Original Paper

Protective Effect of Curcumin Against Oxidative Stress-Induced Injury in Rats with Parkinson's Disease Through the Wnt/ β -Catenin Signaling Pathway

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Key Words

Curcumin • Wnt/ β -catenin signaling pathway • Parkinson's disease • Oxidative stress-induced injury • Dickkopf-1 • Tyrosine hydroxylase • Dopamine transporter • Glial fibrillary acidic protein

Abstract

Background/Aims: The study aimed to investigate the protective effect of curcumin against oxidative stress-induced injury of Parkinson's disease (PD) through the Wnt/ β -catenin signaling pathway in rats. **Methods:** The successfully established PD rat models and normal healthy rats were randomly assigned into the 6-hydroxydopamine (6-OHDA), the curcumin (Cur) and the control groups. Immunohistochemistry was used to detect the positive expression of tyrosine hydroxylase (TH), dopamine transporter (DAT) and glial fibrillary acidic protein (GFAP). Deutocerebrum primary cells were extracted and classified into the control, 6-OHDA, Cur (5, 10, 15 μ mol/L), Dickkopf-1 (DKK-1) and Cur + DKK-1 groups. MTT assays, adhesion tests and TUNEL staining were used to assess cell viability, adhesion and apoptosis, respectively. Western blotting and qRT-PCR were used to examine the protein and mRNA expressions of Wnt3a and β -catenin and the c-myc and cyclinD1 mRNA expressions. **Results:** TH and DAT expressions in the Cur group were elevated and GFAP was reduced compared with the 6-OHDA group. Curcumin enhanced viability, survival and adhesion and attenuated apoptosis of deutocerebrum primary cells by activating the Wnt/ β -catenin signaling pathway. Higher Wnt3a and β -catenin mRNA and protein expressions and c-myc and cyclinD1 mRNA expressions, enhanced superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) contents, decreased malondialdehyde (MDA) content and elevated mitochondrial membrane potential ($\Delta\psi_m$) were found in the 10 and 15 μ mol/L Cur groups compared with the 6-OHDA group. However, opposite tendencies were found in the Cur + DKK-1 group compared to the 10 μ mol/L Cur group. **Conclusion:** This study suggests that curcumin could protect against oxidative stress-induced injury in PD rats *via* the Wnt/ β -catenin signaling pathway.

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Introduction

Parkinson's disease (PD) is a neurodegenerative disorder involving progressive loss of dopaminergic neurons in the substantia nigra pars compacta and the striatum [1]. Currently, PD affects approximately 1% of the population over 65 years old [2]. The clinical symptoms for PD are as follows: rigidity, bradykinesia, resting tremor, and postural instability [3]. Thus far, available PD therapies involve both surgical treatment and pharmacotherapeutic approaches [4-6]. Recently, the role of oxidative stress has attracted increasing attention in neurological abnormalities [7, 8]. An increasing body of evidence has revealed that oxidative stress may be a crucial contributor to the occurrence of other neurodegenerative diseases, such as Alzheimer disease and amyotrophic lateral sclerosis [9, 10]. In addition, oxidative stress is believed to be associated with PD pathogenesis [11].

Curcumin [1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-hepta-diene-3, 5-dione] is a polyphenolic flavonoid and a major coloring agent extracted from *Curcuma longa* [12]. Additionally, it is a common dietary spice that is frequently used in Indian medicine and cuisine [4]. Medically, curcumin has been applied in some cultures for the treatment of diseases correlated with inflammation as well as oxidative stress [13]. Previous evidence has revealed that curcumin possesses multiple properties, such as anticancer, iron-chelating, anti-inflammatory, antioxidant, and neuroprotective activities [14]. For example, curcumin reversed histological, neurochemical and immunohistochemical alterations in global brain ischemia [15]. In addition, curcumin protected against alcohol-induced neurodegeneration in the hippocampal area [16]. Furthermore, curcumin may stimulate neurogenesis and prevent focal cerebral ischemia reperfusion injury [17]. However, the specific underlying mechanism remains unclear.

The wingless-type mouse mammary tumor virus integration site (Wnt) pathway is a critical signaling cascade that modulates various processes in adult and developing tissues, such as neuronal survival, differentiation, axonal extension, neurogenesis, neurotrophin transcription, synapse formation and plasticity, and neuroprotection [18]. These results indicate that the Wnt/ β -catenin signaling cascade plays an essential role in the development of adult nervous system [19]. Wnt/ β -catenin signaling is also critically associated with the regulation of adult neurogenesis [20]. Thus, our study aimed to evaluate whether curcumin protects against oxidative stress-induced injury *via* activation of the Wnt/ β -catenin signaling pathway in a rat model of PD.

Materials and Methods

Animals and Grouping

A total of 40 adult male Sprague-Dawley (SD) rats (Beijing Weitong Lihua Experimental Animal Technology Co., Ltd., Beijing, China) weighing 200 ~ 220 g were obtained and unilaterally injured with 6-hydroxydopamine (6-OHDA) in the left striatum for the establishment of the PD rat model. In total, 28 rat PD models were successfully established, with a successful model rate of 70%. Among the 28 rats, 20 were selected and equally assigned into the model group (6-OHDA group) and the curcumin group (Cur group, 10 μ mol/L), with 10 in each group. Another 10 normal rats were used as the control group. After grouping, the rats were immediately administered treatments. The rats in the control and 6-OHDA groups received 2 mL distilled water by intragastric administration, and the rats in the Cur group received an equal volume of 10 μ mol/L curcumin intragastrically. The treatment was given once each day for 3 consecutive weeks. The experimental rats were all housed in specific pathogen-free (SPF) conditions with free access to food and water. The temperature was controlled at 22 $\pm$ 1°C with a 12-h light/dark cycle and 40 ~ 50% relative humidity. All the animals in the experiment were treated in strict accordance with the Declaration of Helsinki and animal ethics standards. The study was approved by Animal Ethics Committee.

Establishment of the PD Rat Model

The SD rats were anaesthetized with a 40 mg/kg intraperitoneal injection of pentobarbital sodium (P3761, Sigma-Aldrich Chemical Company, St Louis MO, USA). The head was mounted in a stereotaxic frame.

Then, the skin was sliced to expose the anterior fontanel. Based on the Paxinos and Watson brain stereotaxic atlas [21], the substantia nigra compacta and striatum in the left unilateral region were measured with the coordinates as anterior 1.0 mm, left-lateral 3.0 mm, and below-dura 4.0/5.0 mm. The two regions were both injected with 8 μ g 6-OHDA (DG21094, Sigma-Aldrich Chemical Company, St Louis MO, USA) (dissolved in 4 μ L normal saline containing 0.2% ascorbic acid) with an injection speed of 1 μ L/min. The needle was retained for 10 min and withdrawn slowly for 2 ~ 3 min. The cut was conventionally closed after surgery. After model establishment of 7 days, the rats' behaviors were examined. The rats received 0.5 mg/kg intraperitoneal injection of apomorphine (A1147, Sigma-Aldrich Chemical Company, St Louis MO, USA) for rotational behaviors. A stable and constant left-side rotation of over 210 r within 30 min was considered a successful model establishment.

Rotation and Rotarod Experiments

Rotation experiment [22]: the rotational behavior in each group was observed within the 3rd to 5th week before and after treatment. The rotation induced by apomorphine was observed for 30 min once for 2 ~ 3 times. The final index was obtained as the average rotation number within 30 min.

Rotarod experiment [23]: the rotational behavior in each group was observed within the 3rd to 5th week before and after medication. The rotarod experiment was extensively applied to determine the motor coordination. The animals were required to maintain balance in constant motion on the rod, whose diameter was 6 cm and rotation speed was 20 r/min. After a pilot trial of three times, the operation began, and the data were formally recorded. When the rotation rod was set in motion, the rats were placed upon the rod and time was kept. The first falling time and the falling number within 5 min were recorded to reflect the motion coordination. The experiment was conducted 3 consecutive times. The mean value was collected at an interval of 5 min.

Immunohistochemistry

Five rats in each group were treated with an injection of 3% pentobarbital sodium (50 mg/kg, ig) for anesthesia. Following treatment, thoracotomy and arterial cannulation were performed. After a wash with normal saline, 0.1 mol/L phosphate buffer solution (PBS, pH 7.2 ~ 7.4) containing 0.04% paraformaldehyde was injected. The tissues were fixed before the deutocerebrum was severed and immersed in 0.1 mol/L PBS (containing 20% sucrose) at 4°C overnight. On the following day, the ventral midbrain was sliced for frozen coronary serial sections of 20 μ m thickness. The sections were first incubated at 4°C overnight with the primary antibodies [tyrosine hydroxylase (TH), clone TH-16, 1: 500, batch No., T2928, Sigma-Aldrich Chemical Company, St Louis MO, USA; dopamine transporter (DAT), clone CL3123, 1: 200, batch No., AMAB91125, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; glial fibrillary acidic protein (GFAP), clone SP78, 1: 400, batch No., SAB5500113, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA]; then, the sections were reacted with the biotinylated goat anti-mouse IgG (1: 300) secondary antibody for 1 h and with the streptavidin-biotin complex marked by horseradish peroxidase [avidin-biotin-peroxidase complex (ABC), batch No., E2886, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China] for 2 h. Eventually, the buffer solution containing 3, 3'-diaminobenzidine (DAB, batch No., SP-9000-D, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) was used for coloration.

Counting methods of neurons: Five images of the brain slices of rats were randomly selected. Four non-overlapping high-power fields (Biological microscope, model No., CX31-12C04, Olympus Optical Co., Ltd., Tokyo, Japan) were selected in the middle of the intensive region of positive cells on the right substantia nigra (SN) of the right side of the pictures. The TH-, DAT- and GFAP-positive neurons were calculated.

Deutocerebrum Primary Cell Treatment and Grouping

Adult male SD rats (Beijing Weitong Lihua Experimental Animal Technology Co., Ltd., Beijing, China) weighing 200 ~ 220 g were selected, conventionally anesthetized and sterilized. The brain ventral region was obtained from the medium. After removal of the enveloping membrane, the remainder was washed with PBS. Then, 4 mL 0.25% trypsin (Batch No., P7545, Sigma-Aldrich Chemical Company, St Louis MO, USA) was added and absorbed at 37°C for 15 min, which was terminated with Dulbecco's modified Eagle's medium (DMEM, 19-0040, Beijing Tianenze Biotech company, Beijing, China) containing 10% fetal bovine serum (FBS). The supernatant was discarded after centrifugation at 1000 r/min at low temperature for 10 min. The DMEM was added, and the tissue was absorbed and stricken to mix with a dropper until the cells

were completely separated. The cells were re-centrifuged for 5 min and adjusted to 6×10^5 /mL with DMEM. The collected cell suspension was added into the 24-well plate enveloped with polylysine of 0.05 mg/mL (Batch No., 25988-63-0, Sigma-Aldrich Chemical Company, St Louis MO, USA). Each group was assigned 3 replicates. The cells were eventually cultured in a constant-temperature incubator in a 5% CO₂ atmosphere at 37°C. The cell adherence and growth were observed every day with a medium replacement every other day. Primary cells in the control group were processed with no treatment. Primary cells in the 6-OHDA group were treated with 6-OHDA. The Cur group was subdivided into 3 groups based on the different concentration of curcumin (5, 10, and 15 μmol/L). Primary cells in the dickkopf-1 (DKK-1) group were treated with 50 μmol/L DKK-1. Primary cells in the Cur + DKK-1 group were treated with 10 μmol/L Cur and 50 μmol/L DKK-1. On the 5th day of culture, the Cur group was treated with solution containing curcumin at 5, 10, and 15 μmol/L, the DKK-1 group with solution containing DKK-1 at 50 μmol/L, and the Cur + DKK-1 group with solution containing both curcumin at 10 μmol/L and DKK-1 at 50 μmol/L. The solution was replaced every day for 5 consecutive days. The control group and the 6-OHDA group contained no curcumin, while other culture conditions were maintained identical to those of the Cur group. On the 10th day, each group except the control group was treated with 100 μmol/L 6-OHDA for a 2-h reaction. The PD rat model *in vitro* was established. The cells were collected for future use after 10-day culture. The experiment was repeated 3 times, and the mean value was collected for the final results.

Cell Viability

A volume of 1 mL of 6×10^5 /mL cells were seeded on the 24-well plate. On the 10th day of culture, 50 μmol/L 6-OHDA was added for a 2-h reaction. The cells were washed with PBS three times before the medium was replaced with serum-free one. Then, 10 μL MTT at 5 mg/L (No., ST316, Sigma-Aldrich Chemical Company, St Louis MO, USA) was added to each well. The cells were then cultured in constant-temperature incubator containing 5% CO₂ at 37°C for 4 h. The supernatant was gently absorbed and discarded. Then, 100 μmol/L dimethyl sulfoxide (DMSO, B01084003, Amresco, Inc., Solon, Ohio, USA) was added and cultured at 37°C for 10 ~ 20 min until the solution reached uniformity and the particles were completely dissolved. The optical density (OD) value was measured using the microplate reader (Model No., RT-2100C, Rayto Life and Analytical Sciences Co., Ltd., Shenzhen, China) at a wavelength of 570 nm. The cell survival rate = the case group A_{570} / the control group A_{570} . The experiment was repeated 3 times, and the mean value was obtained for the final results.

Cell Adhesion

The cells after treatment were washed with PBS 3 times and digested with 0.25% trypsin. After centrifugation, the cells were prepared into suspension, and 1×10^4 /mL cells in each group were seeded into a 6-well plate connected with fibers and coated with protein. After 30-min culture, when the cells were adherent to the walls, the cells were washed with PBS twice. Only adherent cells were stained by MTT for the detection of cell adhesive ability. One field was calculated by two observers and pictured with an inverted microscope (TMS model, Nikon Corporation, Tokyo, Japan). The control group was set at 100%. The experiment was repeated 3 times, and the mean value was obtained for the final results.

Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP nick End Labeling (TUNEL)

The cells after treatment were removed from the medium and washed with PBS twice. Subsequently, the cells were fixed with 4% paraformaldehyde for 15 min. The paraformaldehyde was then removed, and the cells were re-washed with PBS twice. Next, 50 μL TUNEL solution was added, and the cells were cultured in the dark at 37°C for 60 min. The cells were washed every 5 min three times. The double-staining technique was applied in strict conformity with the instructions in completely dark conditions. After the sections were mounted, the green fluorescence indicated apoptotic nuclei, whereas the blue fluorescence indicated all nuclei. Ten fields under a fluorescence microscope were randomly selected for cell calculation. The cell apoptosis rate = apoptotic cells / total cells × 100%. The experiment was repeated 3 times, and the average value was obtained for the final results.

Mitochondrial Membrane Potential ($\Delta\psi_m$)

The deutocerebrum primary cells at 6×10^5 /mL were inoculated into triplicate wells of a 24-well plate. Curcumin, 6-OHDA and DKK-1 at different concentrations were added. After washing with PBS 3 times,

rhodamine 123 at a final concentration of 10 µg/mL was added. The cells were incubated in an incubator with 5% CO₂ at 37°C for 30 min, washed with PBS three times and digested with 0.125% trypsin to generate a cell suspension. The cells were then centrifuged at 1000 r/min for 5 min. The flow cytometer FC500 (Beckman Coulter, Brea, CA, USA) was used to detect fluorescence intensity (%). The experiment was repeated 3 times, and the average value was obtained for the final results.

Western Blotting

The membrane of treated cells from each group was disrupted, and cytoplasmic and nuclear protein was collected according to the instructions of cytoplasmic and nuclear protein extraction kit (Beyotime Biotechnology Co., Ltd., Shanghai, China). Then, cytoplasmic and nuclear proteins of the same mass were sampled and electrophoresed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A spacer gel of 5% and separation gel of 8% were used for transmembrane. The membrane was washed five times (each for 3 min) before it was sealed with sealing fluid for 1 h. The prepared rabbit antibody β-catenin (SAB4504348, Sigma-Aldrich Chemical Company, St Louis MO, USA) diluted at 1: 1000 and Wnt3a monoclonal antibody (SAB2104343, Sigma-Aldrich Chemical Company, St Louis MO, USA) were added for culture at 4°C overnight. The membrane was washed with cleaning solution three times (3 min each). The secondary antibody, rabbit anti-mouse IgG antibody linked to horseradish peroxidase, was diluted at 1: 1500 and added. The solution was gently oscillated at 37°C for 1 h. The membrane was covered with an equal volume of A and B solution from an Electrochemiluminescence kit (Batch No., 1032, Beijing Shengke Boyuan Biotech Co., Ltd., Beijing, China). Then, the membrane was developed using an X-ray film kit (Guangdong Yuehua Medical Instrument Factory Co., Ltd., Guangdong, China) with exposure in a dark room. The image was analyzed with ImageJ (Bio-Rad Laboratories, Hercules, CA, USA). The experiment was repeated 3 times, and the average value was obtained for the final results.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted from treated cells and tissues, and RNA concentration was measured. After it was transcribed into cRNA, an ABI7500 PCR system was used for the amplification of the required sequences. Each PCR reaction consisted of 20 µL in total, containing 4 µL MgCl₂ of 25 mmol/L, 2 µL reverse transcriptase buffer of 10 ×, 2 µL dNTP of 10 mmol/L, 0.5 µL AMV of 15 U, 0.5 µL nuclease inhibitors of 0.5 U, 0.5 µL oligo (dT), 3 µg template RNA and RNase-free water. The amplification condition was as follows: pre-denaturation at 94°C for 3 min, denaturation at 94°C for 30 s, 57°C for 30 s, annealing at 72°C for 30 s (35 cycles) and elongation at 72°C for 10 min. With glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference and the Ct value as the target gene, the relative expression of target gene was calculated as $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{GAPDH}}$, $\Delta\Delta Ct = \Delta Ct_{\text{target gene}} - \Delta Ct_{\text{GAPDH}}$. The primer design software Primer 5 was used to design the primer sequences of Wnt3a, β-catenin, c-myc and cyclinD1 according to GenBank. The forward and reverse primer sequences were 2 exons to avoid DNA contamination. The primer for the internal reference GAPDH was designed by the ABI Company (Oyster Bay, NY, USA). All primers listed in Table 1 were synthesized by Shanghai Biological Engineering Co., Ltd. (Shanghai, China).

Detection of Superoxide Dismutase (SOD), Glutathione Peroxidase (GSH-Px) and Malondialdehyde (MDA) Contents

A 2% pentobarbital sodium (40 mg/kg, ig) solution was intraperitoneally injected for anesthesia. The rats were beheaded, and the midbrain was removed on ice. The injured tissues were separated and weighed on an accurate electronic scale. Then, cold homogenate was added into the tissues. After the homogenate reached 10%, the tissues were centrifuged at 4°C for 10 min. The supernatant was obtained. The SOD activity was determined by the hydroxylamine method, GSH-Px activity by the dithio-bis-nitrobenzoic acid (DTNB) direct method, and MDA content by the thiobarbituric acid method. The

Table 1. Primer sequences for quantitative real-time polymerase chain reaction, Note: Wnt3a, wingless-type MMTV integration site family member 3a; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR, quantitative real-time polymerase chain reaction

Gene	Forward [5'-3']	Reverse [5'-3']
Wnt3a	ATGGGCGGGAGGGGAGAGAT	CGCCCCATTGGATCCTTAAG
β-catenin	CGTTTCGCCTTCATTATGGACTACCT	GCCGCTGGGTGTCTGATGT
c-myc	CCAGGACTGTATGTGGAGCG	CCTGAGGACCACTGGGCTGT
cyclinD1	GGAGCAGAAGTCCGAAGA	GGGTGGGTTGGAAATGAA
GAPDH	TATCGGACGCCTGGTTAC	CTGTGCCGTTGAACCTGC

analyses were in strict conformity with the instructions of reagent kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

After discarding the medium, the treated cells were washed with PBS twice and digested with trypsin. The centrifugation was performed at 1000 r/min at 4°C for 5 min. The cells were disrupted at 4°C by an ultrasonic cell disruptor. The lysate was obtained, and centrifugation was re-performed at 1000 r/min at 4°C for 10 min. The supernatant was quantitatively assessed using a bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology Co., Ltd., Shanghai, China). The SOD, GSH-Px and MDA contents were determined according to the instructions of the SOD, GSH-Px and MDA kit.

Statistical Analysis

Statistical analysis was performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). The measurement data are presented as the mean $\pm$ standard deviation (SD). Differences among multiple groups were compared by analysis of variance (ANOVA), and differences between two groups were compared by the least significant difference (LSD-t) test. The behavioral indexes at different time points in the groups were compared with repeated measurement ANOVA. The numerical data were analyzed with χ^2 test. $P < 0.05$ was considered statistically significant.

Results

Curcumin Affects the Rotational Behavior of PD rats

The rats in the control group displayed no rotational behaviors. Compared to the control group, the 6-OHDA group and the Cur group exhibited significantly increased rotation numbers within 30 min at each time point (both $P < 0.05$). As time proceeded, the rats in the 6-OHDA group had increased rotation numbers within 30 min; compared to the pretreatment group, the rotation number at the 3rd, 4th and 5th week was significantly increased (all $P < 0.05$). Compared with the 6-OHDA group, the rats in the Cur group displayed lower rotation numbers within 30 min at the 3rd, 4th and 5th week (all $P < 0.05$) in a time-dependent manner. In the Cur group, compared with the pretreatment group, the rotation number within 30 min at the 3rd, 4th and 5th week was notably reduced (all $P < 0.05$, Table 2).

Curcumin Affects the Rotarod Test of PD rats

At the same time point, compared with the control group, the residence time on the rotarod of rats in the 6-OHDA group and the Cur group was significantly shorter, with a shortened motor latency (both $P < 0.05$). Compared with the 6-OHDA group, the residence time on the rotarod of rats in the Cur group was lengthened along with increased motor latency (both $P < 0.05$, Fig. 1).

Curcumin Attenuates Dopaminergic Neuron Death of PD Rats

Immunohistochemistry was used to observe the changes in TH-positive neurons and DAT-positive nerve fibers (Fig. 2). The TH immunoreactive neurons of the SN region of the rats in the control group displayed clear boundaries and morphology, strong immunoactivity, darker perikarya and fibers, higher TH expression and clear neurites. Compared to the control group, the rats in the 6-OHDA group exhibited a 90.28% reduction of TH immunoreactive neurons in the SN region ($P < 0.05$), as well as reduced immunoactivity,

Table 2. Curcumin affects the rotational behavior of PD rats (unit: rotate/30 min), 6-OHDA, 6-hydroxydopamine; PD, Parkinson's disease; Cur, curcumin; *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the 6-OHDA group; &, $P < 0.05$ compared with pretreatment

Group	Pretreatment	After treatment		
		3 rd week	4 th week	5 th week
Control	0	0	0	0
6-OHDA	370 $\pm$ 61*	487 $\pm$ 81*#&	533 $\pm$ 118*#&	554 $\pm$ 129*#&
Cur	365 $\pm$ 73*	342 $\pm$ 97*#&	324 $\pm$ 72*#&	311 $\pm$ 81*#&

Fig. 1. Effects of curcumin on the motor coordination of PD rats. 6-OHDA, 6-hydroxydopamine; PD, Parkinson's disease; Cur, curcumin; *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the 6-OHDA group.

Fig. 2. Curcumin affects the TH and DAT positive expressions. A, TH- and DAT-positive expression in each group; B, Numbers of TH- and DAT-positive neurons; Cur, curcumin; 6-OHDA, 6-hydroxydopamine; TH, tyrosine hydroxylase; DAT, dopamine transporter; *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the 6-OHDA group.

lighter cytoplasmic staining and unclear cell boundaries. Compared with the 6-OHDA group, the TH immunoreactive neurons of the SN region in the Cur group increased 311.6% ($P < 0.05$). The control group had elevated DAT expression, strong immunoactivity, darker-stained cytoplasm, and clear cell boundaries. Compared with the control group, the 6-OHDA group presented decreased DAT expression ($P < 0.05$), weak immunoactivity, lighter-stained cytoplasm, and blurred cell boundaries. The Cur treatment alleviated the 6-OHDA-induced reduction of DAT immunoreactive neurons ($P < 0.05$). The above results indicated that curcumin could alleviate 6-OHDA-induced dopaminergic neuron death of PD rats.

Curcumin Inhibits Activation of Astrocytes

GFAP is a characteristic marker for astrocytes. GFAP showed increased expression and activity during tissue damage and inflammation. As shown in Fig. 3, the control group displayed a small number of scattered GFAP-positive cells with small cell bodies and light-stained cytoplasm. Compared with the control group, the GFAP expression in the 6-OHDA group was significantly elevated ($P < 0.05$), with enlarged cell bodies and deep cytoplasm stained fuchsia; the Cur group exhibited no significant difference compared with the control group ($P > 0.05$). Compared with the 6-OHDA group, the Cur group presented reduced astrocyte number ($P < 0.05$), which indicated that curcumin could inhibit the activation of GFAP astrocytes and alleviate tissue injury.

Fig. 3. Curcumin affects GFAP-positive expression. A, GFAP-positive expression in each group; B, Numbers of GFAP-positive neurons; Cur, curcumin; 6-OHDA, 6-hydroxydopamine; GFAP, glial fibrillary acidic protein; *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the 6-OHDA group.

Fig. 4. Curcumin affects the Wnt3a and β -catenin mRNA and protein expression and c-myc and cyclinD1 mRNA expression among the three groups. A, protein electrophoretogram in each group; B, Relative protein expression of Wnt3a and β -catenin; C, Relative mRNA expression of Wnt3a, β -catenin, c-myc and cyclinD1; Cur, curcumin; 6-OHDA, 6-hydroxydopamine; *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the 6-OHDA group.

Fig. 5. Curcumin affects SOD, GSH-Px and MDA contents of rats. A, SOD and GSH-Px contents in each group; B, MDA content in each group; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; Cur, curcumin; 6-OHDA, 6-hydroxydopamine; *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the 6-OHDA group.

Wnt3a and β -catenin mRNA and Protein Expressions and c-myc and cyclinD1 mRNA Expression in the Brain Tissues of Each Group

The Wnt3a and β -catenin mRNA and protein expressions and c-myc and cyclinD1 mRNA expressions are presented in Fig. 4. Compared with the control group, the 6-OHDA group

Fig. 6. Curcumin affects the morphology of deutocerebrum primary cells at different time points (400 ×). A, Primary cells at 24 h during culture; B, Primary cells at 3 ~ 4 days during culture; C, Primary cells at 5 ~ 6 days during culture.

Fig. 7. Curcumin at different concentrations affects cell viability of deutocerebrum primary cells. 6-OHDA, 6-hydroxydopamine; DKK-1, dickkopf-1; Cur, curcumin; *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the 6-OHDA group; &, $P < 0.05$ compared with the 10 $\mu\text{mol/L}$ Cur group.

and the Cur group showed significantly reduced Wnt3a and β -catenin protein and mRNA expressions and c-myc and cyclinD1 mRNA expression (all $P < 0.05$). Compared with the 6-OHDA group, the above indexes were significantly elevated (all $P < 0.05$).

SOD, GSH-Px and MDA Contents in Brain Tissues of Each Group

As shown in Fig. 5, compared with the control group, the 6-OHDA group and the Cur group exhibited substantially decreased SOD and GSH-Px contents and increased MDA content (all $P < 0.05$). Compared with the 6-OHDA group, the Cur group displayed increased SOD and GSH-Px contents and reduced MDA content (all $P < 0.05$).

Morphology of Deutocerebrum Primary Cells at Different Time Points

The seeded cells adhered to the walls after 5 h. Twenty-four hours later, the cells exhibited uniform growth on the wall. Most cells had a round shape with 1 - 2 stretched neurites. The cell body presented a peripheral halo. After culture on the 3rd to 4th day, the cell body enlarged in a triangular or polygonal shape with interwoven neurites. On the 5th to 6th day, the neurites stretched farther into cubic network. On the 5th to 11th day, the cells reached the most active stage (Fig. 6).

Curcumin at Different Concentrations Affects Cell Survival Rate of Deutocerebrum Primary Cells

The cell survival rate of deutocerebrum primary cells determined by MTT assays is shown in Fig. 7. Compared with the control group, the cell survival rate in the 6-OHDA group was reduced by 29.0% ($P < 0.05$). No significant difference was observed in relative cell survival rate between the 6-OHDA group and the 5 $\mu\text{mol/L}$ Cur group ($P > 0.05$). Compared with the 6-OHDA group, the relative cell survival rate was increased by 15.5% and 23.9% in the 10 $\mu\text{mol/L}$ and 15 $\mu\text{mol/L}$ Cur groups, respectively (both $P < 0.05$). Compared with the

Fig. 8. Curcumin at different concentrations affects cell adhesion of deutocerebrum primary cells. A, Cell adhesion morphology of each group (200×); B, Cell adhesion rate of each group; 6-OHDA, 6-hydroxydopamine; DKK-1, dickkopf-1; Cur, curcumin; *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the 6-OHDA group; %, $P < 0.05$ compared with the 10 μmol/L Cur group.

control group and the 6-OHDA group, the cell survival rate was significantly lower in the DKK-1 group (both $P < 0.05$). Compared with the 10 μmol/L Cur group, the cell survival rate was significantly reduced in the Cur + DKK-1 group ($P < 0.05$).

Curcumin at Different Concentrations Affects Cell Adhesion of Deutocerebrum Primary Cells

The cell adhesion of deutocerebrum primary cells in each group is shown in Fig. 8. Compared with the control group, the cells significantly shrank and displayed weakened adhesive ability in the 6-OHDA group ($P < 0.05$). Compared with the 5 μmol/L Cur group, no significant difference was found about the cell adhesion of the 6-OHDA group ($P > 0.05$). Compared with the 6-OHDA group, the cell morphology and adhesion were enhanced in the 10 μmol/L and 15 μmol/L Cur groups (both $P < 0.05$), with more significant effects shown in the 10 μmol/L Cur group. Compared with the 6-OHDA group, the cells displayed smaller size and weaker adhesive ability in the DKK-1 group ($P < 0.05$). Compared with the 10 μmol/L Cur group, the cells displayed smaller size and weaker adhesive ability in the Cur + DKK-1 group ($P < 0.05$).

Curcumin at Different Concentrations Affects Cell Apoptosis of Deutocerebrum Primary Cells

The cell apoptosis of deutocerebrum primary cells by TUNEL assays is shown in Fig. 9. Compared with the control group, the 6-OHDA group presented more apoptotic cells with significantly higher apoptosis rate ($P < 0.05$). Compared with the 5 μmol/L Cur group, no significant difference was found in cell apoptosis of the 6-OHDA group ($P > 0.05$). Compared with the 6-OHDA group, the apoptotic cells were significantly decreased, with reduced apoptosis rates in the 10 μmol/L and 15 μmol/L Cur groups (both $P < 0.05$); more significant effects were observed in the 10 μmol/L Cur group. Compared with the control group and the 6-OHDA group, the cell apoptosis rate was substantially increased in the DKK-1 group (both $P < 0.05$). Compared with the 10 μmol/L Cur group, the cell apoptosis rate was sharply increased in the Cur + DKK-1 group ($P < 0.05$).

Fig. 9. Curcumin at different concentrations affects cell apoptosis of deutocerebrum primary cells. A, TUNEL staining of each group (400 ×), green represents apoptotic cells and blue represents all cells; B, Cell apoptosis rate; 6-OHDA, 6-hydroxydopamine; DKK-1, dickkopf-1; Cur, curcumin; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling; *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the 6-OHDA group; %, $P < 0.05$ compared with the 10 μmol/L Cur group.

Curcumin Affects Wnt3a and β -catenin mRNA and Protein Expressions and c-myc and cyclinD1 mRNA Expressions

As shown in Fig. 10, compared with the control group, the 6-OHDA group, the Cur group of 5 μmol/L and the DKK-1 group exhibited significant reductions in protein and mRNA expressions of Wnt3a and β -catenin and mRNA expressions of c-myc and cyclinD1 (all $P < 0.05$), while the 10 and 15 μmol/L Cur groups displayed substantial increases in protein and mRNA expressions of Wnt3a and β -catenin and mRNA expressions of c-myc and cyclinD1 (all $P < 0.05$). In addition, the 10 μmol/L Cur group exhibited more significant changes. Compared with the 6-OHDA group, the protein and mRNA expressions of above indexes revealed no significant difference in the 5 μmol/L Cur group, while the 10 and 15 μmol/L Cur groups presented enhanced protein and mRNA expression (all $P < 0.05$) compared with the 6-OHDA group. The protein and mRNA expressions in the DKK-1 group were lower compared to the 6-OHDA group (all $P < 0.05$). Compared with the 10 μmol/L Cur group, the protein and mRNA expressions in the Cur + DKK-1 group were lower (all $P < 0.05$). Inhibition of the Wnt/ β -catenin signaling pathway enhanced the toxic effects of 6-OHDA on PD rats, which was alleviated by the combination of DKK-1 and curcumin. The above results indicated that curcumin protected against injury to PD rats *via* activation of the Wnt/ β -catenin signaling pathway.

Fig. 10. Curcumin affects Wnt3a and β -catenin mRNA and protein expression and c-myc and cyclinD1 mRNA expression. A, Protein electrophoretogram of each group in vitro; B, Protein relative expression of each group in vitro; C, mRNA relative expression of each group in vitro; 6-OHDA, 6-hydroxydopamine; Cur, curcumin; DKK-1, dickkopf-1; *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the 6-OHDA group; &, $P < 0.05$ compared with the 10 $\mu\text{mol/L}$ Cur group.

Fig. 11. Curcumin affects mitochondrial membrane potential. 6-OHDA, 6-hydroxydopamine; DKK-1, dickkopf-1; Cur, curcumin; ψ_m , mitochondrial membrane potential; *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the 6-OHDA group; &, $P < 0.05$ compared with the 10 $\mu\text{mol/L}$ Cur group.

Curcumin Enhances $\Delta\psi_m$ by Activating the Wnt/ β -Catenin Signaling Pathway

Compared with the control group, the 6-OHDA group, the 5 $\mu\text{mol/L}$ Cur group and the DKK-1 group exhibited significantly reduced $\Delta\psi_m$ (all $P < 0.05$), while the 10 $\mu\text{mol/L}$ and 15 $\mu\text{mol/L}$ Cur groups displayed significantly increased $\Delta\psi_m$ (both $P < 0.05$). Compared with the 6-OHDA group, $\Delta\psi_m$ revealed no significant difference in the 5 $\mu\text{mol/L}$ Cur group, while the 10 $\mu\text{mol/L}$ and 15 $\mu\text{mol/L}$ Cur groups presented sharply elevated $\Delta\psi_m$ (both $P < 0.05$). The $\Delta\psi_m$ in the DKK-1 group was lower compared to the 6-OHDA group ($P < 0.05$). In comparison with the 10 $\mu\text{mol/L}$ Cur group, the $\Delta\psi_m$ in the Cur + DKK-1 group was significantly decreased ($P < 0.05$) (Fig. 11). These findings revealed that curcumin could elevate $\Delta\psi_m$ through activation of the Wnt/ β -catenin signaling pathway.

Curcumin Affects SOD, GSH-Px and MDA Contents of Cells in Each Group

Fig. 12 indicated that compared with the control group, the 6-OHDA, the 5, 10 and 15 $\mu\text{mol/L}$ Cur and the DKK-1 groups displayed markedly reduced SOD and GSH-Px contents and markedly increased MDA content (all $P < 0.05$). Compared with the 6-OHDA group, the 5 $\mu\text{mol/L}$ Cur group revealed no significant difference, while the 10 and 15 $\mu\text{mol/L}$ Cur groups presented sharply elevated SOD and GSH-Px contents and significantly reduced MDA content (all $P < 0.05$). The SOD and GSH-Px contents in the DKK-1 group were lower but the MDA content was significantly increased compared to the 6-OHDA group (all $P < 0.05$). In comparison with the 10 $\mu\text{mol/L}$ Cur group, the SOD and GSH-Px contents were lower, and MDA content was sharply enhanced in the Cur + DKK-1 group (all $P < 0.05$). The changes in SOD, GSH-Px and MDA contents in each group *in vivo* and *in vitro* indicated that curcumin could protect against oxidative stress-induced injury in PD rats.

Fig. 12. Curcumin affects SOD, GSH-Px and MDA contents of cells. A, SOD and GSH-Px contents of each group; B, MDA content of each group; Cur, curcumin; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; *, $P < 0.05$ compared with the control group; #, $P < 0.05$ compared with the 6-OHDA group; %, $P < 0.05$ compared with the 10 $\mu\text{mol/L}$ Cur group.

Discussion

Neurodegenerative disorders are neurological diseases that are characterized by extensive neuronal loss in the brain, and degeneration of dopaminergic neurons in the substantia nigra region accelerates the progression of PD [24]. The present study aimed to investigate the effects of curcumin on oxidative stress-induced injury in a rat model of PD. Our findings revealed that curcumin could protect against oxidative stress-induced injury in PD rats through activation of the Wnt/ β -catenin signaling pathway.

First, our data indicated that 6-OHDA induced significant rotational behavior in PD rats, and TH and DAT were reduced in the lesion site of PD rats. TH and DAT expression was diminished in the PD rat model, and the expression of GFAP was enhanced [25]. In our experiment, compared with the 6-OHDA group, the expression levels of TH and DAT in the Cur group were significantly elevated, but the GFAP expression was reduced, indicating that curcumin could alleviate the local tissue damage induced by 6-OHDA. Curcumin, a major active polyphenol component of turmeric, confers various biological effects and possesses antioxidative activity against oxidative stress-induced injury in PD cell and animal models [26, 27]. Curcumin is extensively applied for the treatment of diseases related to inflammation and oxidative stress [13]. Curcumin significantly attenuated rotenone-induced dopaminergic neuronal oxidative stress-induced injury in the substantia nigra region of rats via the activation of the protein kinase B/nuclear factor erythroid 2-related factor 2 signaling pathway [14]. Song et al. demonstrated that curcumin protected against neural impairments induced by 6-OHDA in the substantia nigra region, as evidenced by enhanced memory abilities, elevated levels of SOD and GSH-Px, and reduced concentration of MDA [28]. Yang et al. indicated that curcumin had a neuroprotective function against 6-OHDA-induced hippocampus neurons in rats, and the underlying mechanism promoted neural regeneration of hippocampal tissue by activating the brain derived neurotrophic factor/Tropomyosin receptor kinase B-dependent pathway [29], which was consistent with our results.

Curcumin might enhance viability, survival and adhesive ability and weaken apoptosis of brain cells through the activation of the Wnt/ β -catenin signaling pathway. The Cur group exhibited significantly higher mRNA and protein expression of Wnt3a and β -catenin as well as enhanced activity of SOD and GSH-Px and reduced MDA content. When the Wnt/ β -catenin signaling cascade was inhibited by DKK-1, SOD and GSH-Px contents were reduced, while MDA content was increased. The occurrence of oxidative stress in PD has been reported by studies on nigral tissues, indicating decreased levels of endogenous antioxidant molecules

such as GSH, decreased activity of antioxidant enzymes such as SOD and increased MDA concentration [30, 31]. The endogenous Wnt antagonist DKK-1 increases with age and represses adult neurogenesis and proliferation, whereas DKK-1 knockout mice revealed enhanced Wnt signaling, contributing to increased neurogenesis [32]. Wnt signals are transduced by canonical and non-canonical pathways, relying on the expression profiles of Wnt ligands, coreceptors, the Fzd family receptors, Wnt antagonists, and the activity of cytoplasmic Wnt signaling regulators [18]. Evidence has shown that the Wnt/ β -catenin signaling pathway is involved in PD pathogenesis [33]. *In vivo* and *in vitro* studies have revealed that Wnt3 is present in the hippocampal neurogenic niche and regulates newborn neurogenesis in the dentate gyrus [20]. The findings of Zhou et al. demonstrated that ginsenoside Rg1 exerts anti-apoptotic function in an *in vitro* model of PD, which may be achieved by activating the Wnt/ β -catenin signaling pathway [34]. The inhibition of the Wnt/ β -catenin signaling cascade by DKK-1 aggravates the dopaminergic neuron damage in the substantia nigra region and leads to degeneration in 6-OHDA-lesioned neurons [35].

To conclude, our results demonstrated that curcumin protects against 6-OHDA-induced oxidative stress-related injury in PD rats by activating the Wnt/ β -catenin signaling pathway to enhance viability, survival and adhesive ability and attenuate apoptosis of brain cells. Our study provides evidence for the treatment of PD as well as the underlying mechanism of the neuroprotection function of curcumin. Considering the complexity of molecular and neurological systems, further studies are required to determine the accurate mechanism.

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Disclosure Statement

None.

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