

Chinese Medicine PaBing-II Protects Human iPSC Derived Dopaminergic Neurons from Oxygen Stress

Shouhai Wu

The Second Affiliated Hospital of Guangzhou University of Chinese Medicine

Tongxiang Lin

The Second Affiliated Hospital of Guangzhou University of Chinese Medicine

Yang Xu (✉ yangxu@ucsd.edu)

The Second Affiliated Hospital of Guangzhuo University of Chinese Medicine <https://orcid.org/0000-0001-5574-921X>

Research

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Abstract

Background

PaBing-II Formula (PB-II) is a traditional Chinese medicine developed to treat Parkinson's disease (PD). However, due to the complexity of PB-II and the difficulty of culturing human dopaminergic neurons (DAn) *in vitro*, the mechanism of PB-II to treat PD remains unclear.

Methods

We established the human induced pluripotent stem cells (iPSCs) and derived DAn from hiPSCs to study the protective effects of PB-II on DAn after oxidative stress, which plays an important role in PD pathogenesis.

Results

We found that serum derived from rats that had ingested PB-II significantly protect hiPSC-derived DAn from reactive oxygen species (ROS). In addition, PB-II dependent serum can activate nuclear erythroid-derived factor 2 (Nrf2) responses, which are required for the neutralization of ROS. In addition, PB-II can activate the Nrf2/ARE signal pathway of midbrain dopaminergic neurons of PD rats induced with 6-hydroxydopamine (6-OHDA) injury, rescue DAn cells, and improve the symptoms of PD rats.

Conclusions

PB-II significantly protects the DA neurons from oxidative stress by activating the Nrf2 pathway.

Background

Parkinson's disease is a common progressive neurodegenerative disease characterized by tremor and bradykinesia [1]. As patients age, their symptoms continue to deteriorate [2]. The hallmark of PD is the degeneration of dopaminergic (DA) neurons in the substantia nigra (SN) and the presence of Lewy bodies (LBs) [2, 3]. While the mechanisms underlying PD pathology remains unclear, extensive evidences from postmortem brain tissues suggest that oxidative stress and deficiency of complex I activity are related to PD pathogenesis [4, 5]. Currently there is no cure for PD. The primary treatment of PD is to supplement dopamine or promote endogenous dopamine release. Levodopa, commonly used in the treatment of PD, can significantly improve the motor symptoms of PD, but it cannot prevent the disease progression that eventually leads to the death of DA neurons. The long-term use of L-dopa can also cause movement disorders and insomnia. Serious complications such as anxiety [6].

PaBing-II Formula (PB-II), a Traditional Chinese Medicine remedy, has been developed and used to treat Parkinson's disease (PD) in the Second Affiliated Hospital of Guangzhou University of Chinese Medicine for over 20 years. PB-II is composed of Wumei 20 g, Coptis 3 g, Paeony 20 g, Angelica 10 g, Aconite 10 g, Rehmannia 10 g, Polygonum multiflorum 20 g, Ligusticum chuanxiong 10 g, Pueraria 20 g, Ginseng 10 g,

Acorus 5 g, Gastrodia 10 g, Tortoise plate 10 g, and Roasted licorice 3 g. Wumei nourishes kidney, liver, blood, softens tendons, and balances Yin and Yang to treat the symptoms of involuntary tremor. PaBing-II Formula has significant therapeutic effects on PD patients, especially during the early stage [7, 8]. PB-II can relieve PD patients' motor and non-motor symptoms, improve the therapeutic effects of dopaminergic drugs while reducing drug's side effects, and improve patients' quality of life [9, 10]. Previous studies report that gavaging PD rats induced by 6-hydroxydopamine (6-OHDA) with PB-II improve the rotational behavior [11], and protected the dopaminergic neurons from apoptosis [12, 13]. The 6-OHDA can damage the dopaminergic neurons in substantia nigra of midbrain, and lead to the symptoms of PD in mice. The unilaterally lesioned 6-OHDA-lesioned rat model of PD has proved to be invaluable in advancing our understanding of the mechanisms underlying parkinsonian symptoms, and is widely used in PD research [14]. However, the impact of PB-II on human DAN and mechanisms underlying its therapeutic benefit remain to be established.

Based on the pluripotency of iPSCs to differentiate into all cell types in the body, the iPSC technology has provided an essential model to study human diseases in dishes [15]. Here, we generated iPSC from human fibroblast and differentiated them into dopaminergic neurons. Using this model, we investigated the mechanisms underlying the therapeutic benefits of PB-II on PD patients.

Methods

PB-II medicated serum preparation

For PB-II original recipe, refer to previous reports. We prepared slices of Chinese crude drugs from the pharmacy of Guangdong Province Hospital of Chinese medicine, which were decocted twice with 10 and 8 times of water, filtered, and concentrated with water bath at 80°C to 1.6 kg/L (measured by crude drug weight/volume). Rats took gavage of PB-II twice a day at 32 G•kg⁻¹ (according to the crude drug meter) according to the body surface area converted from clinical dosage. After gavage for 2 weeks, the rats with 10% chloral hydrate anesthesia were bled of arterial blood and separated for medicated serum in accordance with the method reported in the previous studies [16]. The medicated serum and control serum were inactivated at 56 °C for 30 min, then stored at -80 °C before use.

LC-MS

LC-MS was conducted through the combined application of Dionex Ultimate 3000 UHPLC (Thermo Fisher Scientific, Waltham, MA, USA) and Q Exactive Orbitrap mass spectrometer. After screening, the analysis was carried out using the WatersTM UPLCTM HSS T3 C18 (2.1×100 mm, 1.7µm). Chromatographic conditions: Gradient elution was performed with acetonitrile (A) -0.1% formic acid water (B). The elution procedure was: 0 min, 10% A; 5 min, 20% A; 20 min, 60% A; 25 min, 90% A; 28 min 90% A; 29-33min 10% A. The flow rate was 0.2 mL/min. Mass spectrometry conditions: The samples were ionized by ESI ion source and then analyzed by Q ExactiveOrbitrap high-resolution mass spectrometer. The main parameters of the ESI ion source are: spray voltage 3500V (Anion voltage -3500V), capillary temperature

350 °C; Sheath gas: 40, Auxiliary gas, 15. All other parameters are default. The liquid eluent with a retention time of 0.5-29 min was selected by the automatic switching valve for mass spectrometry analysis.

Generation of human DA neurons from iPS cells

We selected an iPS cell line from a healthy human skin cell line preserved in our laboratory. According to the previous reports [17], we differentiated the DA neuron from the iPSC with the necessary media and related cytokines. iPSCs were plated at 4×10^4 cells/cm² on Matrigel (BD)-coated tissue culture dishes for differentiation. N2B27-CDM + bFGF (20ng/ml) was used to culture for 3 days. The differentiation was performed in the KSR medium. On day 3, the cell should be almost confluent (over 80%). The culture was gradually changed to the N2 medium, supplemented at day 0-5 with SB431542 (5 μM)+LDN-193189 (100 nM)+lwp2 (1 μM) to get retinal progenitor, and then split in 1:3 ratio for the next six passages using Accutase. Neural induction media supplemented with 3 μM CHIR99021 and 2 μM on X-ray inactivated MEF feeders or Matrigel-coated plates was used to culture the cells. On day 6-10, induction factors were withdrawn, meanwhile adding PD173074 (0.2 μM)+DAPT (10 μM) for retinal ganglion cell inductions. On day 10-15, the medium was changed to N2, B27 and 300 μg/mL cAMP (Sigma-Aldrich) adding 100 ng/mL SHH (C24) and 100 ng/mL FGF8b. We then added 10 ng/mL BDNF, 10 ng/mL GDNF, 10 ng/mL IGF-1, 1 ng/mL TGF-β, and 0.5 mM db-cAMP and continued culturing cells for 30 days. We collected the cell and detected the cells with dopaminergic neuronal markers, such as TH and TUJ1. We used primary antibodies, which were as follows: Tyrosine Hydroxylase Antibody (CST-2791), β3-Tubulin (TU-20) Antibody (CST-4466), DAPI (Sigma-D9542). The primers of DA neuron-specific genes are shown in the following table.

The immunofluorescence method

Cells were fixed using 4% v/v paraformaldehyde (Alfa Aesar), washed three times with PBS containing 0.2% v/v Tween (PBST) (Fisher Scientific), and permeabilized using 0.15% v/v TritonX-100 (Sigma-Aldrich) in PBS for 1 hr at 25 °C. After gentle removal of PBST, cells were incubated with the primary antibody in PBST overnight at 4 °C. After that, cells were washed three times with PBST and stained with the secondary antibody for 1 hr at 37 °C. The cells were washed three times in PBST, stained with DAPI, and viewed with a Laser scanning confocal microscope (Carl Zeiss-710). Dopaminergic neuron-specific TH antibody, TUJ1 staining, To observe the ratio of dopaminergic neurons, we statistically analyzed the proportion of TH+/TUJ1+ double positive in all the cells.

The establishment of DA neurons oxidation model

Cultured DA neurons were treated with 100 μM H₂O₂ for 12 h in accordance with the method previously reported [18]. After the treatment, we carried out examinations of apoptosis and the ROS levels in the cells. By flow cytometry analysis, we found that ROS and apoptosis increased significantly. And the IF data showed that the ratio of TH/TUJ1 double-positive cells in H₂O₂ treated cells decreased significantly, which is considered to be a DA neuron model of oxidative damage.

Experimental grouping

The neural cell culture and DA neuronal cells were randomly divided into 4 groups, including the control group (Ctrl), oxidative damage model (ODM), blank serum group (BS), and medicated serum group (MS). Control cells were cultured in normal conditions, and the other cultures were treated separately. We added 10% mock serum in the BS group and 10% medicated serum in the MS group but continued normal condition without supplementing the model sample for 24 hours. On the following day, the 3 treatment groups, including the BS group, MS group, and the ODM group, were treated at 100 μM H_2O_2 for another 12 hours. Finally, all cell samples were examined for cell apoptosis, DA neuronal activity, ROS, and Nrf2 signal pathway gene expressions.

Flow cytometry analysis

We used flow cytometry to analyze the TH positive ratio, apoptosis, and ROS levels. For TH detection, we used intracellular staining. The cells were digested, fixed with 4% paraformaldehyde, and blocked with BSA. The TH-antibody (ab75875, 1/100 dilution) was then for 30 min at 22°C. The secondary antibody used was DyLight-488 goat anti-rabbit IgG (H+L) (ab96899) at 1/500 dilution for 30 min at 22°C. Acquisition of >5,000 events was performed. For apoptosis detection, we used the KEYGEN apoptosis kit (#KGA108-1) according to the manufacturer's instructions. We also checked Annexin V-FITC/PI staining through flow cytometry with software (BD). ROS were detected with the Reactive Oxygen Species kit (#KGT010-1) according to the manufacturer's instructions. The brief principle for the detection of ROS was based on the fluorescent probe DCFH-DA. Intracellular ROS can oxidize non-color DCFH into fluorescent DCF. Thus, flow cytometry could be used to detect the fluorescence intensity for ROS levels.

Nrf2/ARE signal detection

Western blotting was carried out for the detection of Nrf2 protein in each group of cells. ImageJ software was used to analyze the protein gray value. The RT-PCR detection was for Nrf2 downstream gene mRNAs, such as HO-1, NQO1, MRP2, and GPX2. The primer sequences are shown in the following table.

Quantification and statistical analysis

Data are represented as mean \pm SEM unless otherwise indicated, and Student's t-test was used for comparing two groups. F-test was used for comparing variances. For comparing multiple groups, one-way ANOVA or two-way ANOVA were used. n was indicated in figure legends. GraphPad Prism 5 software was used for statistical analysis. Differences between two groups were considered significant when the *P*-value was less than 0.05 (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; n.s., not significant. Error bars indicate means \pm s.d.).

Results

Quality control test of medicated serum

We used liquid chromatography and mass spectrometry (LC-MS) method to analyze the active ingredients in the medicated serum, and analyze the main six compounds in the whole recipe (citric acid (Figure 1-1), hyaconitine (Figure 1-2), stilbene glucoside (Figure 1-3), glycyrrhizin (Figure 1-4), paeoniflorin (Figure 1-5), Ginsenoside Rg1 (Figure 1-6)). Perform identification and simultaneous detection to provide a reference for the quality control of PB-II. The detailed map is shown in Figure 1.

Generation of hiPSC and their differentiation into DAn

We derived an iPSC line from healthy human skin fibroblasts as previously described. Briefly, we used a Nucleotransfection Kit (P2 primary cell 4D-Nucleotector X Kit, Lonza) to transfect the Y4 episomal vectors (pCXLE-hOCT3/4-shp53, pCXLE-hSK and pCXLE-hUL, [Okita et al Nat Methods. 2011](#)) to the fibroblasts with the procedure of DT-130. Cells were cultured in ESC medium containing DMEM/F12 (Gibco, 11330), KnockOut™ Serum Replacement (Gibco), MEM non-essential amino acids (Gibco), L-glutamine (Gibco), 55 mM β-mercaptoethanol (Gibco) and 20ng bFGF (Gibco), on feeders which were taken from CF1 mouse embryos and subjected to radioactive irradiation with the dose of 30 Gy advance. Colonies with hESC morphology appeared between day 25 and 45. They were picked and expanded under hESC culture condition. The pluripotency of the hiPSC line was confirmed by the colony morphology, the expression of the pluripotency markers OCT4 and TRA-1-81, and the formation of teratomas in NSG mice (Figure 2A-C). These data confirmed the pluripotency of the hiPSCs.

Using the protocol described previously (Li W et al., 2011, PANS), we differentiated hiPSCs into dopaminergic neurons. We confirmed the presence of dopamine neurons in the differentiating culture using DAn-specific markers. DAn-specific genes such as Tyrosine hydroxylase, TUBB3 (TUJ1), FOXA2, and Engrailed 1 (EN1) were significantly increased in iPSC-derived DAn culture (Figure 2D). In addition, the cell morphology confirmed the morphological characteristics of DA neurons (Figure 2E). Immunofluorescence (IF) analysis demonstrated that about 60% of iPSC-derived cells expressed the neuron-specific markers TUJ1, and about 40% of iPSC-derived neurons were TH⁺/TUJ1⁺, confirming the presence of hiPSC-derived DAn (Figure 2F).

PB-II protects DA neurons from oxidative damage induced by H₂O₂

Immunofluorescence data showed more TUJ1⁺TH⁺ neurons in the PB-II medicated serum (MS) sample than those in the oxidative damage model sample (ODM) which was prepared by H₂O₂ damage and blank serum (BS) samples (Figure 3A). While TUJ1⁺TH⁺ neurons were obviously decreased in the ODM group after the treatment with H₂O₂, MS could significantly increase TUJ1⁺TH⁺ neurons, indicating that MS could protect TUJ1⁺TH⁺ neurons from oxidative stress (Figure 3A). In support of this conclusion, flow cytometric analysis indicated that the percentage of TH⁺ cells in the MS group was significantly higher than that in the ODM or BS group (Figure 3B, 3C). We further analyzed the percentage of apoptosis in each experimental group, indicating that MS protected the neuronal apoptosis after H₂O₂ treatment (Figure 3D, 3E).

PB-II activates the Nrf2/ARE signaling pathway and reduces cellular ROS

In order to explore the mechanism of PB-II to protect DAn from oxidative stress neurons, we examined the ROS levels of hiPSC-derived neuronal culture after various treatments. While the ROS levels were similar between the ODM and BS treatment groups, MS significantly decreased cellular ROS in hiPSC-derived neuronal culture, supporting a role of PB-II in reducing oxidative stress (Figure 4A, 4B). Together, these findings support the notion that PB-II can protect DAn from oxidative stress by reducing cellular ROS levels.

As reported before, the ROS triggers the redox system by activating Nrf2, which induces its downstream genes such as HO-1, NQO1, MRP2, and GPX2. After the treatment with H₂O₂ (100μM, 12h), Nrf2 protein levels and its downstream gene expression was statistically similar to those of the Ctrl group (Figure 4C-F). However, MS treatment significantly increases the expression of Nrf2 protein and its downstream genes such as NQO1 (Figure 4C-F). Therefore, PB-II can activate the Nrf2/ARE signaling pathway to protect DAn from oxidative stress.

PB-II improves the symptoms of PD rats by activating the Nrf2/ARE signaling pathway

To further validate the findings that PB-II can activate the Nrf2/ARE signaling pathway to protect DAn from oxidative stress, we tested the effects of PB-II on PD rat models by injecting 6-OHDA into the substantia nigra striatum of rats to induce the death of midbrain DAn (Model group). The Sham operation group was injected with the same volume of normal saline (Sham group). The PB-II group was given 32g / kg of PB-II by gavage. During the treatment course of 4 weeks, the behavioral symptoms of PD rats were measured weekly. The spinal behavior in PD rats was induced by the subcutaneous injection of APO in the back of the neck, and the number of rotations was recorded within 30 minutes. During the initial stage of treatment (0 weeks), the rats in the Ctrl group and the Sham group had no symptoms of in-situ circles. However, the model group and PB-II group showed serious rotary behavior with more than 210 rotations in 30 minutes, and there was no significant difference between the two groups. During the third week of the treatment, the number of rotations of rats was significantly reduced in the PB-II group (Figure 5E). After 4 weeks of treatment, we euthanized the rats and obtained the tissues of the nigrostriatal region. The number of TH⁺ neurons in the substantia nigra striatum of rats in the PB-II group was significantly higher than those in the Sham group and Model group (Figure 5A). In addition, the levels of Nrf2 protein in midbrain dopamine neurons of rats in the PB-II group were significantly higher than those in the Sham group and Model group (Figure 5B and Figure 5C). The expression of HO-1, NQO1, MRP2, and GPX2 in the midbrain dopaminergic neurons of the PB-II group was also significantly increased, indicating the activation of the Nrf2/ARE signaling pathway by PB-II (Figure 5D). These data confirm that PB-II activates the Nrf2 / ARE signal pathway in the midbrain of PD rats by activating Nrf2 to reduce oxidative stress in DAn, and thus protecting DAn from oxidative stress induced apoptosis.

Discussion

It is commonly believed that oxidative stress can eventually lead to the death of dopaminergic neurons [19-21], and the activation of the endogenous antioxidant system may protect the cells from oxidative damage, which is a research hotspot at present [21]. Multiple studies in various organs have confirmed that the Nrf2- antioxidant response element (ARE) pathway can play a role of endogenous antioxidant to antagonize the oxidative stress injury [22]. In the central nervous system cells, such as dopaminergic neurons, astrocytes, and microglia, Nrf2 maintains the redox balances through the up-regulation of antioxidant gene expression [23]. Previous researches have verified that Nrf2 mostly translocates to the nucleus in the dopaminergic neurons in the substantia nigra of PD patients, while it is present in the cytoplasm in the matched normal control group of the same age [16, 24, 25]. Besides, studies have also demonstrated that overexpression of Nrf2 can reduce the damage of 6-OHDA in dopaminergic neurons [19, 26]. Under physiological condition, Nrf2 protein expression levels were low in cells, mainly in the cytoplasm, where it can interact with Kelch-like ECH associated protein-1 (Keap1) [27, 28]. When the occurrence of oxidative stress, Nrf2 phosphorylation, and Keap1 protein translocation into the uncoupling combine with ARE in the nucleus, regulation on downstream target genes, such as Heme Oxygenase-1 (HO-1) and NAD(P)H quinone dehydrogenase 1 (NQO1), is induced, to enhance the process of detoxification and antioxidant ability of cells [29-31]. *In vitro* experiments have also shown that the up-regulation of HO-1 and NQO1 can protect cells against oxidative damage of glutamic acid, hydrogen peroxide, and amyloid beta-protein [32-34].

Our experiments showed that the PB-II medicated serum could effectively reduce ROS levels in the oxidation model of dopaminergic neurons, protecting dopaminergic neurons from apoptotic death. These results suggested the Nrf2/ARE pathway-mediated antioxidant mechanism might play the role of the PB-II in treating this neurodegenerative disease. When neurotoxic substances, such as H₂O₂, are transported by the dopamine transporter system into neuron cells, and induce oxidative stress and increase DA neuron apoptosis. Compared with the Model group, the Medicated serum group had highly up-regulated nuclear protein Nrf2 and its downstream HO-1, NQO1, MRP2, and GPX2 expression. The results suggest that PB-II plays a protective role in the oxidative stress in neurons through increased nuclear accumulation and phosphorylation of Nrf2, as well as the expression of Nrf2 downstream target genes. PB-II contains 14 kinds of Traditional Chinese Medicine compounds. Although the complex compound composition of these natural Chinese herbal medicine is not very clear, its function has been significantly observed and confirmed by a lot of clinicians in the clinical practice for many years [9, 10, 35, 36]. Furthermore, several researchers have reported its protective effects on midbrain dopaminergic neurons against 6-OHDA toxicity in substantia nigra in rat models [11-13]. PB-II reduces the apoptosis of DA neurons in the PD rats model, promotes cell regeneration, and finally plays a role in improving rats' PD symptoms. This study may provide further evidence in iPSC-derived DA neurons and elaborate reversal of H₂O₂-induced oxidative stress. Furthermore, our experiments provide a useful model for *in vitro* PD studies on complicated Chinese medicine formulas.

Conclusions

PB-II activated Nrf2 signaling pathway in oxidative damaged DA neurons, increasing the expression of antioxidant genes downstream of Nrf2, thereby improving the antioxidant capacity of neurons and reducing the ROS and apoptosis. PB-II might protect DA neurons from functional damage by this mechanism, and thus play a role in the treatment of PD.

Abbreviations

PD: Parkinson's Disease; PB-II: PaBing-II; DAN: Dopaminergic neurons; iPSCs: induced Pluripotent Stem Cells; ROS: Reactive Oxygen Species; Nrf2: Nuclear erythroid-derived Factor 2; 6-OHDA: 6-Hydroxydopamine; SN: Substantia Nigra; LBs: Lewy bodies; LC-MS: Liquid Chromatograph-Mass Spectrometer; Ctrl: the Control group; ODM: Oxidative Damage Model; BS: Blank Serum group; MS: Medicated Serum group; IF: Immunofluorescence; RT-PCR: Reverse Transcription-Polymerase Chain Reaction; TH: Tyrosine Hydroxylase; TUJ1: β 3-Tubulin; HO-1: Heme Oxygenase 1; NQO1: NAD(P)H quinone dehydrogenase 1.

Declarations

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Authors' contributions

XY conceived and designed the work; SHW performed the experiments; TXL analyzed the data and wrote the original draft; XY reviewed and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by the Guangdong Provincial Hospital of TCM Review Board for Ethics. People's cell samples used in our experiments were approved by the board, with all patients providing written informed consent. The animal experiments were approved by the Animal Review Board at Guangdong Provincial Hospital of Chinese Medicine.

Consent for publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

Author details

¹ Center for Regenerative and Translational Medicine, Guangdong Provincial Academy of Chinese Medical Sciences, the Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou 510632, Guangdong, China; ² School of Basic Medical Sciences, Southern Medical University, Guangzhou, Guangdong, 510515, China.

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Tables

Table 1 : The primers of DA neuron-specific genes

Gene	Forward (5' to 3')	Reverse (5' to 3')
Homo-GAPDH	CGGAGTCAACGGATTTGGTC	GACAAGCTTCCCGTTCTCAG
Homo-TUJ1	GCCTCTTCTCACAAGTACGTGCCTCG	GGGCGAAGCCGGGCATGAACAAGTGCA
Homo-TH	GCCCTACCAAGACCAGACGTA	CGTGAGGCATAGCTCCTGAG
Homo-FOXA2	GGGAGCGGTGAAGATGGA	TCATGTTGCTCACGGAGGAGTA
Homo-PITX3	GTGCGGGTGTGGTTCAAGAA	AGCTGCCTTTGCATAGCTCG
Homo-HO-1	AAGACTGCGTTCCTGCTCAAC	AAAGCCCTACAGCAACTGTCC
Homo-NQO1	GAAGAGCACTGATCGTACTGGC	GGATACTGAAAGTTCGCAGGG
Homo-MRP2	AGTGAATGACATCTTCACGTTTG	CTTGCAAAGGAGATCAGCAA
Homo-GPX2	CTGGTGGTCCTTGGCTTC	TG TTCAGGATCTCCTCATTCTG
Rat-GAPDH	CCTCGTCTCATAGACAAGAT	GGGTAGAGTCATACTGGAA
Rat-HO-1	TGCACATCCGTGCAGAGAAT	CTGGGTTCTGCTTGTTTCGC
Rat-NQO1	AGGATGGGAGGTA CT CGAATC	TGCTAGAGATGACTCGGAAGG
Rat-MRP2	GCCCCTCAAGCACTCTGAC	GCTTTGTGTCCCAGATGGACT
Rat-GPX2	GAGCTGCAATGTCGCTTTCC	TGGGTAAGACTAAAGGTGGGC

Figures

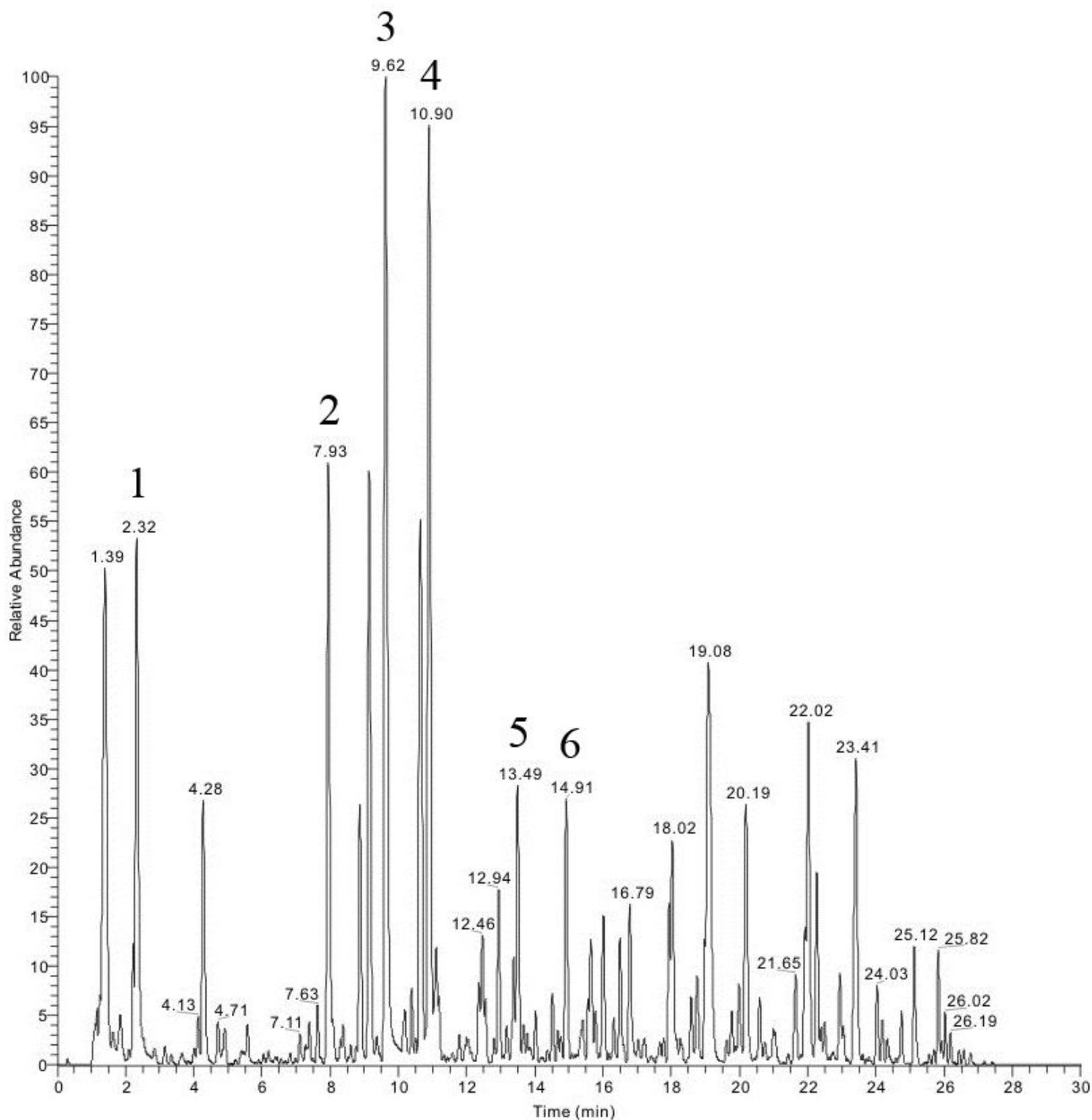


Figure 1

LC-MS spectrum for PB-II. The mass spectrum of PB-II was obtained using the LC-MS in drug-containing serum. 1: Citric acid; 2: Hypaconitine; 3: Stibene glucoside; 4: Liquiritin; 5: Paeoniflorin; 6: Ginsenoside Rg1.

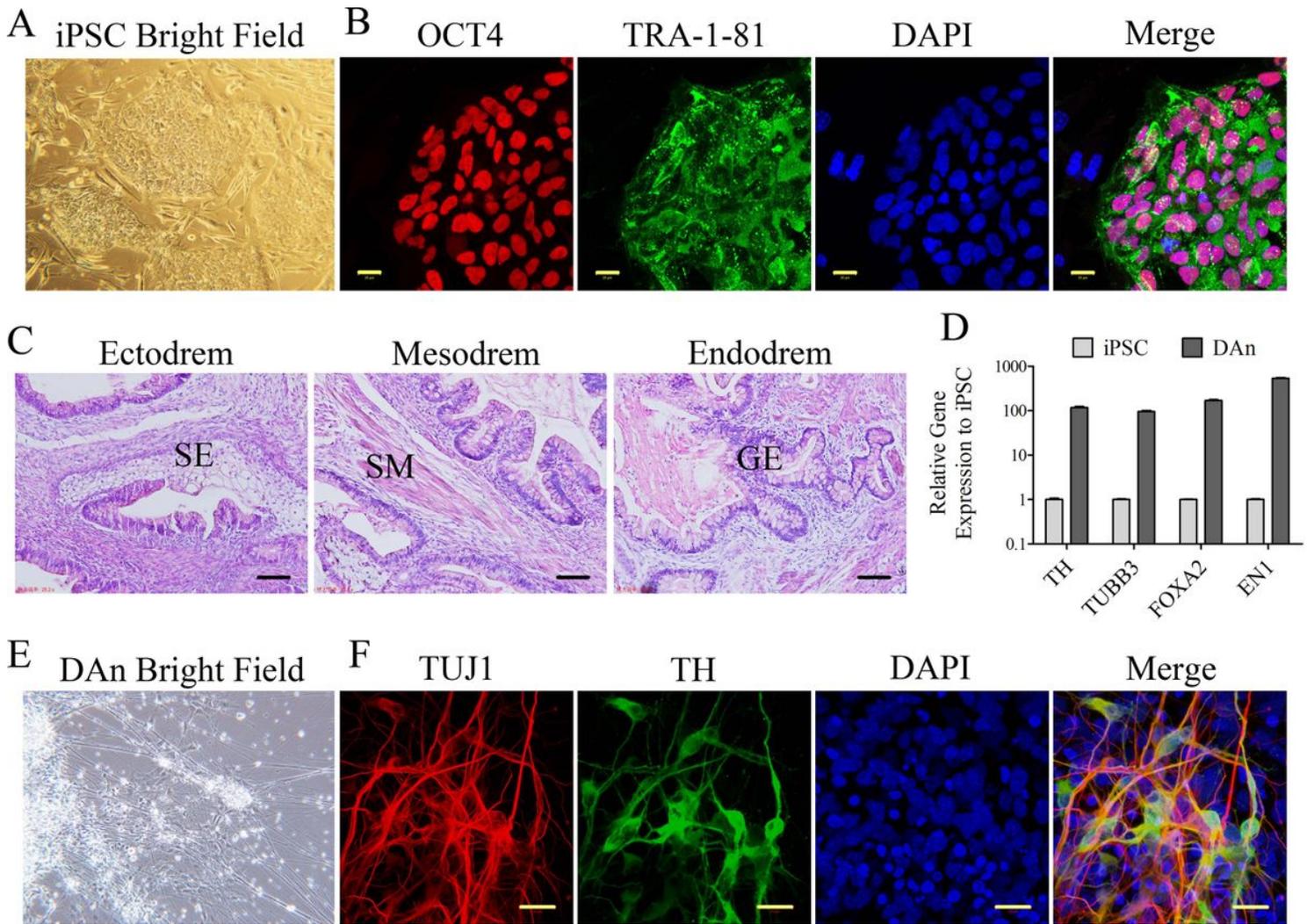


Figure 2

Generation of iPSC and differentiate into the dopaminergic neuron. A-B. Representative colonies of passage-20 iPSC stained positive for the pluripotency-associated markers OCT4 and TRA-1-81 (the scale is 20 μ m). C. Teratomas derived from iPSC have all pluripotency of 3 germ layers. The sections with hematoxylin and eosin staining showed all three germ layers of ectoderm, mesoderm, and endoderm differentiation (the scale is 100 μ m). D. RT-PCR shows that the iPSC-DA neurons expression about 100 times neurons relative genes such TH, TUJ1, FOXA2 and En1 to the iPSC lines. E. Differentiated cells have the morphology of DA neuron. F. Immunofluorescence staining of neuronal cultures derived from iPSC for neuron-specific TUJ1 (red), the DA marker TH (green), and nuclear DAPI (blue) (the scale is 20 μ m).

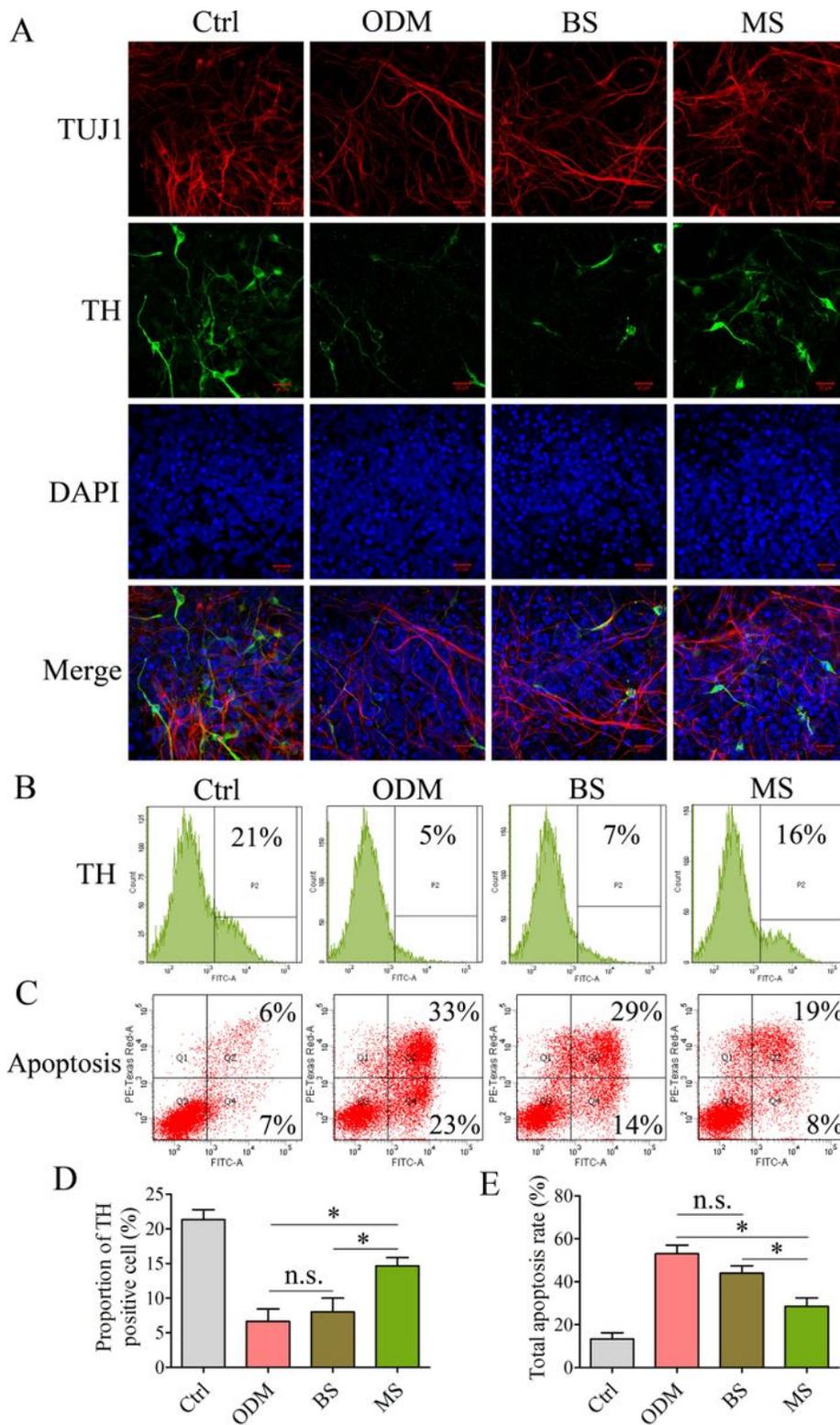


Figure 3

PB-II protects the neuronal cell with activated neuronal expressions. A. Immunofluorescence data showed more TUJ1 and TH neuronal stainings in MS sample than those in the ODM and BS samples (the scale is 20 μ m). B, D. Flow cytometry analysis of the proportion of TH positive cells in each experimental group, compared with the ODM group and the BS group, the MS group had a higher percentage of TH positive cells, with statistically significant differences (n=3, *P \leq 0.05). C, E. Flow cytometry detected the proportion

of apoptosis in each experimental group. Compared with the ODM group and the BS group, the apoptosis in the MS group decreased significantly ($n=3$, $*P\leq 0.05$).

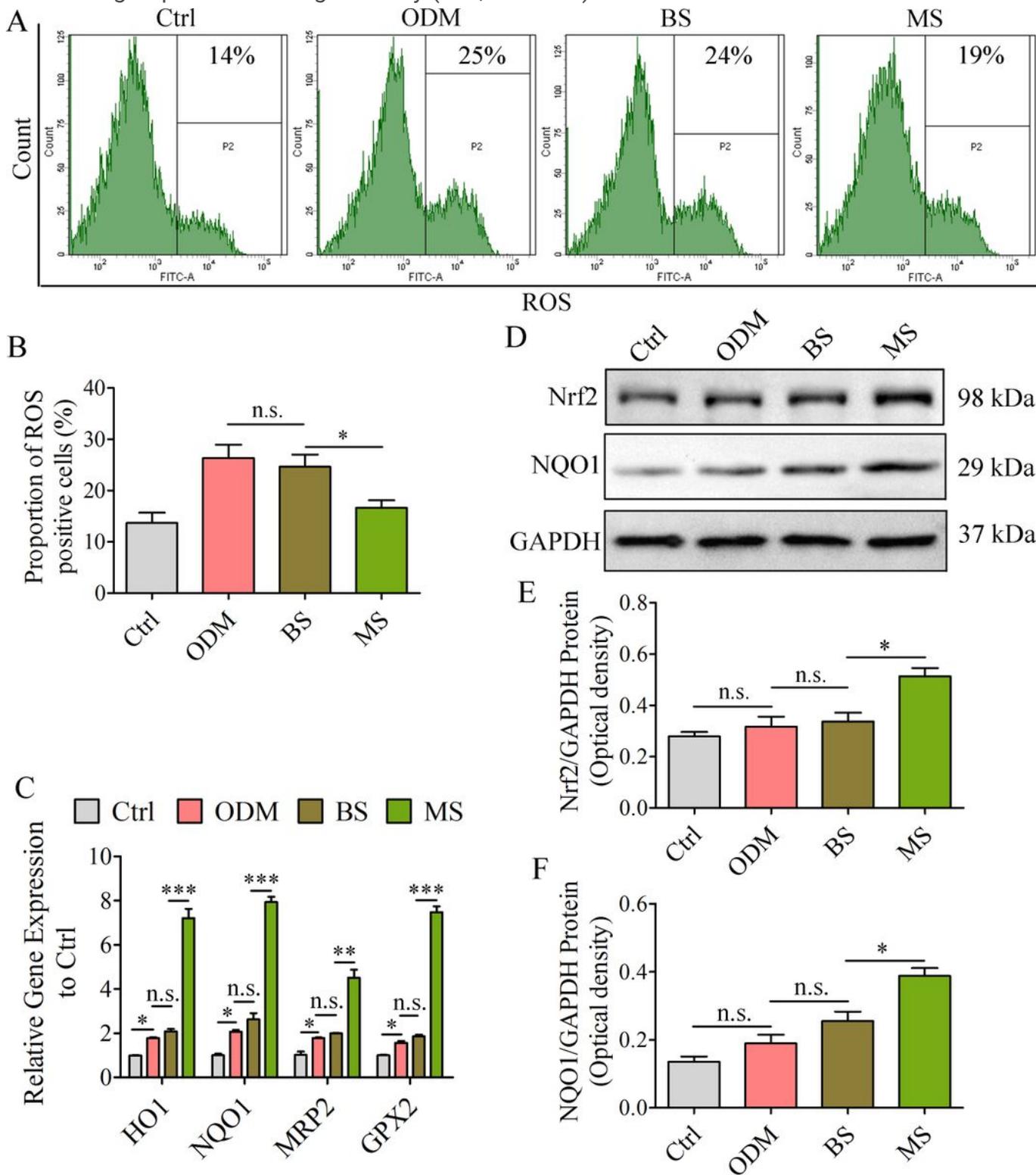


Figure 4

Nrf2 pathway genes are activated in medicated cells and reduce ROS. A, B. The higher ROS level activated under H₂O₂ stress was inhibited by medicated serum. The bar image showed the data and significance ($n=3$, $*P\leq 0.05$). C. The Nrf2 signaling pathway downstream genes, HO-1, NQO1, MRP2, and

GPX2, were found in higher levels in MS samples than those in ODM or BS samples, suggesting they are activated and confirmed the Nrf2 pathway is involved in the protection process. D-F. Because Nrf2 genes are regulated in protein modification ways, we checked their expression by Western blotting, and the data showed that both Nrf2 and NQO1 proteins were present in higher levels in medicated samples. We further analyzed the protein gray value with ImageJ software. The results show that the Nrf2 and NQO1 protein in MS samples are significantly more increased than other samples (n=3, *P≤0.05).

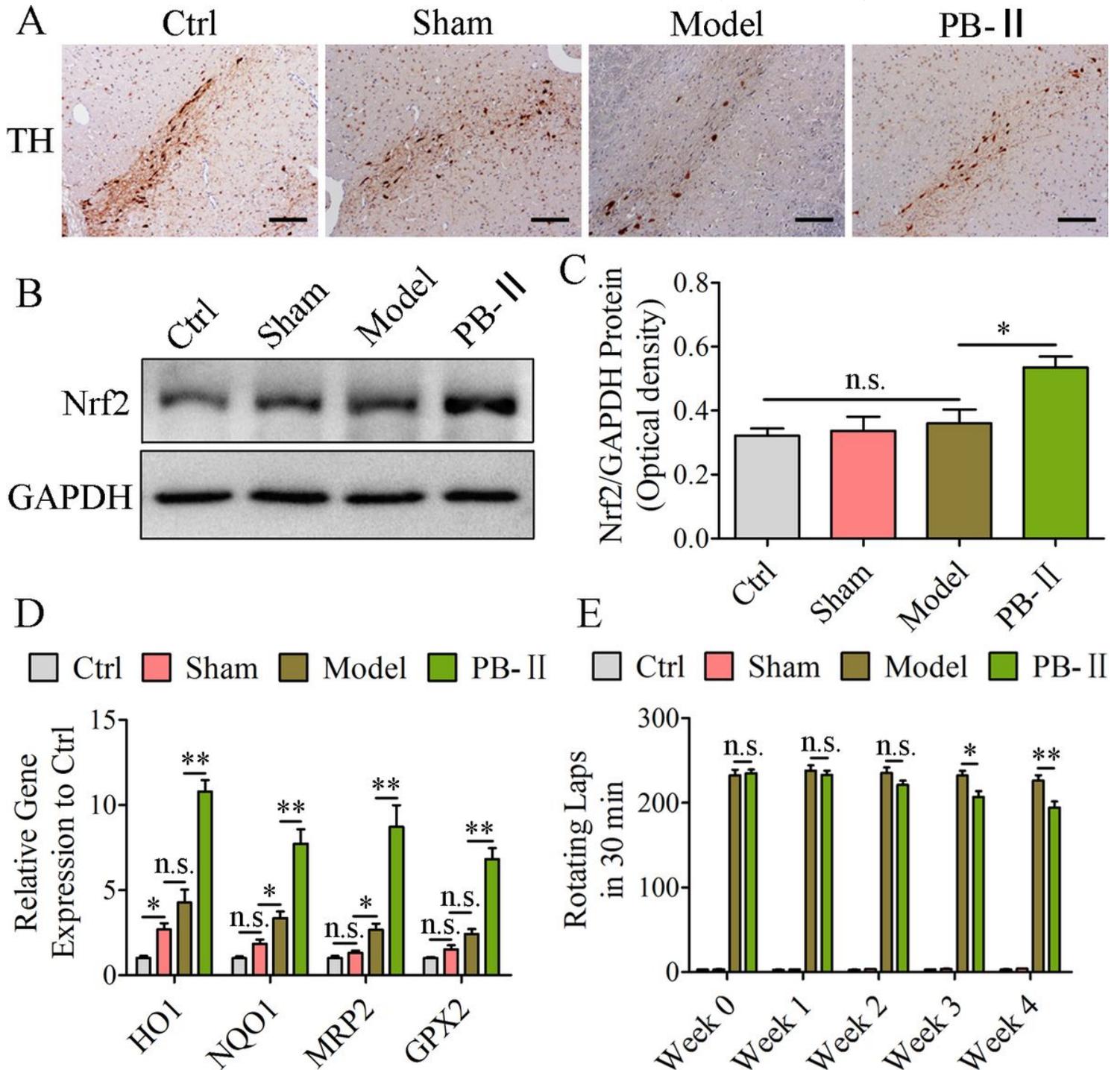


Figure 5

PB-II protects PD rat DA neurons by activating the Nrf2/ARE signaling pathway. A. Immunohistochemical results showed that the TH-positive neurons in the substantia nigra striatum of the PB-II group were significantly increased compared with the Sham and Model groups (the scale is 120 μ m). B, C. Western blotting detection results showed that the expression of Nrf2 protein in cells of the nigrostriatal region of the midbrain in the PB-II group of rats increased significantly (n=3, *P \leq 0.05). D. RT-PCR results showed that the expression of Nrf2 downstream genes in the substantia nigra tissues of rats in the PB-II group was significantly increased, suggesting the activation of the Nrf2 signaling pathway of PB-II on DAN cells under 6-OHDA toxicity (n=3, **P \leq 0.01). E. Calculate the number of rotations of the rats in each experimental group within 30 minutes. From the third week, the number of rotations of the PD-rats in the PB-II group decreased compared with the Model group (n=5, * P \leq 0.05) and reached a very significant difference in the fourth week (n=5, ** P \leq 0.01).