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Isolation of Mesenchymal Stromal Cells from Human Exfoliated Deciduous Teeth

Tamta Jikia[1,ID], Mariam Kakabadze[2,3,ID], Giorgi Kharebava[4,ID], Tatuli Batsatsashvili[5,ID], Nino Bedineishvili[5,ID], Nino Chipashvili[6,ID]

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ABSTRACT

Background: Mesenchymal stromal cells (MSCs) have been intensively studied for clinical applications over the last few decades. Their potential for tissue regeneration has driven high interest in these cells. One source of MSCs is human dental pulp (DPSCs, SHED). Recently, mesenchymal stromal cells derived from human exfoliated deciduous teeth (SHED) have attracted particular attention in part due to their high expression of classic pluripotency/embryonic antigens.
Objectives: Various practical methods for processing, deriving, and proliferating SHED are being investigated for use in regenerative medicine.
Methods: In this work, we demonstrate the establishment of a culture of mesenchymal stromal cells from primary tooth pulp using the explant culture method.
Results: Flow cytometric phenotypic characterization revealed robust expression of mesenchymal surface markers CD90, CD73, and CD105, accompanied by negligible levels of hematopoietic markers CD45 and CD34. These findings suggest that the isolated cells derived from human exfoliated deciduous tooth pulp possess a mesenchymal stromal phenotype.
Conclusions: This optimized protocol may facilitate the widespread cultivation of mesenchymal stromal cells in the laboratory for further use in preclinical research.
Keywords: Mesenchymal stromal cells (MSCs); phenotypic characterization; stem cells derived from human exfoliated deciduous teeth (SHED).

BACKGROUND

Mesenchymal stem cells (MSCs) have gained considerable attention in regenerative medicine due to their multilineage differentiation potential, immunomodulatory effects, and ease of culture. Among the various MSC sources, stem cells from human exfoliated deciduous teeth (SHED) represent an up-and-coming and non-invasive alternative. First identified by Miura et al. in 2003, SHED demonstrate superior proliferative capacity, high plasticity, and enhanced regenerative potential compared to MSCs derived from other dental and non-dental tissues.[1] Their accessibility from naturally exfoliated primary teeth makes them an attractive option for stem cell–based therapies, free from ethical concerns.[2,3]

Given their unique biological properties and high proliferative potential, SHED hold great promise for diverse applications in regenerative medicine, including dental tissue engineering, neural regeneration, and immunomodulation. Optimizing derivation and proliferation protocols is crucial to harness their therapeutic potential and facilitate clinical translation fully.

SHEDs can be isolated through two primary methods: enzymatic digestion and explant culture. Enzymatic digestion uses collagenase and dispase to break down the extracellular matrix, releasing individual stem cells. This method is advantageous for yielding a higher number of viable cells in a shorter time; however, it requires precise enzymatic conditions to prevent cellular damage.[4,5] Conversely, the explant culture method involves placing small pieces of pulp tissue directly in a culture dish, allowing cells to migrate naturally out of the tissue.[6-8] While this technique preserves cell integrity and mimics physiological conditions, it is slower and typically results in a lower initial cell yield.[9]

SHEDs exhibit robust proliferative abilities. Two widely studied proliferation methods include two-dimensional (2D) monolayer culture and three-dimensional (3D) spheroid culture. The traditional 2D culture method involves growing SHED on flat surfaces, which facilitates easy observation and handling. However, this approach may limit cell-cell interactions and reduce stemness over prolonged passages.[10-12] Therefore, it is essential to optimize cell isolation and proliferation methods to achieve the maximum possible cell yield in 2D culture. Additionally, the limited quantity of pulp tissue also underscores the importance of optimizing methods for MSC isolation and proliferation.[13] Here, we were focused on refining the methodology for isolating and cultivating SHEDs.

METHODS

Collection of teeth samples and processing

Teeth were collected at the Tbilisi State Medical University Dental Centre (Apolon Urushadze Dental Clinic). A total of six (n=6) deciduous teeth were collected. Donors were healthy children aged between 7 and 12 years. The criteria for tooth selection included vital deciduous teeth—incisors, canines, and molars—with at least one-third of the root remaining intact. The procedure was performed under local anesthesia. The collected teeth were rinsed with 0.7% saline and then

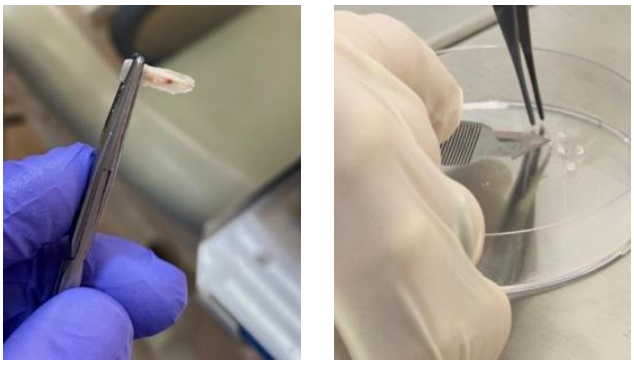
placed in a PBS solution at 4°C. The samples were processed within 3 hours.

Specifically, after disinfecting with a 3% sodium hypochlorite solution for two minutes, the tooth was rinsed with 1X phosphate-buffered saline (PBS). The tooth was sectioned in the sagittal plane using a sterile dental diamond fissure bur (Mani, Inc., USA) along with a high-speed handpiece (NSK, USA) under a water supply to expose the pulp. The study was approved by the TSMU Biomedical Research Ethics Committee (N6-2022/99). Written informed consent was obtained from the children's parents/guardians.

Isolation and cultivation of pulp tissue derived from a deciduous tooth

Pulp tissues were immersed in 70% ethanol for 30 seconds and subsequently rinsed twice with DPBS containing 0.05 mg/mL gentamicin. The tissues were minced into 1–2 mm microblocks using a #20 surgical scalpel (Fig.1).

FIGURE 1. A. Sagittal view of a tooth with exposed pulp; B. The process of pulp tissue fragmentation.

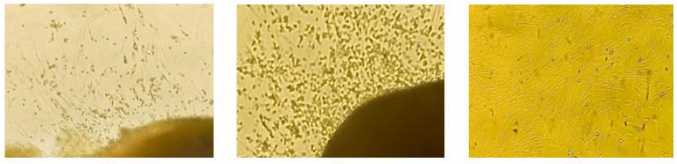


A B

The explants were evenly distributed in two 35 mm Petri dishes, coated with CELLstart™ (Thermo Fisher) solution. Once the samples were secured, 1 mL of culture medium was added to each dish, consisting of DMEM supplemented with 15% FBS, 2 mL of GlutaMAX, and an antibiotic-antimycotic (AA) solution. The cultures were placed at 37°C in a humidified incubator with 5% CO_2. By day 28, explants were surrounded by confluent cells (Fig.2 and Fig.3).

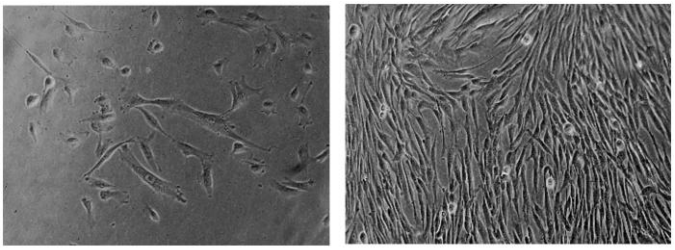
At this point, the plates were washed with PBS, and the cells were detached using TrypLE reagent (Thermo Fisher). To collect mesenchymal stromal (stem) cells, 5 mL of culture medium was added, and the resulting cell suspension was transferred into a sterile 50 mL conical tube. The cells were washed twice, and centrifugation was performed at room temperature at 500 × g for 7 minutes. After centrifugation, the supernatant was carefully discarded without disturbing the cell pellet. The pellet was resuspended in 10 mL DMEM/FBS (15%) and counted. The cells were seeded on freshly coated plates at a density of 2500 cells/cm². Medium was exchanged every 4 days until the cells reached confluency. Cell viability during counting was assessed by Trypan Blue staining.

FIGURE 2. Isolation of stromal cells from a pulp microblock. Passage 0 (A); Day 8th (B); Day 28th (C) (magnification 20x)



A B C

FIGURE 3. Dental pulp-derived mesenchymal stromal cells. Passage 1; Day 1 (A); Day 8th (B) (magnification 20x)



A B

Flow cytometry

The cultured passage 2 DPSCs were collected for a flow cytometry assay. Antibodies against CD34, CD45, CD73, CD90, and CD105 were selected to detect stem cell surface antigens (R&D Systems FMC020). Cells were stained with the above antibodies according to the manufacturer's protocol and analyzed using a BD Accuri C6 Flow Cytometer System.

Cryopreservation

Mesenchymal stromal (stem) cells were washed in 15% DMEM, centrifuged at 500 × g for 7 minutes at 4°C, and then resuspended in cryopreservation medium containing 10% CryoSure-DEX40 at a concentration of 3×10^6 cells/mL. The cryovial aliquots were cryopreserved at −180°C in liquid nitrogen for long-term storage. After a month, a single aliquot was thawed at 37°C, and cell viability was assessed by Trypan Blue staining.

RESULTS

The yield of dental pulp stem cells varies with multiple factors, including donor age, tooth type, isolation technique, and culture conditions. Several studies have provided quantitative data on the number of stromal cells obtained from the pulp of deciduous teeth.14-16 In our hands, passage 1 SHEDs, seeded at 2500 cells/cm², reached greater than 80% confluency in approximately 8 days, requiring one medium exchange every 4 days. Passage two cells (1×10^6, total counts) seeded at the above density in two 225 cm² flasks, reached apparent confluency at day 7, and were harvested at day 8. A total cell yield of 18×10^6 was obtained, indicating a harvest density of 40,000 cells/cm² and four population doublings. Live-dead cell

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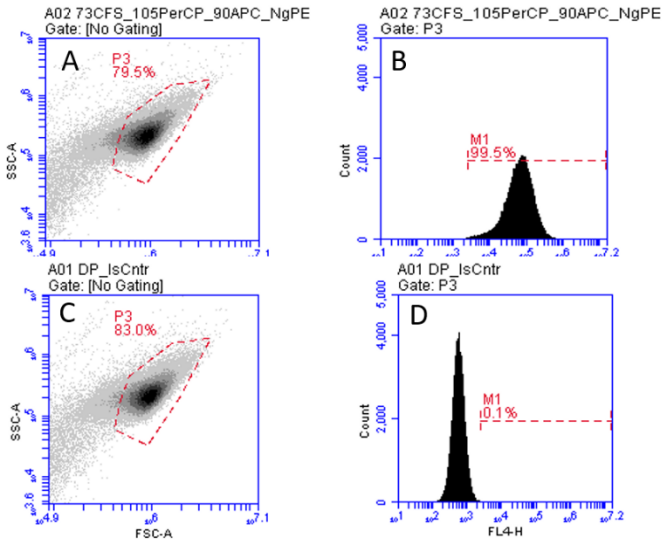
counts, as determined by trypan blue, in the harvest were 98%. Additionally, we tested the post-thaw viability of cryopreserved SHEDs, which was 92% as determined by trypan blue.

Flow cytometric analysis was performed to characterize the immunophenotypic profile of SHED. The expression of key mesenchymal stromal cell markers (CD73, CD90, and CD105) and hematopoietic markers (CD34 and CD45) was assessed. CD73, CD90, and CD105 were abundantly expressed (99.5%, 92.8%, and 64.6%, respectively; only CD90 results are shown in Fig. 4 B), confirming their mesenchymal origin. Additionally, 64.5% of the cells were double-positive for CD105 and CD90, and 89.7% were double-positive for CD73 and CD90.

Analysis also confirmed the absence of hematopoietic contamination, as SHED did not express CD34 or CD45. Specifically, 95.3% of the cells were negative for these markers. Our results confirm the mesenchymal stromal cell identity and their suitability for further in vitro expansion and potential research applications. The isotype control for CD73 was 1.6%, and for CD105, 2.3%. The isotype control for CD90 showed 0.1% (FIG.4D/4E). The isotype double-control for CD105 and CD90 showed 0.9% (Fig.4).

FIGURE 4. SHED Flow Cytometric evaluation of CD90 expression. A. The histogram presents the cell population analyzed by flow cytometry. B. CD90 expression plot (99.5%). C, D Respective isotype control



DISCUSSION

This study describes a method for isolating and proliferating human mesenchymal stromal cells derived from exfoliated deciduous teeth using the explant method. According to the guidelines of the International Society for Cell & Gene Therapy (ISCT) (Viswanathan et al., 2019),[17] the corresponding surface antigens were detected, with positive expression of CD73, CD90, and CD105. However, the expression of CD34 and CD45 was negative.

Cells emerging from the tissue explant gradually formed loosely arranged colonies, predominantly consisting of cells exhibiting a characteristic spindle-shaped morphology. Our findings align with previous studies reporting a homogeneous cell population when using the tissue explant method.[18,19]

A significant challenge in tissue banking worldwide is the degradation of tissue between harvesting and processing, as well as cell death following cryopreservation.[20] To mitigate this, our approach ensures that sample processing is completed within a short timeframe. In our study, the cryopreservation method employed successfully maintained SHED viability above 90%.

The explant culture method represents an effective and gentle approach to deriving SHED while preserving their biological properties. Understanding the advantages and limitations of this method is essential for refining SHED isolation protocols.[21] The explant method significantly reduces the cost of cell culture and enhances the number of viable stem cells obtained. However, the outgrowth of cells from explants is typically slower than that from enzymatic digestion. Despite this, explant cultures offer distinct advantages, including a reduced risk of cell membrane damage, superior preservation of cell viability, and maintenance of the native tissue architecture and microenvironment. These factors help preserve the cells' original phenotypic and functional properties.[22]

CONCLUSIONS

The present study confirms the explant method as an optimal approach for isolating mesenchymal stromal cells. This technique enables the efficient retrieval of bioactive cells in high yield, thereby providing a reliable source for potential applications in tissue and organ regeneration.

AUTHOR AFFILIATION

1Department of Odontology, Tbilisi State Medical University, Tbilisi, Georgia;
2Department of Clinical Anatomy, Tbilisi State Medical University, Tbilisi, Georgia;
3Tbilisi State University, Tbilisi, Georgia;
4Department of Biomedicine, Medical School, Georgian American University, Tbilisi, Georgia;
5Mardaleishvili Medical Center, Tbilisi, Georgia;
6Department of Odontology, Tbilisi State Medical University, Tbilisi, Georgia.

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