Journal of Dental Sciences 20 (2025) 1043–1051

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jds.com

Original Article

Intranasal administration of tissue inhibitor of metalloproteinase 1 extracted from stem cells from human exfoliated deciduous teeth-conditioned medium improves neurological outcomes and alpha-synuclein elimination in Parkinson's disease mice

Yi-No Chen [a,b], Yong-Ren Chen [a,c,d], Bing-Heng Yang [d,e,f], Po-Hui Lee [a], Lu-Ting Kuo [c], Chung-Hsing Li [g,h*]

[a] *Non-invasive Cancer Therapy Research Institute - Taiwan, Taipei, Taiwan*
[b] *Division of Neurosurgery, Department of Surgery, MacKay Memorial Hospital, Taipei, Taiwan*
[c] *Division of Neurosurgery, Department of Surgery, National Taiwan University Hospital, Taipei, Taiwan*
[d] *Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei, Taiwan*
[e] *Division of Clinical Pathology, Department of Pathology, Tri-Service General Hospital, Taipei, Taiwan*
[f] *Trace Element Research Center, Department of Pathology, Tri-Service General Hospital, Taipei, Taiwan*
[g] *School of Dentistry and Graduate Institute of Dental Science, National Defense Medical Center, Taipei, Taiwan*
[h] *Division of Orthodontics and Pediatric Dentistry, Department of Dentistry, Tri-Service General Hospital, Taipei, Taiwan*

Received 10 July 2024; Final revision received 26 November 2024
Available online 12 December 2024

KEYWORDS
SHED-CM;
Parkinson's disease;
Rotenone-induced animal model;
Alpha-synuclein aggregation

Abstract *Background/purpose:* Parkinson's disease (PD) remains a challenging neurodegenerative disorder, requiring the exploration of innovative therapeutic strategies. This study assessed the neuroprotective effects of stem cells from human exfoliated deciduous teeth-conditioned medium (SHED-CM) in a rotenone-induced mouse model of PD.
Materials and methods: The SHED-CM, fractionated and purified via Fast Protein Liquid Chromatography (FPLC) into "yPD01", was administered intranasally to evaluate its impact on motor deficits, olfactory functions, and protein expressions in affected brain regions.

* Corresponding author. School of Dentistry and Graduate Institute of Dental Science, National Defense Medical Center, No. 161, Sec. 6, Minquan E. Rd., Neihu Dist., Taipei City 11490, Taiwan.
E-mail address: chiyenchli@yahoo.com.tw (C.-H. Li).

https://doi.org/10.1016/j.jds.2024.11.029

Results: Upon intranasal delivery, yPD01 produced significant improvements to motor deficits and restoration of protein expressions associated with PD pathology, particularly in the olfactory bulb and substantial nigra. Intriguingly, the intranasal route exhibited efficacy akin to intravenous administration, highlighting its potential as a minimally invasive yet equally effective therapeutic approach. Further investigation indicated key components within yPD01, denoted as peaks P1 and P5, showcasing pivotal therapeutic effects. These components were linked to the interruption of alpha-synuclein aggregation and its clearance within specific brain cells affected by PD.

Conclusion: Intranasally administered yPD01 shows neuroprotective potential for mitigating neurodegenerative symptoms, particularly in PD. The results provide valuable insights into potential mechanisms underlying the neuroprotective effects of SHED-CM and their implications for future therapeutic interventions in PD and related conditions.

Introduction

Mesenchymal stem cells (MSCs) have been widely been proposed for potential therapeutic applications due to its involvement in immunogenicity and the modulation of immune responses, which are possibly related to the regulation of mature hematopoietic cells and optimization of their functions.[1,2] The immunomodulatory effects of MSCs could potentially be applicable to the treatment of neurodegenerative diseases, which is related to molecular events including excessive oxidative stress, deposition of protein aggregates, alteration of autophagy, sustained inflammatory status of the neuronal system, impaired mitochondrial function, and induction of apoptosis. To facilitate immunomodulatory effects, MSCs were elucidated to produce various bioactive molecules such as growth factors, adhesion molecules, angiogenic factors and immunomodulatory molecules, participating the paracrine effects of MSCs on adjacent cells.[3] In addition, MSCs are postulated to express neuroprotective and neurodegenerative effects through various mechanisms, such as producing neurotrophic factors, recruiting and supporting neural progenitors, stimulating angiogenesis, activating microglia, suppressing inflammation, and decreasing apoptosis and free radicals for neurodegenerative disorders characterized by progressive loss of function, structure, or even neuron count.[4] Nevertheless, systemically administered MSCs may contribute to the activation of immune responses leading to graft rejection, hemostasis, prothrombotic phenomena, or tumor cell growth. This alternatively emphasizes the possible application of the conditioned medium of mesenchymal stem cells (MSC-CM), which is believed to accompany the paracrine phenomenon and share similar immunomodulatory effect as the MSCs. The MSC-CM is considered superior in terms of high reproducibility and immunogenicity due to the reduced expression of membrane histocompatibility molecules, and the absence of risk for aneuploidy since MSC-CMs are non-self-replicating.[5,6]

Intranasal (IN) administration can deliver therapeutic substances noninvasively through the blood brain barrier (BBB), initially through exposure to the nasal mucosa. Mostly through respiratory and olfactory epithelium, drugs are distributed to the central nervous system through the innervated olfactory nerves and trigeminal nerves, respectively originating from the cerebrum and pons. Molecules are transported intracellularly and extracellularly. The intracellular pathway is fundamentally comprised of the endocytosis and exocytosis of neuron cells, while the extracellular pathway is composed of translocations of the drug molecules through the absence of tight junction to the lamina propria through the paracellular cleft.[7] Intranasal administration of MSC-CM is postulated as having the following benefits for neurodegenerative diseases: an immunomodulatory effect which mitigates the microglia activation toward a pro-inflammatory phenotype,[8] a neuroprotective effect and neurogenesis through the upregulation of pro-survival genes and alternative downregulation of pro-apoptotic genes. These neuroprotective effects and neurogenesis through the upregulation of pro-survival genes and the alternative downregulation of pro-apoptotic genes alleviate further neurologic damage,[9,10] The intranasal administration also provides a more straightforward route to the injured site than intravenous administration which may distribute the protein or cell products to systemic circulation.

Previous studies have indicated that rotenone could induce pro-inflammatory factors, such as TNF-α, IL-1β, and IL-6, leading to systemic inflammation.[11] However, this effect is modulated by the immunomodulatory properties of MSCs, which produce metabolites, cytokines, growth factors, and chemokines.[12] Rotenone has also been shown to affect the expression of apoptotic mRNA, such as Bcl-2, Bax, and caspase-3 in Swiss mice.[13] Treatment with MSC-CM decreased the expression of γ-H2AX, P53, Bax, and cleaved-caspase-3, while increasing Bcl-2 expression.[14] Using tangential flow filtration (TFF) for fractionation and purification, we maintained proteins within the 5—30 kDa range in the conditioned medium derived from human exfoliated deciduous teeth (SHED-CM). We previously demonstrated the potential therapeutic effects of mitigating cerebral ischemia and neuroinflammation in the ischemic brain with vasospasm, addressing acute brain injury. We also explored the efficacy of a Parkinson's disease (PD) model through the downregulation of microglia,

alpha-synuclein, and motor deficits.[15,16] This article describes the intranasal administration of SHED-CM, processed through fast protein liquid chromatography (FPLC), in a rotenone-induced Parkinson's disease mouse model. We evaluated the recovery of motor deficits, olfactory functions, and tyrosine hydroxylase (TH) levels in the olfactory bulb (OB) and substantia nigra.

Materials and methods

Animals and supplies

Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the National Defense Medical Center Laboratory Animal Center (NDMCLAC)-(IACUC-21-048). Six to eight-week-old male C57BL/6 mice (BioLASCO Taiwan Co., Taipei, Taiwan) were maintained at 24 °C under a 12-h light/dark cycle (lights on 07:00–19:00). Mice were housed in standard laboratory cages and had free access to food and water throughout the study period. All animal experimental procedures and methods were performed following the relevant guidelines and regulations.

Rotenone-induced Parkinson's disease in mice

Twenty-six C57BL/6 mice were used in this study (including pre-testing). The rotenone solution was prepared as a 50X stock with 10 % TWEEN-20 and diluted with 0.5 % carboxymethylcellulose (CMC) (Kosei Pharmaceutical, Osaka, Japan) to produce a final amount of rotenone of 1.5mg/mouse. The solution was stored in a brown vial protected from light and inverted several times before each injection. Rotenone was administered at 50 mg/kg via oral gavage for 21 continuous days. The body weight of each mouse was recorded every day. The PD symptoms were tested using the rotarod running test and the habituation olfactory test.

Stem cells of human exfoliated deciduous teeth (SHED) isolation and culture

All experimental protocols were approved by the Human Subject Research Ethics Board, National Defense Medical Center (Taipei, Taiwan) (TSGHIRB No.: C202125003). The extraction of primary teeth was done without extra procedures or anesthesia, SHED cells were extracted using a syringe from the root of the deciduous tooth and transferred into a 25 cm^2 flask (Corning, Corning, NY, USA). The tissue was then cultured in alpha minimal essential medium (α-MEM) with 10 % fetal bovine serum (FBS) (Gibco, Waltham, MA, USA). Isolation of SHED was not subjected to any type of depletion technique. Upon reaching confluence, the SHED cells were incubated at 37 °C in an atmosphere containing 5 % CO_2 at 100 % humidity. The following antibodies were used: CD73-FITC, CD90-APC, CD105-PerCP-Cy5-5, and CD34-PE (all from BD Biosciences), and isotype controls. 10,000 labeled cells were acquired and analyzed using FACSCalibur and CellQuest (BD Biosciences, Franklin Lakes, NJ, USA) software. The SHED cells used in this study exhibited a fibroblastic morphology with a bipolar spindle shape, expressing MSC markers (CD90, CD73, and CD105) but not endothelial/hematopoietic markers such as CD34.

Preparation of SHED-CM and yPD01

The SHED-CM was generated as follows: when passage 3 SHED cells reached 80 % confluence in a 150 cm^2 cell culture flask, the medium was replaced with fresh medium without FBS in each flask, then incubated for 72 h at 37 °C in an atmosphere containing 5 % CO_2 at 100 % humidity. The medium was collected and centrifuged for 3 min at 2500 rpm. To collect proteins with molecular weights between 5 and 30 kDa, CM was collected in a sterilized beaker, and further concentrated by tangential flow filtration (TFF) and a membrane filter system (Millipore) respectively with a 5 kDa and 30 kDa cut-off unit (Millipore) for 3 h, following the manufacturer's instructions. The protein concentration of the SHED-CM was measured using a PierceTM BCA Protein Assay Kit (Thermo Fisher, Waltham, WA, USA) and adjusted to 100 $\pm$ 2.8 μg/mL with normal saline. Quantitation of each constituent was analyzed by ELISA array using the Quantibody® Human Cytokine Antibody Array 4000 (RayBiotech, Peachtree Corners, GA, USA), following the manufacturer's instructions. This array can detect 200 target proteins, including human inflammatory factors, growth factors, chemokines, receptors, and cytokines. The SHED-CM was subsequently purified with FPLC (ÄKTA™ pure 25, Cytiva, Marlborough, MA, USA). The samples were injected into a 50 mL SUPERLOOP (Cytiva). The elution buffer (50 mM Na_2HPO_4, 150 mM NaCl, pH 7.4) was isolated at a flow rate of 4 mL/min and monitored using a UV detector at 280 nm to separate the SHED-CM proteins. Different fractions were collected and concentrated using a fraction collector (Cytiva), with peaks shown in Fig. 1. The final product, which included peaks 1 and 5, was renamed "yPD01".

yPD01 treatment

The animals were divided into four groups: 1) control group, receiving saline; 2) rotenone group + saline; 3) rotenone group + P1; 4) rotenone group + P5. The latter two groups were used to differentiate the effects of each peak. The solutions were administered either intranasally or intravenously. Each group consisted of 3–6 mice. After 5 days of yPD01 treatment, all mice underwent behavioral evaluation on day 20, and olfactory testing on days 4 and 19. Mice were sacrificed weekly for further histological analysis. In the low-dose group, yPD01 was administered at 46 μg/kg continuously for 5 days, while the high-dose group received five times this amount. Therefore, a dose of 230 μg/kg was used as the treatment dose for subsequent experiments. The treatment protocol for yPD01 in rotenone-induced mice is shown in Fig. 2A.

Behavior evaluation by rotarod system

The Rotarod test was performed as described by Vogel et al.[17] with small modifications. The animals were trained for three consecutive days at the speed of 5 rpm, with three 5-min sessions per day. If mice fell during the

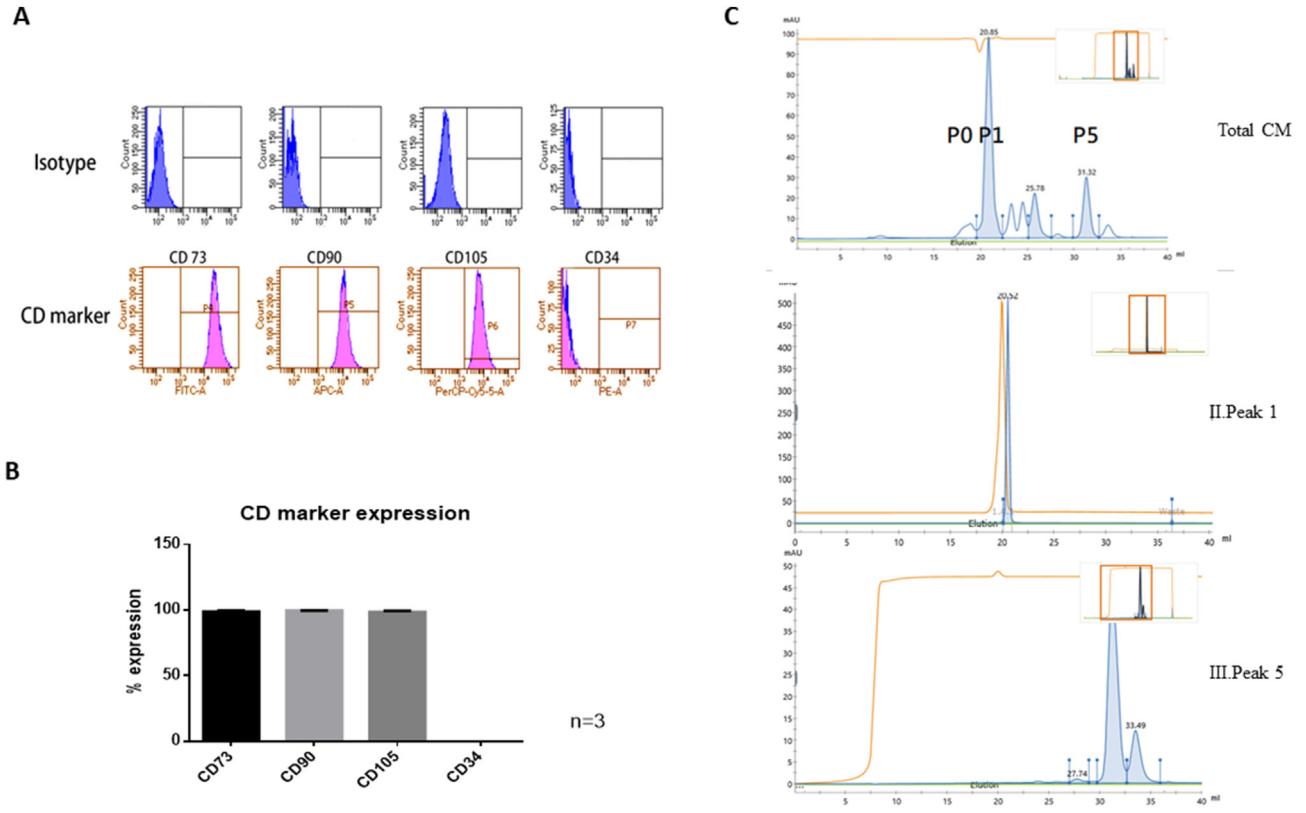

Figure 1 The characterization of SHED and Biological characterization of SHED-CM performance. (A) Stem cells from human exfoliated deciduous teeth (SHED) were stained with anti-CD34, anti-CD73, anti-CD90, anti-CD105, or isotype control, as indicated, and analyzed by flow cytometry. (B) Bar graphs represent the average mean fluorescence intensity as the mean ± SD of CD73, 90, 105 and 34 on SHED cells; n = 3. (C) Fast protein liquid chromatography (FPLC) revealed three dominant compounds in the conditioned medium. *Abbreviations:* CD-cluster of differentiation.

habituation period, it was placed back on the instrument. On the following day, the test trial was performed. The mice were placed on the instrument (Panlab Rota Rod, Harvard Apparatus, Holliston, MA, USA) and rotated at an initial speed of 4 rpm, accelerating at a steady rate of 4 rpm/s to a top speed of 40 rpm over the course of 300 s. The latency to fall was measured during the 5-min test session.

Olfactory test with habituation and dishabituation

Mice were selected for common stimulus response, with a "decrescendo-crescendo" habituation-dishabituation pattern shown in Fig. 4A. The habituation of the first stimulant, 0.02 % isoamyl acetate, prepped by solution, was tested for four times (I1—I4) for an overall duration of 14 min, whilst 0.02 % 2-heptatone diluted solution was administered in the last 2 min after a 2-min rest.

Tissue isolation

Under intraperitoneal injection of 3 % chloral hydrate (10 mL/kg), the experimental mice were sacrificed, followed immediately by isolation of the brain. The OB and substantia nigra were then dissociated and rinsed with PBS, and frozen in −196 °C liquid nitrogen until further use.

Immunohistochemistry staining

The immunohistochemistry (IHC) assay was performed using the Avidin—Biotin complex system (TSA™ kits, PerkinElmer Corp., Norwalk, CT, USA). Mice were sacrificed after behavior evaluation by injection of pentobarbital (200 mg/kg, i.p.) and perfused transcardially with 50 mL of saline followed by 500 mL of a fixative containing 4 % paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.3 for 30 min. Serial brain sections, 10 μm thick, were cut on a cryostat, thaw-mounted onto gelatin-coated slides, and used for immunohistochemical staining. The samples were de-paraffinized by heating at 60 °C for 30 min and application of xylene. They were then rehydrated using a series of decreasing concentrations of ethanol (100 %, 90 %, 70 %, and 50 %) with each step lasting 5 min, and then washed with 0.1 M PBS. The brain sections were incubated in 1 % H_2O_2 to block endogenous peroxidase activity, then washed by PBS and hybridized in blocking buffer (1 % goat serum, 0.5 % Triton-X100) with the primary antibody, anti-alpha-synuclein (1:80, #AB5334P; EMD Millipore, Billerica, MA, USA), anti-tyrosine hydroxylase (1:100, #MAB318; EMD Millipore), at 4 °C, overnight. They were then washed in PBS, hybridized with biotinylated donkey anti-mouse secondary antibody or anti-sheep secondary antibody (1:200, Jackson Immuno Research, West Grove, PA, USA) for 1 h and then transferred to ABC solution for 1 h. After PBS washing,

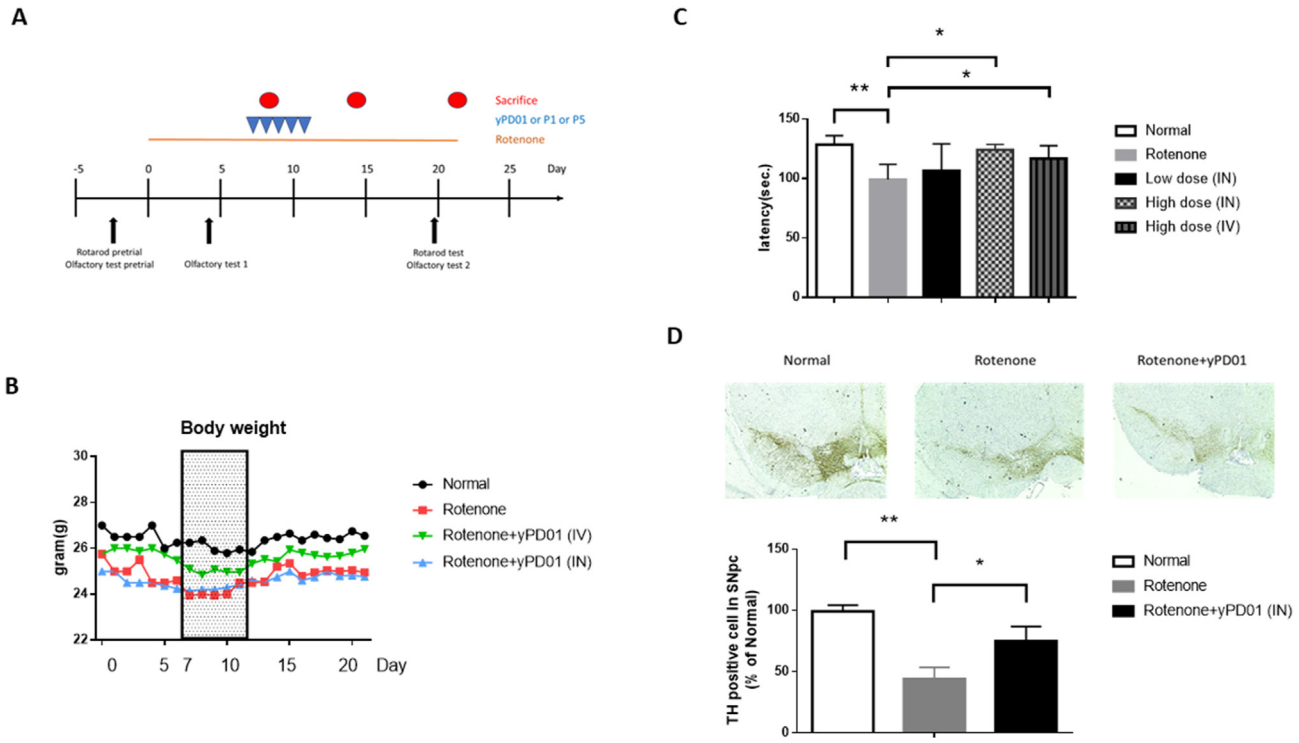

Figure 2 yPD01 prevented rotenone-induced neurotoxicity and motor deficits. (A) Graphical protocol of animal experiment. (B) Body weight comparison between the control, diseased and yPD01 restored group by intravenously and intranasal injection. (C) Motor ability of mice in different groups, with the rescue effect of intravenous or intranasal yPD01. (D) Comparison of the distribution of tyrosine hydroxylase in the substantia nigra of the control, diseased and yPD01 restored group. Data were shown as mean ± SD, $*P < 0.05$, $**P < 0.01$.

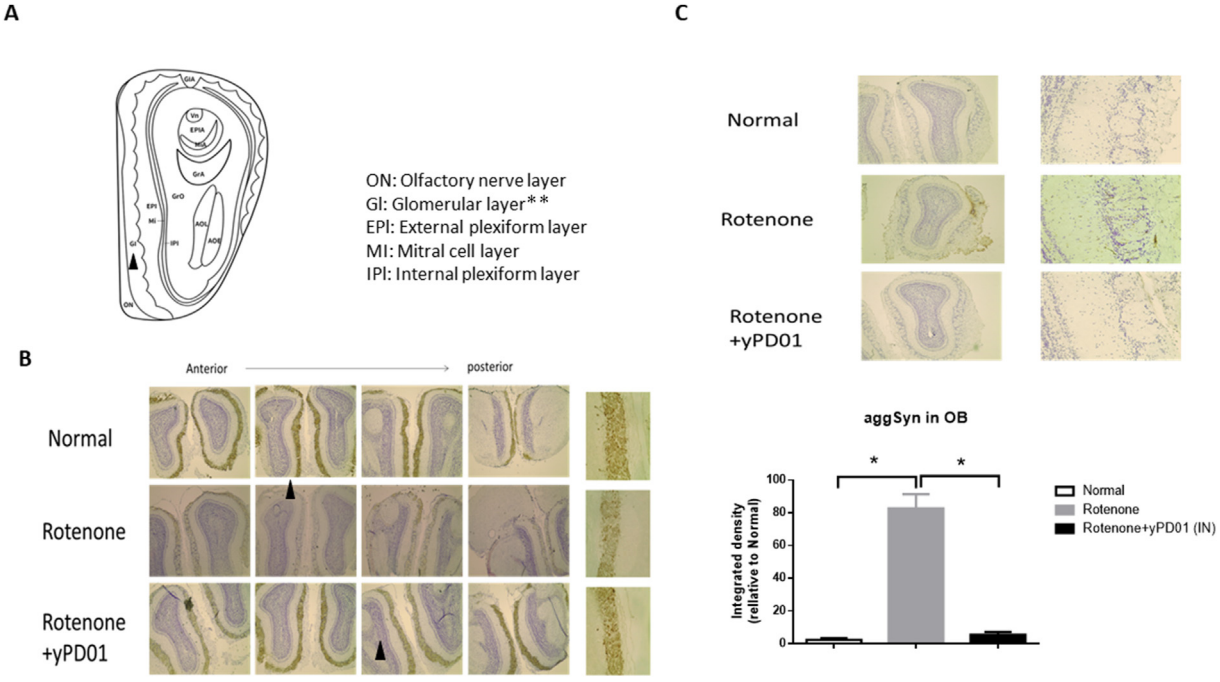

Figure 3 yPD01 prevented rotenone-induced TH neurons reduction and alpha-synuclein aggregation. (A) Tyrosine hydroxylase positive cells are mostly localized in the glomerular cell layer of OB. Comparison of the distribution of tyrosine hydroxylase (B) and alpha-synuclein (C) in the OB of the control, diseased and yPD01 restored group. Data were shown as mean ± SD, $*P < 0.05$.

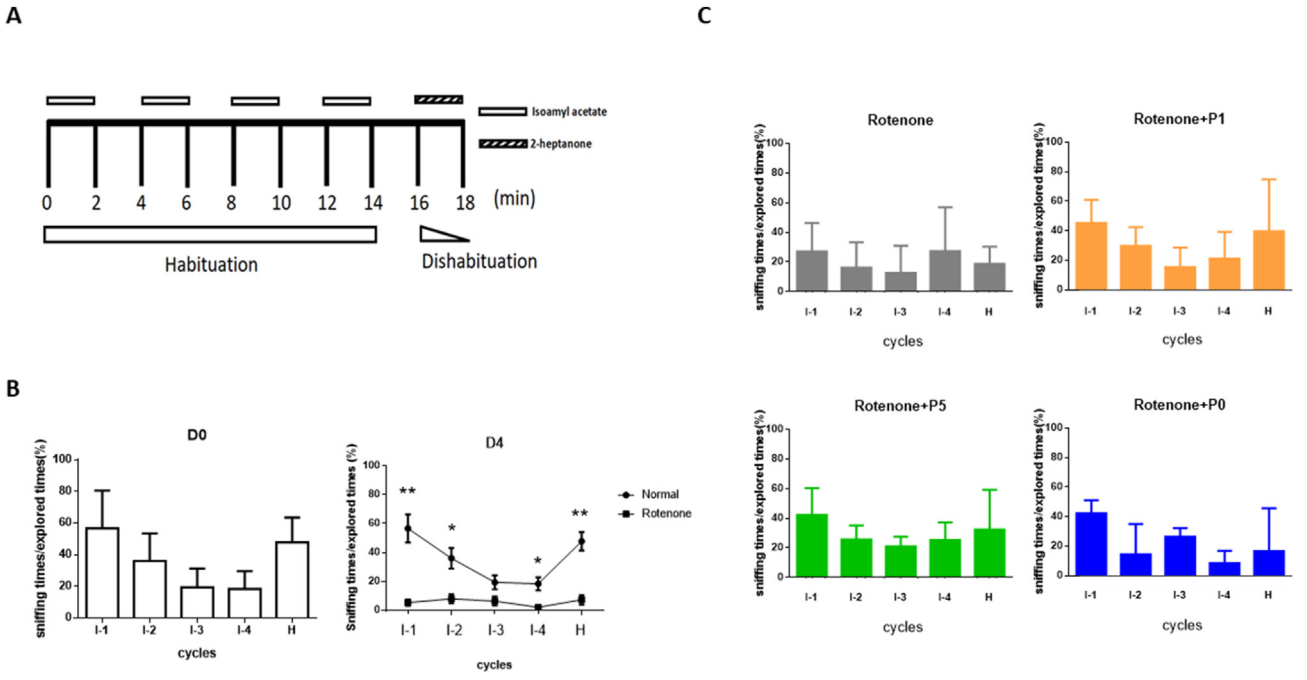

Figure 4 Mouse sniffing behavior during odor habituation/dishabituation. (A) Mice were exposed to two odors as indicated on the X-axis: isoamyl acetate (I1—I4) and 2-heptanone (H). The habituation and de-habituation protocol involved the use of isoamyl acetate during the first 14 min, followed by heptanone in the last 2 min after a 2-min resting period. A normal 'decrescendo-crescendo' sniffing/exploration pattern was observed on Day 0 (D0) and Day 4 (D4) following rotenone induction (B). The sniffing patterns on Day 21 (D21) after rotenone induction were compared among the disease group, and groups treated with P0, P1, and P5 (C). Data were shown as mean ± SD, $*P < 0.05$, $**P < 0.01$.

0.05 % 3,3′-diaminobenzidine (DAB) solution was added by gentle shaking to establish staining. The brain sections were then immersed in PBS to stop the reaction. Finally, the sections were fixed on a slide and observed with a microscope. The photos were analyzed and quantified by Image J software (version 1.52, National Institute of Health). The IHC images were converted to grayscale and the grey threshold defined. We then measured the gray integrated density of the staining exceeding the threshold (defined as the gray level value when the stained cells were completely removed, leaving only the background). For immunofluorescence staining, Cy5 conjugated to anti-mouse immunoglobulin antibody (Vector, Burlingame, CA, USA) and was used as a secondary antibody.

TIMP-1 levels in SHED-CM

ELISA for tissue inhibitor of metalloproteinase 1 (TIMP-1) was performed using an ELISA kit (#E0552h; Amsbio, Milton Park Abingdon, UK) following the manufacturer's instructions. Protein concentrations were determined using a standard curve.

Statistical analysis

Statistical data are generally presented as the mean ± standard deviation (SD) of at least three biological individuals. Statistical analysis was performed using unpaired two-tailed t-tests and one-way ANOVA with GraphPad Prism 8.2.1 (GraphPad Software, San Diego, CA, USA), where a P-value <0.05 was considered a significant difference. With ordinary one-way ANOVA, Tukey's multiple comparison test was used for post hoc multiple comparison testing.

Results

SHED, flow cytometry and cell markers

SHED cells were prepared and analyzed using flow cytometry, which revealed that the majority of markers expressed on SHED are CD73, CD90, and CD105, with no detection of CD34 (Fig. 1A and B). We further used FPLC to separate and characterize the SHED-CM, identifying three prominent compounds: (1) P0, with a peak absorbance of about 3 mAU and a retention time of approximately 18 min; (2) P1, with a peak absorbance of about 35 mAU and a retention time of approximately 20.78 min; and (3) P5, with a peak absorbance of about 12 mAU and a retention time of approximately 32.37 min (Fig. 1C). ELISA test results revealed that the predominant component of P1 is TIMP-1 (Supplemental data).

Difference in rotenone-induced PD mice and the treatment of yPD01

We established an experimental PD model by administering 50 mg/kg/day of rotenone orally for 21 consecutive days to replicate the pathological hallmarks of PD (Fig. 2A). Induction resulted in a mild decrease in body weight,

particularly between days 7 and 11 (Fig. 2B), but this was recovered following the administration of yPD01. However, unlike previous studies that observed weight loss in PD mice, this effect was not found in the current study. Each body weight was approximately 26 g. Motor ability was assessed using the Rotarod test, a well-known system for evaluating brain functions. The mice in the PD group exhibited significant postural instability and reduced latency on the Rotarod device, but improvements were noted after the introduction of yPD01, administered either intranasally at different doses or intravenously (Fig. 2C). Finally, TH levels were compared among the normal, PD, and post-treatment groups. TH is known to mediate the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), the precursor of dopamine. The substantia nigra of the PD mice showed significantly lower levels of TH, but these levels were restored after the administration of yPD01 (Fig. 2D).

The OB of the rotenone-treated PD mice were isolated and collected for analysis, with TH-positive cells primarily localized in the OB glomerular cell layer (GL; Fig. 3A). Immunohistochemistry analysis of the OB showed that rotenone administration resulted in severe shrinkage of the GL with decreased TH expression, while also facilitating the accumulation of alpha-synuclein, consistent with previous results. Restoration of TH expression was observed following the intranasal administration of yPD01 (Fig. 3B), which also halted the accumulation of alpha-synuclein in the OB (Fig. 3C).

Olfactory function evaluation model

Experiments were conducted on fledgling mice, which were grouped based on their response to stimulants following a unitary 'decrescendo-crescendo' pattern. The habituation phase was carried out over the first 14 min using 0.02 % isoamyl acetate (I), while de-habituation was conducted with 0.02 % 2-heptanone (H) during the final 2 min, following a 2-min rest period (Fig. 4A). Rotenone induction led to impaired olfactory detection and a diminished response to stimuli after four days of administration (Fig. 4B). Regarding sniffing duration, we observed that mice with rotenone-induced olfactory dysfunction tended to increase their exploration time for new odors after treatment with P1 and P5, although no significant differences were noted among the groups (Fig. 4C).

Intranasal administration of yPD01 stopped the intraneuronal aggregation of alpha-synuclein

Following IN administration, double immunofluorescence staining was conducted to observe the quantity and density of targeted cell expression. In Fig. 5, the red color indicates TH-containing cells, while the green color represents alpha-synuclein aggregates. In PD diseased cells, alpha-synuclein (green) begins to accumulate by week 2, showing maximum performance in the week 3 and merging with the red area to produce yellow coverage. In the yPD01-treatment group (bottom), no green is observed in weeks 1 or 2, and it appears only minimally in week 3. This indicates that alpha-synuclein was not expressed inside the TH-containing cells, suggesting that yPD01 prevented the dopaminergic intraneuronal aggregation of alpha-synuclein (Fig. 5).

Discussion

The SHED-CM used in the study was limited to proteins within 5—30 kDa, removing smaller compounds such as protease and peptidase, and larger particles which were considered cell debris or impurities, thereby avoiding non-specific effects and allowing the absorption of active constituents in CM.[15] The purified solution, renamed as "yPD01", consisted of two substantial compounds, which probably figured prominently in the reversal of neurological deficits in the PD models (Fig. 1).

We modulated the dosage and route of yPD01 administration and found that intranasal administration achieved motor neurological improvements comparable to those achieved by intravenous administration (Fig. 2C). We evaluated olfactory function using the habituation-dishabituation chart (Fig. 4A), the first such before/after comparison for the intranasal administration of cell products in PD models. The reduced sniffing/exploration time ratio indicated that the mice either suffered from attenuation of olfactory function due to a reduction of dopaminergic neurons caused by rotenone administration, or neurodegeneration of cranial neurons leading to motor dysfunction and thus increased exploration time (Fig. 4B). Two proteins, P1 and P5, were found to be vital compounds in the conditioned medium, as treatment with these two proteins improved the olfactory function pattern in these two specific groups (Fig. 4C and D).

Most previous studies on the intravenous administration of stem cell products in Parkinson's disease models have focused on motor deficits and brain tissue sampling, particularly in terms of the location of TH-positive cells in the striatum and substantia nigra.[18] Several articles have mentioned olfactory epithelium regeneration via intravenous treatment of stem cell products after neuron transection.[19,20] However, intravenous administration is still associated with the targeting of peripheral organs, pulmonary entrapment, and a lack of significant cell engraftment in the brain parenchyma, phenomena not associated with intranasal administration which provides more concentrated delivery to the brain and thus greater therapeutic efficacy for PD and other diseases models.[21] The study found reduced alpha-synuclein accumulation and increased tyrosine hydroxylase expression in both the intravenous and intranasal yPD01 administration groups (Figs. 3—5), systematic intranasal delivery may provide a relatively efficient route for PD treatment.

Previous studies using ELISA arrays had identified high levels of insulin-like growth factor binding protein 6 (IGFBP-6), tissue inhibitor of metalloproteinase 2 (TIMP-2), and TIMP-1, as active constituents in SHED-CM.[15] Through ELISA and Cytokine Array assays, we confirmed that TIMP-1 is the main component of P1, while the composition of P5 remains unknown. Accumulating evidence implicates matrix metalloproteinases (MMPs) as major contributors to the pathogenesis of PD. The expression of MMPs, such as MMP-1, -2, and -9, as well as

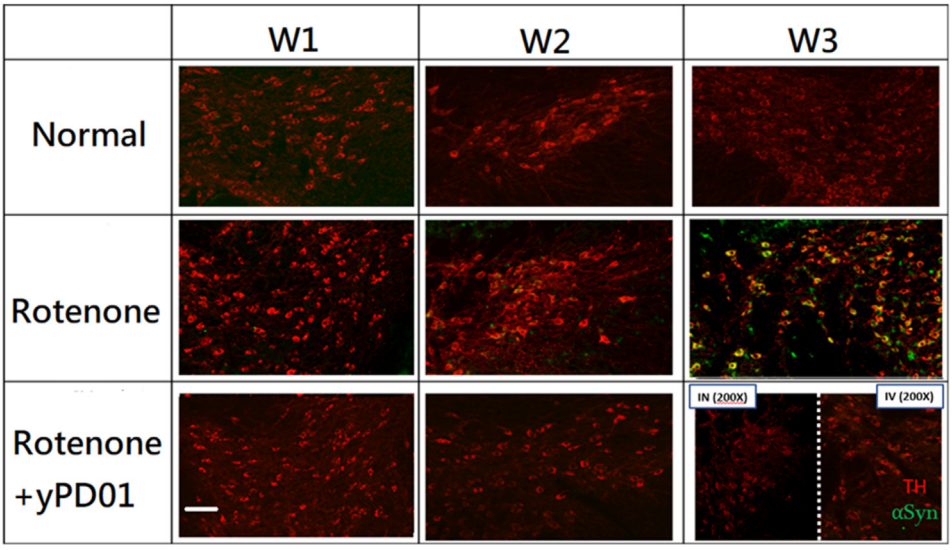

Figure 5 Double-staining in substantia nigra after intranasal and intravenous administration of yPD01. The red color indicates the TH-containing cells, whereas the green color represents alpha-synuclein aggregates. Yellow content shows intraneuronal aggregation of alpha-synuclein. *Abbreviations:* W- week, Rot-rotenone, aSyn-alpha-synuclein. TH - tyrosine hydroxylase. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

TIMP-1 and -2, in the substantia nigra of postmortem PD brain tissue was first reported by Lorenzl et al., who observed alterations in MMP-2 and TIMP-1 in the substantia nigra of PD patients.[22] Since then, numerous studies have demonstrated that MMPs are involved in various pathophysiological processes of PD, including microglial activation, inflammation, direct dopaminergic apoptosis, disruption of the BBB, and modulation of alpha-synuclein cleavage. Therefore, TIMP-1 represents a potential clinical target for therapeutic intervention in PD.

As shown in Fig. 5, both intranasal and intravenous administration halted dopaminergic intraneuronal aggregation of alpha-synuclein in the OB. Alpha-synuclein accumulation was proposed to be a pathological hallmark of PD or Lewy Body Dementia (LBD), as both can be distinguished in terms of the onset of Parkinsonism and cognitive impairment, and are both affected by the presence of alpha-synuclein rich Lewy Bodies (LBs) and Lewy Neurites (LNs) in neurons.[23] The water-selective channel aquaporin-4 (AQP4) is related to water homeostasis and the functioning of the glymphatic system, which eliminates various metabolites and amyloidogenic proteins from the brain tissue.[24] However, the specific role of AQP4 in these mechanisms remains unclear. A recent study has shown that alpha-synuclein clearance and propagation are mediated by glymphatic function and AQP4 mislocalization may contribute to the development and propagation of Lewy body pathology in human synucleinopathy such as Lewy Body Dementia and Parkinson's disease.[25] Braak et al. elucidated the impact of the alpha-synuclein accumulation in different brain regions, suggesting that sporadic PD progresses in a caudo-rostral pattern. This was later followed by the dual-hit hypothesis, which implies that sporadic PD can originate in the neurons of the nasal cavity and in the gut.[26,27] The solution yPD01 administered in our experiment could potentially interrupt the dual-hit effect by preventing or slowing the spread of alpha-synuclein aggregation and reducing the overall burden of pathology in the OB.

This study demonstrates that yPD01 exhibits the potential to improve behavioral and histological neurodegenerative symptoms: (1) P1 and P5 proteins were vital compounds in the conditioned medium that contribute to improving neurological functions, particularly in olfactory recovery, with TIMP1 being confirmed as the main component of P1. (2) Both intravenous and intranasal administration of yPD01 showed significant therapeutic effects, as evidenced by reduced alpha-synuclein accumulation and increased tyrosine hydroxylase expression. The intranasal administration route may offer a more effective and less invasive therapeutic approach to PD treatment.

yPD01, a conditioned medium of SHED subsequent to purification with FPLC, exhibits promise for alpha-synuclein clearance and the improvement of motor deficits in a rotenone-induced animal model of PD. The solution can be effectively applied intranasally to achieve neuroprotective and potentially regenerative outcomes for olfactory dysfunction related to PD.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgements

The authors gratefully acknowledge the support of National Taiwan University Hospital, the laboratory animal center of Taipei Medical University, and National Science and Technology Council which provided facilities, equipment, and grants. We would like to thank Young Cell Bank Ltd. Co. for

technical support. This study was supportted by grant MOST 110-2622-B-016-001, NSTC 111-2622-B-016-001.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2024.11.029.

References

1. Zhao L, Chen S, Yang P, Cao H, Li L. The role of mesenchymal stem cells in hematopoietic stem cell transplantation: prevention and treatment of graft-versus-host disease. *Stem Cell Res Ther* 2019;10:182.
2. Andrzejewska A, Lukomska B, Janowski M. Concise review: mesenchymal stem cells: from roots to boost. *Stem Cell* 2019; 37:855—64.
3. Kupcova Skalnikova H. Proteomic techniques for characterisation of mesenchymal stem cell secretome. *Biochimie* 2013;95: 2196—211.
4. Wang Y, Ji X, Leak RK, Chen F, Cao G. Stem cell therapies in age-related neurodegenerative diseases and stroke. *Ageing Res Rev* 2017;34:39—50.
5. Yu B, Zhang X, Li X. Exosomes derived from mesenchymal stem cells. *Int J Mol Sci* 2014;15:4142—57.
6. Moll G, Rasmusson-Duprez I, von Bahr L, et al. Are therapeutic human mesenchymal stromal cells compatible with human blood? *Stem Cell* 2012;30:1565—74.
7. Erdő F, Bors LA, Farkas D, Bajza Á, Gizurarson S. Evaluation of intranasal delivery route of drug administration for brain targeting. *Brain Res Bull* 2018;143:155—70.
8. Losurdo M, Pedrazzoli M, D'Agostino C, et al. Intranasal delivery of mesenchymal stem cell-derived extracellular vesicles exerts immunomodulatory and neuroprotective effects in a 3xTg model of Alzheimer's disease. *Stem Cells Transl Med* 2020;9:1068—84.
9. Ma X, Huang M, Mengna Z, et al. ADSCs-derived extracellular vesicles alleviate neuronal damage, promote neurogenesis and rescue memory loss in mice with Alzheimer's disease. *J Contr Release* 2020;327:688—702.
10. Sahay A, Scobie KN, Hill AS, et al. Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. *Nature* 2011;472:466—70.
11. Zhao Z, Ning J, Bao XQ, et al. Fecal microbiota transplantation protects rotenone-induced Parkinson's disease mice via suppressing inflammation mediated by the lipopolysaccharide-TLR4 signaling pathway through the microbiota-gut-brain axis. *Microbiome* 2021;9:226.
12. Li P, Ou Q, Shi S, Shao C. Immunomodulatory properties of mesenchymal stem cells/dental stem cells and their therapeutic applications. *Cell Mol Immunol* 2023;20:558—69.
13. Khalil WK, Assaf N, ElShebiney SA, Salem NA. Neuroprotective effects of bee venom acupuncture therapy against rotenone-induced oxidative stress and apoptosis. *Neurochem Int* 2015; 80:79—86.
14. Huang Y, Mei X, Jiang W, et al. Mesenchymal stem cell-conditioned medium protects hippocampal neurons from radiation damage by suppressing oxidative stress and apoptosis. *Dose Response* 2021;19:1559325820984944.
15. Chen YR, Lai PL, Chien Y, et al. Improvement of impaired motor functions by human dental exfoliated deciduous teeth stem cell-derived factors in a rat model of Parkinson's disease. *Int J Mol Sci* 2020;21:3807.
16. Chen TF, Chen KW, Chien Y, et al. Dental pulp stem cell-derived factors alleviate subarachnoid hemorrhage-induced neuroinflammation and ischemic neurological deficits. *Int J Mol Sci* 2019;20:3747.
17. Vogel RW, Ewers M, Ross C, Gould TJ, Woodruff-Pak DS. Age-related impairment in the 250-millisecond delay eyeblink classical conditioning procedure in C57BL/6 mice. *Learn Mem* 2002;9:321—36.
18. d'Angelo M, Cimini A, Castelli V. Insights into the effects of mesenchymal stem cell-derived secretome in Parkinson's disease. *Int J Mol Sci* 2020;21:5241.
19. Franceschini V, Bettini S, Pifferi S, et al. Human cord blood CD133+ stem cells transplanted to nod-scid mice provide conditions for regeneration of olfactory neuroepithelium after permanent damage induced by dichlobenil. *Stem Cell* 2009;27: 825—35.
20. Kim YM, Choi YS, Choi JW, et al. Effects of systemic transplantation of adipose tissue-derived stem cells on olfactory epithelium regeneration. *Laryngoscope* 2009;119:993—9.
21. Danielyan L, Beer-Hammer S, Stolzing A, Schäfer R, Siegel G, Fabian C. Intranasal delivery of bone marrow-derived mesenchymal stem cells, macrophages, and microglia to the brain in mouse models of Alzheimer's and Parkinson's disease. *Cell Transplant* 2014;23(Suppl 1):S123—39.
22. Lorenzl S, Albers DS, Narr S, Chirichigno J, Beal MF. Expression of MMP-2, MMP-9, and MMP-1 and their endogenous counter-regulators TIMP-1 and TIMP-2 in postmortem brain tissue of Parkinson's disease. *Exp Neurol* 2002;178:13—20.
23. Shults CW. Lewy bodies. *Proc Natl Acad Sci USA* 2006;103: 1661—8.
24. Lapshina KV, Ekimova IV. Aquaporin-4 and Parkinson's disease. *Int J Mol Sci* 2024;25:1672.
25. Braun M, Simon MJ, Jang J, et al. Aquaporin-4 mis-localization slows glymphatic clearance of α-synuclein and promotes α-synuclein pathology and aggregate propagation. *bioRxiv [Preprint]* 2024;19:2024. 08.14.607971.
26. Braak H, Del Tredici K, Rüb U, de Vos RA, Jansen Steur EN, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol Aging* 2003;24:197—211.
27. Hawkes CH, Del Tredici K, Braak H. Parkinson's disease: the dual hit theory revisited. *Ann N Y Acad Sci* 2009;1170:615—22.