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Piperine increases striatal levels of DA and TH and decreases α-syn and $Aβ_{42}$ deposition in PDD mice by regulting autophagy: downexpression Beclin-1 and LC3B and upexpression p62

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Abstract

Piperine, the major pharmacological ingredient of pepper, can delay the procession of neuropharmacological effects, but its effects and mechanisms on Parkinson's disease dementia (PDD) mice is still unclear, we investigated whether piperine could help treating PDD mice. Here, PDD mice were randomly divided into eight groups (n = 12/group): a normal control group, a PDD model group, a madopar group, an autophagy inhibitor group, an autophagy activator group, and groups receiving low, medium or high doses of piperine respectively. The normal control and PDD model mice were injected with saline. Treatments were administered to the mice once per day continuously for 30 days. The behavioral tests were assessed. Dopamine (DA), Monoamine Oxidase-B (MAO-B), DOPA decarboxylase (DDC), β-secretase, acetylcholinesterase (AChE), amyloid $β_{42}$ ($Aβ_{42}$), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels were detected. α-synuclein (α-syn), tyrosine hydroxylase (TH), HSP90, Beclin-1, LC3B, p62 mRNA levels and miRNA-99a-5p expression were determined. Neuronal histology was observed. The behavior of PDD mice improved significantly after peperine treatment compared with the PDD model mice. In addition, our results also showed that peperine treatment increased DA, TH, DDC and p62 levels, decreased MAOB, β-secretase, AChE, $Aβ_{42}$, TNF-α, IL-6, Beclin-1 and LC3B levels, and down-regulated α-syn, HSP90, Beclin-1, LC3B mRNA levels and miR-99a-5p expression. These findings suggest that piperine may reduce the expression of mmu-miR-99a-5p and autophagy-related factors (HSP90, Beclin-1, LC3B and p62) to alleviate the neurological impairment of PDD mice, which is shown to slow down the process of DA metabolism and Aβ production and resist neuroinflammation.

Keywords: Piperine, Parkinson's dementia, Autophagy, Dopamine, miRNA-99a-5p

Introduction

Parkinson's disease (PD) is a neurodegenerative disease that disproportionately affects elderly people and is known as the "cancer that never dies" [1]. The WHO predicts that by 2030, the number of PD patients in the United States will reach 1.2 million [2]. With the prolonged course of PD, most patients will be accompanied by inattention, cognitive decline and other dementia manifestations. According to incomplete statistics, 70–80% of PD patients eventually develop Parkinson's disease with dementia (PDD), which seriously affects the quality of life and increases the disability rate and mortality rate [3]. The typical neuropathological basis of PDD is the massive deposition of α-synuclein (α-syn)

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and β-amyloid (Aβ) in the brain tissue, which overlays with Alzheimer's disease (AD) in etiology, pathogenesis, pathological features and clinical manifestations [4]. At present, the treatment of PDD is very limited, although cholinesterase inhibitors and monoaminergic proved effective in clinical trials, but the efficacy is far from meeting the expectations of patients, family members and clinicians [5]. Therefore, it is important to develop new, highly effective and natural anti-PDD drugs with low toxicity and side effects.

Autophagy is widely present in eukaryotic cells and is closely related to cell development and neurodegenerative diseases [6]. Autophagy is a powerful process for removing such proteins and maintaining mitochondrial homeostasis, so as to maintain the stability of the internal environment of cells [6]. α-syn and Aβ are important pathological products in the process of PDD [4]. Inhibition of α-syn and Aβ in the disorder of abnormal aggregation and degradation has been proved to be a breakthrough in the prevention and treatment of PDD, which is the main mechanism of neuron injury. Previous studies have found that regulating macrophage or chaperone-mediated autophagy (CMA) can alleviate the symptoms of PD or AD, reduce the expression of α-syn and Aβ, and protect dopaminergic and hippocampal neurons [7]. In order to investigate the effect of autophagy on PDD, we hypothesized that regulating autophagy can contribute to PDD treatment, which has not been reported yet.

Piperine, an alkaloid isolated from piper nigrum, has previously been demonstrated to inhibit inflammation, analgesic effects, anticonvulsant, antioxidant and cognitive [8]. Piperine also significantly reduced the immobility time of forced swimming and tail suspension mice and antagonized the damage of PC12 cells induced by corticosterone [9]. It also inhibited the activity of monoamine oxidase and increased the level of monoamine neurotransmitters, thus producing antidepressant activity in the depressed model mice [10]. However, it is not clear whether piperine can produce neuroprotective effects on PDD mice. Therefore, we studied the neuroprotective effect and mechanism of piperine in PDD mice. To test this hypothesis and evaluate the effects of piperine treatment on PDD mice, we performed the behavioral tests and determined α-syn, $A\beta_{42}$, dopamine (DA), monoamine oxidase-B (MAO-B), dopa decarboxylase (DDC) and other related gene and protein expressions. In addition, to assess the neuron damage of piperine, the histopathology of brain was conducted. Immunohistochemistry, ELISA, immunofluorescence staining, RT-PCR and western blot were used to estimate the damage of dopaminergic and hippocampal neurons of PDD mice.

Materials and methods

Animals

In this study, 96 C57BL/6 male mice (6–8 weeks old, weight 18–22 g) were obtained from SPF (Beijing) Biotechnology Co., Ltd (Beijing, China). Animals were housed in an animal house with a temperature of 20–24 °C, a humidity of 65-70%, and a light/dark cycle of 12 h, 5 animals were housed in each cage with free access to food and water. All experiments were conducted and approved by the Animal Ethics Committee of Guangdong Hospital of Traditional Chinese Medicine (No. 2020062).

Animal model and experimental groups

The mice were randomly divided into eight groups (n = 12), normal control group, model group, piperine low-dose group (10 mg/kg, ig, CAS:94–62-2, Sichuan Weikeqi Biological Technology Co., Ltd.), piperine medium-dose group (20 mg/kg, ig) and piperine high-dose group (40 mg/kg, ig), autophagy inhibitor group (3-methyladenine, 3-MA, 30 mg/kg, ip, CAS: S2767, Selleck, USA), autophagy activator group (rapamycin, 1 mg/kg, ip, CAS: S1039, Selleck, USA), and madopar group (112.5 mg/kg, ig, CAS: SH2443, Shanghai Roche Pharmaceutical Co., Ltd.) for 40 days, once a day. Except for the normal group, reserpine was subcutaneously injected with 0.1 mg/kg once 48 h for 40 days [11]. In addition, on the 20th day of drug-administrated, except for the normal group, bilateral common carotid artery ligation operation was performed under 10% chloral hydrate [350 mg/kg, ip, CAS: A600288-0250, Sangon Biotech (Shanghai) Co., Ltd.] anesthesia and lasted 2 h in all the other groups. When the mice were anesthetized, bilateral common carotid arteries were simultaneously clamped with artery clips for 10 min, then loosened and repeated for 3 times [12]. Finally, the mice were sutured and the PDD mice were established.

Open field test

After the last administration, mice were put into a self-made opaque open box (height 30 cm, bottom side length 72 cm, bottom side was divided into 64 small squares on average, side length of small squares was 9 cm, center 16 grids were central grids, and other grids were edge grids). The experiment lasted for 5 min, and only one mouse was tested at a time. Place the mouse gently in the center square of the self-made open box, and press the stopwatch at the same time to observe and record the behavior of the mouse, including: (1) the residence time of the center grid (the time when the mouse was placed in the center grid until all its four paws left); (2) cross the grid number; (3) the number of grooming (licking feet, scratching and washing face); (4) The number of hind legs standing (the two front paws were vacated at

the same time and the hind paws landed on the ground for 1 time). Remove the excrement from the box before the next round of experiments. Calculate the distance of the central motion and the distance of the edge motion according to the number of the crossing lattice.

Pole test

After the last administration, a ball with the diameter of 2.5 cm was fixed at the top of the wooden pole which was at the length of 50 cm and at the thickness of 1 cm. The ball was wrapped with gauze to increase friction, and the wooden pole was placed vertically in a square plastic bowl. At the beginning of the experiment, the mice were placed on the ball, and the time spending on getting down from the ball and the time to climb the wooden pole was recorded. Each mouse was tested for 3 times and the average value was taken.

Sample collection

After the behavioral test, the mice were anesthetized and fixed with 10% chloral hydrate (350 mg/kg, intraperitoneal injection), and then perfused and fixed with 0.9% normal saline successively through the heart. The mice were killed with their heads severed, and the whole brain was removed within 1 min. The cortical, striatum and midbrain tissues were rapidly separated in ice water, weighed, and stored at − 80 °C for index detection.

ELISA analysis

The striatum from mice were homogenized according to the ratio of striatum to ice-normal saline 1:9, centrifuged at 12,000 r/min at 4 °C for 10 min, and the supernatant was extracted. The striatal of DA, MAOB, DDC, β-secretase, acetylcholinesterase (AChE), $A\beta_{42}$, TNF-α and interleukin-6 (IL-6) levels of mice were determined by mouse ELISA kit (LOT: 07/2021, Shanghai Enzyme-linked Biotechnology Co., Ltd.), respectively. All kits were conducted according to the manufacturer's protocol.

Immunohistochemistry

The mesencephalon was fixed in 4% paraformaldehyde overnight. The mesencephalon was dehydrated by 80% (1 min × 2 times), 95% (3 min × 2 times) and 100% (5 min × 2 times) ethanol, followed by paraffin impregnation, paraffin embedding, and conventional paraffin section with thickness of 4 μm. Slices were immersed in 0.3% H_2O_2 for 30 min, then hot repaired in citrate buffer (0.01 mol/L, pH 6.0), washed with PBS buffer (5 min × 3 times). Slices were blocked in 5% BSA and incubated overnight at 4℃ with tyrosine hydroxylase (TH) primary antibody (CAS: ab137869, 1:100, Abcam, USA). This was followed by incubation with HRP-labeled secondary antibody (cas: ab205718, 1:1000, Abcam, USA) for 30 min at 37 °C. Then nuclear redyeing was done with hematoxylin, washed and dried with distilled water, sealed with neutral resin, and stored at 37 °C. The positive expression of TH was observed under light microscope, and the images were taken.

Immunofluorescence staining

The mesencephalon was fixed in 4% paraformaldehyde for 24 h, then dehydrated, paraffin embedded and then cut into 30-μm sectioned. Dried the sections until dewaxing, rehydrated and repaired with hot antigen. After cooling, washed with PBS for 3 times, 5 min each time. Dropped 5% BSA (cas: ST025, Shanghai Biyuntian Biotechnology Co., Ltd.) into the sections and sealed at 37 °C for 2 h and then with the Beclin-1, LC3B, p62 and GFAP antibodies (1:100; cas: ab62557, ab192890, ab109012 and ab7260, Abcam, USA) overnight at 4 °C. The next day was incubated in fluorescent secondary antibody (1:1000, cas: ab150081 and ab150117, Abcam, USA) and incubation at 37 °C for 30 min. And added DAPI solution (cas: C1002, Shanghai Biyuntian Biotechnology Co., Ltd.) incubated at 37 °C for 5 min, sealed the sections by fluorescence anti-quenching agent (cas: P0123, Shanghai Biyuntian Biotechnology Co., Ltd.) and observed under fluorescence microscope. The expression rate of fluorescent proteins was calculated by Image-J analysis software.

HE staining

The sections were dewaxed to water after conventional dewatering and embedding treatment. Stain with hematoxylin aqueous solution for 15 min, rinse with water and then stain with alcohol eosin solution for 3 min. After dehydration and transparency, seal the slices with neutral gum, and observe the changes of the histopathological structure of the midbrain under light microscope.

Real-time polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from the right striatum in each group using Trizol reagent (cas: 15596018, Invitrogen, USA) and reverse transcribed into cDNA according to standard protocol (cas: AG11706 and AG11701, Hunan Aikerui Biological Engineering Co., Ltd.). RT-PCR was performed by the reaction conditions were as follows: pre-denaturation at 94 °C for 30 s, denaturation at 94 °C for 10 s, annealing at 64 °C for 30 s, extension at 72 °C for 30 s, maintained a total of 40 cycles. The quantitative analysis of each mRNA level calculated by using $2^{-\Delta\Delta Ct}$ method after normalizing (cycle threshold) values with that of β-actin. The primers were used as (Table 1 and Table 2).

Table 1 Gene sequence

Gene name	Primer sequence (5′–3′)	Primer length (bp)
α-syn	F: CCGAGTATCGTTTCCCGGTT	78
	R: CATGAATCAACCGCTGCCAC	
HSP90	F: TGCTAAGTCTGGCACGAAGG	78
	R: ACACCAAACTGCCCGATCAT	
TH	F: GGACCACCAGCTTGCACTAT	86
	R: GTATCCTGCTCTGAGACGGC	
	R: GTGAGGACTCCAGCCACAAA	
Beclin-1	F: GCTGTAGCCAGCCTCTGAAA	80
	R: AATGGCTCCTGTGAGTTCCTG	
LC3B	F: GGGACCCTAACCCCATAGGA	111
	R: TCTCCCCCTTGTATCGCTCT	
p62	F: ACTGCTCAGGAGGAGACGAT	77
	R: CCGGGGATCAGCCTCTGTAG	
β-actin	F: ACACTCTCCCAGAAGGAGGG	147
	R: TTTATAGGACGCCACAGCGG	

Table 2 miRNA sequence

miRNA name	miRNA sequence
mmu-miR-99a-5p	AACCCGUAGAUCCGAUCUUGUG
hsa-U6	Omitted due to length (Accession: NR_002752.2)

Western blot analysis

The right striatum slice in each group were homogenized with RIPA lysis buffer (containing protease inhibitor) (cas: P0013, Shanghai Biyuntian Biotechnology Co., Ltd.) was added at a rate of 1:5 (m:v). After homogenized in ice bath and centrifuged at 14,000*g* for 20 min at 4 °C. Protein concentration was determined by a BCA protein quantitation kit (cas: P0012, Shanghai Biyuntian Biotechnology Co., Ltd.). Then, lysates containing 50 μg proteins were subjected to polyacrylamide gel electrophoresis at a constant voltage of 80 V and separated gel electrophoresis at a constant voltage of 110 V. Subsequently, transferred onto PVDF membranes and blocked with 5% BSA (cas: ST025, Shanghai Biyuntian Biotechnology Co., Ltd.) for 60 min on shaker platform at 37 °C. The blots were probed with the corresponding primary antibodies: TH, α-syn, HSP90, Beclin-1, LC3B, p62 and GAPDH (1:1000, cas: ab137869, ab212184, ab13492, ab62557, ab192890, ab109012 and ab8245, Abcam, USA). The next day, the blots were incubated with HRP-labeled secondary antibody (1:1000 dilution, cas: ab205719 and ab205718, Abcam, USA) for 2 h at 37 °C. ECL chromogenic agent (cas: P0018AM, Shanghai Biyuntian Biotechnology Co., Ltd.) was added and detected by gel imaging analysis system, the gray value of each target strip was analyzed, the ratio of each group to GAPDH gray value was calculated, and the differences among groups were calculated.

Statistical analysis

SPSS17.0 software was used for statistical analyses. Measurement data were represented as mean ± standard deviation. All the data were normally distributed and had homogeneous variances. One-way analysis of variance was used for comparison between groups. The rank-sum test was used to compare between groups for non-normal distribution or variances. Significance is determined on a criterion of $P<0.05$.

Results

Piperine treatment elevate activity of PDD mice in the behavioral testsOpen field test

As shown in Fig. 1 (I–VI), the PDD mice showed an increase in the center grid dwell time and length of the distance along the central area ($P<0.01$), a decrease in the number of crossings, hindlimb standing and modifying, and distance along the edge ($P<0.01$) when compared to the normal control mice. In addition, the mice of 3-MA, piperine and madopar treated groups showed a decline in the center grid dwell time and length of the distance along the central area ($P<0.05$) and an increase in the number of crossing, hindlimb standing and modifying, and distance along the edge ($P<0.05$) when compared to PDD mice, however, the rapamycin treated mice were the contrary. The mice of piperine and rapamycin treated groups showed an increase in the center grid dwell time and length of the distance along the central area ($P<0.05$) and a decline in the number of crossings, hindlimb standing and modifying, and distance along the edge ($P<0.01$) as compared to the mice of 3-MA. Finally, the comparison of piperine and rapamycin treated mice to madopar treated mice, center grid dwell time and length of the distance along the central area were augmented ($P<0.05$) and the number of crossings, hindlimb standing and modifying, and distance along the edge were reduced($P<0.01$). These data suggest that the motor activity and exploration of PDD mice were elevated after piperine treatment.

Pole test

As shown in Fig. 1 (VII–VIII), the time of turning head down and total climbing time of the pole were significantly prolonged in the PDD mice when compared to the normal control mice ($P<0.01$). The time of turning head down and total climbing time of the pole were significantly shortened ($P<0.01$) in the 3-MA, piperine and madopar treated mice when compared with that in

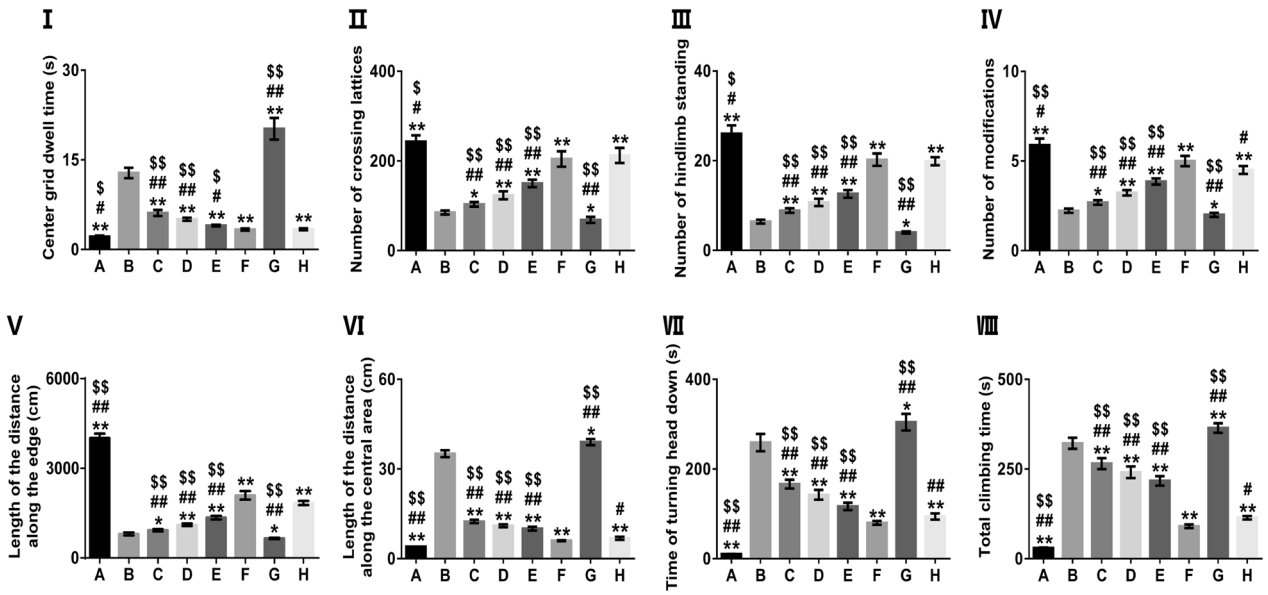

Fig. 1 Results of behavioral tests in treated and untreated PDD mice. In open field and pole tests, all showed a better locomotor, exploration and coordination activities than the PDD model mice. As compared to the model group, the center grid dwell time (I) and length of the distance along the central area (VI) decreased ($P<0.05$), time of turning head down (VII) and total climbing time (VIII) shorten ($P<0.01$), numbers of crossing lattices (II), hindlimb standing (III), modifications (IV) and length of the distance along the edge (V) increased ($P<0.05$) in all doses of piperine treated groups. As compared to 3-methyladenine (3MA) and madopar treated groups, the center grid dwell time (I) and length of the distance along the central area (VI) prolonged ($P<0.05$), time of turning head down (VII) and total climbing time (VIII) extended ($P<0.05$), numbers of crossing lattices (II), hindlimb standing (III), modifications (IV) and length of the distance along the edge (V) reduced ($P<0.05$) in all doses of piperine treated groups. $^{*}P<0.05$, $^{**}P<0.01$ vs model group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs 3MA group; $^{\$}P<0.05$, $^{\$\$}P<0.01$ vs madopar group. Values are mean ± SD of 12 mice. **A** normal control group; **B** model group; **C** 10 mg/kg piperine group; **D** 20 mg/kg piperine group; **E** 40 mg/kg piperine group; **F** 3MA group; **G** rapamycin group; **H** madopar group

the PDD mice, however, the mice in rapamycin treated group were on the contrary. In addition, as compared to the 3-MA treated mice, the time of turning head down and total climbing time of the pole were significantly prolonged ($P<0.05$) in the piperine, madopar and rapamycin treated mice. Moreover, as compared to the madopar treated mice, the time of turning head down and total climbing time of the pole were also prolonged ($P<0.01$) in the piperine and rapamycin treated mice. These data suggest that the motor coordination ability of mice was improved after piperine treatment.

The cortex levels of DA, MAOB, DDC, β-secretase, AChE, $A\beta_{42}$, TNF-α and IL-6 in the PDD mice

Elisa analysis revealed that the cortex levels of DA and DDC were reduced ($P<0.01$, Fig. 2I and III), MAOB, β-secretase, AChE, $A\beta_{42}$, TNF-α and IL-6 were increased ($P<0.01$, Fig. 2II, IV, V, VI, VII and VIII) in the PDD mice when compared with the normal control mice. The cortex levels of DA and DDC were elevated ($P<0.05$, Fig. 2I and III), MAOB, β-secretase, AChE, $A\beta_{42}$, TNF-α and IL-6 were decreased ($P<0.05$, Fig. 2II, IV, V, VI, VII and VIII) in the 3-MA, piperine treated mice as compared to the PDD mice, but those changes of rapamycin treated mice were on the opposite. Furthermore, a significant decrease in DA and DDC levels and a significant increase in MAOB, β-secretase, AChE, $A\beta_{42}$, TNF-α and IL-6 levels were observed in the low and medium dose of piperine and rapamycin treated mice compared with the 3-MA treated mice ($P<0.05$, Fig. 2). Finally, a significant reduction in DA and DDC levels in high dose of piperine treated ($P<0.05$, Fig. 2I and III) and rapamycin treated mice ($P<0.05$) and a significant augment in MAOB, β-secretase, AChE, $A\beta_{42}$, TNF-α and IL-6 levels in rapamycin treated mice ($P<0.05$, Fig. 2II, IV–VIII) and a significant decrease in MAOB, β-secretase, AChE, $A\beta_{42}$, TNF-α and IL-6 levels in the high dose of piperine treated mice ($P<0.05$, Fig. 2II, IV–VIII) were observed compared with the madopar treated mice. The results suggested that piperine could increase the level of DA and decrease the level of $A\beta_{42}$ by affecting the activities of related enzymes and the levels of inflammatory factors.

Piperine increases striatal level of TH

As shown in Fig. 3, striatal level of TH was markedly attenuated in the PDD mice when compared with the

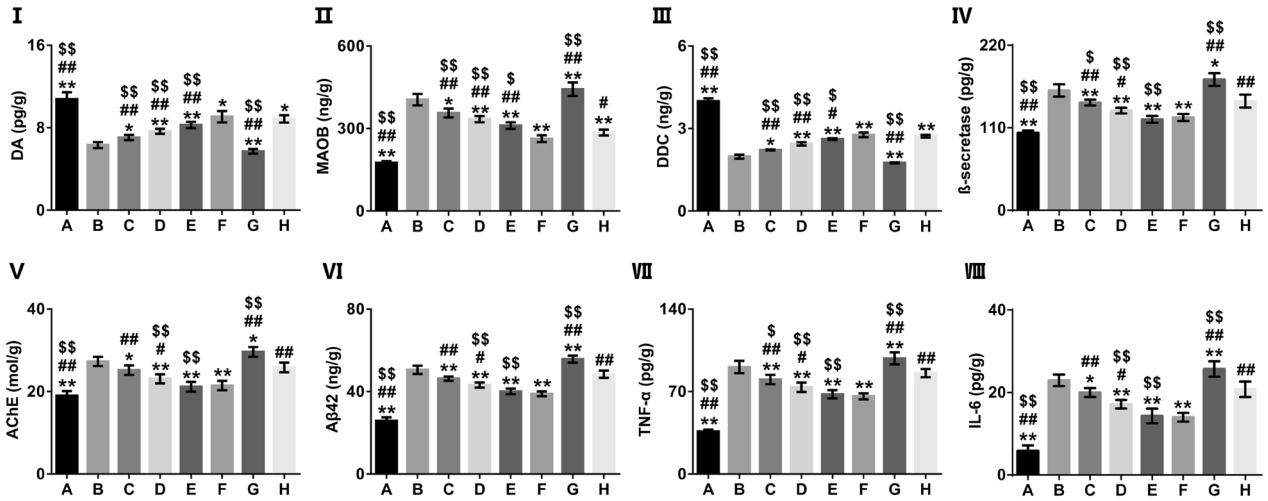

Fig. 2 DA, MAOB, DDC, β-secretase, AChE, $A\beta_{42}$, TNF-α and IL-6 levels in treated and untreated PDD mice. Piperine has effects on regulating DA metabolism and $A\beta_{42}$ and anti-inflammation.Compared to the model group, DA (I) and DDC (III) levels increased ($P<0.05$), MAOB (II), β-secretase (IV), AChE (V), $A\beta_{42}$ (VI), TNF-α (VII) and IL-6 (VIII) levels reduced($P<0.05$) significantly in all doses of piperine treated groups. In addition, DA (I) and DDC (III) levels was less ($P<0.05$), MAOB (II), β-secretase (IV), AChE (V), $A\beta_{42}$ (VI), TNF-α (VII) and IL-6 (VIII) levels higher ($P<0.05$) in low and medium doses of piperine treated groups than that of the 3-methyladenine (3MA) group. Besides, DA, MAO-B, DDC, β-secretase, AChE, $A\beta_{42}$, TNF-α and IL-6 levels in high dose of piperine treated group was lower than that of the madopar group. $^{*}P<0.05$, $^{**}P<0.01$ vs model group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs 3MA group; $^{\$}P<0.05$, $^{\$\$}P<0.01$ vs madopar group. Values are mean ± SD of 12 mice. **A** normal control group; **B** model group; **C** 10 mg/kg piperine group; **D** 20 mg/kg piperine group; **E** 40 mg/kg piperine group; **F** 3MA group; **G** rapamycin group; **H** madopar group

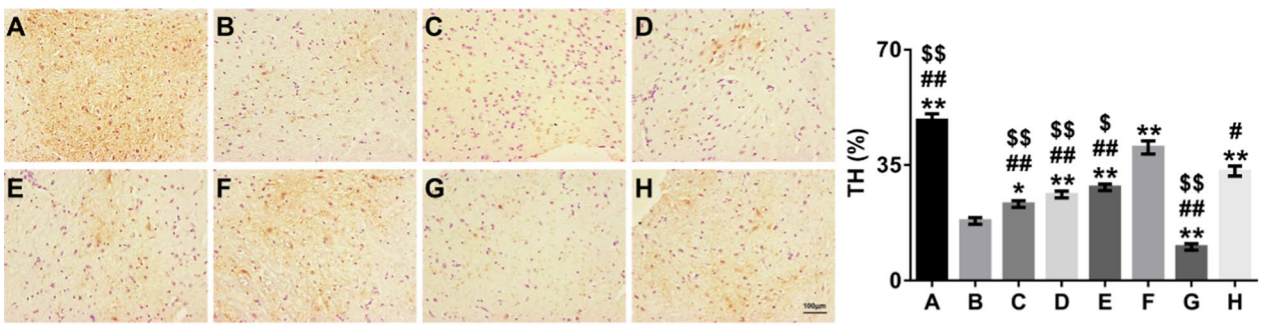

Fig. 3 Expression of TH positive neuron in the midbrain of PDD mice (× 200) (n = 3). Piperine could increase expression of TH positive neuron. Expression of TH positive neuron in all doses of piperine treated groups was more than that of the PDD model group ($P<0.05$). Expression of TH positive neuron in all doses of piperine treated groups was less than that of the 3-methyladenine (3MA) group ($P<0.05$). In addition, expression of TH positive neuron in all doses of piperine treated groups was decreased compared with that of madopar group ($P<0.05$). The bar is 100 μm. The brown part is the cells with positive expression. $^{*}P<0.05$, $^{**}P<0.01$ vs model group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs 3MA group; $^{\$}P<0.05$, $^{\$\$}P<0.01$ vs madopar group. **A** normal control group; **B** model group; **C** 10 mg/kg piperine group; **D** 20 mg/kg piperine group; **E** 40 mg/kg piperine group; **F** 3MA group; **G** rapamycin group; **H** madopar group

normal control mice ($P<0.01$). Striatal level of TH in the 3-MA, piperine and madopar treated mice was higher than that in the PDD mice ($P<0.05$), by contrast, striatal level of TH was reduced in the rapamycin treated mice ($P<0.01$). As compared to the 3-MA mice, striatal level of TH was decreased in all doses piperine, madopar and rapamycin treated mice ($P<0.05$). As compared to the madopar treated mice, striatal level of TH was also declined in all doses piperine and rapamycin treated mice ($P<0.05$). This result suggested that piperine could promote the positive expression of TH neurons in PDD mice.

Piperine suppress autophagy of PDD mice

Most neurodegenerative diseases that afflict humans are associated with the intracytoplasmic deposition of

aggregate prone proteins in neurons and autophagy dysfunction. Therefore, to test whether piperine could affect DA or $A\beta_{42}$ levels by regulating autophagy, we employed autophagy inhibitor (3-MA) and activator (rapamycin) were used as controls. Figure 4 shows that Beclin-1 and LC3B expressions were increased ($P<0.01$), but p62 expression was decreased in PDD mice compared to normal control mice ($P<0.01$). The mice treated with 3-MA, piperine and madopar have reduced Beclin-1 and LC3B expressions ($P<0.05$) and elevated p62 expression ($P<0.05$) compared to the untreated PDD mice, however, the mice treated with rapamycin showed a contrary change of Beclin-1, LC3B and p62 expressions. In addition, the mice treated with piperine, madopar and rapamycin exhibited an increase of Beclin-1 and LC3B expressions ($P<0.05$) and a decrease of p62 expression ($P<0.01$) as compared with the mice treated with 3-MA. Furthermore, the high dose of piperine treated mice

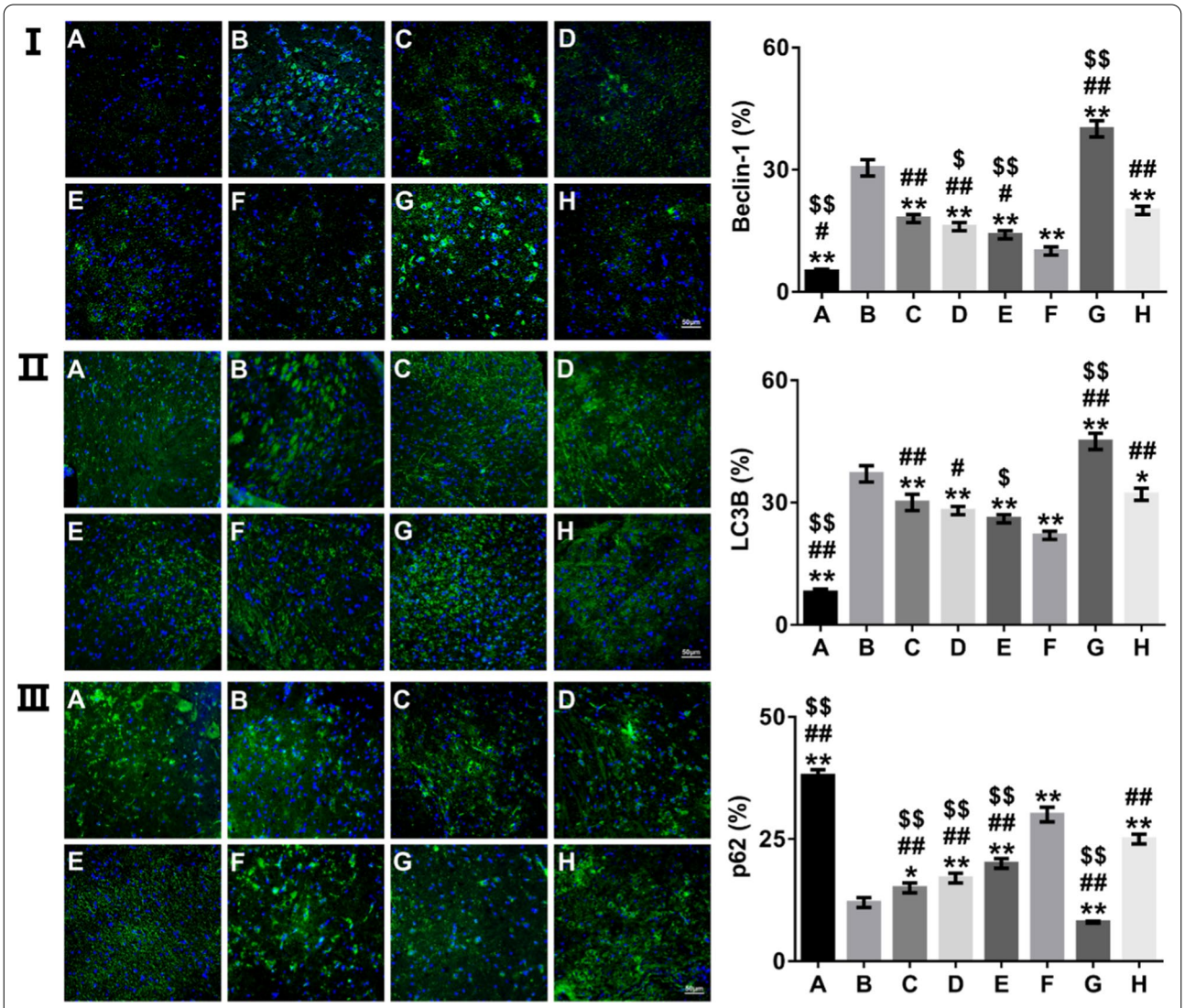

Fig. 4 Representative immunofluorescent assays of Beclin-1, LC3B and p62 levels (× 400) (n = 3). The Beclin-1 (I) and LC3B (II) expressions were decreased ($P<0.01$), p62 (III) expression was increased ($P<0.05$) in all doses of piperine treated groups compared with the model group.The Beclin-1 (I) and LC3B (II) expressions were increased ($P<0.05$), p62(III) expression was declined ($P<0.01$) in low and medium doses of piperine treated groups compared with 3-methyladenine (3MA) group.In addition, the Beclin-1 (I) and LC3B (II) expressions were reduced ($P<0.05$), p62 (III) expression was augmented ($P<0.01$) in high dose of piperine treated group compared with madoapr group. The Bar is 50 μm. The green part is the cells with positive expression. The blue part indicates the nucleus. $^{*}P<0.05$, $^{**}P<0.01$ vs model group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs 3MA group; $^{\$}P<0.05$, $^{\$\$}P<0.01$ vs madopar group. **A** normal control group; **B** model group; **C** 10 mg/kg piperine group; **D** 20 mg/kg piperine group; **E** 40 mg/kg piperine group; **F** 3-MA group; **G** rapamycin group; **H** madopar group

showed an increase of Beclin-1 and LC3B expressions and a reduction of p62 expression; nevertheless, Beclin-1, LC3B and p62 expressions of the rapamycin treated mice showed an opposite change. The results suggested that piperine may exert anti-PDD activity by inhibiting autophagy.

Neuronal histology of PDD mice

As shown in Fig. 5, neurons were arranged normally with clear morphology and structure without injury. Eosinophilic injured neurons were observed in the mesencephalon of PDD model and rapamycin treated mice. The eosinophilic injured neurons showed nuclear pyknosis and irregular shape of cell body. The cytoplasm and nucleus showed eosin staining and inflammatory cell infiltration. However, the above lesions were significantly alleviated in the mice treated with 3-MA, piperine and madopar, which showed less eosinophilic injury neurons and less inflammatory cell infiltration.

α-syn, TH, HSP90, Beclin-1, LC3B, p62 mRNA levels and miRNA-99a-5p expression in the control and the treated PDD mice

In the cortex, α-syn, HSP90, Beclin-1, LC3B mRNA levels and miRNA-99a-5p expression were higher ($P<0.01$, Fig. 6 I, III, IV, V and VII) and TH and p62 were lower ($P<0.01$, Fig. 6II and VI) in the PDD mice compared to that in the normal control mice. As compared to the PDD mice, α-syn, HSP90, Beclin-1, LC3B mRNA levels and miRNA-99a-5p expression (Fig. 6 I, III, IV, V and VII) were clearly decreased and TH and p62 (Fig. 6 II and VI) were increased in the 3-MA, piperine and madopar treated mice, however, the mice treated with rapamycin showed a contrary change of α-syn, TH, HSP90, Beclin-1, LC3B mRNA levels and miR-99a-5p expression. As compared to the 3-MA treated mice, α-syn, HSP90, Beclin-1, LC3B mRNA levels and miR-99a-5p expression (Fig. 6 I, III, IV, V and VII) were clearly augmented and TH and p62 (Fig. 6 II and VI) were reduced in the piperine, rapamycin and madopar treated mice($P<0.05$). As compared to the madopar treated mice, α-syn, HSP90, Beclin-1, LC3B mRNA levels and miR-99a-5p expression (Fig. 6 I, III–V and VII) were significantly elevated and TH and p62 (Fig. 6 II and VI) were dropped in the piperine and rapamycin treated mice($P<0.05$). These results suggested that the anti-PDD activity of piperine may be closely related to autophagy related factors and miR-99a-5p.

α-syn, TH, HSP90, Beclin-1, LC3B and p62 expressions in the control and the treated mice

As shown in Fig. 7, levels of α-syn, HSP90, Beclin-1 and LC3B were significantly increased ($P<0.01$), while the levels of TH and p62 were significantly decreased ($P<0.01$) in the PDD mice when compared with the normal control mice. As compared to the PDD mice, levels of α-syn, HSP90, Beclin-1 and LC3B were significantly less ($P<0.01$), whereas the levels of TH and p62 were significantly higher ($P<0.01$) in the high dose of piperine treated mice, and the change of α-syn, TH, HSP90, Beclin-1, LC3B and p62 expressions were on the

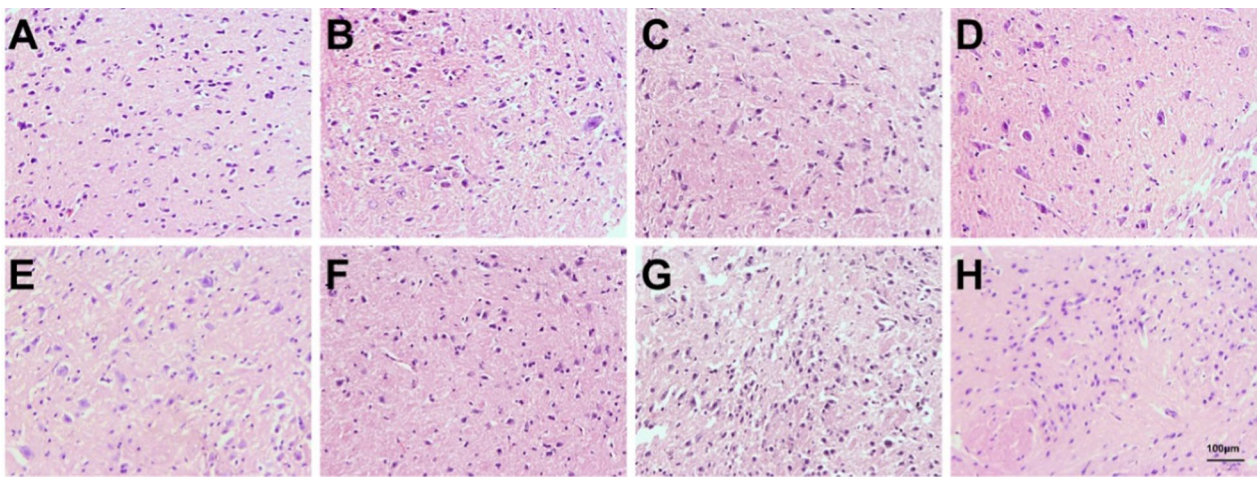

Fig. 5 Results of midbrain histopathology (HE, × 200). Histopathology findings showed that the eosinophilic injured neurons showed nuclear pyknosis, irregular shape of cell body, eosin staining of cytoplasm and nucleus, and infiltration of inflammatory cells were observed in the model group compared to the normal control group. In addition, there was less eosinophilic neuron injury and less inflammatory cell infiltration in the 3-methyladenine (3MA), all doses of piperine and madopar treated groups. The blue part indicates the nucleus. The red part indicates the cytoplasm. **A** normal control group; **B** model group; **C** 10 mg/kg piperine group; **D** 20 mg/kg piperine group; **E** 40 mg/kg piperine group; **F** 3-MA group; **G** rapamycin group; **H** madopar group

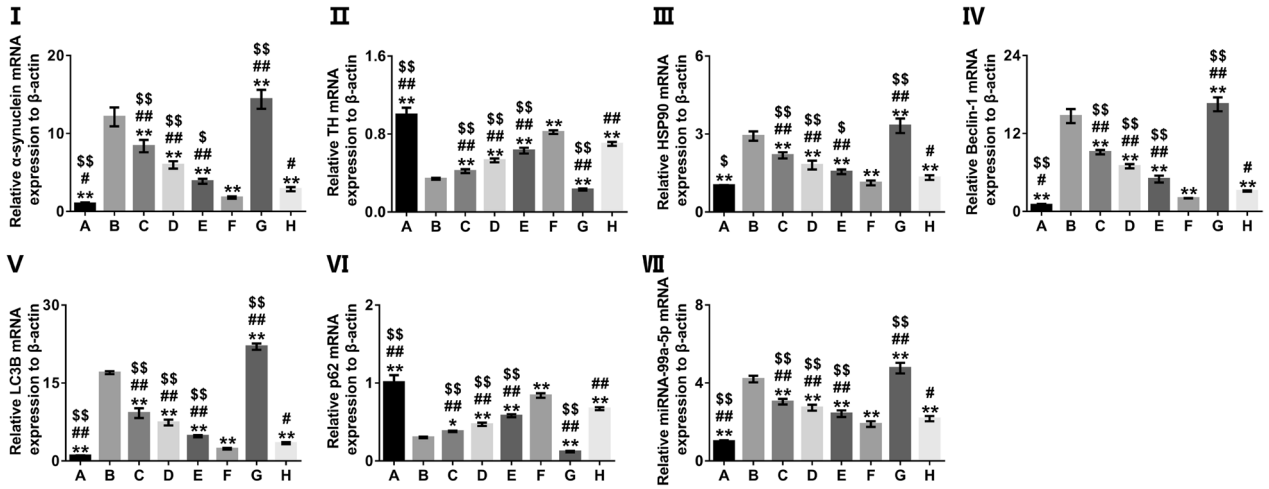

Fig. 6 α-syn, TH, HSP90, Beclin-1, LC3B and p62 mRNA levels and miRNA-99a-5p expression in the brain of PDD mice (n = 12). As compared to the model group, α-syn (I), HSP90 (III), Beclin-1 (IV), and LC3B (V) mRNA levels and miRNA-99a-5p (VII) expression were decreased ($P < 0.01$), however, TH (II) and p62 (VI) mRNA levels were increased ($P < 0.05$) in all doses of piperine treated groups. As compared to 3-methyladenine (3MA) group, α-syn (I), HSP90 (III), Beclin-1 (IV) and LC3B (V) mRNA levels and miRNA-99a-5p (VII) expression were increased ($P < 0.05$), TH (II) and p62 (VI) mRNA levels were reduced ($P < 0.05$) in all doses of piperine treated groups. As compared to madopar group, α-syn (I), HSP90 (III), Beclin-1 (IV),and LC3B (V) mRNA levels and miRNA-99a-5p (VII) expression were augmented ($P < 0.05$), TH (II) and p62 (VI) mRNA levels were dropped ($P < 0.01$) in all doses of piperine treated groups. $^{*}P < 0.05$, $^{**}P < 0.01$ vs model group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs 3MA group; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ vs madopar group. **A** normal control group; **B** model group; **C** 10 mg/kg piperine group; **D** 20 mg/kg piperine group; **E** 40 mg/kg piperine group; **F** 3-MA group; **G** rapamycin group; **H** madopar group

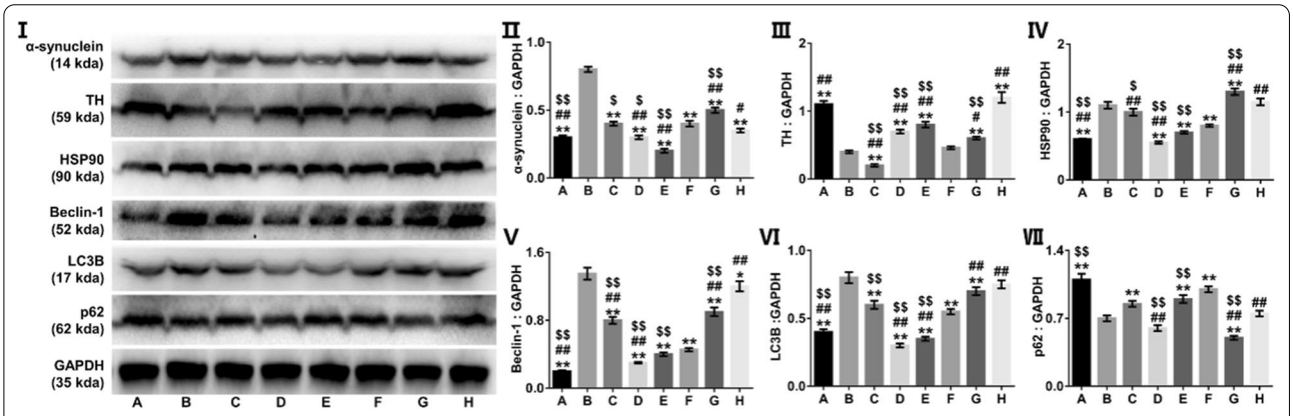

Fig. 7 Representative western blot assays of α-syn, TH, HSP90, Beclin-1, LC3B and p62 expressions (× 400) (n = 3). The α-syn (I, II), HSP90 (I–IV), Beclin-1 (I–V), LC3B (I–VI) expressions were decreased ($P < 0.01$), TH (I–III) and p62 (I–VII) expressions were increased ($P < 0.01$) in high dose of piperine treated group compared with the model group. In addition, α-syn (I, II), HSP90 (I–IV), Beclin-1 (I–V) and LC3B (I–VI) expressions were lower ($P < 0.01$) in the medium dose of piperine group, TH (I–III) expression was higher ($P < 0.01$) than that of 3-methyladenine (3MA) group. Besides, the α-syn (I&II), HSP90 (I–IV), Beclin-1 (I–V), LC3B (I–VI), TH (I–III) and p62 (I–VII) expressions were reduced ($P < 0.05$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs model group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs 3MA group; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ vs madopar group. **A** normal control group; **B** model group; **C** 10 mg/kg piperine group; **D** 20 mg/kg piperine group; **E** 40 mg/kg piperine group; **F** 3MA group; **G** rapamycin group; **H** madopar group

contrary in the rapamycin treated mice. In addition, the medium dose of piperine treated mice showed a decrease of levels of α-syn, HSP90, Beclin-1 and LC3B ($P < 0.01$), whereas levels of α-syn, HSP90, Beclin-1 and LC3B were increased in the rapamycin treated mice ($P < 0.01$); Likewise, the high dose of piperine treated mice exhibited a higher TH expression ($P < 0.01$), but rapamycin treated mice showed a decrease p62 expression ($P < 0.01$) as compared to those of 3-MA treated mice. Finally, the expressions of α-syn, TH, HSP90, Beclin-1, LC3B and p62 in the medium dose of piperine treated mice were lower than that of madopar treated mice ($P < 0.05$). These results

further suggested that the anti-PDD activity of piperine is mainly related to autophagy.

Discussion

Related studies have shown that the incidence of dementia in PD is about 40%, and the incidence of dementia in PD is significantly higher compared with healthy people [3]. The clinical symptoms of dementia patients with PDD are mainly manifested as impairment of attention, cognitive and executive decline, memory loss, visual space destruction, etc., [13]. The typical pathological features are a sharp decline of the striatal DA level and dominance of cholinergic system activity, thus damaging the brain tissue [14]. The pathological mechanism of PDD is complex and varied, and there is no effective treatment in clinic [15]. Madopar is a compound preparation of levodopa and benserazide, and levodopa is an effective ingredient for PD treatment [16]. However, long-term use of madopar will be accompanied by drug efficacy decline, movement disorders, symptom fluctuations, and even cognitive and mental disorders [17]. In addition, memantine hydrochloride is regarded as a commonly used drug to improve the activity and cognitive function of PDD patient, mainly because it blocks the neuron damage caused by the increase of glutamate level, protects nerve cells and promotes the improvement of cognitive function. However, there are still different degrees of adverse reactions, which affect the compliance of PDD patients [18]. Since there are very few drugs for PDD treatment, it is of great clinical significance to search a natural, highly effective and low toxic anti-PDD drug.

It has been found that the alkaloids extracted from plants have significant neuropharmacological activities, which are characterized by multiple targets, less side effects, low toxicity and strong action, and are suitable for long-term stable treatment [19]. Clinical use of alkaloids in the treatment of central nervous system diseases has gradually become the mainstream, and has achieved good results, its pathogenesis is related to most of the brain neurotransmitters, nutritional factors, receptors, ion channels and other mechanisms [20]. Piperine belongs to the cinnamalamide alkaloid group. In animal models of AD, piperine has been found to have potent anti-depressant and anti-cognitive impairment effects [21]. In many studies, piperine has been shown to have anti-monoamine oxidase B effects, increasing levels of monoamine neurotransmitters [22]. In addition, piperine has been reported to protect against MPTP-induced mitochondrial dysfunction and cell death in PC12 cell models [9, 23]. However, the effects of piperine in PDD mice and the underlying mechanisms remain unknown. Therefore, this study mainly explored the effects and mechanism of piperine on PDD mice.

In the current study, the PDD mice in this study was a combination of reserpine injection plus dual vascular occlusion [24, 25]. The method of PDD model mouse is as follows: we first used reserpine to make a PD mouse, and then a common vascular dementia method, dual-vessel occlusion was operated on PDD mouse [25]. After modelling, PDD mice showed cognitive impairment and bradykinesia, which was consistent with the clinical manifestations of PDD, indicating that the model was successfully prepared [25]. In our behavioral tests, a classical approach of the open-field test was used to evaluate the anxiety-like behavior, locomotor and exploratory abilities of PDD mice in an unfamiliar environment [26]. We found that the piperine treated mice exhibited significantly less anxiety related behaviors with stronger locomotor and exploratory activities in the new environment. With regard to the pole test, the time of turning head down and total climbing time of the pole were used as an indicator to judge the coordination of PDD mice [27]. We also found that the motor coordination ability of PDD mice was better following the piperine treatment. All of these findings hinted that the piperine treatment could significantly improve the behavioral competence of PDD mice.

Additionally, abnormal DA system plays an important role in PDD progress. Dopaminergic neurons in the substantia nigra degenerate and die and dopamine synthesis decreases is characteristic lesions of PD patients [3]. α-syn is an important pathological product in the pathological process of PD, which eventually leads to the degeneration and necrosis of dopaminergic neurons [4]. Levodopa is decarboxylated into DA by DDC, and TH is the rate-limiting enzyme for DA synthesis [28]. Recent studies have found that inhibiting the activity of MAO-B can slow down the degradation and reuptake of DA in the brain, thus increasing DA level in the brain and significantly improving the clinical symptoms of PD [29]. Using this PDD mice, we found that piperine could increase TH, DDC and DA levels, and decrease α-syn level in the brain, indicating that piperine could alleviate PDD symptoms by increasing the generation and transformation of DA, however, the effect of high-dose piperine treated mice is slightly weaker than that of madopar treated mice.

Some studies suggest that induce autophagy is associated with impairments in learning and memory ability of animals after neuron injury, and inhibit autophagy can protect the damaged nerve cells and significantly improve the learning and memory function [6]. The role of Beclin-1 could be mediated by autophagy activation, resulting the formation of autophagosomes [30]. Looking at autophagy activation, LC3B is a membrane protein of autophagosomes, it can reflex a consequence of

autophagosomes and is used to evaluate the activity of autophagy relatively quantitatively [30]. p62 is an important autophagy substrate protein, and its main function is to participate in protein ubiquitination and the recycling of aging and damaged organelles [30]. Furthermore, we confirmed that high dose piperine intervention could be successfully applied on the PDD mice, in which the focal Beclin-1 and LC3B expression was suppressed and p62 was elevated, and thus could well inhibit the autophagy in line with the 3-MA intervention.

PDD is a neurodegenerative disease mostly occurring in elderly PD patients [3]. PDD patients have high levels of pro-inflammatory cytokines IL-6 and TNF-a, which enhance the immune response, stimulate the inflammatory response and accelerate the speed of cell apoptosis [31]. HSP90 is an intracellular protein with molecular chaperone and protective function, which can protect cells from apoptosis as a signal molecule of intercellular stress, the elevated HSP90 level can trigger immune response [32]. MicroRNAs (miRNAs) are small noncoding RNAs with a full length of 19–24 nucleotide fragments [33]. They mainly regulate the expression of cell genes by silencing target genes through RNA interference [33]. Recent research has revealed that the HIV-derived miR-99 can cause macrophages to produce large amounts of TNF-a inflammatory cytokines when HIV-1 infects the body [34]. We also found that piperine treated mice showed a decline of TNF-α, IL-6, HSP90 and miR-99a-5p expressions compared with the untreated PDD mice. This suggests that piperine may have an inhibitory effect on neuroinflammation, in which piperine is slightly stronger than madopar in the expression of inflammatory cytokines.

In conclusion, our study demonstrated that the PDD mouse model was successfully established by a combination of reserpine injection plus dual vascular occlusion operation. Piperine treated mice improved the locomotor and cognitive abilities of the PDD mice. The effects of piperine may be involved in the increase of DA production, the decrease of Aβ and inflammatory factors production, and autophagy inhibition in PDD mice.

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Author contributions
LH, MD and XZ participated in research design, LH provided funding, MD, YX,YC and XZ performed experiments, MD performed data analysis, LH and MD wrote the manuscript, LH and MD critically revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data presented in this published article are available from the corresponding authors on reasonable request.

Declarations

Ethical approval and constent for publication
This article does not contain any studies with human participants or animals performed by any of the authors. For this type of study formal consent is not required.

Competing interests
The authors declare no competing interest.

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