Original Paper

Salidroside Protection Against Oxidative Stress Injury Through the Wnt/ β -Catenin Signaling Pathway in Rats with Parkinson's Disease

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Key Words

Salidroside • Parkinson's disease • Wnt/ β -catenin signaling pathway • Oxidative stress • 6-OHDA • Apoptosis

Abstract

Background/Aims: Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease, and recent studies suggested that oxidative stress (OS) contributes to the neurodegeneration leads to dopamine cell degeneration in PD. In this study, we hypothesized that salidroside (SDS) offers protection against OS injury in 6-hydroxydopamine (6-OHDA) unilaterally injected rats as well as the underlying mechanism. **Methods:** SDS and LiCl (activators of the Wnt/ β -catenin signaling pathway) administration alone and in combination with 6-OHDA injection in rats was performed 3 days before modeling for 17 consecutive days to verify the regulatory mechanism by which SDS affects the Wnt/ β -catenin signaling pathway as well as to evaluate the protective effect of SDS on PD in relation to OS *in vivo*. In addition, pheochromocytoma 12 (PC12) cells were incubated with 10 μ mol/L SDS or LiCl alone or with both in combination for 1 h followed by a 24-h incubation with 100 μ mol/L 6-OHDA to obtain *in vitro* data. **Results:** *In vivo* the administration of LiCl was found to ameliorate behavioral deficits and dopaminergic neuron loss; increase superoxide dismutase (SOD) activity, glutathione peroxidase (GSH-Px) levels, and glycogen synthase kinase 3 β phosphorylation (GSK-3 β -Ser9); reduce malondialdehyde (MDA) accumulation in the striatum and the GSK-3 β mRNA level; as well as elevate β -catenin and cyclinD1 mRNA and protein levels in 6-OHDA-injected rats. This SDS treatment regimen was found to strengthen the beneficial effect of LiCl on 6-OHDA-injected rats. *In vitro* LiCl treatment decreased the toxicity of 6-OHDA on PC12

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cells and prevented apoptosis. Additionally, LiCl treatment increased SOA activity, GSH-Px levels, and GSK-3 β -Ser9 phosphorylation; decreased MDA accumulation in the striatum and GSK-3 β mRNA levels; as well as increased β -catenin and cyclinD1 mRNA and protein levels in 6-OHDA-treated PC12 cells. Additionally, SDS treatment increased the protective effect of LiCl on 6-OHDA-treated PC12 cells. **Conclusion:** Evidence from experimental models suggests that SDS may confer neuroprotection against the neurotoxicity of 6-OHDA in response to OS injury and showed that these beneficial effects may be related to regulation of the Wnt/ β -catenin signaling pathway. Therefore, SDS might be a potential therapeutic agent for treating PD.

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Introduction

Parkinson's disease (PD) is a progressive neurological disease that is linked to the progressive loss of nigrostriatal dopaminergic neurons [1]. PD affects approximately 1% of the population aged 55 years and older; a positive correlation was found between the risk of PD and age [2]. PD is characterized by motor symptoms including motor disturbance, akinesia, tremor, rigidity, and hypokinesia as well as non-motor symptoms including sleep disturbance, pain, apathy, depression, and constipation [3]. Several cellular abnormalities underlying neurodegeneration are present in sporadic PD, including oxidative stress (OS), proteasomal stress, mitochondrial dysfunction, and excitotoxicity, which are also involved in mutations of some familial PD genes [3]. Although multiple risk factors are involved in the occurrence and progression of PD, the pathogenesis remains unclear [2]. However, in many PD cases, OS seems to be a common potential mechanism that gives rise to cell dysfunction and death [4]. OS is regarded as an imbalance between the production of reactive oxygen species and the ability of the cell to elicit an effective antioxidant response [5]. The role of OS in PD is well understood, despite the limited treatment therapies [6]. Unfortunately, current therapy targets the symptoms of PD but fails to arrest the development of neurodegenerative processes [7].

Salidroside (SDS), derived from the medicinal plant *Rhodiola rosea*, which mainly grows in high mountains, is a phenolic glycoside that has been used as an anti-fatigue herb in China for a long time [8]. SDS exhibits a wide spectrum of pharmacological effects [9], including anti-oxidative, anti-fatigue, anti-cancer, anti-apoptosis, and anti-inflammatory effects [10]. In addition, SDS has been shown to have cytoprotective and angiogenic effects on human bone marrow-derived mesenchymal progenitor cells through the Akt/mammalian target of rapamycin (mTOR)/p70S6K/MAPK signaling pathways [11]. As a key molecular pathway, the Wnt/ β -catenin signaling pathway regulates neuronal survival and development of the central nervous system [12]. Previously, the pathway was related to research on the treatment of another neurodegenerative disorder, Alzheimer's disease (AD) [13]. Increasing evidence suggests that this pathway that once exhibited abnormalities can also participate in the potential mechanism of PD [12]. Beta-catenin is a crucial molecule in the Wnt signaling pathway and plays a significant role in cell adhesion, growth, invasion, and metastasis [14]. Glycogen synthase kinase 3 β (GSK-3 β) has been shown to participate in PD, since it can affect the central nervous system and neuronal apoptosis [15, 16]. Additionally, cyclin D is involved in the occurrence of several neurodegenerative diseases [17]. With these factors associated with neuronal disorders serving as indicators in this study, we explore how SDS, by mediating the Wnt/ β -catenin signaling pathway, protects against OS injury in rats with PD.

Materials and Methods

Ethics statement

All animal experiments involved in this study were permitted by the Ethics Committee of Key Laboratory for Biotechnology on Medicinal Plants of Jiangsu Province, School of Life Science, Jiangsu Normal University.

Experimental animals

A total of 40 male and female Wistar rats (Shanghai Jia Ke Biotechnology Co., Ltd, Shanghai, China, SCXK-Shanghai: 2015-0006) weighing 180 ~ 220 g were selected as experimental animals. One week before the experiment, all rats were raised in a standard animal room at 25°C with a 12 h light-dark cycle stimulated by light. The rats were raised with free access to food and water. Rats were deprived of food 12 h before surgery.

Animal treatment

Forty Wistar rats were randomly assigned to 5 groups: sham, PD, SDS, SDS + lithium chloride (LiCl) and LiCl groups. The rats in the sham group were directionally injected with the same volume of saline as a replacement of 6-hydroxydopamine (6-OHDA) solution at two points in the right striatum. The rats in the PD group received no treatment after 6-OHDA modeling. The rats in the SDS group were modeled with 6-OHDA and treated with intraperitoneal injection of SDS (250 mg/kg, purchased from the Jiangsu Biotechnology company, Nanjing, China) [18] once a day, starting 3 days before modeling to the 14th day after modeling. The rats in the SDS + LiCl group were modeled with 6-OHDA and intraperitoneally injected with SDS (250 mg/kg) and LiCl (1 mEq/kg, purchased from Sigma Aldrich company, St. Louis, Missouri, USA) once a day, starting 3 days before modeling to 14 days after modeling. The rats in the LiCl group were modeled with 6-OHDA and treated with intraperitoneal injection of LiCl (1 mEq/kg) once a day, starting 3 days before modeling to 14 days after modeling.

Establishment of PD models

The rat models of Parkinson's disease were constructed in accordance with the research of Romuk et al. [2], and the behavior test was conducted 8 weeks after 6-OHDA injection.

Rotation experiment and Morris water maze test

Rotation experiment: before rats were killed, apomorphine (APO, 0.25 mg/kg, purchased from Sigma Aldrich company, St. Louis, Missouri, USA) was subcutaneously injected, and rotation laps were counted manually 10 ~ 40 min after injection. The number of constant rotation laps toward the right side in 30 s was also recorded. A rotation of 360 degrees was counted as a lap.

Morris water maze test [19]: The Morris water maze is a cylindrical pool made of stainless steel, with a diameter of 200 cm and height of 50 cm. Four points were marked on the pool wall for water entry, dividing the pool into 4 quadrants (named A, B, C, and D in a clockwise direction). The water depth was approximately 30 cm, and the temperature was kept between 22 and 25°C. A platform with a diameter of 11 cm and height of 29 cm was fixed in one quadrant, 1 cm below the water level. Cameras were installed above the water maze and connected to computers to automatically input the swimming trace of rats for analysis. The place navigation test was conducted for three days, once in the morning and once in the afternoon each day. All rats facing the platform were placed into the 4 quadrants separate from the water entry site, and the time spent finding the platform (within 60 s) was recorded as the escape latency. If the rats were unable to find the platform in 60 s, they were led to the platform with the assistance of a researcher's hand and were kept there for 10 s before being placed back into the cage. This test indicated the learning ability of the rats. The spatial probe test was performed on the fourth day of the place navigation test, the platform was removed, and each rat was released from the quadrant that is opposite to the designated quadrant. The designated quadrant is the quadrant that had been containing the platform. The time spent by the rats to find and enter the designated quadrant was recorded to assess their spatial learning/memory abilities.

Immunohistochemistry

Brain tissue was obtained after the behavioral tests and detected by immunohistochemistry. After the intraperitoneal injection of 10% chloral hydrate (400 mg/kg) in rats for anesthesia, brain tissues were taken out immediately and fixed with paraformaldehyde for 48 ~ 72 h. The substantia nigra was embedded in paraffin, and 5 µm coronal slices were obtained. Tyrosine hydroxylase (TH) immunohistochemistry was conducted using streptomycin peroxide (SP). The slices were dewaxed by xylene, dehydrated by ethanol, and the endogenous peroxidase was sealed with 3% hydrogen peroxide and repaired by antigen. Then, the primary antibody of TH (1:5000, Cell Signaling Company, Danvers, MA, USA) was added and incubated for 1 h at 37°C. The second antibody labeled by horse radish peroxidase (Beijing Zhongshan Jinqiao Biotechnology

Co., Ltd, Beijing, China) was added and incubated for 30 min at 37°C, then fresh 3,3'-diaminobenzidine (DAB) was added for coloration for 1 ~ 2 min. The slices were then washed with phosphate buffer solution (PBS) 3 times for 2 min. Then, they were counterstained with hematoxylin for 1 min, dehydrated, transparentized, and sealed using neutral gum. The slices were observed with an optical microscope, and pictures were taken for analysis.

Terminal-deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) staining of substantia nigra neurons

Paraffin sections were dewaxed routinely in distilled water, washed with PBS 3 times for 3 min, and repaired in a microwave through the following procedures: the beaker containing 0.01 M citrate buffer (pH 6.0) and 1 mmol ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0) was heated to boiling (98°C ~ 100°C) for 8 to 10 min. After natural cooling, sections were washed with distilled water for 3 min, and sections were removed and then washed with 0.1 M PBS 2 times for 3 min. The amount of neuronal apoptosis in the substantia nigra was measured based on the instructions of the apoptosis kit (Wuhan Wuhan Biological Technology Co., Ltd, Wuhan, China).

Cell culture

Rat adrenal pheochromocytoma (PC12) cell line (obtained from the Institute of Cell Biological Sciences, Chinese Academy of Sciences, Shanghai, China) was inoculated in a 25 cm² bottle with Dulbecco's minimum essential medium (DMEM, high glucose) containing 15% fetal bovine serum. Cells were cultured in the incubator with 5% CO₂ at 37°C, and the medium was replaced every 3 days. When the convergence of monolayer cells reached 80%, cells were digested with 0.25% trypsin, which was terminated by adding the medium containing serum, and the solution was then blown into a single cell suspension. The concentration of PC12 cells was adjusted to 1 × 10⁵/ml and inoculated in a 96-well plate, with 100 µl in each well. Cells were incubated in an incubator with 5% CO₂ at 37°C.

Cell treatment

Cells were divided into five groups: control group, 6-OHDA, SDS, SDS + LiCl and LiCl groups. The cells in the control group were cultured normally. The final concentration of 100 µmol/L 6-OHDA was added into the culture medium of the 6-OHDA group, and the cells were cultured for 24 h. Ten µmol/L SDS was added to the SDS group and reacted for 1 h, and then 100 µmol/L 6-OHDA was added to the final concentration of 100 µmol/L, and PC12 cells were cultured for 24 h. Ten µmol/L SDS + LiCl was added to the SDS + LiCl group and reacted for 1 h, and 6-OHDA was then added to the final concentration of 100 µmol/L, and the cells were cultured for 24 h. LiCl was added to the LiCl group and reacted for 1 h, and 6-OHDA was then added to the final concentration of 100 µmol/L, and the cells were cultured for 24 h.

3-(4,5-dimethylthiazol-2-yl)-3,5-di-phenyltetrazoliumromide (MTT) assay

PC12 cells were inoculated to 96-well plates and treated based on the cell grouping. The culture medium was removed, and 20 µl of MTT solution (5 mg/ml, Sigma Company, St. Louis, Missouri, USA) was added to each well. After incubation for 4 h, the liquid in each well was removed, and 150 µl dimethyl sulfoxide (DMSO, Sigma Co., St. Louis, Missouri, USA) was added. Plates were shaken for the full dissolution of purple crystals, and a microplate reader was used to measure optical density (OD) at 540 nm. Cell survival rate = (the OD value of the experimental group/the OD value of the control group) × 100%.

TUNEL staining of PC12 cells

PC12 cells were made into cell slides and washed with PBS 2 times for 1 min, fixed with 1 ml of 4% polyformic acid at 4°C for 30 min and washed (4 min per wash) with Tris-buffered saline (TBS) 3 times. Then, the slides were digested with proteinase K (20 µg/ml, Merck Co., Darmstadt, Germany) without DNase at 37°C for 30 min to remove the nucleases, and then washed with PBS 3 times for 4 min. A mixture of Terminal deoxynucleotidyl transferase (TdT) (Roche Co., Alameda, CA, USA) and Biotin-dUTP (Roche Co., Alameda, CA, USA) was added to the samples, and the cells were cultured in darkness for 60 min at 37°C, and then washed with PBS. Then, the stop buffer was added, and cells were incubated at room temperature for 10 min and washed with PBS. Subsequently, a mixture of 10 µl Streptavidin-HRP (Roche Co., Alameda, CA, USA) and 490 µl Biotin-dUTP was added to the samples, followed by incubation at 37°C in darkness for 60 min.

and washed with PBS. Cells were colored by DAB (Roche company, Alameda, CA, USA), and restained with hematoxylin (Solarbio Co. Ltd., Beijing, China). Then, cells were washed with PBS, dehydrated, sealed using neutral gum and photographed. In each group, 5 fields ($\times 200$) were randomly selected, and the number and proportion of apoptotic cells in every 100 cells was calculated to represent the apoptotic index (AI) [20]. Apoptotic cells were usually singular and characterized by shrinking membranes, no inflammatory reaction and compact nuclei with brownish-yellow particles or fragments.

Detection of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) activity and malondialdehyde (MDA) content

Brain tissue was obtained after behavioral tests. After the intraperitoneal injection of 10% chloral hydrate (400 mg/kg) for anesthesia, brain tissue was removed immediately and placed on a dry ice to isolate substantia nigra tissue. The water on the surface was removed, weighed and homogenized (Shanghai Botonyc Chemical Technology Co., Ltd, Shanghai, China). The procedure was as follows: substantia nigra was shredded using an ophthalmic scissor and normal saline (1:10) of 4°C was added to produce 10% brain tissue homogenate. Then, the homogenate was placed into a refrigerated centrifuge and centrifuged for 15 min at 3000 r/min. The supernatant was removed and saved for future SOD, GSH-Px activity, and MDA contents were detected by the colorimetry method using a microplate reader (BECKMAN-Coulter Co., Miami, FL, USA) according to the kit instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Cells from each group were collected and then centrifuged for ultracentrifugation. A total of 100 μ l supernatant was obtained to detect the OD values using a microplate reader according to the SOD, GSH-Px and MDA kit instructions, and the activity was calculated. The experiment was repeated a total of three times.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The substantia nigra from all groups was processed and total RNA was extracted by the TRIzol method (TaKaRa Co. Ltd., Dailian, Liaoning, China). RNA concentration and purity were measured. Complementary DNA (cDNA) was synthesized by reverse transcription according to the reverse transcription kit instructions (DRR047S, TaKaRa Co. Ltd., Dailian, Liaoning, China) with a reaction system of 10 μ l. Then, 65 μ l diethyl pyrocarbonate (DEPC) was added to the reaction system and mixed fully. The real time-PCR reaction system was as follows: 5 μ l SsoFast Green Supermix (1708882, BIO-RAD, Hercules CA, USA), 0.5 μ l forward primer (10 μ M), 0.5 μ l reverse primer (10 μ M), and 4 μ l cDNA. The PCR conditions were as follows: pre-degeneration at 95°C for 1 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 5 s, extension at 72°C for 5 s. Primers were synthesized by Shanghai Invitrogen Bioengineering Company, Shanghai, China (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference, and the concentration of each gene in each sample was repeated a total of 3 times. The melting curve was used to evaluate the specificity of qRT-PCR results, and the threshold cycle (Ct) value was acquired. Δ Ct = Ct_(target gene) - Ct_(internal reference), Δ Ct_(experiment group) - Δ Ct_(control group), and the relative expression of target genes was calculated according to the $2^{-\Delta\Delta Ct}$ [21]. The experiment was repeated a total of 3 times.

Western blot analysis

The substantia nigra was shredded on ice using ophthalmic scissors, and single detergent lysate (containing protease inhibitors and phosphatase inhibitors, 1:5) was added. After ultrasonication, substantia nigra was lysed overnight at 4°C and centrifuged at 4°C for 10 min at 12000 r/min. The supernatant was aspirated and stored at -20°C. Twenty-four h after treatment, the culture medium was washed with ice-cold PBS 2 times and then reacted in ice-cold RIPA containing protease inhibitors and phosphatase inhibitors for 30 min. Following centrifugation at 12000 r/min (4°C, 20 min), the supernatant was aspirated and stored at -20°C. PBS was used to dilute the 2 μ g/ μ l bovine serum albumin (BSA) into 20 μ g/ml, 15 μ g/ml, 10 μ g/ml, 5 μ g/ml, 2.5 μ g/ml and 0 μ g/ml BSA solutions. Protein concentration was determined

Table 1. Primer sequences for qRT-PCR. Notes: qRT-PCR, quantitative real-time polymerase chain reaction; GSK-3 β , glycogen synthase kinase 3 beta; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Gene	Primer Sequence
β -catenin	Forward: GCCAGTGGATTCCGTAAGT Reverse: GAGCTTGCTTTCCTGATTGC
GSK3 β	Forward: GACGCTCCCTGTGATTATGTC Reverse: GTTAGTCGGGCAGTTGGTGTAT
cyclinD1	Forward: GCCCTCGGTGTCCTACTTC Reverse: CTCCTCCTCGCACTTCTGTT
GAPDH	Forward: AACGGATTGGTGGTATTGGG Reverse: TCGCTCTGGAAGATGGTGAT

by the bicinchoninic acid assay (BCA, Thermo Co. Ltd., Waltham, MA, USA) according to the instructions and the number of samples. Protein electrophoresis was carried out in the chromatography cabinet at 4°C. The separation gel was conducted at 120 V, and the stacking gel was conducted at 80 V. The protein on the gel was transferred onto the polyvinylidene fluoride (PVDF) membrane and blocked with 5% skimmed milk with Tris-Buffered Saline Tween 20 (TBST) at room temperature for 2 h. Then, the primary antibodies, cyclinD1 (1:1000, #2978, Cell Signaling Technology, CST, Boston, USA), β -catenin (1:1000 dilution, #9587, CST, Boston, USA), t-GSK-3 β (1:1000 dilution, ab32391, Abcam, Cambridge, England), phosphorylated GSK-3 β -Ser9 (1:1000, ab75814, Abcam, Cambridge, England) and GAPDH (1:1000, #5174, CST, Boston, USA) were added and incubated overnight at 4°C. TBST was used to wash the membrane 3 times for 10 min. Secondary antibodies were added and incubated at room temperature for 1 h. TBST was used to wash the membrane 3 times for 10 min. Finally, chemiluminescence, X-ray filming, color-development and fixation were conducted, and the results were analyzed.

Statistical analysis

All data were processed with SPSS 22.0 (IBM Corp. Armonk, NY, USA). Measurement data were expressed as the mean $\pm$ standard deviation (SD). Concerning the place navigation test during the Morris water maze, the data were analyzed by repeated measures ANOVA and least significant difference (LSD) post hoc test. In other experiments, comparisons among multiple groups were analyzed by one-way ANOVA, and comparisons between two groups were examined by *t* tests. $P < 0.05$ indicated statistical significance.

Results

PD rats treated with SDS and LiCl in combination inhibit smallest number of rotations

Approximately 10 min after the injection of APO, rats in each group showed excited behaviors, such as different levels of agitation and freezing, for 30 min, which gradually ceased. The number of rotations is shown in Figure 1. Compared with the PD group, the SDS, SDS + LiCl and LiCl groups showed significant differences ($P < 0.05$). Compared with the SDS and LiCl groups, the SDS + LiCl group showed statistically fewer rotations (degree of freedom (F) (4, 35) = 1781 ($P < 0.0001$)).

PD rats treated with SDS and LiCl in combination show strengthened abilities of learning and spatial learning memory

The results of the place navigation test during the Morris water maze test are shown in Table 2. The data were analyzed by repeated measures ANOVA and LSD post hoc test, with F values achieved on 1st day: F (4, 35) = 7.310 ($P = 0.0002$); 2nd day: F (4, 35) = 9.014 ($P < 0.0001$); 3rd day: F (4, 35) = 11.170 ($P < 0.0001$). The escape latency differed when the PD, SDS, and LiCl groups were independently compared with the Sham group ($P < 0.05$). However, the PD rats treated with SDS and LiCl in combination showed similar escape latency as the rats receiving sham-operation ($P > 0.05$). In addition, after SDS and LiCl treatment in combination, the PD rats exhibited decreased escape latency ($P < 0.05$). After SDS or LiCl treatment, the PD rats showed similar escape latency as before treatment ($P > 0.05$).

The results obtained from the spatial probe test are also shown in Table 2. The PD rats spent significantly less time for swimming in the training quadrant (F (4, 35) = 96.580 ($P < 0.0001$)), with fewer times crossing the removed hidden platform (F (4, 35) = 56.920 ($P < 0.0001$)), than the rats

Fig. 1. Number of rotations in each group after injection of APO. Notes: *, compared with the PD group, $P < 0.05$; #, compared with the SDS group, $P < 0.05$; APO, apomorphine; PD, Parkinson's disease; SDS, salidroside; LiCl, lithium chloride.

receiving sham-operation ($P < 0.05$). After SDS and LiCl treatment in combination, the PD rats exhibited more time for swimming in the training quadrant ($P < 0.05$). After SDS or LiCl treatment, the PD rats showed similar time for swimming in the training quadrant as before treatment ($P > 0.05$).

SDS improves TH deletion in PD rats through activation of Wnt/ β -catenin signaling pathway

Immunohistochemical staining results showed that TH was localized in the cytoplasm, showing brown staining in each group, but there were differences in the number of positively stained cells and degree of staining ($F(4, 35) = 294.900$ ($P < 0.0001$)). A large number of positive cells with chocolate-brown cytoplasm were found in the sham group. The PD group had few TH-positive cells with weakly stained cytoplasm, and the cell structure was irregular and unclear. The number of TH-positive cells significantly increased in the PD groups, SDS + LiCl and LiCl groups, especially in the SDS + LiCl group. Compared with the PD group, the cells in the groups treated with SDS showed clearer cell structures and staining ($P < 0.05$) (Fig. 2). These results demonstrated that the activation of the Wnt/ β -catenin signaling pathway improved the lack of TH in the substantia nigra of PD rats, especially those treated with SDS.

SDS protects neurons against apoptosis in PD rats through activation of the Wnt/ β -catenin signaling pathway in vivo

The results of the TUNEL assay are shown in Fig. 3 ($F(4, 35) = 126.400$ ($P < 0.0001$)). In comparison to the sham group, the TUNEL-positive cell number in each group of PD rats

Table 2. The PD rats receiving SDS and LiCl treatment in combination exhibited decreased escape latency, with more time spent for swimming in the training quadrant and more times crossing the removed hidden platform. Notes: The data were presented as mean $\pm$ standard deviation (SD); PD, Parkinson's disease; SDS, Salidroside; LiCl, lithium chloride; *, $P < 0.05$ compared with the sham group; #, $P < 0.05$ compared with the PD group

Group	Escape latency (s) during place navigation test			Spatial probe test (%)	
	1 st day	2 nd day	3 rd day	Time spent for swimming in the training quadrant (%)	Number of times crossing the removed hidden platform 5 th day
Sham	12.63 $\pm$ 3.07	11.95 $\pm$ 2.71	8.81 $\pm$ 2.45	57.59 $\pm$ 3.29	4.86 $\pm$ 1.08
PD	27.23 $\pm$ 8.27*	26.37 $\pm$ 7.51*	24.08 $\pm$ 6.97*	37.91 $\pm$ 2.43*	0.94 $\pm$ 0.33*
SDS	23.41 $\pm$ 6.14*	21.98 $\pm$ 5.42*	19.02 $\pm$ 5.14*	39.34 $\pm$ 1.66*	1.05 $\pm$ 0.41*
SDS + LiCl	18.81 $\pm$ 4.42#	17.27 $\pm$ 3.15#	15.02 $\pm$ 2.87#	55.23 $\pm$ 2.76#	3.89 $\pm$ 0.97#
LiCl	20.53 $\pm$ 4.94*	20.12 $\pm$ 4.12*	19.10 $\pm$ 3.45*	41.07 $\pm$ 3.18*	1.02 $\pm$ 0.31*

Fig. 2. Number of TH-positive cells in the substantia nigra of rats in each group

Fig. 3. Comparisons of the apoptotic rate of rat substantia nigra neurons in each group. Notes: *, compared with the sham group, $P < 0.05$; #, compared with the PD group, $P < 0.05$; &, compared with the SDS group, $P < 0.05$; PD, Parkinson's disease; SDS, salidroside; LiCl, lithium chloride.

Fig. 4. Comparison of SOD, GSH-Px and MDA contents in the substantia nigra of rats in each group.

Fig. 5. Expression of Wnt/ β -catenin signaling pathway-related factors in rats of each group. Notes: A, mRNA expression of β -catenin, GSK-3 β and cyclinD1 detected by qRT-PCR; B, relative protein expression of β -catenin, GSK-3 β , GSK-3 β -Ser9 and cyclinD1 detected by western blot; C, histogram of protein expression of β -catenin, GSK-3 β , GSK-3 β -Ser9 and cyclinD1 in each group; *, compared with the sham group, $P < 0.05$; #, compared with the PD group, $P < 0.05$; &, compared with the SDS group, $P < 0.05$; GSK-3 β , glycogen synthase kinase 3 beta; PD, Parkinson's disease; SDS, salidroside; qRT-PCR, quantitative real-time polymerase chain reaction; LiCl, lithium chloride.

increase ($P < 0.05$). Compared with the PD group, the TUNEL-positive cell number in SDS, SDS + LiCl and LiCl groups decreased significantly ($P < 0.05$). The TUNEL-positive cell number in the SDS + LiCl group was decreased compared to the SDS and LiCl groups ($P < 0.05$). These results indicated that the activation of the Wnt/ β -catenin signaling pathway inhibited neuronal apoptosis in the substantia nigra of PD rats, especially those treated with SDS.

SDS strengthens the anti-oxidant capacity in PD rats through activation of the Wnt/ β -catenin signaling pathway in vivo

Compared with the sham group, the contents of SOD and GSH-Px were decreased by varying degrees, while the content of MDA was increased in groups of PD rats ($P < 0.05$). Compared with the PD group, the SOD and GSH-Px activity decreased, and the MDA content increased in the groups treated with SDS or LiCl ($P < 0.05$). Compared with the SDS and LiCl groups, the SOD, GSH-Px activity and MDA content in the SDS + LiCl group showed statistical significance ($P < 0.05$) (Fig. 4). These results showed that the anti-oxidant capacity of PD rats

Fig. 6. PC12 cell viability in each group detected by MTT assay. Notes: *, compared with the sham group, $P < 0.05$; #, compared with the 6-OHDA group, $P < 0.05$; &, compared with the SDS group, $P < 0.05$; PC12, pheochromocytoma 12; MTT, 3-(4,5)-dimethylthiazol(-z)-2-yl)-3,5-di-phenyltetrazolium bromide; 6-OHDA, 6-hydroxydopamine; SDS, salidroside; LiCl, lithium chloride.

Fig. 7. Apoptotic rate of PC 12 cells in the control, 6-OHDA, SDS, SDS + LiCl and LiCl groups. Notes: *, compared with the control group, $P < 0.05$; #, compared with the 6-OHDA group, $P < 0.05$; &, compared with the SDS group, $P < 0.05$; 6-OHDA, 6-hydroxydopamine; SDS, salidroside; LiCl, lithium chloride.

improved through the activation of the Wnt/ β -catenin signaling pathway; furthermore, this capacity was further improved with the addition of SDS.

SDS treatment activates the Wnt/ β -catenin signaling pathway in vivo

The results of qRT-PCR and western blot analysis indicated that compared with the sham group, mRNA expression and the extent of GSK-3 β phosphorylation increased, while the extent of GSK-3 β -Ser9 phosphorylation and protein expression of β -catenin and cyclinD1 decreased in each group of rats ($P < 0.05$). Compared with the PD group, mRNA expression and the extent of GSK-3 β phosphorylation decreased, while the extent of GSK-3 β -Ser9 phosphorylation and protein expression of β -catenin and cyclinD1 mRNA and protein expression increased in the SDS, SDS + LiCl and LiCl groups ($P < 0.05$). These differences in mRNA expression and the extent of GSK-3 β phosphorylation, in β -catenin and cyclinD1 mRNA and protein expression, and the extent of GSK-3 β -Ser9 phosphorylation reached statistical significance in the SDS + LiCl, SDS and LiCl groups ($P < 0.05$) (Fig. 5). These results demonstrated that SDS reversed the effect of LiCl on the expression of Wnt/ β -catenin signaling pathway-related factors.

SDS promotes PC12 cell survival in vitro through activation of the Wnt/ β -catenin signaling pathway

The protective effect of SDS on the injury of PC12 cells caused by 6-OHDA was detected by MTT assay. The results showed that after treatment with SDS or LiCl for 24 h, survival rate of PC12 cells in the SDS, SDS + LiCl and LiCl groups increased compared with the rate in the 6-OHDA group ($P < 0.05$), with the highest increase occurring in the SDS + LiCl group (Fig. 6). This indicated that LiCl had a protective effect on cell injury caused by 6-OHDA to some extent and that the protective effect was improved after treatment with SDS.

SDS prevents PC12 cells from apoptosis through activation of the Wnt/ β -catenin signaling pathway in vitro

Three fields were selected randomly for cell counting. The results showed that compared with the control group, the number of apoptotic cells caused by 6-OHDA markedly increased in the 6-OHDA group ($P < 0.05$). Compared with the 6-OHDA group, the number of apoptotic cells significantly decreased in the SDS, SDS + LiCl, and LiCl groups ($P < 0.05$), especially in the SDS + LiCl group (Fig. 7). The results showed that 6-OHDA can increase PC12 cell apoptosis and LiCl can inhibit PC12 cell apoptosis caused by 6-OHDA. In addition, treatment with SDS can enhance this therapeutic effect.

Fig. 8. Effect of SDS on contents of SOD, GSH-Px and MDA caused by 6-OHDA in PC12 cells. Notes: A, SOD content in PC12 cells among groups; B, GSH-Px content in PC12 cells of each group; C, MDA content in PC12 cells of each group; *, compared with the control group, $P < 0.05$; #, compared with the 6-OHDA group, $P < 0.05$; &, compared with the SDS group, $P < 0.05$; SDS, salidroside; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; PC12, pheochromocytoma 12; qRT-PCR, quantitative real-time polymerase chain reaction; 6-OHDA, 6-hydroxydopamine; LiCl, lithium chloride.

Fig. 9. Protein and mRNA expression of Wnt/ β -catenin pathway-related factors in rats after treatment with SDS. Notes: A, mRNA expression of β -catenin, GSK-3 β and cyclinD1 in each group as detected by qRT-PCR; B, relative protein expression of β -catenin, GSK-3 β and cyclinD1 in each group as detected by western blot; C, histogram of the protein expression of β -catenin, GSK-3 β -Ser9 and cyclinD1 in each group; *, compared with the control group, $P < 0.05$; #, compared with the 6-OHDA group, $P < 0.05$; &, compared with the SDS group, $P < 0.05$; SDS, salidroside; qRT-PCR, quantitative real-time polymerase chain reaction; LiCl, lithium chloride; 6-OHDA, 6-hydroxydopamine.

SDS strengthens the anti-oxidant capacity through activation of the Wnt/ β -catenin signaling pathway in PC12 cells

Compared with the control group, SOD and GSH-Px contents decreased, while MDA contents increased in the 6-OHDA group ($P < 0.05$). Compared with the 6-OHDA group, the contents of SOD and GSH-Px were increased, while MDA contents were decreased in the SDS, SDS + LiCl and LiCl groups ($P < 0.05$). Compared with the SDS + LiCl group, there were statistically significant differences in the contents of SOD, GSH-Px and MDA in the SDS and LiCl groups ($P < 0.05$) (Fig. 8). The results suggested that LiCl could increase the SOD and GSH-Px contents and decrease the MDA content induced by 6-OHDA in PC12 cells. The effect was weakened after treatment with SDS.

SDS treatment activates Wnt/ β -catenin signaling pathway in vitro

The effect of SDS on the expression of the Wnt/ β -catenin signaling pathway-related proteins in cells of each group was detected by qRT-PCR and western blot analysis. The results showed that compared with the control group, mRNA and the extent of GSK-3 β phosphorylation increased, while the extent of GSK-3 β -Ser9 phosphorylation, mRNA and protein expressions of β -catenin and cyclinD1 significantly decreased in PC12 cells, caused by 6-OHDA ($P < 0.05$). Compared with the 6-OHDA group, a reverse trend was observed in the SDS, SDS + LiCl, and LiCl groups ($P < 0.05$). The above comparisons all reached statistical significance ($P < 0.05$) (Fig. 9). The results implied that SDS enhances the effect of LiCl on activating the expression of Wnt/ β -catenin signaling pathway-related factors in PC12 cells.

Discussion

As the second most common neurodegenerative disease, PD is usually accompanied by a slow, progressive degeneration of dopaminergic neurons in the substantia nigra [7]. PD is a frequently occurring disorder worldwide that has been highlighted as having an uncertain pathogenesis [3]. OS contributes to diseases associated with the central nervous system and is involved in the initiation and progression of PD due to the reactive oxygen species generated by dopamine metabolism, mitochondrial malfunction and neuro-inflammation [1, 22]. A previous study demonstrated the positive role of SDS in attenuating OS [23]. Marchetti et al. uncovered a novel factor in astrocyte–neuron crosstalk in PD and found that the Wnt/β-catenin signaling cascade was the common final pathway for neuroprotection and self-repair [24]. However, there remains a lack of investigations on the protective effect of SDS on OS as well as on the anti-apoptotic effect of the SDS-mediated Wnt/β-catenin signaling pathway on neurons in PD.

Initially, we performed the rotation test and the Morris water maze test and found that the administration of LiCl to activate the Wnt/β-catenin signaling pathway ameliorated the behavioral deficits seen in PD rats. In addition, SDS treatment strengthened the beneficial effect of LiCl on 6-OHDA-injected rats. SDS has been proven capable of promoting anti-fatigue, anti-cancer, anti-oxidant and anti-aging effects [25]. It has been found to exhibit taste avoidance and conditioned disgust reactions in combination with LiCl [26]. Concerning *in vivo* and *in vitro* TUNEL experiments, we found that the administration of LiCl improved dopaminergic neuron loss and prevented PC12 cells from apoptosis, and SDS treatment increased the protective effect of LiCl on 6-OHDA-treated PC12 cells. PD results from the degeneration of substantia nigra neurons [7]. A study showed that apoptosis was closely related to the occurrence and development of PD, and the discharges of substantia nigra neurons in normal rats were at a higher level than that in PD rats [28]. SDS has been demonstrated to weaken apoptosis in neurons, thus acting as a protector of neuronal cells *in vitro* [29]. LiCl may also inhibit cell apoptosis [30]. Moreover, activation of the Wnt/β-catenin signaling pathway suppressed the apoptosis of neurons [31], which is consistent with our results indicating that activation of the Wnt/β-catenin signaling pathway mediated by SDS blocked neuronal apoptosis in the substantia nigra of PD rats.

In addition, we determined the SOD activity, GSH-Px content, and MDA accumulation in PD rats and 6-OHDA-treated PC12 cells and found that activation of the Wnt/β-catenin signaling pathway by LiCl mitigated OS injury in PD. Additionally, we demonstrated that SDS promoted the activation of the Wnt/β-catenin signaling pathway, thus preventing PD rats and 6-OHDA-treated PC12 cells from OS injury. A study observed decreased SOD and GSH-Px activity, as well as increased MDA content of the substantia nigra in PD rats, which was similar to the findings of [32]. The activation of the Wnt/β-catenin signaling pathway results in imbalance of oxidative homeostasis as evidenced by the relationship between inhibited OS and the activated Wnt/β-catenin signaling pathway in another study [33, 34]. In terms of the RT-PCR and western blot assays, the results showed that in comparison with the sham operation control groups, mRNA and protein expression of GSK-3β increased, while GSK-3β-Ser9 phosphorylation, β-catenin expression and β-catenin and cyclinD1 mRNA and protein expression decreased in the other groups. Noticeably, the involvement of SDS and LiCl further enhances the expression of Wnt/β-catenin signaling pathway-related factors such as GSK-3β and β-catenin. Lowly expressed GSK-3β has pivotal significance in inducing neuronal apoptosis, especially in neurodegenerative disorders [16]. It was previously demonstrated that the inhibited GSK-3β expression and enhanced cyclinD1 expression contributed to the activation of this pathway [35].

Additionally, we found that 6-OHDA could cause injury to PC12 cells, while SDS and LiCl could provide protective effects against damage to PC12 cells. As a widely used neurotoxin in the construction of PD models, 6-OHDA increases intracellular oxidants and functional cell damage and leads to cell death [36, 37]. Previous studies showed that LiCl can greatly inhibit cell apoptosis, and SDS shows similar positive effects [23, 30], further supporting our results.

Conclusion

The current study indicates that SDS plays a protective role against OS injury in a rat model of PD through activation of the Wnt/ β -catenin signaling pathway. Our study suggests that activation of the Wnt/ β -catenin signaling pathway may block the apoptosis of substantia nigra neurons in PD rats and improve the anti-oxidant capacity of PD rats with the combination of SDS. The combination of SDS and LiCl shows a better effect on reducing the cell damage and cell apoptosis caused by 6-OHDA. The results of the study may provide potential therapeutic strategies in the future treatment of OS injury involved in PD and the combination of SDS and the Wnt/ β -catenin signaling pathway. Additionally, LiCl may provide a new therapy for OS injury in PD. However, since rats in the SDS, SDS + LiCl, and LiCl groups received daily intraperitoneal injection of SDS or LiCl starting 3 days before modeling, on the 14th day after modeling, it is more appropriate to inject equal volumes of these solvents daily in the PD and control groups, as in the other groups. Therefore, further efforts are needed to confirm our findings. In addition, the protective mechanism of SDS in PD must be explored in future studies.

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Disclosure Statement

The authors have declared that no competing interests exist.

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