso-tang promotes autophagy enhancement in models of Parkinson’s disease,” Journal of Ethnopharmacology, vol. 134, no. 2, pp. 313–322, 2011.

[15] Y. Li, J. Huang, S. Pang et al., “Novel and functional ATG12 gene variants in sporadic Parkinson’s disease,” Neuroscience Letters, vol. 643, pp. 22–26, 2017.

[16] B. Spencer, R. Potkar, M. Trejo et al., “Necitin 1 gene transfer activates autophagy and ameliorates the neurodegenerative pathology in alpha-synuclein models of Parkinson’s and Lewy body diseases,” The Journal of Neuroscience, vol. 29, no. 43, pp. 13578–13588, 2009.

[17] N. Fujita, T. Itoh, H. Omori, M. Fukuda, T. Noda, and T. Yoshimori, “The Atg16L1 complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy,” Molecular Biology of the Cell (MBtC), vol. 19, no. 5, pp. 401–407, 2012.

[18] J. S. Amenta, T. J. Hlivko, A. G. McBee, H. Shinozuka, and S. Aguilar, “Are dopamine oxidation metabolites involved in the loss of dopaminergic neurons in the nigrostriatal system in Parkinson’s disease?” ACS Chemical Neuroscience, vol. 8, no. 4, pp. 702–711, 2017.

[19] K. Liu, J. Huang, R. Chen et al., “Protection against neurotoxicity by an autophagic mechanism,” Brazilian Journal of Medical and Biological Research, vol. 45, no. 5, pp. 401–407, 2012.

[20] S. N. Suresh, A. K. Chavalmane, V. Vidyadhara et al., “A novel autophagy modulator 6-Bio ameliorates SNCA/α-synuclein toxicity,” Autophagy, vol. 13, no. 7, pp. 1221–1234, 2017.

[21] P. Anglade, S. Vyas, F. Javoy-Agid et al., “Apoptosis and neuroprotection of cannabidiol against MPP+-induced toxicity in SH-SY5Y cells,” Biochemistry, 2016, vol. 75, pp. 13–18, 2015.

[22] F. Liu, J. Zhao et al., “Effect of Trehalose on PC12 cells for anti-fibrosis effect by inhibition of mammalian target of rapamycin,” Journal of Cellular and Molecular Medicine, vol. 21, no. 3, pp. 359, 2016.

[23] G. Chen, X. Hu, W. Zhang et al., “Mammalian target of rapamycin regulates isoloquiquetininduced autophagic and apoptotic cell death in adenoid cystic carcinoma cells,” Apoptosis, vol. 17, no. 1, pp. 90–101, 2012.

[24] N. A. G. Santos, N. M. Martins, F. M. Sisti et al., “The neuroprotection of cannabinoids at MPP+-induced toxicity in PC12 cells involves trkA receptors, upregulation of axonal and synaptic proteins, neuroinflammation, and might be relevant to Parkinson’s disease,” Toxicology in Vitro, vol. 30, no. 1, pp. 231–240, 2015.

[25] P. Jiang and N. Mizushima, “LC3- and p62-based biochemical methods for the analysis of autophagy progression in mammalian cells,” Methods, vol. 75, pp. 13–18, 2015.

[26] P.-F. Wu, C.-C. Chiu, C.-Y. Chen, and H.-M. D. Wang, “7-Hydroxycyclomucrine induces human melanoma death via triggering autophagy and apoptosis,” Experimental Dermatology, vol. 24, no. 12, pp. 930–935, 2015.