Neuroprotective effects of curcumin via autophagy induction in 6-hydroxydopamine Parkinson’s models

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ABSTRACT

Curcumin, a polyphenolic compound extracted from curcuma longa, acts as a nontoxic matter with anti-oxidant and anti-inflammatory effects as well as antiproliferative activities. Here, our research aimed to explore the neuroprotective effects of curcumin both in the 6-hydroxydopamine (6-OHDA)-lesioned rat model of Parkinson’s disease (PD) in vivo and 6-OHDA-lesioned PC12 cells in vitro. In vitro, 6-OHDA caused a distinct decrease in cell viability of PC12 cells (150 μM). With the incubation of curcumin (1 μM), 6-OHDA-induced apoptosis was suppressed, increasing the autophagy markers (LC3-II/LC3-I, Beclin-1) and inhibiting phosphor-AKT/AKT, phosphor-mTOR/mTOR. In vivo, curcumin (50 mg/kg) reduced the accumulation of α-synuclein and led to higher parkinsonian disability scores in 6-OHDA-lesioned PD rats, contributing to induction of autophagy through inhibiting AKT/mTOR signal pathway. Moreover, treatment with autophagy inhibitors, such as 3-MA and chloroquine, abolished the neuroprotective effects of curcumin as evidence by compromised autophagy and declined motor behavior in PD rats. In conclusion, the present study demonstrated that curcumin repressed PC12 cell death in vitro and improved parkinsonian disability scores in vivo by inhibiting AKT/mTOR signaling pathway which mediated by autophagy, indicating a potential value of curcumin in the therapeutic intervention of Parkinson’s disease.

1. Introduction

Parkinson disease (PD) is a widespread, age-related, second most common neurodegenerative disease (NDD), featured by progressive degeneration of dopaminergic neurons at the substantia nigra pars compacta (SNpc) (Bloem et al., 2021; Lees et al., 2009). The presence of Lewy bodies (LBs), intraneuronal and axonal inclusions composed of aggregated α-synuclein (α-syn) protein, is one of the pathological hallmarks of PD (Spillantini et al., 1998). Accumulation of α-syn has been related to damaged operating of protein degradation mechanisms (Xilouri et al., 2013). One of the main routes in the mammalian cells for the degradation of α-syn is autophagy-lysosome pathway (ALP) (Pan et al., 2008). Autophagy (Greek, “self-eating”) is an extremely conserved mechanism, through which cells transfer unessential or underlying dangerous intracytoplasmic ingredients by double-membranated structures called autophagosomes to the lysosome for degradation (Noda and Inagaki, 2015), including micro-autophagy, chaperone-mediated autophagy (CMA) and macro-autophagy (herein referred to as autophagy in this paper). Autophagy degrades cellular metabolism products like damaged mitochondria and oxidative stress protein aggregates, resulting in cytotoxicity upon accumulation, which is necessary for cellular homeostasis, organelle quality control and organisinal accommodation to environmental stress (Green et al., 2011). Piles of intracellular pathways have been implicated as contributors to PD pathogenesis.

Of note, autophagy is a cellular deterioration approach that...
participates in multifarious processes both in physiological and diseased cells (Komatsu et al., 2006), shifting the multidinous components into the lysosome for degradation (Nixon, 2013). Recently, emerging evidence indicates that autophagy is tightly associated with the pathogenesis of PD and inspections on autophagy in PD models may provide new targets for PD therapy (Cheng et al., 2020; Moors et al., 2017). Moreover, autophagy impairment has been found in the brains of PD subjects and also in vivo models of PD (Klionsky et al., 2011). Inhibition of autophagy by knocking down the indispensable autophagy gene Atg7 predisposes to PD occur in animal models, illustrating the neuroprotective role of autophagy in the development of PD (Lachenmayer and Yue, 2012). Besides, several previous researches reported that autophagy inducers alleviated PD related dopaminergic neurons damage, suggesting the underlying therapeutic effects of autophagy enhancers in PD (Decressac et al., 2013; Xiong et al., 2011). According to the preceding researches, it has been supposed that autophagy is the acquiescent backup pathway when an aggregate-prone cell substrates (e. g. damaged mitochondria) unable to be efficiently cleared by the proteasome (Iae et al., 2015). Therefore, modulating autophagy in PD animal models in which autophagy was dysregulated can help relieve the symptoms.

Curcumin, which is widely used in India, Southeast Asia and China in food preparation or for medical purposes (Aggarwal et al., 2007). The neuroprotective functions of curcumin have been established for therapeutic intervention in PD, reported in our previous research, that curcumin, as a neuroprotective compound utilized in PD models, displayed anti-inflammatory, anti-oxidant and anti-apoptosis properties, and has been shown to improve neurological functions (Wang et al., 2017). Cellular distribution of curcumin has not attracted much interest, even though curcumin fluorescence can be utilized to penetrate membranes and localize it within cells. Recently, there are several studies demonstrated the various targets of curcumin connected with PD. For example, compared with placebo group, pre or post-treatment of curcumin in 6-OHDA-lesioned rats contributed to the decline of DA neuron activity (Wang et al., 2009, 2017). It also showed a neuroprotective effect in SYSY N2 cells by attenuating the cytotoxicity of accumulated α-syn, decreasing apoptosis, reducing the intracellular reactive oxygen species (ROS) and inhibiting the activation of caspase 3, being against the α-syn-induced cytotoxicity (Yavapour-Bali et al., 2019). In addition, curcumin also has been proofed the neuroprotective effects against cerebral ischemia or traumatic brain injury (Wu et al., 2006). Excepting for the foregoing functions, Pei et al. found that curcumin suppressed hepatocellular carcinoma tumor growth through reducing GPC3-wnt-β-catenin signaling pathway, which was partially mediated by activation of autophagy (Hu et al., 2019). Zhang et al. showed that curcumin improved age-related and surgically induced osteoarthritis by promoting autophagy in mice via Akt/mTOR signaling pathway (Zhang et al., 2018). Furthermore, one study suggested that elevated lung cancer cell autophagy competence abduced by curcumin even induced autophagic death, indicating an underlying tumor treatment method (Zhang et al., 2010).

Based on the aforementioned researches, curcumin played a vital role in the process of autophagy. Of note, low doses of natural chemical products (e.g. curcumin) often activate an adaptive stress response, whereas high doses initiate an opposite response leading to cell death, which is often referred to as hormesis. At higher doses of curcumin (>15 μM), cells undergo mitochondrial destabilization due to calcium release from the endoplasmic reticulum and reduce the acetylation of cytoplasmic proteins, and finally die (Rainey et al., 2020). However, to best of our knowledge, there was no research that ever reported the neuroprotective effects of autophagy, which was induced by curcumin, in 6-OHDA-lesioned PD PC12 cell and rats models. Thus, in the present study, we attempt to investigate the therapeutic effect of curcumin in a rat model of 6-OHDA-lesioned PD by elucidating its impact on autophagy so as to provide new ideas for the treatment of PD.

2. Materials and methods

2.1. Herbal materials and reagents

Curcumin and 6-OHDA were obtained from Sigma-Aldrich and dissolved in corn oil or saline containing 0.2% ascorbic acid, respectively. Apomorphine hydrochloride was purchased from Wako Co. Ltd and also dissolved in saline with 0.2% ascorbic acid.

2.2. Cell culture and treatment

Rat adrenal pheochromocytoma PC12 cells were provided by Stem Cell Bank of the Chinese Academy of Sciences, maintained in DMEM (Gibco), 10% fetal bovine serum (FBS, Gibco), supplemented with 1% antibiotic (Gibco), cultured in a constant humidified incubator with 5% CO2 at 37 °C. PC12 cells were treated with 6-OHDA (0, 25, 50, 100, 150 and 200 μM) for 24h to induce the model of PD. To evaluate the neuroprotective effects and find appropriate concentration of curcumin on PD in vitro, we pretreated with curcumin dissolve in DMSO (0.5, 1.0, 2.0, 5.0 and 10.0 μM) for 6h and then co-operated with the appropriate 6-OHDA concentration for 24h.

2.3. Determination of cell viability and oxidative stress levels

Cell viabilities were tested by CCK8 (Beyotime, China). PC12 cells were seeded in a 96-well plate at a roughly density of 5 × 103 cells per well. After treatment, cells were incubated with CCK8 for 4h at 37 °C. Then optical density was measured by spectrophotometry at 450 nm. The viability indicated as absorbance, or as a percentage of viable cells from the total number of cells in certain well. Finally, the values were expressed as percentage of control. MDA and ROS levels were determined using a corresponding commercial kit (Beyotime, China) according to the manufacturer’s instructions. Protein concentration of the sample was determined using a BCA protein assay kit (catalog no.P0012; Beyotime, China).

2.4. Animals and treatment

Adult male Sprague-Dawley (SD) rats were used in this study (weight: 180–250g), which were randomly distributed between the experimental groups. Curcumin dissolved in vegetable oil daily was intragastric once daily for 2 week (Curcumin-L group; 25 mg/kg; Curcumin-H group; 50 mg/kg) upon the PD rats model success. After one week of Curcumin injection, 3-MA (10 mg/kg), chloroquine (40 mg/kg), a kind of autophagy inhibitor, were respectively injected into the abdominal cavity half an hour before the administration of curcumin initiate from the second week. Several studies displayed that 3-MA and chloroquine could across the Blood-Brain Barrier (BBB), play a role as autophagy inhibitors (Tang et al., 2014; Vodicka et al., 2014). The dose of curcumin was based on some previous publication papers (Song et al., 2016; Yang et al., 2014).

2.5. Induction of 6-OHDA-lesioned PD model and behavior test

The procedure of the PD model according to our previous papers (Wang et al., 2019; Zhang et al., 2019b). To assess general motor behavior, a quantitative assessment of locomotor activity using forelimb functional test (FFT) was performed every day for one week, which was performed as our previous study (Wang et al., 2019). During a period of 60 min following curcumin or saline treatment, we counted the number of time that the right and the left forepaws of the rats contacted the weight-bearing walls every 20 min (Schallert et al., 2000). Moreover,
Apomorphine (0.5 mg/kg, i.p.) test was also carried out for one week and rotations were quantified for half an hour following injection. Meanwhile, to evaluate the motor incoordination, we carried out the rotarod test. Rats were placed on the rotating rod and given 30s to adapt it, then started the instrument. The rats were crawled on the rotating rod for 90s at a rate of 5 rpm, then the acceleration function was activated. The time from the start of the acceleration to the time of the fall was the score of the rats. Each rat was tested 3 times with an interval of 15 min. Before the formal test, continuous training is required for 3 days.

2.6. Immunoblot analysis and immunofluorescence (IFC)

The next day after the behavior tests, immunoblot analysis was performed based on our published papers (Wang et al., 2018; Zhang et al., 2019a). The primary antibodies used in this paper as fellows: polyclonal rabbit anti-Tyrosine Hydroxylase antibody (1:1000; Millipore), polyclonal rabbit anti-LC3 antibody (1:1000; Abcam), monoclonal rabbit anti-a-syn (1:1000; CST), monoclonal rabbit anti-Beclin1 (1:1000; CST), monoclonal rabbit anti-p62 (1:1000; CST) and polyclonal rabbit anti-LAMP1 (1:1000; Sigma-Aldrich) and anti-GAPDH antibody (1:1000; Bioworld Technology), anti-mTOR (1:1000; CST), anti-p-mTOR (1:1000; CST), anti-AKT (1:1000, CST), anti-p-AKT (1:1000, CST). The detailed protocol of IFC was shown in our previous published papers (Wang et al., 2019; Xie et al., 2014). Slices were incubated with primary antibodies for anti-Tyrosine Hydroxylase antibody (1:200) at 4 °C overnight in a humidified chamber. After rewarming for 45 min at 37 °C and washing in Phosphate-buffered saline (PBS) for 3 times, sections were incubated in secondary goat anti-rabbit antibody for 1 h, and then colorized in DAB mixed solution.

Fig. 1. Neuroprotective effect of curcumin on neurotoxic chemical-induced injury in PC12 cells. Effects of different concentration of 6-OHDA on PC12 cells viability. The cells were incubated with 6-OHDA for 4h (A), 8h (B), 12h (C), 16h (D), 20h (E) and 24h (F); 6-OHDA decreased the cell viability as a dose and time dependent manner. (G) After pretreated with different concentration of curcumin, the cells were incubated with 6-OHDA (150 μM) and different concentration of curcumin for 24h. Cell viability assessed using the CCK8 assay. Assessment of the oxidative markers with or without curcumin treatment, such as MDA (H) and ROS (I). Data are expressed as mean ± SEM. n = 5-6 wells for each group. *P < 0.05 compared to the control, ***P < 0.0001 compared to the control, #P < 0.05 compared to the 6-OHDA (150 μM) treated group, ###P < 0.001 compared to the 6-OHDA (150 μM) treated group (ANOVA followed by Bonferroni multiple-comparison post hoc tests).
2.7. Transmission electron microscope (TEM)

All rats were decapitated under 1% pentobarbital sodium, and their brains were collected quickly. According to the stereotactic map, found the substantia nigra in the midbrain, cut into smaller pieces with less than 1mm³ with sharp blades. Then place the brain in the electron microscope fixing solution at 4 °C for 2–4h. After fixation, 1% agarose was wrapped, and 0.1M phosphate buffer PB (PH 7.4) was rinsed 3 times for 15 min each time. 1% citric acid and 0.1M PB (PH 7.4) were fixed at room temperature (20 °C) for 2h. 0.1M PB (PH 7.4) was rinsed 3 times, each time for 15 min. The tissue was sequentially dehydrated by 50%–70%-80%-90%-95%-100% alcohol-100% acetone-100% acetone for 15 min each time. Staining of uranium lead and the sections were dried overnight at room temperature. Finally, observation under transmission electron microscope.

2.8. Statistical analysis

All descriptive results in this paper are expressed as group means ± s.e.m. A two-way analysis of variance ANOVA was used to analyze behavioral data followed by Bonferroni multiple-comparison post hoc tests. Neurochemical data conformed to normal distribution were analyzed by one-way ANOVA followed by Bonferroni multiple-comparison post hoc tests when appropriate. Data were analyzed using Graphed Prism or SPSS 17.0 with correction p values < 0.05 considered significant.

3. Results

3.1. Neuroprotective effect of curcumin on neurotoxic chemical-induced injury in PC12 cells

PC12 cells were treated with 6-OHDA at various concentrations (25, 50, 100, 150 and 200 μM) and different times (4, 8, 12, 16, 20, 24 h). We found that 6-OHDA treatment leaded to a significant decline in cell viability at 150 μM for 24 h (Fig. 1A–F). The survival ratio of PC12 cells treated with 6-OHDA (150 μM) was 60.28 ± 2.46% compared to the control cells (P < 0.01). Meanwhile, PC12 cells were pre-treated with curcumin (0.5, 1, 2, 5 and 10 μM) for 6 h followed by the addition of 6-OHDA for an additional 24 h. The survival ratio of cells pre-treated with curcumin increased obviously at a dose of 1 and 2 μM compared with 6-OHDA only treated cells (124.32 ± 6.56%, 126.61 ± 7.72%, respectively, Fig. 1G), as well as no toxic effects of curcumin in the control group up to 10 μM. Strangely, pre-treatment with 5 and 10 μM curcumin did not attenuate cell injury, indicating the protective effect of curcumin on 6-OHDA-induced injury in PC12 cell is not dose-dependent. Of note,
pre-treatment with curcumin (1 μM) could obviously reduce the oxidative stress levels in terms of ROS and MDA (Fig. 1H and I).

### 3.2. Pharmacological induction of autophagy by curcumin in PC12 cells

To determine whether curcumin has the autophagic effect on PC12 cells, the cells were pretreated with curcumin (1 μM) for 6 h. An immunoblot assay revealed that the conversion ratio of LC3-II to LC3-I was lower in cells treated with 6-OHDA compared to control group but no statistic difference, and the cells treated by curcumin obviously increase the ratio compared to 6-OHDA lesioned cells (p < 0.01, Fig. 2A), which is parallel to previous results. Moreover, the level of autophagy marker Beclin-1 was also obviously increased by curcumin administration when compared to PD groups (Fig. 2B). Immunoblotting was next

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**Fig. 3.** Curcumin improved the neurochemical and parkinsonian disability scores in 6-OHDA-leisioned PD rats. (A) Schema of the two parts experiments: at the beginning of the studies, the rats were injected unilaterally with 6-OHDA in the right medial forebrain bundle (MFB). After two weeks, lesion efficacy was assessed by the rotations after the use of apomorphine for the following intraperitoneal injection of curcumin-L (25 mg/kg) and curcumin-H (50 mg/kg) for two weeks. Rats were rated for contralateral rotation (B), forelimb function (C) and rotarod test (D) on days 1, 2, 3, 4, 5, 6 and 7. (E) The protein level of α-synuclein/GAPDH in the lesioned striatum; (F) The protein level of TH in the lesioned striatum (n = 4*4 = 16); (G) Extent of the dopaminergic denervation induced by 6-OHDA lesions in each group in the striatum (4X, 6 random fields to obtain the figures, n = 4*2 = 8), Scale bar is 100 μm. Data are expressed as Mean ± SEM. n = 4 for each group. *P < 0.05 compared to PD group; ****P < 0.0001 compared to PD group (two-way ANOVA was used to analyze behavioral data followed by Bonferroni multiple-comparison post hoc tests).
performed to analyze the protein expressions of phosphor-Akt(ser473)/pan-Akt, phosphor-mTOR(ser2448)/pan-mTOR. Expression analysis demonstrated that curcumin treatment prompted a significant reduction in phosphor-AKT and phosphor-mTOR in comparison to the 6-OHDA lesioned cells (Fig. 2C and D). These findings indicated that inducing autophagy levels in relation to curcumin administration could result from the inhibition of AKT/mTOR signal.

3.3. Curcumin improved the neurochemical and parkinsonian disability score in 6-OHDA-lesioned PD rats

In vivo, we tested the possible neuroprotective effects of curcumin in 6-OHDA-lesioned PD model. Fig. 3A shows the schema of the experiments. We found that curcumin obviously reduced contralateral rotation in 6-OHDA-lesioned rats in all time points (F5,20 = 23.45, p < 0.01,
Fig. 3B). With regards to the curcumin-L (25 mg/kg) and curcumin-H
groups (50 mg/kg), we found that the protective effect of curcumin on
contralateral rotation in PD rats was dose-dependent. Parallely, as
depicted in Fig. 3C, forelimb function score was obviously ameliorated
by curcumin treatment (F2,110 = 3.26, p < 0.01). In addition, it pro-
moted the number of lesioned forelimbs utilized versus PD rats after
the second day. Meanwhile, the improvement in forelimb function was more
apparent in the curcumin-H than curcumin-L group (p < 0.01, Fig. 3C).
In terms of rotarod test, curcumin-H treatment could increase the time
on the rotarod after the third day injection compared with PD rats
(F4,10 = 36.76, p < 0.01, Fig. 3D). However, there was no difference
between the curcumin-L and PD group (p > 0.01, Fig. 3D), indicating
low dose of curcumin (25 mg/kg) did not influence the rotarod test
performance. In brief, based on the behavior results, the 6-OHDA-
induced parkinsonian disability scores were significantly reversed by
the curcumin daily treatment. Furthermore, western blotting analysis
demonstrating that there was a distinct increase the level of a-syn in the
PD rats in striatum probably owing to the dysfunction of autophagy, and
the expression of a-syn was significantly reduced after curcumin
administration by 29% in curcumin-L group, 36% in curcumin-H group
compared with PD group (n = 4 for each group, p < 0.01, Fig. 3E).
Simultaneously, Tyrosine hydroxylase (TH) protein levels and positive
neurons in the striatum were dramatically decreased by nearly 60%–
80% in the PD or curcumin groups (n = 4 for each group, p < 0.05,
Fig. 3F and G). Interestingly, no obvious changes were observed in terms
of TH levels between PD and curcumin rats. Therefore, We speculated
the potential reason, at least in part, resulted from too many dopami-
nergic neurons death induced by the 6-OHDA.

3.4. Autophagy-mediated neuroprotection by curcumin in PD rats

To determine the induction of curcumin on neuronal autophagy,
LC3-II/LC3-I, Beclin-1 and p62, as autophagic flux indexes, were
measured in striatum by Western blot. We found that treated with
curcumin caused a significant upregulation of LC3-II/LC3-I ratio, Beclin-1
and LAMP-1 levels in the PD plus curcumin group (p < 0.05,
Fig. 4A–C). Furthermore, induction of autophagy was much more
remarkable in curcumin-H group (50 mg/kg) than curcumin-L group
(25 mg/kg) according to the aforementioned protein levels, indicating
exist a dose-dependent. To determine more accurate quantification of
curcumin effects on autophagy process, we next examined levels of p62
in the cytosolic fraction. We detected that p62 expression increased in
PD group, suggesting that autophagic influx was impaired by 6-OHDA
injection. Treatment with curcumin could reduce p62 expression in the
curcumin group (p < 0.05, Fig. 4D). As shown in Fig. 4E, a significant
increase both in autophagosome and autolysosome were exhibited in the
curcumin group compared with that of control group and PD group (p <
0.01). All the controls in Figs. 2–4 are the sham groups, not the
contralateral side.

3.5. The effect of curcumin in PD rats was offsetted by autophagy
inhibitors

As shown in Fig. 5A, curcumin (50 mg/kg) overly reduced contra-
lateral rotation compared with PD group, which was fully reversed by
an autophagy inhibitor 3-MA (F4,110 = 120.35, p < 0.05, Fig. 5A). In
addition, the effects of curcumin in forelimb functional performance
and rotarod test were also abolished by 3-MA (F8,20 = 12.43, p < 0.05,
Fig. 5B; F6,10 = 36.35, p < 0.05, Fig. 5C, respectively). These findings
illustrating that 3-MA could abolish the neuroprotective effects of cur-
cumin in PD rats. We further utilized another autophagy inhibitor
chloroquine to repeat aforementioned tests. Similarly, we found chlo-
roquine could reverse the function of curcumin as 3-MA in terms of
contralateral rotation, forelimb functional and rotarod test (p < 0.05,
Fig. 5D–F). Together, these data indicated that curcumin improved the
impairment of autophagy induced by 6-OHDA and played a significant
role in neuroprotective effects in PD rats. Meanwhile, examination of the
infected midbrain revealed that 3-MA could counterbalance the effect of
curcumin on the levels of LC3-II/LC3-I and Beclin-1 (n = 4 for each
group, p < 0.05, Fig. 5G and H) as well as increasing expression of p62
(p < 0.05, Fig. 5I). In parallel, chloroquine existed the similar effect to
the 3-MA, namely decreased the LC3-II/LC3-I and Beclin-1 (n = 4 for
each group, p < 0.05, Fig. 5J and K) and also unregulated p62 expres-
sions (p < 0.01, Fig. 5L).

3.6. AKT/mTOR pathways were involved in curcumin-induced
neuroprotection in PD rats

Up to date, it is still unclear whether AKT/mTOR pathway is
participated in curcumin-mediated neuroprotection in PD rats. There-
fore, we assessed the level of p-AKT and mTOR by Western blot in PD
striatum (Fig. 5M–N). We found curcumin (50 mg/kg) significantly
reduced p-AKT and mTOR levels in PD striatum (p < 0.05, Fig. 5M–N)
when compared with sham group. Based on the existent research, AKT
and mTOR inhibition is supposed to trigger autophagy, our results
indicated curcumin-induced autophagy was dependent on AKT/mTOR
pathway inhibition. Meanwhile, we found that treatment with auto-
phagy inhibitors 3-MA and chloroquine both abolished the inhibition of
AKT/mTOR, compared with curcumin group (p < 0.05, Fig. 5G and H).
Hence, these data indicated AKT/mTOR signaling are probably involved
in curcumin-induced neuroprotection. We also summarized the working
model of curcumin in the 6-OHDA-lesioned PD models in Fig. 6.

4. Discussion

The present research targeted to investigate the neuroprotective ef-
ects of curcumin in the PD models and possible underlying mechanisms
related to autophagy. Our results displayed that in vitro pretreatment 6
h with curcumin (1 μM) alleviated cellular injury and death ratio
induced by 6-OHDA in PC12 cells, which are well established PD-related
toxins. Meanwhile, we initially revealed that activation of autophagy
through inhibiting Akt/mTOR pathway probably contributes to the
neuroprotective effects of curcumin in the cellular PD model. Moreover,
consistent with the findings in vitro that curcumin (50 mg/kg) could
improve α-syn toxicity and parkinsonian disability scores in 6-OHDA-
lesioned PD rats, such effects can be abolished by autophagy inhibi-
tors either 3-MA or chloroquine. Curcumin administration decreased
α-syn expression, but there was no improvement in TH activity.
One potential explanation is that curcumin was administrated after 3
weeks of 6-OHDA infusion, at this stage, majority of the dopami-
nergic neurons have already lost, unable to regenerate. Interestingly, we
found that curcumin supplementation substantially increased LC3-II/
LC3-I, Beclin-1 and reduced p62 protein expression, indicating auto-
phagy played an active role in PD. Similarly, such neuroprotection also
worked via modulating Akt/mTOR pathway in vivo. This report
demonstrated the protective effects of curcumin against damage in both
the 6-OHDA-lesioned PD rats and PC12 cells through induction of
autophagy. In this study, we chose the PC12 cells as a PD model. PC12
cells are made as a cell line from an induced, transplantable rat pheo-
chromocytoma, could be differentiated into neuronal-like cells. It is
observed that when maintained in growth medium they could synthet-
size, store, and release catecholamines. The capacity to undergo
neuronal differentiation under nerve growth factor is a useful and vital
trait for PD studies. Among potential advantages of this, one for PD
research is that the neuronally differentiated cells directly model sym-
pathetic neurons, which are one of the neuron types affected by the
disease (Malagelada and Greene, 2008). All these features make PC12
cells widely used to model PD.

Of note, curcumin has been proven that can cross the blood brain
barrier (BBB) (Maiti and Dunbar, 2018). However, one major obstacle to
curcumin’s clinical efficacy is its poor bioavailability (Permeability rates
(Pe) is 0.60 ± 0.67 × 10-6 cm/s) (Lee et al., 2019). In order to develop
new curcumin formulations with stronger bioavailability and various tissues distribution, there exists plenty of derivative of curcumin displaying clinical perspective, such as liposomal curcumin, curcumin nanoparticles, and curcumin phospholipid complexes (Lopresti, 2018). Nevertheless, one shortage of this experiment was that we did not test the curcumin across the BBB to function. Two standard experimental measures of BBB permeability are logBB (the concentration of drug in the brain divided by concentration in the blood) and logPS (permeability surface-area product). In addition, a battery of drug-sensitive central nervous system (CNS) tests, called NeuroCart, can also be utilized to test whether the compound reaches its intended target in the CNS or not (Groeneveld et al., 2016). With continual increases in computer power and improvements in molecular simulations, silico methods may provide viable alternatives. Using Molecular Dynamics Simulations is also a method to predict B permeability of Drug-Like Compounds (Carpenter et al., 2014).

Interestingly, α-syn overexpression inhibits autophagy at a very early stage of autophagosome formation, hence RNAi depletion of α-syn increases autophagy (Winslow et al., 2010). Furthermore, mutant α-syn directly couples TFEB and holds back its nuclear translocation in vivo model of PD, cutting down the transcriptional level of TFEB-dependent autophagy-lysosome pathway (Decressac et al., 2013). Recently, Han and colleagues reported that small molecule Ka promoted neuroinflammatory inhibition via the induction of autophagy for the treatment of PD. Knockdown of Atg5 or autophagy inhibitor significantly inhibited the Ka-mediated NLRP3 inflammasome inhibition and deteriorated the phenotype of PD (Han et al., 2019). These findings demonstrated the autophagy dysfunction in PD. In support of this assertion, in this study, we found that the level of autophagy marker Beclin-1 and the ratio of LC3-I to LC3-II were lower (but no statistic difference) in PD group compared with sham group in vivo and in vitro, and curcumin could obviously reverse such conditions. However, numerous studies have shown that such neurotoxins as MPP+ and 6-OHDA can increase mitophagy and autophagy in cultured cells. Possible reasons includes different autophagy stage exists individual markers expression levels. At the late autophagy stage, the LC3-II and Beclin1 can be incorporated into autophagosomes and then degraded by the lysosome, leading to the reduction of the contents. Moreover, the levels of the LC3- II/LC3- I and Beclin1 at this time point by Western blot are unable to draw the conclusion that 6-OHDA inhibits the autophagy. Both our results and previous studies demonstrated that autophagy dysfunction is a hallmark pathology involved in PD pathogenesis. However, one limitation in this part is that detecting autophagy flux (refers to the entire dynamic process of autophagy) is hard to be achieved by this method. Mammalian LC3 is an autophagosome marker that is processed by Atg4 at its C-terminus to become LC3-I. LC3-I is lipidoside through PE conjugation to form LC3-II. Monitoring the conversion of LC3-I to LC3-II by change in molecular weight is a common method to monitor changes in the autophagic pathway. There are several methods for monitoring autophagosome formation and autophagic flux. For example, the most common method would likely be the treatment with Bafilomycin A1 and subsequent western blotting (Baeken et al., 2021). Moreover, using the number of autophagosomes determined by GFP-LC3 localization is a common method for measuring autophagic activity and measuring the protein levels through Western blot. However, each method has its limitations and more than one method should be used to validate autophagic activity.

In terms of initiation of autophagy, the initial vesicles are derived from a variety of membrane sources, including endoplasmic reticulum, the Golgi, mitochondria and certain endosomal intermediates et al. (Hailey et al., 2010). Nutrient deprivation, growth factor depletion, and/or low cellular energy levels are well established primordial autophagy inducers, which mediated mTORC1 and AKT inhibition (Hurley and Young, 2017). Recent data have demonstrated that dysregulation of mTOR is implicated in the pathogenesis of PD (Lan et al., 2017). For instance, heterogeneous deletion of En1 in PD mouse model, which is vital for the survival of dopaminergic neurons, results in up-regulation of mTOR signaling in dopaminergic neurons (Nordstroma et al., 2015). Malagelada et al. reported that elevated REDD1 expression, as an inhibitor of mTOR, suppresses mTOR signaling while is neuroprotective in cellular and animal models of PD (Malagelada et al., 2010). Additionally, accumulating evidence suggested that inhibition of mTOR with rapamycin or its derivatives could exert a neuroprotective effect in cross-species PD models (Maihese, 2016). Collectively, these findings are also supported by our present study showing that curcumin could obviously downregulate the phosphor-AKT or phosphor-mTOR levels. The next question is whether curcumin directly interacts with any of the participants in the PI3K/Akt/mTOR/p70S6K signaling cascade or it may have some additional partner/target upstream of this pathway. To our knowledge, curcumin induced autophagy by upregulating PS3 and P21, inhibiting PI3K/Akt/mTOR, as well as via a plenty of signaling pathways, including AMPK, MAPK/ERK1/2, Bcl-2 signaling cascade, and Rab GTPase network (Shakeri et al., 2019).
In conclusion, the present study demonstrated that curcumin reduced PC12 cell death in vitro and improved parkinsonian disability score in vivo probably by inhibiting AKT/mTOR signaling, which might be mediated by autophagy.

Author contributions
XZR and LLC carried out the experiments and analyzed the data. XX and STZ made substantial contributions to conception and design and replenished the required data. CLX and HJH involved in drafting the manuscript. HJH and XMZ helped to revise the paper.

Ethics statement
The whole animal experiments carried out on the basis of the Guide for the Care and Use of Laboratory Animals and also approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University.

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Declaration of competing interest
No commercial or financial relationships that could be construed as a potential conflict of interest.

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