

p-hydroxybenzaldehyde ameliorates Parkinson's disease by regulating the PI3K/Akt/Nrf2 pathway

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Abstract

p-hydroxybenzaldehyde (p-HBA) is a natural phenolic component, which is an active ingredient in traditional Chinese medicines such as *Gastrodia elata* Bl. This study aimed to examine the pharmacodynamic effects of p-HBA and its mechanism in PD. A two-point method was used to establish a rat model of PD via stereotactic injection of 6-hydroxydopamine (6-OHDA) into the brain and BV2 cells were treated with MPP⁺ to induce oxidative stress to clarify the target and pathway of p-HBA. The results indicated that p-HBA improved the behavior of PD rats treated with p-HBA. And p-HBA significantly increased the number of TH- and NeuN-positive neurons while reducing the number of α -synuclein-positive neurons in the substantia nigra and improved neuronal cell morphology. ELISA showed that p-HBA decreased the secretion of NO, an oxidative stress-related factor, and increased the expression of GSH-Px, a peroxidase, in the serum of rats with PD. p-HBA reduced LDH and ROS secretion in MPP⁺-induced oxidative stress damage in BV2 cells and increased SOD and GSH-Px levels. Immunofluorescence and western blotting analysis showed that p-HBA increased the expression of p-PI3K/PI3K, p-AKT/AKT, Nrf2 and HO-1. The above experimental results suggest that, p-HBA may regulate the PI3K/AKT/Nrf2 pathway, alleviating oxidative stress in nerve cells, and preventing the development of PD.

1.Introduction

The occurrence of Parkinson's disease (PD) involves many factors, and commonly occurs in middle-aged and elderly individuals [1]. According to epidemiological studies, the global prevalence of PD is 0.1–0.3%, with that among individuals aged > 65 years being approximately 0.95%. The prevalence of PD increases by 1% each year after 60 years of age and 4% each year after 80 years of age [2]. Studies have shown that the lesion in PD include degeneration of the substantia nigra (SN) and dopaminergic neurons and the formation of Lewy bodies [3]. PD is caused by the relative or absolute insufficiency of dopamine (DA) secretion, a relative increase in acetylcholine levels, and dominant cholinergic nerve function, all of which result in increased muscle tone and other symptoms of PD [4]. The etiology and pathological mechanisms of PD are extremely complex. Degeneration and necrosis of DA neurons lead to insufficiency of DA secretion, which is considered the main pathological mechanism of PD [5]. Currently, the treatment of PD is based on drugs that enhance the function of the central DA nerve or weaken the function of the cholinergic nerve [6]. Commonly used first-line drugs include levodopa, selegiline hydrochloride (SEL), amantadine, and anticholinergics [7]. Nevertheless, long-term use of these drugs leads to bad reactions. For instance, levodopa and amantadine cause side effects such as nausea, vomiting, and loss of appetite and serious adverse reactions such as hallucinations and mental disorders [8]. Although SEL is well tolerated, they may cause gastrointestinal ulcers and angina pectoris [9]. Anticholinergic drugs may cause memory loss, dry mouth, constipation, and other symptoms [10]. Therefore, developing more effective and safer drugs for the prevention and treatment of PD is necessary.

Tall gastrodia tuber (*Gastrodia elata* Bl.), sweet in taste and smooth in texture, is a traditional medication native to Yunnan Province, China. Studies have shown that tall gastrodia tuber can reduce the loss of tyrosine hydroxylase (TH)-positive neurons in pars densa SN, consequently ameliorating PD [11]. In addition, it increases the expression of glial cell-derived neurotrophic growth factor (GDNF) and decreases that of tumor necrosis factor- α (TNF- α) and caspase-3, exerting therapeutic effects against PD [12]. p-hydroxybenzaldehyde (p-HBA), a natural phenolic component of tall gastrodia tuber, has several neuroprotective activities, including anti-inflammatory and antioxidant activities [13, 14]. A study on oxidative stress in mouse brain microvascular endothelial cells (bEnd.3) showed that p-HBA improved the expression of nuclear factor erythroid-2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1), thereby alleviating oxidative stress and nerve damage, inhibiting neuronal apoptosis, and improving neurological function [15]. Studies have shown that Nrf2 serves as a target protein of AKT and that inhibitors of AKT and PI3K can decrease Nrf2 activity, thus regulating the oxidative stress response and promoting the progression of PD [16]. In this study, the pharmacodynamic effects of p-HBA against PD were investigated using rat models of PD *in vivo* and BV2 cells with MPP⁺-induced oxidative damage *in vitro*. In addition, the mechanism through which p-HBA regulates the PI3K/Akt/Nrf2 pathway to alleviate oxidative stress was examined.

2. Materials

2.1 Drugs

p-HBA was purchased from Chengdu Aikeda chemical reagent company (Batch No.: 202005171). Its structural formula is shown in Fig. 1A SEL was purchased from Orion Corporation of Finland (Batch No.: 2018581). It can inhibit the activity of MAO-B, preventing the degradation of DA and enhancing the function of dopaminergic neurons, and can be used as monotherapy for the clinical treatment of PD [17]. SEL was used as the positive control drug in our study.

2.2 Reagents

LDH, SOD, ROD and GSH-Px assay kits (Batch No.: A003-1-2, A001-3-2, A006-2-1 and A005-1-2, respectively) were purchased from Nanjing Jiancheng Bioengineering Co., LTD. An NO assay kit (Batch No.: S0021S) was purchased from Shanghai Biyuntian Biotechnology Co., LTD. Anti-PI3K, anti-p-PI3K, anti-AKT, anti-p-AKT, anti-HO-1 and anti-Nrf2 (Batch No.: R22768, 341468, 342529, 310021, R24541 and R380773, respectively) were bought from Zhengneng Bio-Technology Co., LTD. Anti- β -actin antibodies (Batch No.: AC026) was bought from Cell Signaling Technology Inc.

LY294002 (Batch No.: A133122) was purchased from Beijing AmBeed Biotechnology Co., Ltd. and is a specific inhibitor of PI3K.

2.3 Experimental animals and cells

SPF male SD rats (age, 8 weeks; body weight, 200 $\pm$ 20 g) were bought from Chengdu Dashuo Laboratory Animal Co., LTD. (license number: [Chuan] SCXK 2020-030). The rats were maintained in a controlled

environment at $23 \pm 2^{\circ}\text{C}$ with humidity of $50 \pm 5\%$ and were allowed free access to food and drinking water. Animals were handled in accordance with the Animal Welfare and Management Act. All animal experiments were executed in accordance with the Guidelines for the Care and Use of Laboratory Animals by the National Research Council and were approved by the Ethics Committee of the Yunnan University of Chinese Medicine (R-06201975). The research was conducted in accordance with European community guidelines for laboratory animal use and care.

Mouse microglia (BV2), batch number CX0103, provided by Wuhan Doctor Bioengineering Co., Ltd., China. Cells were cultured in DMEM medium containing 10% fetal bovine serum and 10% double antibody at 37°C in a 5% CO_2 incubator.

3.Methods

3.1 Drug Preparation

(1) Preparation of low-, medium-, and high-dose p-HBA solutions: p-HBA was weighed on a 1/10,000 electronic balance and dissolved in double steamed water. Subsequently, p-HBA solutions were prepared at concentrations of 1, 2, and 4 mg/mL, which were used as low, medium, and high doses, respectively, and were stored at room temperature away from light.

(2) Preparation of the SEL solution: SEL was weighed on a 1/10,000 electronic balance and dissolved in double steamed water. The concentration of SEL was adjusted to $56.25 \mu\text{g/mL}$, and the solution was stored at room temperature away from light.

(3) Preparation of the 6-OHDA solution: 6-OHDA was weighed and dissolved in 0.2% ascorbic acid to obtain $5\text{-}\mu\text{g}/\mu\text{L}$ 6-OHDA. The solution was wrapped with tin foil to avoid exposure to light.

3.2 Establishment of PD model

3.2.1 Anesthesia Induction and Fixation

☒ Healthy SD male rats were selected, no rotation behavior was detected by APO. The rats were anesthetized via intraperitoneal injection of 2% pentobarbital sodium (40 mg/kg).

☒ After narcosis, the head of each rat was shaved with a shaving machine, the tongue was moved to one side of the tooth to prevent bites, and the head was fixed on a stereoscope. The blunt end of the metal rod was inserted through the left and right ear canals, respectively, and the skull was fixed so that the left and right scale values were similar. After the metal rod was fixed, the ears were gently pulled and shaken to confirm the immobility of the head. In addition, the jaw was maintained in a horizontal plane without tilting.

3.2.2 Modeling

☒ The skin and periosteum of the rat head were cut with surgical scissors, and the position of the fontanelle was determined at the intersection of sagittal and coronal sutures. The coordinates of the fontanelle as the origin were zero. The right dense part of SN (SNC) and the ventral tegmental area (VTA) of the midbrain were injected using a two-point method. The location of SNC was determined based on the following coordinates: AP = -5.0 mm; ML= -1.5 mm; DV= -8.2 mm. The location of VTA was determined based on the following coordinates: AP = -5.0 mm; ML= -1.5 mm; DV= -8.2 mm.

☒ A 10- μ L microsampler was washed with 0.2% ascorbic acid 2–3 times. Discharge the bubbles, and inhale 8 μ L of 6-OHDA solution (5 μ g/ μ L).

☒ The position of the injection pump was adjusted, and 4 μ L of 6-OHDA solution was injected into SNC and VTA at the injection rate of 1 μ L/min. The needle was kept in place for 5 minutes after injection and withdrawn slowly.

☒ The wound was cleaned with a cotton ball soaked in iodophor, and the drill hole was sealed with bone wax. After the wound was closed using an Angle needle and surgical suture, the suture and surrounding region were cleaned with a cotton ball soaked in iodophor.

3.2.3 Validation of PD models

After 2 weeks of modeling, 0.5-mg/kg apomorphine was intraperitoneally injected into rats, and the number of rotations within 30 minutes was recorded after 5 minutes of adaptive rotation. Rats with more than 120 rotations were considered successful models of PD [18].

3.3 Grouping and treatment

Rats with PD were divided into the following 5 groups, with 6 rats in each group randomly: model, SEL, low-dose p-HBA, medium-dose p-HBA, and high-dose p-HBA. In addition, 6 healthy male SD rats comprising the control group (Saline containing 0.2% ascorbic acid was injected into the blank group of rats in the same way as in 3.2.) were administered double steamed water intragastrically once a day for 6 weeks (Fig. 1B). Based on pre-determined doses and related studies [19], the low, medium, and high doses of p-HBA were set to 10 mg/kg, 20 mg/kg, and 40 mg/kg, respectively. The doses of treatment in all groups are shown in Table 1.

Table 1
Treatment dose in each group.

Group	Dose
Control	Equal amount of double steamed water
Model	Equal amount of double steamed water
SEL	562.5 µg/kg
Low p-HBA	10 mg/kg
Medium p-HBA	20 mg/kg
High p-HBA	40 mg/kg

3.4 APO-induced rotation test

After 4 and 6 weeks of treatment, 0.5-mg/kg apomorphine was intraperitoneally injected into rats, and the number of rotations within 30 minutes was recorded after 5 minutes of adaptive rotation. The double-blind principle was used to evaluate the number of rotations in rats.

3.5 Morris water maze test

In this study, before establishing the model, the rats were trained adaptively to the water maze environment and familiar with the experimental procedure. For the Morris water maze test, a pool with a diameter of 1.6 m and a height of 0.5 m was used. The pool was grouped into four quadrants. In the target quadrant (third quadrant), a well-configured circular transparent platform was placed, and four easily distinguishable markers were pasted on the black pool wall. The platform was overwhelmed 1 cm below the water surface, and the temperature of the water was maintained at 20°C. During the experiment, light exposure was stable and the movement of rats was recorded using a camera placed above the pool. The results were processed and analyzed using supporting software [20]. The experiment was carried out after 6 weeks of administration.

3.5.1 Memory Training Period (day 1–5)

On day 1, whether the rats located the platform within 60 s was observed, and the required time was recorded. If the rats did not locate the platform, they were led to the platform and permitted to stay for 60 s to observe and learn. Subsequently, the rats were removed from the pool and dried. This training procedure was repeated for the next 4 days.

3.5.2 Space Exploration Experiment (day 6)

The platform was removed, and the rats were placed in the water maze in the order of the first 5 days. Given that the number of times the rats located the platform is proportional to their spatial memory [21], the number of times the rats bridged the location of the platform within 60 s was recorded to evaluate the cognitive function of rats.

3.6 Open field test

The open field test is a commonly used neurobehavioral experimental method for evaluating mental depression based on the spontaneous motor behavior of rats. Depression is the most common non-motor symptom of PD, with a high incidence rate in the early stages of the disease. Long-term depression can exacerbate the motor symptoms of PD [22]. For the open field test, a black box of 100 cm × 100 cm × 40 cm was used and a camera was placed above it to record the movement of rats in a quiet and dark place. The test was performed after 4 and 6 weeks of treatment, and the results were processed and analyzed using supporting software. The number of grids crossed at the level of the open field experiment reflected the level of spontaneous activity of the rats, and the number of grids crossed decreased when the rats became depressed. The resting time reflected the depression level and motor function of rats. The longer the resting time, the higher the degree of depression and the worse the motor function. The standing time reflected the exploratory action and curiosity level of rats in a new environment. The shorter the standing time, the more depressed the animal [23].

3.7 Suspension test

The two forelimbs of the rats were suspended on a horizontal wire (diameter, 3 mm) fixed 60 cm above the ground. The suspension time of only one forelimb was not within the normal time range. The test was performed after 4 and 6 weeks of treatment. The test was performed once every 5 minutes 3 times. The longer the suspension time, the stronger the grip strength, balance, and coordination [24]. The average suspension time of both forelimbs was evaluated to examine the effects of p-HBA on limb movement disorder in rats with PD.

3.8 Tape removal test

The tape removal test was performed after 4 and 6 weeks of treatment. Studies have shown that the time required to tear the tape is inversely proportional to the balance and coordination ability of rats [25]. Two researchers cooperatively conducted the test. One researcher was responsible for fixing the rat, whereas the other pasted a medical tape (0.5 cm × 0.5 cm) on the hairless part of the nose tip of the rat and gently pressed it with fingertips to ensure it was firmly glued. The first group released the tape and placed the rats on a horizontal table, whereas the second group recorded the time required to remove the tape completely. Each rat was tested three times, with an interval of at least 2 min and the longest observation time of 60 s. The average time was calculated to evaluate the effects of p-HBA on the balance, coordination, and perception abilities of rats with PD.

3.9 Effects of p-HBA on serum levels of NO and GSH-Px in rats

Blood was collected from the abdominal aortas of rats and centrifuged at 3500 r/min for 15 min to gain serum. The expression of NO and GSH-Px in rat serum was tested using colorimetry assay kits according to the instructions.

3.10 HE staining

Rat brain tissues were fixed in 4% paraformaldehyde for 24 h, dehydrated, embedded in paraffin, and sectioned. The sections were dewaxed, stained with hematoxylin and eosin successively, dehydrated, and sealed with neutral resin. Finally, pathological changes in rat brain tissues were observed using a pathological section scanner.

3.11 Immunofluorescence analysis for the detection of TH, NeuN, and α -synuclein

Dehydrated rat brain tissues were sliced and incubated with primary antibodies against TH (1:1000), NeuN (1:1000), and α -synuclein (1:1000). Subsequently, the tissue slices were incubated with goat anti-rabbit antibody (1:5000) for 10 min in the dark (FITC) and treated with DAPI for 10 min to stain the nucleus. The slices were sealed, stored in the dark, and photographed. Microscopically, the cytoplasm and nucleus of positive cells appeared green and blue, respectively.

3.12 Effect of p-HBA on PI3K, P-Akt and Nrf2 protein expression detected by immunofluorescence assay

The brain tissues of rats in the control group, model group and p-HBA 40 mg/kg group were taken for immunofluorescence study. The tissue sections of brain with good dehydration were observed and incubated with primary antibody PI3K (1:200), P-Akt (1:100) and Nrf2 (1:200). Following this, goat anti-rabbit secondary antibody (1:400) tagged with red fluorophore was introduced. Cell nuclei were retained using DAPI, followed by sealing the slides. The expressions of PI3K, P-Akt and Nrf2 were then observed using a fluorescence microscope.

3.13 The effect of p-HBA on oxidatively damaged BV2 cells

3.13.1 p-HBA concentration investigation

Mouse microglia (BV2) were purchased from Wuhan Doctor Bioengineering Co., Ltd. The cells were cultured in DMEM medium containing 10% fetal bovine serum and 10% double antibody at 37°C in a 5% CO₂ incubator. When the cell count reaches 80% to 90%, it can be used for subsequent experiments.

Take BV2 cells in the logarithmic growth phase and inoculating them at a density of 1×10^5 cells/mL into a 96-well plate, 100 μ L per well, incubating overnight in an incubator. Cells were divided into a control group and p-HBA dose groups (final concentrations of 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.0625 mg/mL, and 0.03125 mg/mL, prepared using DMEM medium containing 1/1000 DMSO). The p-HBA dose groups were replaced with 100 μ L of the aforementioned drug-containing medium, while the control group was replaced with an equal volume of DMEM medium. The cells were cultured in a incubator for 24 hours, the supernatant was discarded, and each well was added with 10 μ L of CCK-8 solution and 100 μ L of DMEM basal medium. The cells were cultured in a CO₂ incubator for 30

minutes, and the OD value was measured at a wavelength of 450 nm using a microplate reader. Calculate the cell survival rate using the following formula.

cell viability (%) =	$\frac{\text{Sample OD value}-\text{Average OD value of zero hole}}{\text{Control mean}-\text{Average OD value of zero hole}} \times 100\%$
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3.13.2 MPP⁺ concentration investigation

BV2 cells in the logarithmic growth phase were seeded in a 96-well plate at a density of 1×10^5 cells/mL (100 μ L per well) and incubated overnight. Wells were assigned to a blank group and MPP⁺ treatment groups (final concentrations: 1200, 1000, 800, 600, and 400 μ mol/L; prepared in DMEM). The MPP⁺ treatment groups received 100 μ L of MPP⁺-containing medium at the specified final concentrations, while the blank group was given an equal volume of DMEM basal medium. After 24 h of conventional incubation, the supernatant was discarded, and 10 μ L CCK-8 solution plus 100 μ L DMEM basal medium were added to each well. Following 30-min incubation in a 5% CO₂ incubator, OD₄₅₀ values were measured using a microplate reader. Cell survival rate was then calculated.

3.13.3 Establishment of an MPP⁺ in vitro-induced oxidative damage model in BV2 cells

Cells were divided into control group, MPP⁺ group, MPP⁺ + p-HBA groups (0.03125, 0.0625, 0.125 mg/mL), and MPP⁺ + LY294002 (PI3K inhibitor) + p-HBA groups (0.03125, 0.0625, 0.125 mg/mL). After overnight incubation, supernatants were discarded. The MPP⁺ + LY294002 and MPP⁺ + LY294002 + p-HBA groups were treated with LY294002 solution (10 μ mol/L), while other groups received basal medium. Following 1-h incubation, supernatants were discarded. p-HBA treatment groups were administered p-HBA (0.03125, 0.0625, 0.125 mg/mL), whereas control group, MPP⁺ group,, and MPP⁺ + LY294002 groups received basal medium. After 4-h incubation, supernatants were discarded. All groups except control group were exposed to MPP⁺ solution (1000 μ mol/L) for 24 h. At 24 h post-MPP⁺ treatment, measurements were performed: LDH release by microplate assay, intracellular ROS by chemifluorescence, SOD activity by WST-1 method, and GSH-Px activity by colorimetric assay.

3.13.4 Effects of p-HBA on the levels of LDH, SOD, ROS and GSH-Px in culture supernatants

Take BV2 cells in the logarithmic growth phase and seed them at a concentration of 1×10^5 cells/mL in a 6-well plate, with 1 mL per well, and incubate overnight in an incubator. Follow the methods described in section “3.13.3 Establishment of an MPP⁺ in vitro-induced oxidative damage model in BV2 cells” for grouping, modeling, and drug administration. After 24 hours, collect the cell lysate and determine protein content using the BCA assay kit. Simultaneously, follow the instructions of the LDH, ROS, SOD, and GSH-Px assay kits to measure the levels of LDH, ROS, SOD, and GSH-Px in the samples.

3.13.5 Determination of the effects of p-HBA on the protein expression of p-PI3K, PI3K, p-AKT, AKT, Nrf2 and HO-1 in BV2 cell lysates via western blotting

Take BV2 cells in the logarithmic growth phase and inoculate them at 1×10^5 cells/mL into a 6-well plate, 1 mL per well. Cells were divided into control group, MPP⁺ group, MPP⁺ + LY294002 (PI3K inhibitor) group, MPP⁺ + p-HBA (0.0625 mg/mL) dose group, and MPP⁺ + LY294002 + p-HBA (0.0625 mg/mL) dose group. The model was established and drugs were administered according to the method described in section “3.13.3 Establishment of an MPP⁺ in vitro-induced oxidative damage model in BV2 cells”. 24 hours after administration, remove the culture medium, wash once with PBS, add 100 μ L of mixed solution (RIPA lysis buffer: PMSF = 100:1) to lyse the cells and extract the total protein. Concurrently perform constant-voltage electrophoresis (180 V, 40 min) to separate proteins, then transfer to a PVDF membrane (110 V, 70 min). Block with a protein-free rapid blocking solution for 15 min, followed by incubation with primary antibodies against PI3K (1:500), p-PI3K (1:500), AKT (1:500), p-AKT (1:500), Nrf2 (1:500), HO-1 (1:500), and β -actin (1:10,000) at 4°C overnight. The next day, the membrane was incubated with goat anti-rabbit secondary antibody (1:5,000) at room temperature for 1 hour. The expression levels of target proteins were assessed by calculating the ratio of optical density between target protein bands and internal control protein bands.

4. Statistical analysis

The SPSS Statistics (version 26.0) software was used for statistical analysis. The Shapiro–Wilk test was used to check the normality of the data. For normally distributed data, one-way ANOVA was used to compare the data among multiple groups, the LSD method was used to assess the homogeneity of variances, and Dunnett’s T3 method was used to assess the heterogeneity of variances. Non-parametric tests were used to compare non-normally distributed data. A P-value of < 0.05 indicated statistically differences.

5. Results

5.1 p-HBA lowered the number of rotations induced by APO

To investigate whether p-HBA alleviated the damage caused by 6-OHDA, the number of rotations induced by APO in rats with PD was evaluated. Compared with the model group, the number of rotations in SEL group, P-HBA10mg /kg, 20mg/kg and 40mg/kg dose groups had no significant change before administration, and the difference was not significant ($P > 0.05$, Fig. 1C). After 4 weeks of treatment, the number of rotations was significantly decreased in the SEL, low-dose p-HBA, and medium-dose p-HBA groups ($P < 0.05$, Fig. 1D). Although the number of rotations in the high-dose p-HBA group showed a decreasing trend, it was not significantly different from that in the model group ($P > 0.05$, Fig. 1D). After 6 weeks of treatment, the number of rotations was notably decreased in the SEL, low-dose p-HBA,

medium-dose p-HBA, and high-dose p-HBA groups ($P < 0.01$, Fig. 1E). Rats in the control group did not exhibit rotation behavior after injection of APO; therefore, the control group is not labeled in Fig. 1C-Fig. 1E.

5.2 p-HBA increased the number of platform crossings in the Morris water maze test

To investigate whether p-HBA improved the spatial memory of rats with PD, the number of platform crossings was evaluated in the Morris water maze test. Compared with the control group, the model group had a lower number of platform crossings ($P < 0.01$, Fig. 1F). Compared with the model group, the SEL, medium-dose p-HBA, and high-dose p-HBA groups had a significantly higher number of platform crossings ($P < 0.05$, Fig. 1F).

5.3 Open field test

5.3.1 Number of horizontal grid crossings

After 4 weeks of treatment, the number of horizontal grid crossings was significantly lower in the model group than in the control group ($P < 0.01$, Fig. 1G). Compared with the model group, the SEL, medium-dose p-HBA, and high-dose p-HBA groups had a notably higher number of horizontal grid crossings ($P < 0.05$ or $P < 0.01$, Fig. 1G). After 6 weeks of treatment, the number of horizontal grid crossings was significantly lower in the model group than in the control group ($P < 0.01$, Fig. 1H). Compared with the model group, the SEL, medium-dose p-HBA, and high-dose p-HBA groups had a significantly higher number of horizontal grid crossings ($P < 0.05$ or $P < 0.01$, Fig. 1H). The experimental results suggest that p-HBA can increase the autonomous exploratory behavior of PD rats.

5.3.2 Resting time

After 4 weeks of treatment, the resting time was significantly longer in the model group than in the control group ($P < 0.01$, Fig. 1I). Compared with the model group, the high-dose p-HBA group had a notably shorter resting time ($P < 0.05$, Fig. 1I). Although the resting time of the SEL, low-dose p-HBA, and medium-dose p-HBA groups showed a decreasing trend, it was not significantly different from that of the model group ($P > 0.05$, Fig. 1I). After 6 weeks of treatment, the resting time was significantly longer in the model group than in the control group ($P < 0.01$, Fig. 1J). Compared with the model group, the SEL, medium-dose p-HBA, and high-dose p-HBA groups had a significantly shorter resting time ($P < 0.05$, Fig. 1J). Although the resting time of the low-dose p-HBA group showed a decreasing trend, it was not significantly different from that of the model group ($P > 0.05$, Fig. 1J). These results collectively indicated that p-HBA enhanced the autonomous exploratory activity of rats with PD.

5.3.3 Standing time

After 4 weeks of treatment, the standing time was significantly shorter in the model group than in the control group ($P < 0.01$, Fig. 1K). Compared with the model group, the SEL, medium-dose p-HBA, and high-dose p-HBA groups had a significantly longer standing time ($P < 0.05$, Fig. 1K). After 6 weeks of

treatment, the standing time was significantly lower in the model group than in the control group ($P < 0.01$, Fig. 1L). Compared with the model group, the SEL, low-dose p-HBA, medium-dose p-HBA, and high-dose p-HBA groups had a significantly longer standing time ($P < 0.05$, Fig. 1L). Altogether, these results indicated that p-HBA increased the autonomous exploratory activity of rats with PD.

5.4 Suspension test

The suspension test was performed to evaluate the grip strength, balance ability, and coordination ability of rats with PD. After 4 weeks of treatment, the suspension time was significantly shorter in the model group than in the control group ($P < 0.05$, Fig. 1M). Compared with the model group, the SEL, low-dose p-HBA, medium-dose p-HBA, and high-dose p-HBA groups had a significantly longer suspension time ($P < 0.05$, Fig. 1M). After 6 weeks of treatment, the suspension time was significantly shorter in the model group than in the control group ($P < 0.05$, Fig. 1N). Compared with the model group, the SEL, medium-dose p-HBA, and high-dose p-HBA groups had a significantly longer suspension time ($P < 0.05$ or $P < 0.01$, Fig. 1N). Although the suspension time of the low-dose p-HBA group showed an increasing trend, it was not significantly different from that of the model group ($P > 0.05$, Fig. 1N).

5.5 Tape removal test

The tape removal test was performed to examine the effects of p-HBA on the balance and coordination abilities of rats with PD. After 4 and 6 weeks of treatment, the time required to remove an adhesive tape from the nose tip was significantly longer in the model group than in the control group ($P < 0.05$ or $P < 0.01$; Fig. 1O, 1P). Compared with the model group, the SEL, low-dose p-HBA, medium-dose p-HBA, and high-dose p-HBA groups required significantly less time to remove the tape ($P < 0.05$ or $P < 0.01$; Fig. 1O, 1P).

5.6 p-HBA improved the serum levels of NO and GSH-Px in rats with PD

Compared with the control group, the model group had significantly higher serum levels of NO and significantly lower serum levels of GSH-Px ($P < 0.05$, Fig. 1Q, 1R). Compared with the model group, the SEL, low-dose p-HBA, medium-dose p-HBA, and high-dose p-HBA groups had significantly lower serum levels of NO, whereas the SEL and low-dose p-HBA groups had significantly higher serum levels of GSH-Px ($P < 0.05$ or $P < 0.01$, Fig. 1Q, 1R). The serum levels of GSH-Px in the medium-dose and high-dose p-HBA groups showed an increasing trend ($P > 0.05$, Fig. 1R).

5.7 p-HBA had protective effects on neurons in the substantia nigra

HE staining was performed to assess the morphological characteristics and quantity of neurons in the SN. Normal neurons in the SN of the control group were purple-red with an irregular shape, larger cell body, and higher abundance. Compared with the control group, the model group had a lower number of neurons in the SN and more cell shrinkage. Compared with the model group, the SEL, low-dose p-HBA,

medium-dose p-HBA, and high-dose p-HBA groups showed an increase in the number of neurons and cell size (Fig. 2A).

5.8 p-HBA improved the expression of TH, NeuN, and α -synuclein in rats with PD

Under UV excitation, the nucleus appeared blue, whereas TH, NeuN, and α -synuclein appeared green. Compared with the control group, the model group had significantly lower expression of TH and NeuN but significantly higher expression of α -synuclein in the SN ($P < 0.05$ or $P < 0.01$; Fig. 2B). Compared with the model group, the SEL, low-dose p-HBA, medium-dose p-HBA, and high-dose p-HBA groups had significantly higher expression of TH but significantly lower expression of α -synuclein in the TN ($P < 0.05$ or $P < 0.01$; Fig. 2B). In addition, the expression of NeuN in the SN of rats was significantly higher in the high-dose p-HBA group than in the model group ($P < 0.05$, Fig. 2B).

5.9 p-HBA increased the protein expression of PI3K, Nrf2, and p-Akt

The results showed that the protein expression of P-AKT was significantly lower and that of P13K and Nrf2 was significantly higher in the model group than in the control group ($P < 0.05$; Fig. 3). The significant increase in the expression of P13K and Nrf2 may explain the activation of oxidative stress response in the model group. Compared with the model group, the p-HBA 40mg/kg group had significantly higher protein expression of Nrf2 and p-AKT ($P < 0.05$; $P < 0.01$, Fig. 3), PI3K protein expression showed an upward trend ($P > 0.05$, Fig. 3). These results suggested that the antioxidative effects of p-HBA are associated with the regulation of the PI3K/AKT/Nrf2 pathway.

5.10 p-HBA had protective effects on BV2 cells with oxidative damage

5.10.1 p-HBA concentration examination

Based on preliminary experiments, We hypothesized that p-HBA exerts protective effects against PD by alleviating oxidative stress-induced damage in nerve cells by regulating the PI3K/AKT pathway. To verify this hypothesis, MPP⁺ was used to induce oxidative damage in BV2 cells and the targets of p-HBA were identified. The p-HBA concentration examination showed that, compared with the blank group, the cell survival rate decreased significantly ($P < 0.05$) when the p-HBA dosage was 0.25 mg/ml, indicating that when the p-HBA concentration exceeded 0.25 mg/ml, it produced drug toxicity and severely damaged the growth of BV2 cells (Fig. 4A). Therefore, the dosing concentrations of p-HBA were selected as 0.03125 mg/ml, 0.0625 mg/ml, and 0.125 mg/ml for the small, medium, and large doses, respectively.

5.10.2 MPP⁺ concentration examination

The experimental results showed that compared with the blank group, the survival rate of BV2 cells decreased significantly at MPP⁺ doses of 600 µmol/L, 800 µmol/L, 1000 µmol/L, and 1200 µmol/L ($P < 0.05$; $P < 0.01$, Fig. 4B). The cell survival rate decreases with increasing dose. When the modeling dose is 1000 µmol/L, the cell survival rate is approximately 50%. Therefore, 1000 µmol/L was selected as the modeling dose of MPP⁺ for subsequent experiments.

5.10.3 p-HBA reduced LDH and ROS levels of oxidative damage in BV2 cells; increased SOD and GSH-Px levels

To verify the protective effects of p-HBA against oxidative damage, the levels of the oxidative stress-related factors LDH, SOD, ROS and GSH-Px in the culture supernatant of BV2 cells were evaluated. The results showed that, compared with the blank group, the MPP⁺ group had significantly higher LDH and ROS levels and significantly lower GSH-Px and SOD levels ($P < 0.01$; Fig. 4C, D, E, F); Compared with the MPP⁺ group, the LDH levels in the MPP⁺+p-HBA (0.03125 mg/mL) dose group showed a decreasing trend ($P > 0.05$) (Fig. 4C), while the LDH levels in the MPP⁺+p-HBA (0.0625 mg/mL, 0.125 mg/mL) dose groups were significantly reduced ($P < 0.05$) (Fig. 4C); The ROS levels were significantly reduced in the MPP⁺+p-HBA dose groups (0.03125 mg/mL, 0.0625 mg/mL, 0.125 mg/mL), while the levels of GSH-Px and SOD were significantly increased ($P < 0.05$; $P < 0.01$, Fig. 4D, E, F). Additionally, we observed that following administration of the inhibitor LY294002, compared to the MPP⁺+p-HBA (0.03125 mg/mL, 0.0625 mg/mL, 0.125 mg/mL) dose groups, the MPP⁺+LY294002 + p-HBA (0.03125 mg/mL, 0.0625 mg/mL, 0.125 mg/mL) showed a decreasing trend in LDH levels ($P > 0.05$), a significant increase in ROS levels, and a significant decrease in GSH-Px and SOD levels ($P < 0.05$). These results suggest that p-HBA protects BV2 neurons, regulates the expression of oxidative stress factors, and has neuroprotective effects. Since LY294002 is a specific inhibitor of PI3K, the antioxidant stress-protective effect of p-HBA was antagonized by the addition of LY294002. Therefore, we speculate that p-HBA may mediate the release of the aforementioned oxidative stress factors by activating the PI3K signaling pathway. Consequently, we conducted the following experiments to validate this hypothesis.

5.10.4 p-HBA increased the protein expression of p-PI3K/PI3K, p-AKT/AKT, Nrf2 and HO-1 in BV2 cells with oxidative damage

Western blotting was performed to investigate the effects of p-HBA on the protein expression of PI3K, p-PI3K, p-AKT, AKT, Nrf2 and HO-1 in BV2 cells with oxidative damage. The effects of p-HBA on the expression of PI3K, p-PI3K, AKT, p-AKT, Nrf2, and HO-1 proteins in oxidatively damaged BV2 cells were detected using Western blotting. The results show that, compared with the control group, the MPP⁺ dose group exhibited significantly downregulated expression of p-PI3K/PI3K, p-AKT/AKT, Nrf2, and HO-1 ($P < 0.05$; $P < 0.01$, Fig. 5A-E), confirming that MPP⁺ induces neurotoxicity via suppression of the PI3K/AKT/Nrf2 pathway. Relative to the MPP⁺ group, the MPP⁺ + p-HBA (0.0625 mg/mL) dose group showed significantly upregulated expression of p-PI3K, p-AKT, Nrf2, and HO-1 ($P < 0.05$; $P < 0.01$, Fig. 5A-E). However, compared with the MPP⁺ + p-HBA (0.0625 mg/mL) group, the MPP⁺ + LY294002 + p-HBA

(0.0625 mg/mL) dose group demonstrated marked downregulation of these proteins ($P < 0.05$; $P < 0.01$, Fig. 5A-E), indicating that LY294002 abolishes p-HBA-mediated activation of the PI3K/AKT/Nrf2 pathway. These findings indicate that p-HBA anti-PD is associated with the regulation of the PI3K/Akt/Nrf2 signaling pathway. After adding the inhibitor LY294002, the PI3K/Akt phosphorylation activation induced by p-HBA was eliminated, and the downstream Nrf2 nuclear translocation and HO-1 expression were more significantly inhibited, ultimately reversing the protective effect of p-HBA.

6. Discussion

PD is a degenerative disease of the nervous system. Its pathogenesis is due to the degeneration and death of dopaminergic neurons in substantia nigra, which may be related to many factors such as heredity, environmental factors and aging of nervous system [26]. In our study, the effects of p-HBA against PD and its mechanism in regulating the PI3K/Akt/Nrf2 pathway were investigated using a rat model of PD *in vivo* and a BV2 cell model of oxidative damage *in vitro*.

Dopaminergic neurons are primarily located in the dense belt of SN in the midbrain. After successful modeling with 6-OHDA, the decrease in DA levels on the injured side leads to a compensatory reaction. Subsequently, the D2 receptor on the injured side is upregulated, resulting in hypersensitivity [27]. When DA receptor agonists, such as APO, are used, the D2 receptor leads to stronger DA activity than that on the healthy side. This increased activity manifests as rotation behavior toward the healthy side, with the number of rotations being proportional to the degree of damage caused to dopaminergic neurons [28]. In this study, after 6 weeks of treatment, the number of rotations was significantly lower in the three p-HBA groups, indicating that p-HBA had a certain protective effect on dopaminergic neurons.

In the central nervous system, the hippocampus and striatum are associated with spatial memory and programmed memory, respectively. DA in the SN reaches the hippocampus and striatum through specific pathways and participates in motor regulation of the basal ganglia. Therefore, insufficient secretion of DA can impair memory function in the hippocampus and striatum [29]. The Morris water maze test is used to assess spatial learning and memory in animals [30]. On day 6 of the exploration experiment, the number of times the rats located the platform was higher in the three p-HBA groups than in the model group. This improvement in cognitive function may be attributed to the protective effects of p-HBA on dopaminergic neurons in the SN [31].

The open field test is commonly used to assess the state of depression in animals based on their autonomous motor behavior [32]. Depression peaks in the early stages of PD, and its long-term persistence can exacerbate the motor symptoms of PD [33]. In the open field test, a reduction in movement and standing time indicates that depression can lead to a decrease in the autonomous spatial exploration activity of animals [34]. Consistently, this study revealed similar results. In addition, p-HBA significantly increased the number of grid crossings and standing time and decreased the resting time in rats with PD, suggesting that p-HBA can alleviate depression in rats with PD and increase their spatial exploration activity.

Suspension and tape removal tests are used to evaluate the grip strength and balance and coordination abilities of animals [35]. In this study, the suspension time of rats in the p-HBA groups was significantly higher than that of rats in the model group after 4 and 6 weeks of treatment. In addition, the time required to remove an adhesive tape from the nose tip was significantly lower in the three p-HBA groups than in the model group. These findings suggest that p-HBA can effectively improve the grip strength and balance and coordination abilities of rats with PD.

6-OHDA rapidly generates a large amount of ROS after entering neurons in the SN, leading to oxidative stress and decreasing the number of neurons [36]. In this study, pathological analysis showed that p-HBA can increase the number of neurons in the SN and improve their morphological characteristics, indicating that it has protective effects on neurons in the SN.

TH is a rate-limiting enzyme synthesized by DA. The number of dopaminergic neurons can be evaluated by calculating the number of TH-positive cells in the SNc [37]. In addition, the expression of NeuN can directly reflect the maturity status of neurons [38]. α -synuclein, the main component of Lewy bodies, is involved in many aspects of nerve plasticity, learning and memory, and regulation of DA uptake [39]. α -synuclein is readily accumulated in damaged neurons. Its excessive accumulation and folding can directly activate microglia and cause oxidative stress, which is a key factor in the pathogenesis of PD [40]. In this study, the number of TH- and NeuN-positive cells was significantly higher and that of α -synuclein-positive cells was significantly lower in the SN of rats in the drug-treated groups, suggesting that p-HBA can alleviate neuronal damage in the SN.

As an important free radical-scavenging enzyme in the body, GSH-Px can suppress the formation of lipid peroxides and enhance the defense against oxidative damage [41]. NO is an extremely unstable free radical with strong oxidation property. It is a small molecule with a simple structure that rapidly diffuses through biofilms. Excess NO can cause oxidative damage to cells [42]. In this study, p-HBA was found to decrease the expression of NO and increase the expression of GSH-Px in the serum of rats with 6-OHDA-induced PD. These findings suggest that the therapeutic mechanism of action of p-HBA in PD is related to its protective effects against oxidative stress.

Existing studies have shown that rats with 6-OHDA-induced PD have a significantly higher number of abnormally activated microglia in the substantia nigra densa, suggesting that microglia-mediated oxidative stress and inflammation play a key role in the progression of PD [43]. Reactive oxygen species (ROS) are the end products of cell respiration, protein folding, and various metabolic activities. ROS are well-known inflammatory signaling molecules and inflammatory mediators. Long-term or chronic exposure to ROS, including OH^- , ROO^- and NO, is considered a key factor in the progression of inflammatory diseases. MPP^+ acts as a specific inhibitor of mitochondrial complex I, blocking the electron transport chain and inducing explosive accumulation of superoxide anions (O_2^-), which are then converted into highly toxic OH^- through enzymatic reactions, causing protein/lipid oxidative damage and activating pro-inflammatory transcription factors such as NF- κ B[44]. Cell death caused by the direct

application of MPP⁺ is similar to oxidative damage caused by 6-OHDA. Therefore, BV2 cells were treated with MPP⁺ to establish an *in vitro* model of oxidative damage.

SOD is an antioxidant metal enzyme widely distributed in microorganisms, plants, and animals, and is broadly classified into three types. Among these, Cu/Zn-SOD is primarily found in the cytoplasm of eukaryotic cells, where it plays a role in regulating the balance between cellular oxidation and antioxidant processes. It catalyzes the dismutation of superoxide anion radicals into O₂ and H₂O₂, making it a key component in antioxidant mechanisms[45]. LDH is a stable cytoplasmic enzyme that exists in all cells. It participates in intracellular redox reactions by converting lactic acid to pyruvate. When the permeability of the cell membrane is altered, a large amount of LDH is released, leading to cell damage [46]. GSH-Px as a key antioxidant enzyme that removes lipid peroxides, has reduced activity, which can cause SOD to catalyze O₂⁻ into H₂O₂, H₂O₂ accumulates and converts into more toxic -OH free radicals, triggering ROS bursts and lipid peroxidation damage[47]. In this study, in MPP⁺-induced oxidative stress in BV2 cells, LDH and ROS levels were significantly reduced, while SOD and GSH-Px levels were significantly increased, indicating that p-HBA can alleviate cellular damage by reducing oxidative stress.

LY294002 is a highly specific PI3K inhibitor that acts by competitively binding to the ATP-binding site of PI3K, inhibiting its phosphorylation activity, and thereby blocking the transmission of downstream signals[48]. In this study, the complete blockade of the protective effect of p-HBA by LY294002 strongly confirms the central role of the PI3K/Akt/Nrf2 pathway in the mechanism of action of p-HBA. Notably, in the LY294002-treated group (MPP⁺+LY294002), although there was a downward trend in pathway protein expression compared to the MPP⁺ group, the difference was not statistically significant ($P > 0.05$). This may be because MPP⁺ had already strongly inhibited pathway activity, rendering the additional inhibitory effect of LY294002 insufficiently evident statistically.

The PI3K/Akt signaling pathway serves as an important pathway for improving cognitive function in the brain [49]. Phosphoinositide 3-kinase (PI3K) requires interaction with receptors or linkers to be activated. Owing to its different structure and substrate specificity, type I PI3K, which can be activated by cell surface receptors, is the most widely investigated kinase. AKT is a serine/threonine protein kinase that requires the activation of two sites, Thr308 and Ser473, for complete activation. It is involved in regulating cell survival, growth, proliferation, metabolism, and migration [50]. Nrf2 is an important protein involved in antioxidant defense. It contains 6 Neh functional domains; among which, Neh2 is the binding site of Keap1 and Neh3 is closely associated with the activation of ARE. Nrf2 can be targeted to regulate inflammation and oxidative stress responses, delaying the progression of PD [51]. The activity of Nrf2 is regulated by the PI3K/Akt pathway, which further phosphorylates Nrf2 by regulating the activity of Nrf2-ARE, enhancing the activity of Nrf2 and the endogenous antioxidant capacity of cells. The results of this study suggest that the antioxidant defense mechanism of p-HBA is related to the PI3K/Akt/Nrf2 signaling pathway. The increased expression of P13K and Nrf2 in model group may be attributed to the activation of oxidative stress response in model group. A study showed that the expression of Nrf2 and its downstream protein HO-1 was higher in mouse midbrain dopaminergic neurons (MN9D) with 6-

OHDA-induced damage than in control cells. However, treatment with icariin increased the expression of both factors in MN9D cells with 6-OHDA induced damage [52]. Another study showed that the expression of PI3K was higher in differentiated rat adrenal pheochromocytoma cells (PC12) with 6-OHDA-induced injury than in control cells, whereas treatment with asiaticoside increased the expression of PI3K in PC12 cells with injury [53]. These findings are consistent with those of the present study.

In conclusion, this study showed that p-HBA exerted protective effects against PD both *in vivo* and *in vitro*. Mechanistically, p-HBA regulates PI3K, AKt, and Nrf2 proteins and alleviates inflammation and oxidative stress, consequently preventing the progression of PD. These findings offer a valuable reference for the use of p-HBA in the clinical treatment of PD and offer novel insights into the development of new drugs for PD.

Abbreviations

DA
dopamine
GDNF
glial cell-derived neurotrophic growth factor
MPP⁺
MPP⁺ IODIDE
HO-1
heme oxygenase-1
Nrf2
nuclear factor erythroid-2-related factor 2
6-OHDA
6-hydroxydopamine
PD
Parkinson's disease
p-HBA
p-hydroxybenzaldehyde
PI3K
Phosphoinositide 3-kinase
SOD
superoxide dismutase
LDH
lactatedehydrogenase
ROS
Reactive oxygen species
GSH-Px
glutathione peroxidase

SEL
selegiline hydrochloride
SN
substantia nigra
TH
tyrosine hydroxylase
TNF- α
tumor necrosis factor-alpha
VTA
ventral tegmental area.

Declarations

Ethics approval and consent to participate

All animal experiments were executed in accordance with the Guidelines for the Care and Use of Laboratory Animals by the National Research Council and were approved by the Ethics Committee of the Yunnan University of Chinese Medicine (R-06201975). The research was conducted in accordance with European community guidelines for laboratory animal use and care.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contribution

Peixin Guo, Yuhuan Xie, Qinghua Chen and Xue Wu designed the experiments. Xue Wu, Linao Zhang, Qing Li, Shifang Luo, Feifan Liu, Zifang Chen, Wentao Chen, Jiying Wang, Yan Wan, Yuanmei Bai and Rong Chen conducted the experiments, while Xue Wu, Linao Zhang, Qing Li and Peixin Guo analyzed the data and discussed the results. Xue Wu, Linao Zhang and Qing Li wrote the manuscript.

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Data Availability

Data will be made available on request.

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Figures

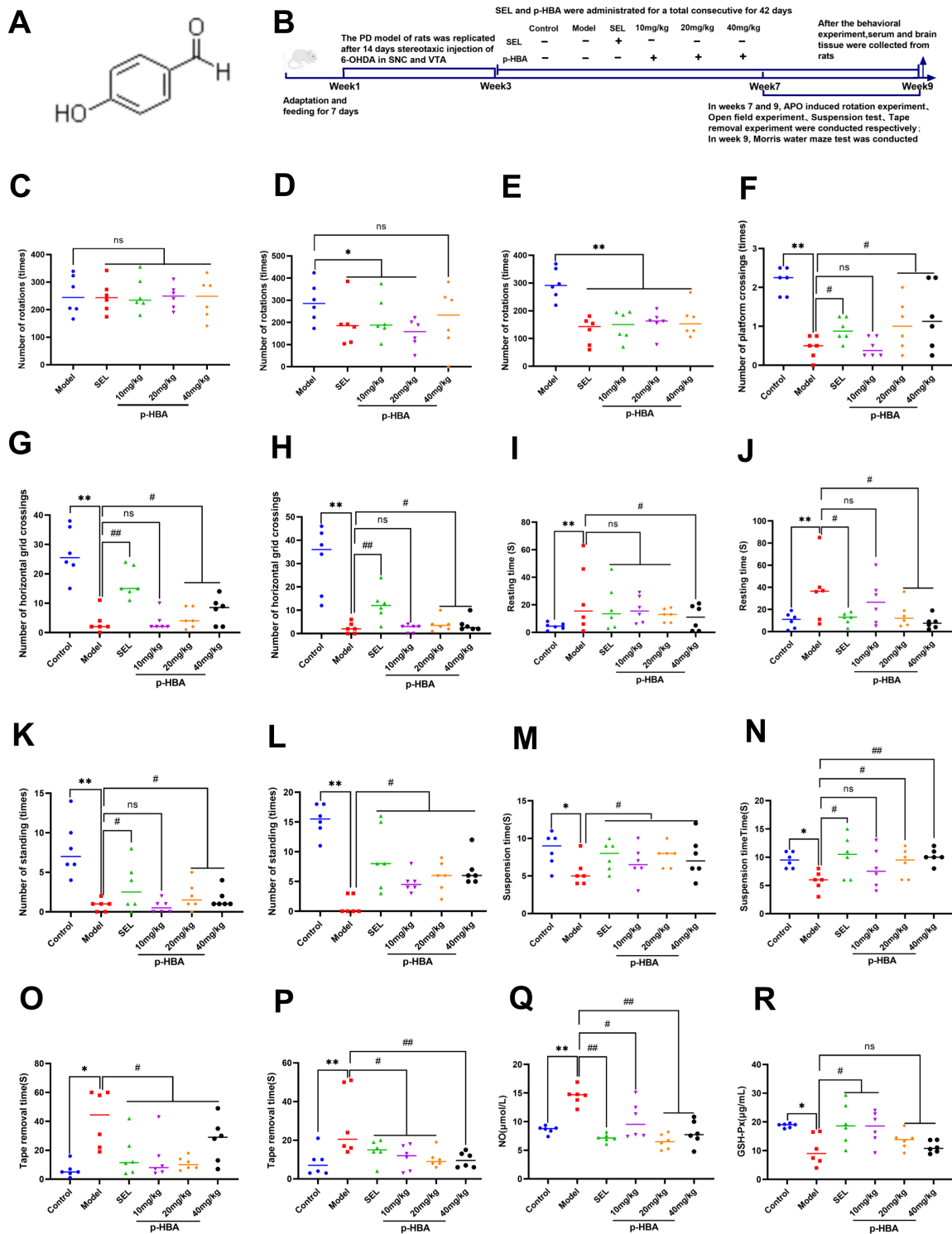


Figure 1

. (A)Structural formula of p-HBA. (B)Establishment of rat models of PD and their treatment timeline. (C)p-HBA reduced the number of rotations induced by APO in rats (n = 6). Number of rotations before treatment. (D)p-HBA reduced the number of rotations induced by APO in rats (n = 6). Number of rotations after 4 weeks of treatment. (E)p-HBA reduced the number of rotations induced by APO in rats (n = 6). Number of rotations after 6 weeks of treatment. (F)p-HBA increased the number of platform

crossings in rats with PD (n = 6). (G)p-HBA increased the number of horizontal grid crossings in rats with PD (n = 6). Number of horizontal grid crossings after 4 weeks of treatment. (H)p-HBA increased the number of horizontal grid crossings in rats with PD (n = 6). Number of horizontal grid crossings after 6 weeks of treatment. (I)p-HBA decreased the resting time of rats with PD (n = 6). Resting time of rats after 4 weeks of treatment. (J)p-HBA decreased the resting time of rats with PD (n = 6). Resting time of rats after 6 weeks of treatment. (K)p-HBA increased the standing time in rats with PD (n = 6). Standing time of rats after 4 weeks of treatment. (L)p-HBA increased the standing time in rats with PD (n = 6). Standing time of rats after 6 weeks of treatment. (M)p-HBA increased the suspension time of rats with PD (n = 6). Suspension time of rats after 4 weeks of treatment. (N) p-HBA increased the suspension time of rats with PD (n = 6). Suspension time of rats after 6 weeks of treatment. (O)p-HBA reduced the time required to remove an adhesive tape from the nose tip in rats with PD (n = 6). Time required to remove the tape after 4 weeks of treatment. (P)p-HBA reduced the time required to remove an adhesive tape from the nose tip in rats with PD (n = 6). Time required to remove the tape after 6 weeks of treatment. (Q) p-HBA improved the levels of NO and GSH-Px in the serum of rats with PD induced by 6-OHDA (n = 6). NO levels. (R)p-HBA improved the levels of NO and GSH-Px in the serum of rats with PD induced by 6-OHDA (n = 6). GSH-Px levels. (* P < 0.05; ** P < 0.01 versus the control group; # P < 0.05; ## P < 0.01 versus the model group; Ns, no statistical significance).

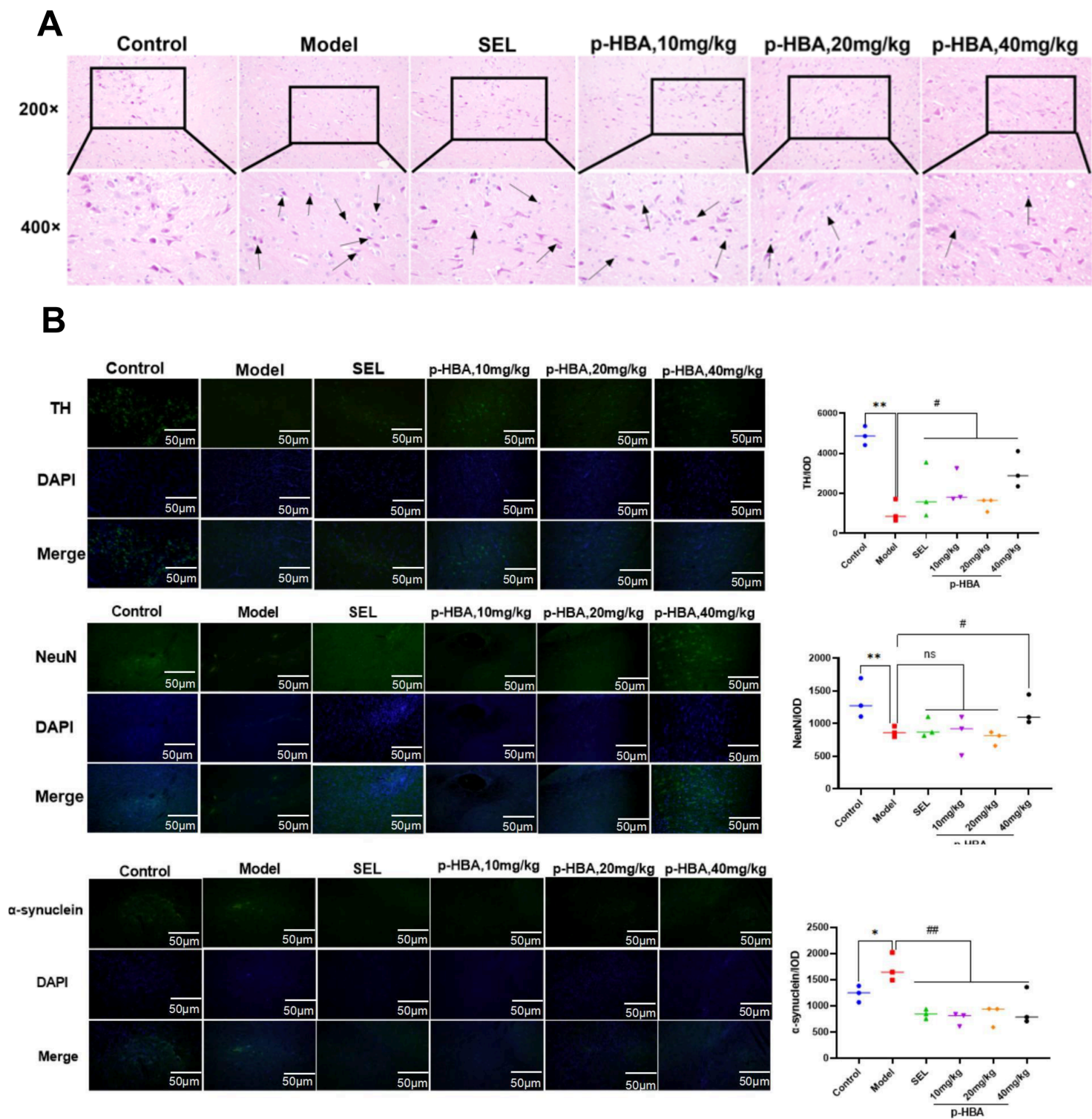


Figure 2

(A) p-HBA had protective effects on neurons in the substantia nigra of rats with PD ($n = 6$). HE staining (200 x and 400 x). The black arrow represents cellular atrophy. (B) Expression of TH, NeuN and α -synuclein in the substantia nigra of rats with PD (100x) ($n = 3$). Green, TH, NeuN and α -synuclein protein; blue, 4,6-diamino-2-styrenol (DAPI). (* $P < 0.05$ versus the control group; ** $P < 0.01$ versus the control

group; # $P < 0.05$ versus the model group; ## $P < 0.05$ versus the model group; Ns, no statistical significance)

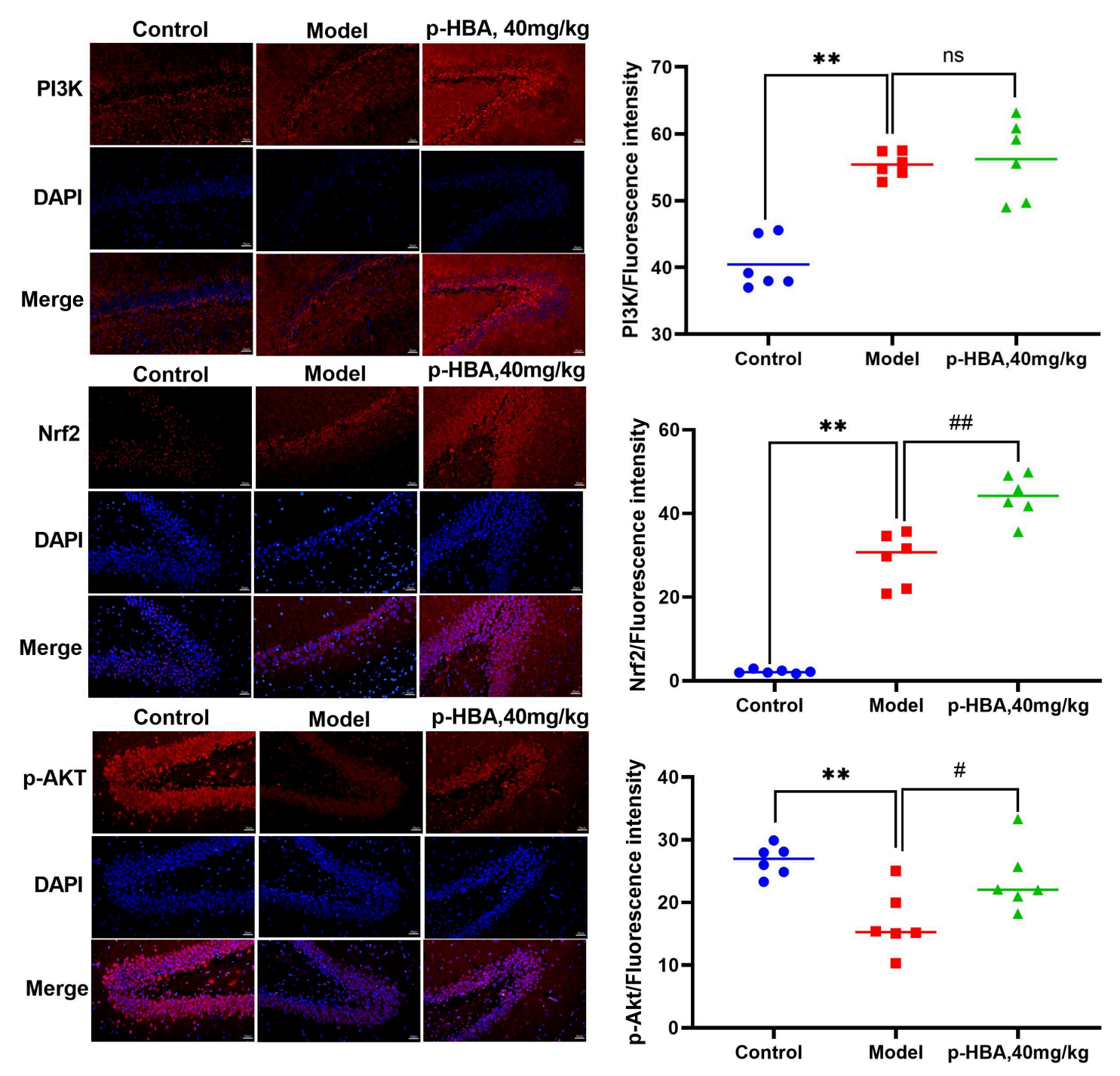


Figure 3

p-HBA increased the protein expression of PI3K, Nrf2, and p-Akt($n = 6$). ** $P < 0.01$ versus the control group; # $P < 0.05$ versus the model group; ## $P < 0.01$ versus the model group.

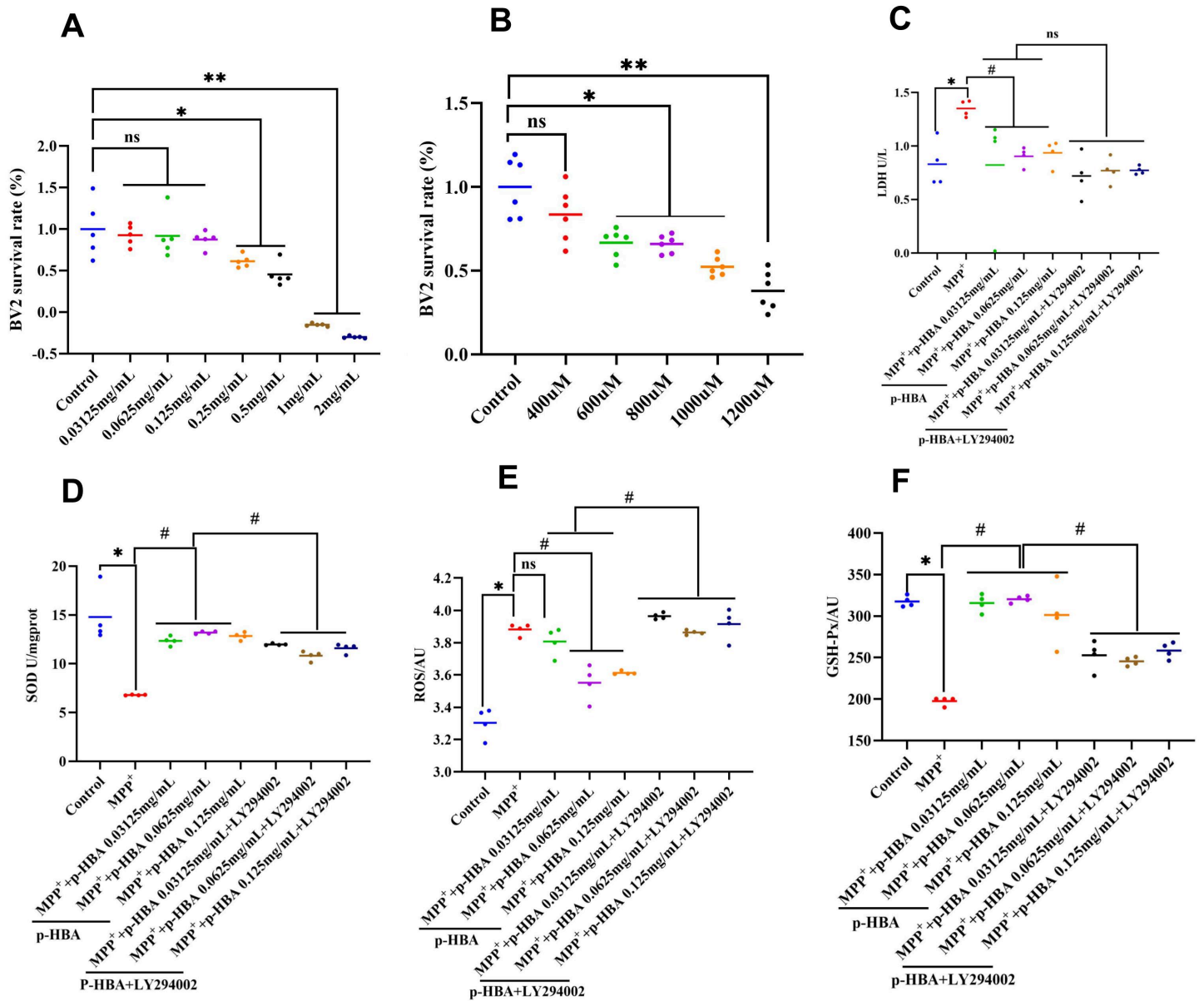


Figure 4

(A) The effect of different concentrations of p-HBA on the survival rate of normal BV2 cells (n=4). (B) The effect of different concentrations of MPP⁺ on the survival rate of normal BV2 cells (n=4) (**P* < 0.05 versus the control group). (C-F) p-HBA reduced LDH and ROS levels of oxidative damage in BV2 cells; increased SOD and GSH-Px levels (n = 4). (C) LDH levels; (D) SOD levels; (E) ROS levels; (F) GSH-Px levels (***P* < 0.01 versus the control group; #*P* < 0.05; ##*P* < 0.01 versus the model group).

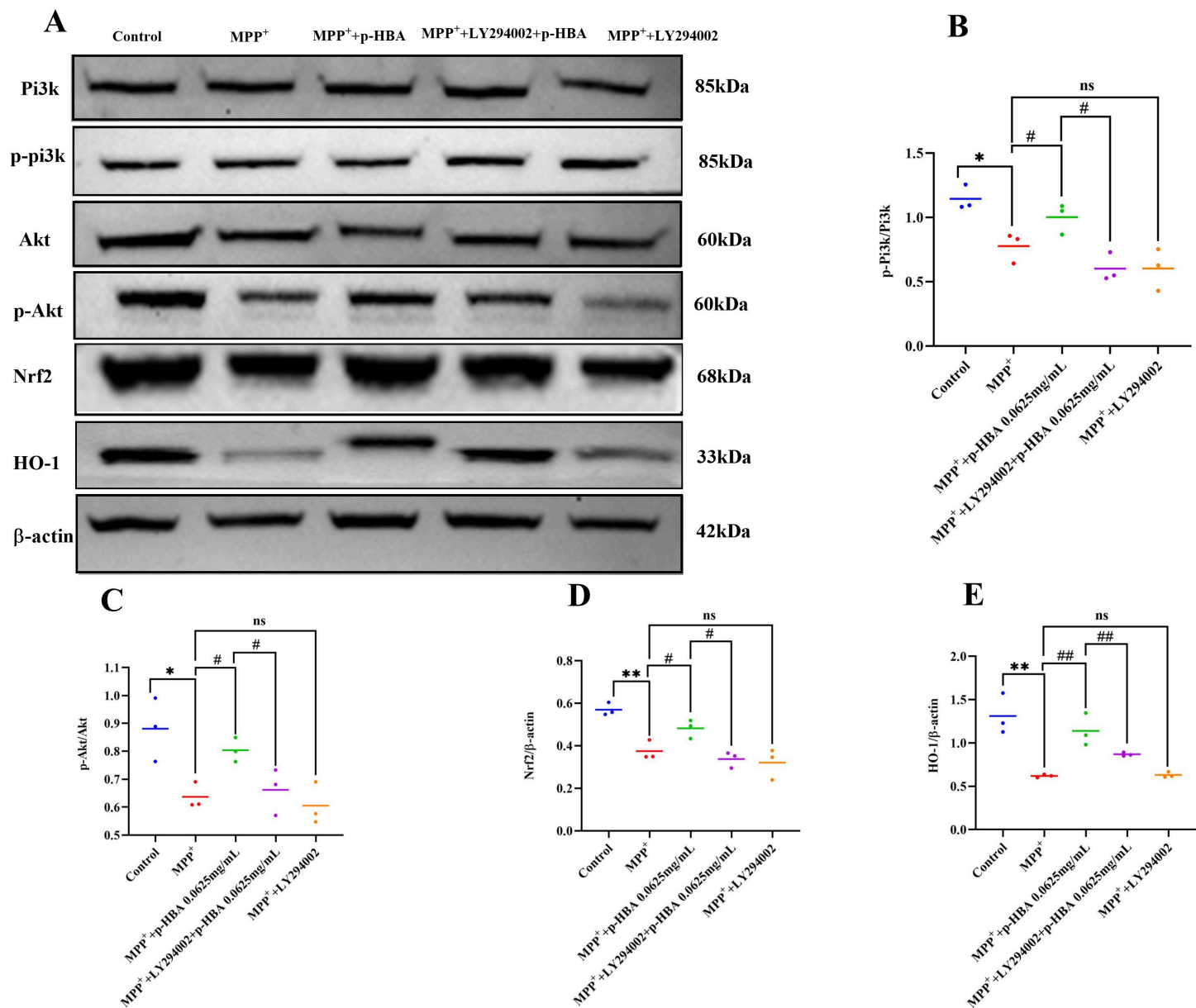


Figure 5

p-HBA increased the protein expression of PI3K, p-PI3K, AKT, p-AKT, Nrf2 and HO-1 in BV2 cells with oxidative damage (n = 3). (A) Western blotting of p-PI3K/PI3K, p-AKT/AKT, Nrf2 and HO-1 proteins. (B) Protein expression of p-PI3K/PI3K. (C) Protein expression of p-AKT/AKT. (D) Protein expression of Nrf2. (E) Protein expression of HO-1. * $p < 0.05$; ** $p < 0.01$ versus the control group; # $p < 0.05$; ## $p < 0.01$; ns: $p > 0.05$ versus the model group.