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Original Article

Factors derived from human exfoliated deciduous teeth stem cells reverse neurological deficits in a zebrafish model of Parkinson's disease

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KEYWORDS
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Abstract *Background/purpose:* Mesenchymal stem cells exhibit therapeutic efficacy for brain injury. This study examined the effect of mesenchymal stem cells derived from human exfoliated deciduous teeth (SHED) on alleviating symptoms of Parkinson's disease (PD).
Materials and methods: SHED were isolated to examine the biosafety and bioavailability of stem cells derived from human exfoliated deciduous teeth-derived conditioned medium (SHED-CM) for the alleviation of PD symptoms in a 6-hydroxydopamine (6-OHDA)-induced PD zebrafish model.

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Results: SHED-CM administration did not induce neurological, skin or muscle toxicity in control zebrafish at any dose, and estrogen equivalent testing showed no chronic toxicants. Induction of PD with 6-OHDA suppressed zebra SHED-CM was administered to zebrafish treated with 6-OHDA to induce PD symptoms. Similar to nomifensine, a drug with proven anti-PD potential, SHED-CM repaired the motor deficiencies in the zebrafish PD model.
Conclusion: Our results indicate the biosafety of SHED-CM and its therapeutic potential in treating PD in a zebrafish model.

Introduction

Parkinson's disease (PD) is a chronic progressive neurodegenerative disease,[1] the pathogenesis of which is associated with disruption of the dopamine transporter pathway, mitochondrial dysfunction,[2] neuroinflammation in substantia nigra,[3] environmental toxins,[4] lipoprotein transporter mutation[5] and familial factors. The precise mechanisms and curative treatment of PD remain poorly understood, but mammalian PD models involving complicated neuroanatomical networks may not be ideal platforms for the initial stages of evaluation and development of novel drugs or therapeutics. Zebrafish are widely seen as an ideal model for investigating neurodegenerative diseases due to their: i) easy handling and maintenance; ii) well-studied and understood central nervous system; iii) brains that exhibit high regenerative capacity; iv) high PD-related gene identity with humans; and v) high translational value.[6] Among drugs that can induce PD-like symptoms, 6-hydroxydopamine (6-OHDA) is a selective catecholamine neurotoxin and is the most common drug used to induce PD in animal models.[7] 6-OHDA inhibits mitochondrial function and induces inflammation, leading to damage of dopaminergic terminals and cell bodies, while causing the loss of dopaminergic neurons in zebrafish,[8] making 6-OHDA-treated zebrafish an excellent animal model for the development and screening of potential drug/therapeutics for PD.

Our stem cell-based therapy was conducted using cells from the normal pulp of human exfoliated deciduous teeth (SHED). SHEDs originate from the cranial neural crest and expresses early MSC markers and are easily obtained without raising ethical issues. SHEDs have promising potential in immunomodulatory and regenerative medicine. The stem cells derived from human exfoliated deciduous teeth-derived conditioned medium (SHED-CM) has been reported to effectively treat autoimmune encephalitis.[9] Based on our previous study in Parkinson's disease (PD) rats[10] and other reports focusing on amyotrophic lateral sclerosis (ALS)[11] and allodynia[12] animal models, we identified significant neuroprotective effects and improvements in motor function as the major findings of this research. SHED-CM treatment could represent a promising new strategy for treating neurodegenerative diseases. In the present study, SHEDs were isolated and SHED-CM was prepared with a focus on the spectrum of proteins present, and the biosafety and effectiveness of SHED-CM on alleviating the symptoms of PD was examined in a zebrafish PD model.

Material and methods

Isolation and culture of stem cells from SHED

Exfoliated deciduous teeth were obtained from children aged 5—9, with informed consent from donors' parents. The study was approved by the Institutional Review Board of Tri-Service General Hospital, National Defense Medical Center (Taipei, Taiwan) (approval no. TSGH IRB No.: 2-108-05-103). The SHED cells were extracted by syringe from the root of the deciduous tooth and transferred to a 25 cm^2 flask (Corning, Inc., NY, USA). Isolation was performed as described previously.[13] SHED cells were cultured in MSC NutriStem® XF Basal Medium supplemented with MSC NutriStem® XF Supplement mix (both from Biological Industries, Beit Haermek, Israel). The SHED cells were not subjected to any depletion techniques and, upon confluence, were detached using Accutase® and subcultured in 75 cm^2 flasks at a density of 1.5×10^6 cells/cm^2, and then sub-cultured in a 150 cm^2 flask (all from Corning, Inc., Corning, NY, USA) at a density of 3×10^6 SHED cells at passage 3. The cells were incubated at 37 °C in an atmosphere containing 5% CO_2 at 100% humidity. The SHED cells used in the present study exhibited a fibroblast-like morphology with a bipolar spindle shape, and expressed the MSC markers CD73, CD90 and CD105, but not endothelial/hematopoietic markers such as CD34 and CD45.[14]

Identification of SHED cells by flow cytometry

SHED cell characteristics were confirmed using flow cytometry (CD34-, CD73+, CD90+ and CD105+).[14] Zhang et al. reported the differentiation capacity of SHED cells.[15] For cell surface marker analysis, SHED cells were incubated with antibodies against MSC markers for 30 min at room temperature (FITC-CD90, PerCP-Cy™5.5-CD105 and APC-CD73) (#562245, BD Biosciences, Bergen, NJ, USA), negative markers (endothelial/hematopoietic markers) followed by PE-CD34 and PE-CD45 (#562245, BD Biosciences). The cells were then analyzed using a FACSCalibur flow cytometer (BD Biosciences), with data analysis conducted using BD FACSDiva (v8.0.1, BD Biosciences).

Preparation of conditioned medium

When passage 3—5 (P3-P5) SHED cells reached 80% confluency in a 150 cm^2 cell culture flask, the medium was replaced with 10 ml DMEM/F12 (Cat. 21041025, Gibco, Waltham, MA, USA) and 10 ml PBS and then incubated for 72 h. The medium was collected and centrifuged at 1400*g* for 3 min at room temperature. To enrich proteins ranging between 5 and 30 kDa in weight, CM was collected in a sterilized beaker and further concentrated using a Tangential Flow Filtration (TFF) membrane filter system (EMD Millipore, Burlington, MA, USA) with 5 and 30 kDa cut-off points (EMD Millipore) for 3 h following the manufacturer's protocol.[16] The protein concentration in SHED-CM was measured using a Pierce™ bicinchoninic acid protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) and adjusted to 100.0 ± 7.8 μg/ml using normal saline. To ensure consistent efficacy, the data shown were generated using a single batch of SHED-CM from the standardized procedure for further experiments.

The protein concentration of the CM was adjusted to 10 mg/ml (10 ng/nl) for experiments (the initial CM volume was 100 ml with a concentration of 100.0 ± 7.8 μg/ml). After lyophilization and dissolution of the constituents with distilled water, a protein solution with a final concentration of 10 mg/ml was established, achieving a 100-fold concentration. The maximum injection volume into the yolk sac and muscle in zebrafish larvae was respectively 40 and 20 nl/fish, with respective maximal doses for yolk sac injection and intramuscular injection of 400 and 200 ng/fish. Quantification of each constituent was analyzed using a Qubit Fluorometric Quantification and an ELISA array, 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.

Animal supplies

Fig. 1 shows a flow chart of the CM procedure and experiments in the PD zebrafish model. Animal experiments were approved by the Institutional Animal Care and Use Committee or Panel (IACUC/IACUP) of the Taipei Medical University (Taipei, Taiwan) (approval no. LAC2022-0257). All experiments and protocols were performed in compliance with human and animal ethical regulations. Adult zebrafish were raised at 27.5—28.5 °C on a 14-h light, 10-h dark cycle in an Aquaneering recirculating water system (Vitargent Biotechnology, Shatin, Hong Kong). All zebrafish used for experiments were wild type, AB strain.[17]

Creation of the Parkinson's disease animal model in zebrafish

Embryos of AB strain wild-type zebrafish were collected after natural spawning, staged, and raised synchronously at 28.5 °C in Hank's buffer (13.7 mM NaCl, 540 mM KCl, 25 mM Na_2HPO_4, 300 mM $CaCl_2$, 100 mM $MgSO_4$, 420 mM $NaHCO_3$, pH 7.4). After retrieving the fertilized ova, zebrafish larvae 2 days post fertilization (dpf) were treated with 6-OHDA (250 μM), either with or without the assessed drugs for 4 days in a 24-well plate. At 5 dpf, zebrafish were transferred into 10-cm dishes (10 fish/dish), and swimming behavior was evaluated as described previously.[7]

Survival test

30 wild-type AB zebrafish from each group were transferred into a 6-well microplate and treated with 0—400 ng/fish CM

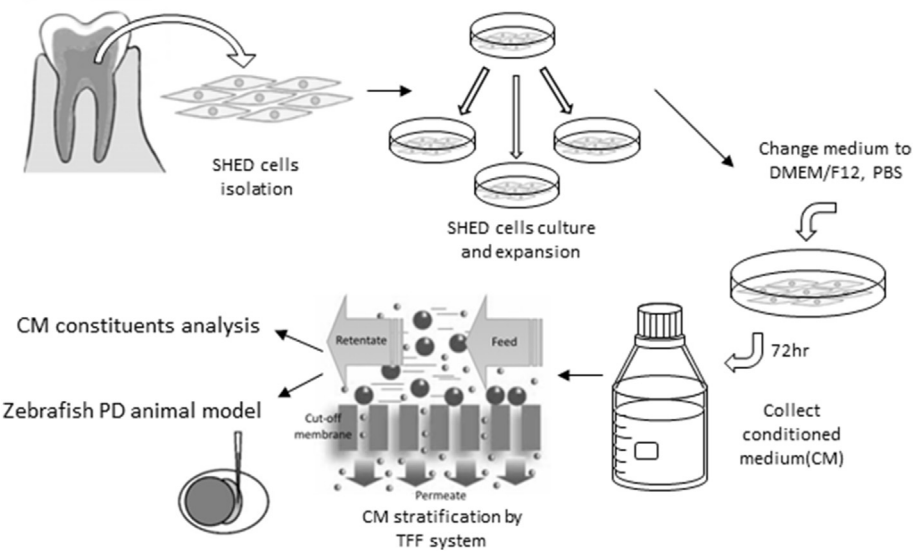

Figure 1 Flow chart of stem cells derived from human exfoliated deciduous teeth-derived condition medium (SHED-CM) preparation and anti-Parkinson's disease (anti-PD) efficacy in the zebrafish model of PD. Instead of using all the elements inside the SHED-CM, we treated the PD-zebrafish with a relatively purified and quantified protein to achieve high quality control and reproducibility of the experiments. *Abbreviations:* DMEM/F12 - Dulbecco's Modified Eagle Medium/F12, PBS - Phosphate buffered saline, TFF - Tangential Flow Filtration.

by yolk sac injection, with an injection volume of 40 nl/fish. Zebrafish mortality was observed for 14 days. The yolk sac microinjection technique was performed as described previously.[18] Microinjection needles (outer diameter (O.D.) = 20 μm; BM100T-10, BioMedical Instruments, Shenzhen, Guangdong, China) were filled with 40 μl CM using a Microloader (Eppendorf, Hamburg, Germany). The needle was placed in the manipulator (Narishige MN-151; Narishige International, Ltd., London, U.K) and connected to a pneumatic pump for microinjection (Eppendorf). Injections were performed under 20× magnification using an inverted light microscope. Zebrafish embryos were fixed at the edge of a microscope slide set in a petri dish. Excess water was removed using a mesh to immobilize the eggs during this procedure.

Skin, pigmentation and muscle toxicity test

50 wild-type AB zebrafish larvae were transferred to a 6-well microplate and treated with SHED-CM (44, 133 and 400 ng/fish) by yolk sac injection. SHED-CM was diluted in physiological saline as the vehicle control. After treatment, zebrafish were stained with acridine orange (AO, acridinium chloride hemi-[zinc chloride], (Sigma—Aldrich, Sofia, Bulgaria) at room temperature. 10 zebrafish from each group were photographed under a fluorescence microscope to quantify skin fluorescence intensity while observing morphology and pigmentation changes.

Neurotoxicity test

50 wild-type AB zebrafish were transferred into a 6-well microplate and treated with SHED-CM (22, 67 and 200 ng/fish) by muscle injection with an injection volume of 20 nl/fish. Subjects were anesthetized by adding drops of tricaine methanesulfonate (4 mg/ml stock solution (MS222, Sigma—Aldrich) into the culture medium until operculum movement slowed. Intramuscular injection was performed via a 26G micro-syringe at an angle of 45° into the dorsal lateral musculature.[19] The whole procedure was performed with the left side facing up. SHED-CM was diluted in physiological saline, and physiological saline was used as the vehicle control. After treatment, the average movement speed of 10 zebrafish from each group was monitored for 60 min using an automated video tracking system (TM-01, Singa Technology Co., Taoyuan City, Taiwan).

Estrogen equivalent (EEQ) test

Embryos (3 embryos per group per test) were treated with different doses of CM plus 3 parts per billion estradiol-17β (E2) (Perkin Elmer, Inc., Waltham, MA, USA). Estrogen activity was tested and GFP signal was induced using a stable transgenic system containing the promoter of the estrogen-dependent liver-specific choriogenin H (ChgH) gene regulating enhanced green fluorescence protein (eGFP) gene coding region (ChgH-eGFP).[20] For the EEQ dose response test, the *P*-value of the T-test for different concentrations was compared with the estradiol-17β (E2) control.

Behavior test for Parkinson's disease

A total of 70 (10 for each group) 6-OHDA treated and wild-type (control) zebrafish were injected with vehicle (physiological saline; negative control), SHED-CM (22, 44, 133 and 400 ng/fish) or nomifensine (1.5 μg/ml), a drug for treatment of PD (positive control) by yolk sac injection (40 nl/fish), and then transferred to a 96-well microplate. The plate was loaded into a zebrafish-specific behavior analysis system (Viewpoint, Inc., Portland, OR, USA), and the motility of each larva was recorded for 30 min using an automated video tracking system. The average movement speed (mm/min) of the larva was measured as the endpoint to assess PD treatment efficacy.

Western blotting

Fish brain tissues were collected in lysis buffer (100 mM HEPES pH 7.5, 5 mM magnesium chloride, 150 mM sodium chloride, 1 mM EDTA, 1% (v/v) Triton X-100 and 1% (v/v) protease inhibitor cocktail (Calbiochem, San Diego, CA, USA)) and centrifuged at 12,000 g at 4 °C for 10 min. We took 30 μg proteins resolved by 15% SDS-PAGE as supernatant. The proteins were transferred onto a nitrocellulose membrane using an electric blotting apparatus (Biorad, Taipei, Taiwan), blocked with washing buffer A (150 mM sodium chloride plus 13 mM Tris—hydrochloric acid, pH 7.5, and 0.1% (v/v) Tween 20) containing primary antibody for at least 1 h at room temperature. Each membrane was washed 10 × 3 min with washing buffer A and subsequently incubated with the secondary antibody (anti-TH Tyrosine Hydroxylase Antibody (NBP2-42212)) for 1 h at room temperature. The blots were developed using the enhanced chemiluminescent kit (Pierce) on Kodak CL-Xposure™ film.

Statistical analysis

Results are presented as the mean ± standard deviation. Statistical comparisons were performed using unpaired Student's *t*-test (EEQ data), or one-way ANOVA followed by Tukey's post-hoc test (all other data). $P < 0.05$ indicated statistical significance.

Results

Preparation of SHED derived mesenchymal stem cells

SHED stem cells were isolated to examine their MSC-specific features, revealing a fibroblast-like morphology (Fig. 2A). Flow cytometry results showed the SHED stem cells were negative for hematopoietic markers (CD34 and CD45) (Fig. 2B), and highly positive for MSC markers (CD73, CD90 and CD105) (Fig. 2C). Taken together, these data confirm the mesenchymal features and morphology of SHED stem cells.

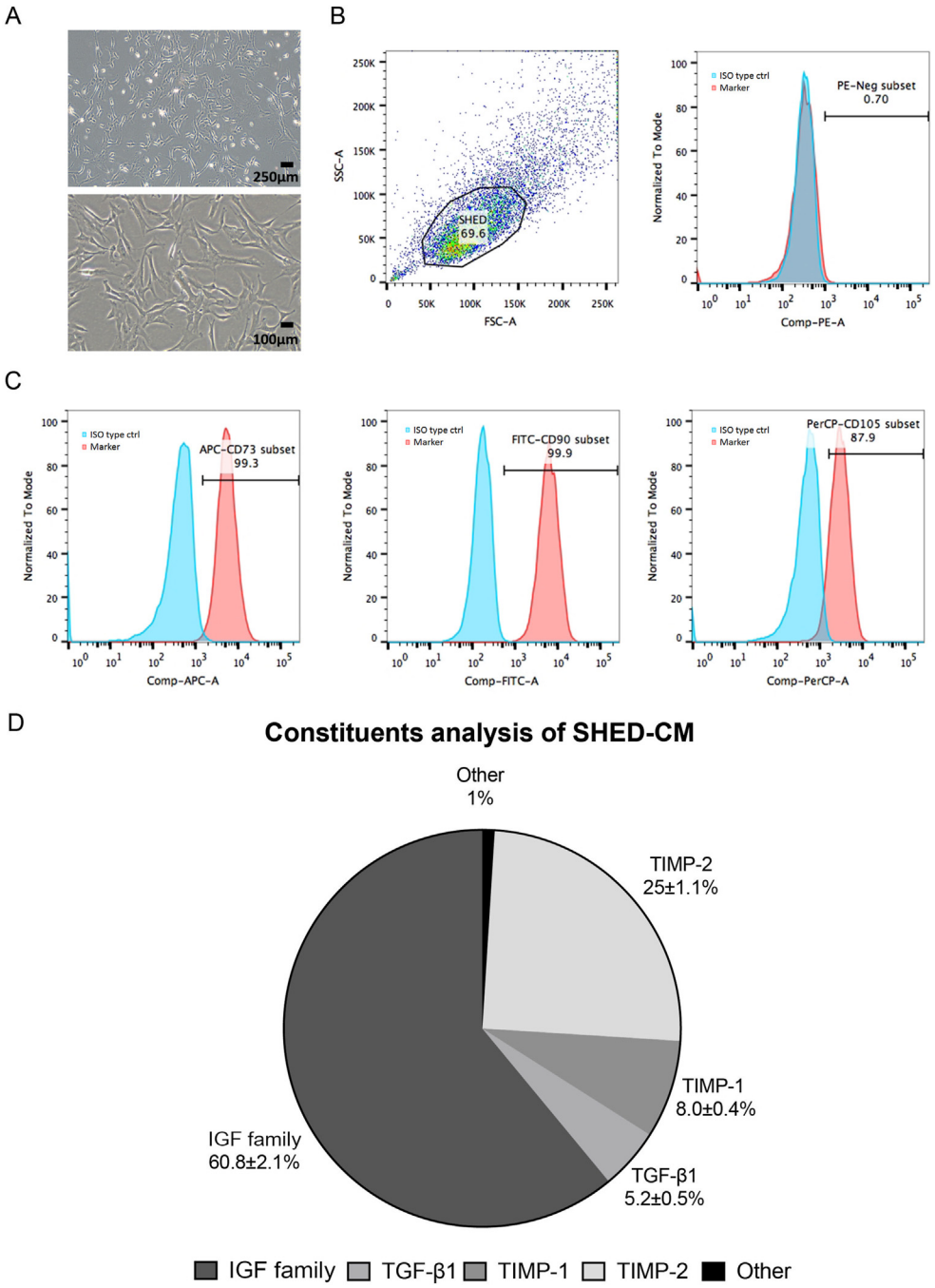

Figure 2 Characterization of the mesenchymal features of stem cells derived from human exfoliated deciduous teeth (SHED) and active constituents in the SHED-derived conditioned medium (SHED-CM). (A) Microscopic examination of SHED at passage 2 shows the fibroblast-like SHED morphology after cultivation. Flow cytometry analysis shows SHED were negative for (B) hematopoietic markers (CD34 and CD45) and positive for (C) mesenchymal stem cells markers, CD73, CD90 and CD105. (D) Quantibody cytokine arrays show IGF family and, TIMP-2 levels were the highest among all active constituents. *Abbreviations:* Insulin-like growth factor (IGF), Tissue inhibitor of metalloproteinases (TIMP), Transforming growth factor (TGF), Cluster of differentiation (CD).

SHED-CM major constituents

Studies of paracrine regulation of MSCs face challenges in terms of quality control and proper selection of the size range of proteins in the CM. Small molecules <5 kDa are considered impurities in the CM, thus the spectrum of these particles was excluded to avoid any non-specific effects following administration in zebrafishes. Proteins with a molecular weight >30 kDa or <5 kDa were extracted using TFF to illustrate the beneficial paracrine effects of specific portions of the SHED-CM which exhibit effective treatment capacity in PD zebrafish, instead of using the total paracrine mixture for treatment. Following TFF, the total SHED-CM was separated into 3 portions (<5 kDa, 5—30 kDa and

>30 kDa). The protein content of each portion was quantified using Qubit. The total protein amount in the 500 ml SHED-CM was 14.16 mg, including 4.78 mg (33.8%) with an apparent molecular weight >30 kDa, as opposed to 7.72 mg (54.5%) for <5 kDa, and 1.67 mg (11.7%) for 5–30 kDa. Thus, following TFF, 11.7% of SHED-CM contents were retrieved to examine their potential benefits for PD zebrafish treatment.

The defined range of SHED-CM (5–30 kDa) was respectively subjected to Quantibody Cytokine Arrays and multiplex ELISA antibody arrays for screening and comparison of the expression profiles of various cytokines. 3 clinically harvested milk teeth were used to generate all the SHED stem cells, each group of which was used to produce SHED-CM production (500 ml) using TFF procedures. The resultant SHED-CM constituents are presented in Fig. 2D. The total protein concentration of SHED-CM was 100.0 ± 7.8 μg/ml, with active constituents including IGF family (IGF binding protein 2, 4, 6 and IGF-1), tissue inhibitor of metalloproteinases-2 (TIMP2), tissue inhibitor of metalloproteinases-1 (TIMP1) and transforming growth factor β (TGF-β). The IGF family and TIMP-2 levels respectively accounted for 60.8 ± 2.1% and 25 ± 1.1% of total protein, followed by TIMP1 (8.0 ± 0.4%), TGF-β (5.2 ± 0.5%) and other proteins (1%) (Fig. 2D).

Biosafety evaluation of SHED-CM: Survival test

SHED-CM biosafety was evaluated based on its effect on the viability of the cultivated zebrafish. 30 wild-type AB larval zebrafish (day 3 post-hatching) were cultivated in 6-well microplates and treated with the SHED-CM via yolk sac injection in doses ranging from 6.25 to 400 ng/fish. No zebrafish death was noticed during the 14-day observation period (Table 1), indicating that SHED-CM did not contain substances noxious to cultivated larval zebrafish.

SHED-CM biosafety evaluation for skin and muscle, dermal and neurotoxicity during development

Yolk sac injection was used to examine the effect of SHED-CM on the normal development of skin and muscle. Fig. 3A and B respectively show no significant changes to the skin and muscle patterns or pigmentation patterns in the 14 days following injection with various SHED-CM doses. These results suggest that SHED-CM did not disrupt the development of skin and muscle from the larva to adult stage in zebrafish.

To determine whether SHED-CM induced neurotoxicity in zebrafish, we examined swimming capacity following intramuscular injection at doses of 22, 67 and 200 ng/fish, finding respective average moving speed (mm/min) of 115.2 ± 10.2, 129.8 ± 7.0 and 137.2 ± 8.2 (Fig. 3C). No significant difference was found between the control and SHED-CM groups, but higher dose treatment (67 and 200 ng/fish) was found to significantly increase movement speed compared with the 22 ng/fish group.

Adult zebrafish were tested for skin toxicity by dermal injection of various doses of SHED-CM, followed by staining with acridine orange, producing no specific response (Control, 38.754 ± 0.8253 vs. Vehicle, 37.8039 ± 0.7927, no statistical significance). Dermal injection of SHED-CM showed no detectable skin toxicity at the three administered dose. (44 ng/fish, 39.6228 ± 1.0229; 133 ng/fish, 36.441 ± 1.1167; 400 ng/fish, 38.5423 ± 1.3837) and no statistical significance between control and SHED-CM groups (Fig. 3D).

Table 1 Survival test of cultivated larval zebrafish treated with various doses of stem cells derived from human exfoliated deciduous teeth-derived condition medium (SHED-CM) via yolk sac injection (n = 30).

Dose (ng/fish)	Number of deaths	Mortality (%)
Control	0	0
Vehicle	0	0
6.25	0	0
12.5	0	0
25	0	0
50	0	0
100	0	0
200	0	0
400	0	0

SHED-CM biosafety evaluation: Estrogen equivalent (EEQ) test

Disruption of the estrogen system is associated with tumorigenesis and chronic toxicity, thus an EEQ test was performed to determine whether SHED-CM induced any estrogenic activity in the estrogen-related pathways. Calculating the 17β-estradiol equivalents in various doses of SHED-CM extracts showed no significant estrogenic activity (Table 2), suggesting that SHED-CM does not disrupt estrogen-related pathways.

Evaluation of anti-parkinsonism efficacy of SHED-CM in 6-OHDA-treated adult zebrafish

After excluding any potential biohazard or toxicity concerns during zebrafish development, the anti-PD potential of SHED-CM was then examined in the zebrafish model of PD. PD was induced by administering 6-OHDA, resulting in the death of dopaminergic neurons. Zebrafish were then injected with either vehicle or different doses of SHED-CM, (22, 44, 133 or 400 ng/fish). The swimming capacity of the treated zebrafish was analyzed and recorded using a zebrafish-specific behavior analysis system (Viewpoint, Inc.), and presented as both swimming distance (mm) and travelling speed (mm/min) (Fig. 4). Compared with the control zebrafish, administration of 6-OHDA significantly decreased swimming ability (swimming distance: control, 4366 ± 373 mm vs. 6-OHDA 1611 ± 270 mm; travelling speed: control 145.5 ± 12.4 mm/min vs. 6-OHDA 53.7 ± 9.0 mm/min). Nomifensine, a drug that can ameliorate PD symptoms, was used as a positive control and moderately improved swimming ability in 6-OHDA-injured zebrafishes (Fig. 4; swimming distance, 6-OHDA, 1611 ± 270 mm vs. 6-OHDA + Nomifensine, 2738 ± 165 mm; travelling speed: 6-OHDA 53.7 ± 9.0 mm/min vs. 6-

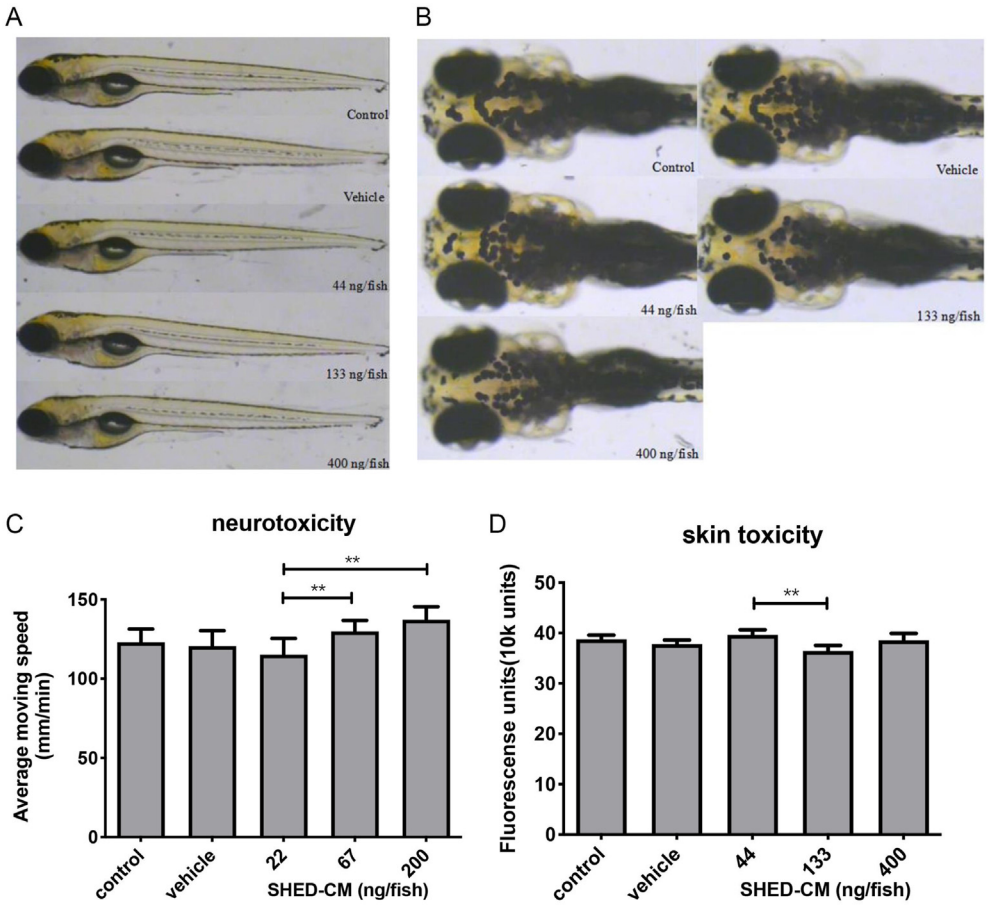

Figure 3 Stem cells derived from human exfoliated deciduous teeth-derived conditioned medium (SHED-CM) biosafety evaluation for developmental toxicity, neurotoxicity and skin toxicity. (A) Wild-type AB larval zebrafish (day 3 post-hatching) cultivated in 6-well microplates and treated with different doses of SHED-CM via yolk sac injection exhibited regular skin and muscle development over a 14-day observation period (n = 10). (B) Different doses had no effect on pigmentation. (C) Neurotoxicity and skin toxicity tests of SHED-CM in adult zebrafish (day 17). Neurotoxicity test, determined by calculating the zebrafish average movement speed (mm/min) (n = 10). (D) Skin toxicity of zebra fish treated with SHED-CM, stained with acridine orange, quantified by zebrafish skin fluorescence intensity (10 K units; n = 10). $^{**}P < 0.01$.

Table 2 Estrogen equivalents test to exclude the estrogenic activity of stem cells derived from human exfoliated deciduous teeth-derived conditioned medium (SHED-CM) in cultivated zebrafish (larva stage) (n = 10).

Estrogen Equivalent (EEQ) Dose Response Test	SHED-CM dose (mg) + 3ppb E2	*P* value[a] (Compare with 3ppb E2, control)		
	20	0.88	0.78	0.86
	9.09	0.51	0.92	0.30
	4.13	0.41	0.88	0.56
	1.88	0.78	0.01[b]	0.50
	0.85	0.63	0.32	0.61

[a] The *P* value of T-test for different doses were compared with the 17β-estradiol (E2) control.
[b] $P < 0.05$ was regarded as significant difference.

OHDA + Nomifensine 91.3 ± 5.5 mm/min). SHED-CM administration also remarkably improved impaired motor symptoms in the presence of 6-OHDA (swimming distance: 6-OHDA 1611 ± 270 mm; 6-OHDA + SHED-CM 22 ng/fish, 2087 ± 193 mm; 6-OHDA + SHED-CM 44 ng/fish, 2686 ± 202 mm; 6-OHDA + SHED-CM 133 ng/fish, 3178 ± 311 mm; and 6-OHDA + SHED-CM 400 ng/fish 2652 ± 160 mm; travelling speed: 6-OHDA + SHED-CM 22 ng/fish, 69.6 ± 6.4 mm/min; 6-OHDA + SHED-CM 44 ng/fish, 89.5 ± 6.7 mm/min; 6-OHDA + SHED-CM 133 ng/fish, 105.9 ± 10.4 mm/min; and 6-OHDA + SHED-CM 400 ng/fish 88.4 ± 5.3 mm/min. SHED-CM also significantly improved

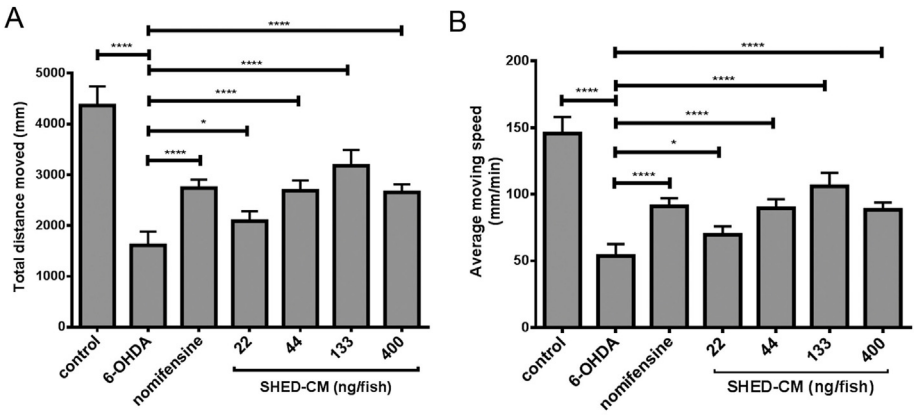

Figure 4 Anti-Parkinson's disease (anti-PD) efficacy of stem cells derived from human exfoliated deciduous teeth-derived conditioned medium (SHED-CM). (A) Total swim distance of larva measured as the endpoint to assess PD treatment efficacy. (B) Average larva movement speed (mm/min) measured as the endpoint to assess PD treatment efficacy. Compared with nomifensine, SHED-CM significantly improved the impaired motor symptoms in a dose-dependent manner between 0 and 133 ng/fish ($n = 10$). *$P < 0.05$, ****$P < 0.0001$. *Abbreviations:* 6-hydroxydopamine (6-OHDA).

impaired motor symptoms. A significant dose-dependent stimulatory effect of SHED-CM on motor behavior was observed as the dose ranged from 0 to 133 ng/fish. The efficacy of SHED-CM decreased slightly at 400 ng/fish, suggesting that the highest dose may elicit some additional effects. These data suggest that SHED-CM has potential for alleviating PD-related symptoms in 6-OHDA-treated zebrafish.

Finally, Fig. 5A and B shows the results of anti-parkinsonism, expressed via Tyrosine hydroxylase (TH) cells. We used the ratio of TH and β-Actin to show the expression of our protein of interest. The PD-zebrafish (6-OHDA/6-OHDA with N/S) showed significant decrease in TH cell expression, as different concentrations of the conditioned medium show outstanding rescue effects. Normal saline probably enervates the 6-OHDA through dilution.

Discussion

The 6-OHDA-treated zebrafish PD model is closely associated with microglial activation and neuroinflammation. Tissue regeneration accounts for the microenvironment conditions at the injury sites. Inflammatory reactions occur immediately following administration of 6-OHDA and dysregulation of astrocytes and/or microglia activation may reduce regenerative capacity, leading to severe injury that hinders neural repair.[21] Based on our previous report in a PD rat animal model,[10] we hypothesize that SHED-CM may provide an anti-PD effect in 6-OHDA-treated zebrafish via the promotion of a rapid transition from the inflammatory phase at the neural injured site to the regenerative phase.

In the present study, SHED-CM exhibits optimal biosafety and can effectively ameliorate 6-OHDA-induced neurological and motor ability deficits in zebrafishes with PD-like symptoms treated with 6-OHDA in a dose-dependent manner. The maximal effect was achieved at a SHED-CM dose of 133 ng/fish. The constituent components of SHED-CM were purified using staged TFF to narrow down the spectrum of proteins, including the IGF family (IGFBP2,4,6 and IGF1), TIMP1, TIMP2 and TGF-β. The PD-zebrafish were treated using a purified and quantified protein solution to achieve high-quality control and reproducibility in experiments. ELISA examination showed minimal differences between SHEM-CM batches in terms of constituent composition. While SHED-CM produced notable treatment outcomes, further studies are needed to clarify the mechanisms of motor improvement, and the development of novel anti-PD therapeutics requires the identification of the purified/isolated functional proteins.

PD patient post-mortems show upgraded TIMP1 in the neurons and reactive glial cells in the substantia nigra.[22] TIMP2 inhibits the functions of MMPs and has also been associated with hippocampal function and cognitive function.[23] In addition, TIMP1 coordinates with TIMP2 to suppress MMP3 production, resulting in microglia inactivation and a microenvironment suitable for neuronal cell survival and tissue regeneration. IGF-1 serves a range of functions in the central nervous system, including regulation of early brain development, myelination, formation of synapses, neurogenesis and cognition.[24] Importantly, IGF-1 has also been shown to have a wide spectrum of neuroprotective effects against neuro-inflammation and oxidative stress in the brain.[25] Our previous study of an animal model of subarachnoid hemorrhage showed that neurological outcome improvements were closely associated with anti-neuroinflammatory effects primarily through the IGF-1 signaling pathway.[16] In the present study, quality-controlled conditions were used to cultivate SHED stem cells and collect CM with a defined protein spectrum (Fig. 2D). We speculate that IGF1, IGFBPs, TIMP1 and TIMP2 have synergistic effects in ameliorating inflammatory response and suppress oxidative stress.

A report focusing on amyotrophic lateral sclerosis (ALS)[11] concluded SHED-CM significantly suppressed the mutant SOD1-induced intracellular aggregates and neurotoxicity. In allodynia[12] animal models, SHED-CM could protect and/or repair DRG neurons damaged by nerve transection. Taken together with our results, SHED-CM could be a promising

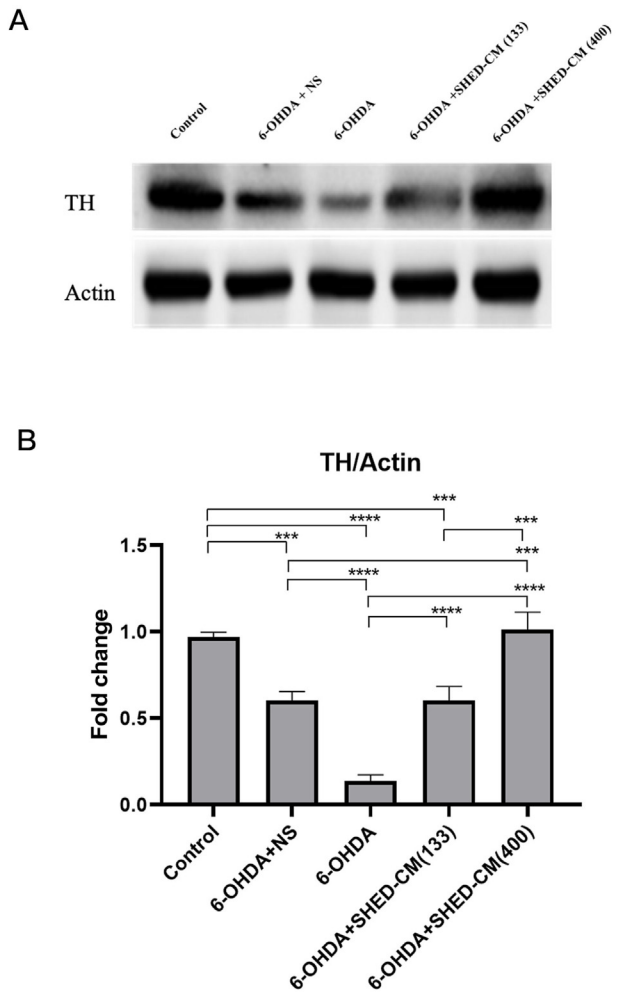

Figure 5 Determination of tyrosine hydroxylase (TH) cells in zebrafish brain tissue. Western blotting using lysates from healthy controls, individuals with induced Parkinson's disease and the post stem cells derived from human exfoliated deciduous teeth-derived conditioned medium (SHED-CM) treatment groups. (A) Western blotting using healthy zebrafish brain tissue, induced Parkinson group and the groups treated with SHED-CM. (B) Ratio of tyrosine hydroxylase expression and β-Actin. ***$P < 0.001$; ****$P < 0.0001$. *Abbreviations:* 6-hydroxydopamine (6-OHDA).

treatment for neuronal damage diseases. There are some limitations to our research. We think it is necessary to study the contribution of each SHED-CM component. Additional other *in vivo* PD models should be examined to produce definitive conclusions. Moreover, in future research, the efficacy of SHED-CM should be evaluated not only in neurons but also in other neural cells *in vivo* prior to preclinical studies.

In conclusion, various assays were performed in present study to evaluate the biosafety of SHED-CM and demonstrate its therapeutic potential in a zebrafish model of PD. The improvement of motor abilities in the zebrafish model of PD treated with SHED-CM suggests that it may serve as a novel means of managing PD in the future. Further analysis is required to elucidate the major effective constituents that contribute to SHED-CM efficacy in the zebrafish model of PD.

Declaration of competing interest

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

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