

# Collection and culture of stem cells derived from dental pulp of deciduous teeth: Technique and clinical case report

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## Abstract

**Introduction:** Stem cells (SCs) are capable of inducing tissue regeneration and are, therefore, potentially therapeutic. Similarly to bone marrow and umbilical cords, dental pulp is one of the available sources of SCs. The fact that these cells are easily accessible and that deciduous teeth are not vital organs, and are normally discarded after exfoliation, make them particularly attractive for use in safety and viability tests. **Objective:** To describe the collection, isolation and culture of SCs obtained from the pulp of deciduous teeth as well as their characterization by flow cytometry, and the induction of differentiation into osteogenic and adipogenic lineages. **Methods:** SCs were obtained in a relatively straightforward manner and showed good proliferative capacity, even from a small amount of pulp tissue. **Results:** Analysis by flow cytometry confirmed the characteristics of mesenchymal SCs with low expression of CD34 and CD45 antigens, which are markers for hematopoietic cells, and high levels of expression of CD105, CD166, CD90 and CD73 antigens, which are markers for mesenchymal SCs. Cell plasticity was confirmed by identifying calcium deposits in cultures that received osteogenic medium, and intracellular lipid accumulation in adipogenic cultures that received adipogenic medium. **Conclusions:** SCs collected from deciduous teeth show promising potential for application in tissue regeneration. Therefore, it is important that knowledge about the existence and characteristics of this source of stem cells be disseminated among dentists and that the technique, its limitations and possible indications are highlighted and discussed.

**Keywords:** Stem cells. Tissue therapy. Cell culture techniques. Deciduous teeth.

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## INTRODUCTION

Science has taken a keen interest in stem cells (SCs) given their ability to stimulate tissue regeneration, which raises exciting and promising therapeutic prospects.<sup>1-13</sup> This fact makes SCs a viable alternative in dentistry.<sup>14</sup> However, there are still limitations in how they are obtained, grown and controlled in terms of proliferation and differentiation, which encourages the search for new sources, techniques and applications.

SCs can have an embryonic or adult origin.<sup>2,6,15</sup> Adult SCs are present in a wide range of tissues such as the pancreas, bone marrow, adipose tissue and umbilical cord. Because they are obtained from the patients themselves, these cells have the advantages of not triggering immune rejection, responding to growth factors inherent in the host, and not incurring ethical or moral objections.<sup>6</sup> The foundation for cell therapy is the isolation of high quality adult human SCs from different sources as these cells feature peculiar characteristics, and there may be preferred SCs sources for each specific need.<sup>12,16,17</sup>

Recently, it was discovered a new source of SCs deriving from the pulp of deciduous teeth. The fact that these cells are easily accessible and that deciduous teeth are not vital organs, being normally discarded after exfoliation, make them attractive for safety and viability tests. Studies conducted with these cells underscore their outstanding capacity to proliferate and induce tissue regeneration,<sup>12,17,18,19</sup> although limitations still exist regarding the amount of available cells as well as the techniques used for SC collection and culture.<sup>19,20</sup>

Although there is still the need to standardize techniques and to conduct clinical studies in order to determine their potential application, this study aims to disseminate among dentists knowledge about SCs obtained from the pulp of deciduous teeth, discussing the technique, its limitations and possible therapeutic potential through the description of a technique and a clinical case report.

This will allow professionals to inform patients and/or their legal guardians that although this is still an experimental technique it shows promise and that this tissue — which is usually discarded — can be collected and cryopreserved for future use.

## TECHNIQUE AND CLINICAL CASE REPORT

### Tissue collection

An eight-year-old female patient with mixed dentition has been monitored since five years old by an orthodontist and was selected as pulp tissue donor after authorization by her legal guardians. Radiographic and photographic exams were requested and, in conjunction with the orthodontist, teeth 6.3, 7.3, 7.5 and 8.3 were defined as targets as they were in the phase of exfoliation and did not show any carious lesions.

The surgery was performed in two stages with a 15-day interval. The procedure requires strict control of the aseptic chain due to widespread presence of microorganisms in the oral environment. Extraoral asepsis and intraoral prophylaxis were performed and 2% chlorhexidine rinses applied. Next, infiltrating and gingival anesthesia was applied, followed by syndesmotomy and removal of the teeth with a pedodontic forceps as quickly as possible to avoid saliva contamination. Soft tissue remnants were removed and the teeth were immediately placed in individual containers filled with Dulbecco Modified Eagle medium (DMEM, Sigma Chemical Co. St. Louis, Mo, USA) culture medium with 50 mg/ml of gentamicin (Novafarma, Anápolis, Brazil), stored under controlled temperature, between 4 °C and 8 °C, and sent to a laboratory for cell isolation.

### Isolation, selection and expansion of mesenchymal SCs

The entire procedure of pulp removal and cell culture was performed in a vertical laminar flow unit. Whenever necessary, access to the pulp chamber was made using diamond discs (KG Sorensen, São Paulo, Brazil) at low speed, under

constant irrigation, and the pulp tissue removed with the aid of currettes and endodontic files. Only a small amount of pulp tissue was obtained. One of the four deciduous teeth extracted no longer contained pulp tissue and the others had a small amount of pulp. Therefore, few cells were available to begin cultivation. Once collected, the tissue was immediately placed in culture bottles containing DMEM supplemented with 10% fetal bovine serum (SBF, Cultilab, Campinas, Brazil) and stored at 37 °C and 5% CO<sub>2</sub> for cell proliferation and adherence to the bottle. The medium was completely replaced every three days during a period of approximately 10 days, when culture reached about 80-90% confluence. The culture was monitored by means of an inverted optical microscope. After adherence to the plastic surface, SCs initially exhibited an ovoid shape that evolved early during the first 24 hours to a fibroblastoid form, which remained until confluence (Fig 1). As the culture was replaced, the adherent mesenchymal SCs were selected while cells suspended in culture medium were gradually discarded.

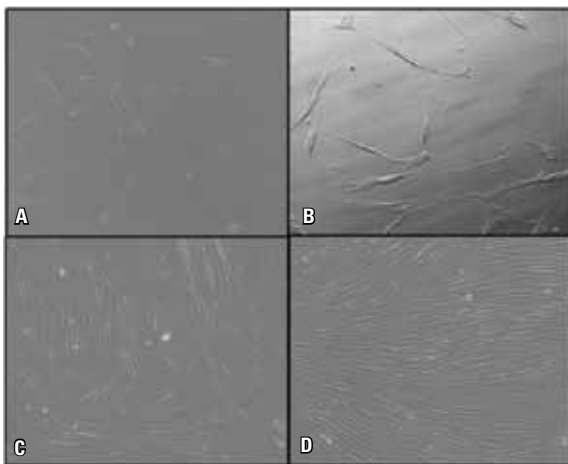


FIGURE 1 - Optical microscopy follow-up of primary culture of SCs derived from deciduous teeth. **A)** Scattered cells still showing a rounded shape. **B)** Cells with fibroblastoid features after 72 hours of culture. **C)** Stabilization of cell morphology and increased proliferation. **D)** Culture with total confluence.

After culture confluence cells were released from the plastic surface to allow the continued proliferation and cryopreservation of part of the cells. To release the cells, the medium was replaced twice with sterile saline solution and, after removing the saline solution, 0.25% trypsin (Invitrogen, São Paulo, Brazil) was added for 2-5 minutes to allow the connections between cells and extracellular matrix to be broken, thereby enabling the detachment of cells from the surface of the plastic culture flask.

After ascertaining that cells had been released through observation with an inverted optical microscope, the enzyme was inactivated by adding a complete medium. The medium of the bottle containing the cells was then collected using a pipette and centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded and the cell pellet resuspended in 1 mL of DMEM supplemented with 10% fetal bovine serum (SBF). At this point, some of the cells were set aside for cryopreservation in liquid nitrogen for further studies and potential therapeutic uses, while the remaining was used for continuity of culture, allowing *in vitro* characterization.

For cryopreservation, the maximum concentration of 10<sup>6</sup>-10<sup>7</sup> cells per tube was observed, with a final volume of 1 mL being added, i.e., 900 µL of complete medium containing cells and 100 µL of dimethyl sulfoxide (DMSO). Cryopreservation tubes had their cryostatic temperature lowered, ranging from 4 to -80 °C for 24 h, with subsequent storage in liquid nitrogen.

### Characterization of mesenchymal SCs

Cell characterization was performed by analysis of flow cytometry and confirmation of cell plasticity through the induction of differentiation into osteogenic and adipogenic lineages.

### Flow cytometry

The sample cells were labeled with specific monoclonal antibodies linked to fluorochromes. Reading was performed in a flow cytometer.

The following antibodies were used: FITC anti-human CD90, APC anti-human CD45, PE anti-human CD166, PE anti-human CD73 (BD Pharmingen), PE anti-human CD34 (Becton Dickinson) and FITC anti-human CD105 (R&D Systems).

Cells with less than 100% confluence were trypsinized as described before. Immediately after detachment, cells were resuspended in DMEM supplemented with 10% SBF and remained at rest in the oven for 2 hours. After the rest period, the cells were washed twice with saline at 4 °C, at 3000 rpm for 2 minutes at 10 °C and resuspended in 1 mL of saline solution. Two-hundred  $\mu$ L of the solution were placed in tubes which received 2  $\mu$ L of antibodies. The tubes were incubated at 4 °C in the dark for 30 minutes and washed twice with saline at 4 °C (1 mL) by centrifugation at 2000 rpm, 10 °C for 2 minutes. After this procedure, CellQuest software was used for data acquisition and analysis with a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA, USA). At least 50,000 events were collected and analyzed.

Results confirmed the characteristics of mesenchymal SCs (Table 1) with low level expression of CD34 and CD45 antigens, which are markers for hematopoietic cells, and high levels of expression of CD105, CD166, CD90 and CD73 antigens, which are markers for mesenchymal SCs.<sup>20</sup>

TABLE 1 - Analysis by flow cytometry of SCs collected from deciduous teeth. Percentage of cells positive for CD90, CD73, CD34, CD45, CD166 and CD105 in the total population.

MARKERS	CYTOMETRY
CD 90	99.92%
CD 73	99.95%
CD 34	0.08%
CD 45	2.4%
CD 166	96.8%
CD 105	97%

### Osteogenic differentiation

The cells were cultivated in a 24-well plate in DMEM supplemented with 10% fetal bovine serum for two days until 50% confluence was reached. From this point on, osteogenesis inducing medium was employed, which is composed of: DMEM containing 10% fetal bovine serum, 100 nM dexamethasone, 0.05 mM of L-ascorbic acid 2-phosphate and 10 mM of  $\beta$ -glycerophosphate (Sigma-Aldrich, USA). Culture time was 21 days. Control group cells were grown in DMEM supplemented with 10% fetal bovine serum. The experiments were conducted in triplicate, culture medium was changed every three days and the evolution of differentiation was monitored daily by optical microscopy.

Osteogenic differentiation capacity was verified by alizarin red staining, which identifies the deposition of calcium in the culture. After 21 days of cultivation, the cultures were washed with PBS, fixed with 4% paraformaldehyde (Electron Microscopy Sciences, USA) for 30 minutes, washed twice with distilled water, and 2% alizarin red staining was applied (Sigma-Aldrich, USA) for three minutes. After removing the alizarin, the cultures were washed three times with distilled water to remove residue and the stained areas were analyzed with optical microscopy to confirm color and quality evaluation.

In all cultures that received osteogenic medium, red calcium deposition was found due to staining with alizarin, whereas in the negative control group calcium deposits were not found in any of the wells, confirming the plasticity of the cultured cells (Fig 2).

### Adipogenic differentiation

The cells were cultivated in a 6-well plate in DMEM supplemented with 10% fetal bovine serum until 100% confluence was reached. They were then stimulated for up to three weeks with modified DMEM containing: 10% fetal bovine serum, 60  $\mu$ M of indomethacin (Sigma-Aldrich),

0.5  $\mu$ M of isobutylmethylxanthine (Sigma–Aldrich) and 0.5  $\mu$ M hydrocortisone (Sigma–Aldrich). Throughout the experiments a control group was maintained cultivated in DMEM supplemented with 10% fetal bovine serum. The culture medium was replaced every three days.

The evolution of differentiation was monitored daily under an inverted microscope. To allow observation of fat deposition the wells were washed with PBS, cells were fixed with 4% PFA for 1 hour at room temperature, stained with Oil Red solution (Sigma–Aldrich) (3 volumes of 3.75% Oil Red O in isopropanol and 3 volumes of distilled water) for 5 minutes and washed with distilled water to remove residue.

After 14 days intracellular lipid accumulation was found in the culture, confirming the plasticity of the cultured cells (Fig 3), whereas in the negative control group droplets were not found in any of the wells.

## DISCUSSION

The scientific community has conducted research that underscores the importance and prospects of therapy with SCs in several areas, such as medullary damage,<sup>2,3</sup> neurological changes, such as in Parkinson's<sup>8</sup> and Alzheimer's diseases,<sup>21</sup> autoimmune diseases such as diabetes type 1,<sup>5,15</sup> liver diseases,<sup>13</sup> kidney damage,<sup>7</sup> and retina degeneration.<sup>22</sup> In some cases, such as coronary heart disease, consistent clinical results have already been found,<sup>9,10</sup> indicating SCs' safety and viability.

In dentistry, experiments have focused on the use of cell therapy in oral tissue regeneration<sup>12,14,23</sup> and on the collection, isolation, culture and characterization of SCs derived from the pulp of teeth.<sup>16-19,24</sup> Some studies indicate that the SCs obtained from deciduous teeth have greater regenerative and proliferative potential when compared to permanent teeth,<sup>16,17,19</sup> besides being more easily accessible. Furthermore, deciduous teeth are not vi-

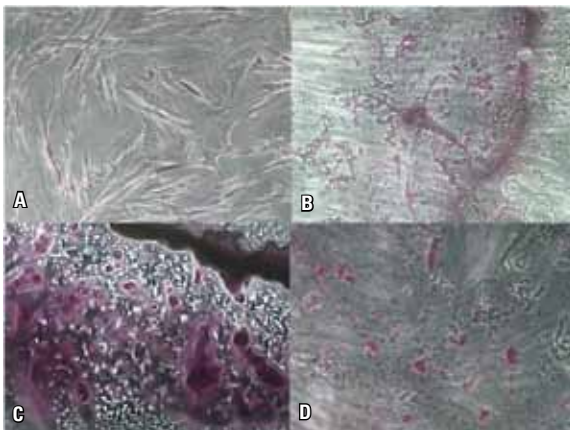


FIGURE 2 - Induction of differentiation of SCs collected from deciduous teeth cultured with osteogenic medium. Analysis by optical microscopy of calcium deposits stained with alizarin red after 21 days of culture. **A)** Negative control with DMEM, with no calcium deposits. **B-D)** Cell differentiation in osteogenic induction medium, confirmed by presence of calcium deposits stained with alizarin red.

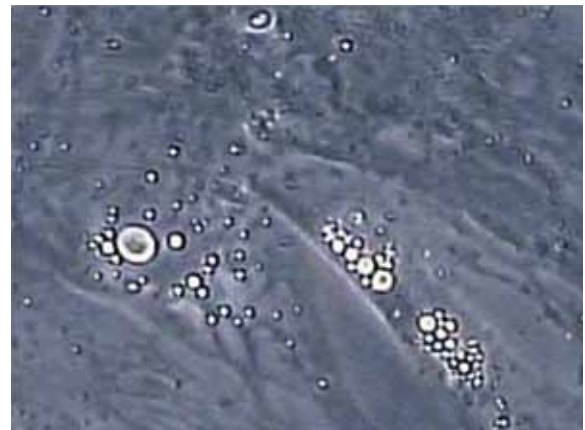


FIGURE 3 - Induction of differentiation of SCs collected from deciduous teeth cultured with adipogenic medium. Analysis by optical microscopy of intracellular lipid accumulation after 14 days of culture, confirming differentiation.

tal organs and are usually disposed after exfoliation. It is conjectured that SCs from different sources may have different characteristics and therefore specific indications for therapeutic application.

Exfoliation of deciduous teeth, however, is one of the factors hindering their use as it limits the time during which deciduous teeth remain available, i.e., from 6 to about 12 years of age.<sup>25</sup> This hurdle can be circumvented by informing the patient's legal guardian that tissue can be collected during the exfoliation period while the cells can be cultivated and kept by cryopreservation in liquid nitrogen. This technique is well established and described in the literature, and allows the maintenance of SC characteristics.<sup>26,27</sup>

There have been recent attempts at cryopreservation of dental pulp, or even of the whole tooth — instead of just SCs — with the purpose of thawing and growing them at a later date.<sup>28,29</sup> Once proved that these cells do not change with time, either in quality or quantity in this process of tissue (not SCs) cryopreservation, storage would

be simpler and cheaper as lab cultures would only be performed when the use of cells is indicated, thereby saving time, reagents and personnel, requiring a simpler laboratory structure.

In this study, the technique for collecting and cultivating SCs obtained from deciduous teeth proved to be relatively simple and fast. However, there was only a small amount of pulp tissue to start the cell culture. This condition can pose an obstacle to the effective therapeutic use of SCs since a longer time may be required for cell culture and divisions, which can lead to changes in the characteristics of these cells.<sup>20</sup> Thus, an alternative solution to this shortcoming would be to request the evaluation and monitoring of patients with mixed dentition by an orthodontist, who would indicate a greater number of tooth extractions without interfering with the normal development of dentition.

Another problem to be avoided given the characteristics of the oral environment is the risk of contamination of cultures by microorganisms.<sup>28,29</sup> In addition to the typically careful handling

inherent in cell culture techniques, it is necessary to observe criteria such as absence of extensive carious lesions in the selected teeth, control of the aseptic chain during surgical procedure, and preventing that the pulp from the removed deciduous teeth has contact with oral fluids. Selected teeth should be in an advanced stage of root resorption, but with intact junctional epithelium to avert prior contamination of the pulp tissue.

The American Academy of Pediatric Dentistry recently published a text<sup>30</sup> advising dentists to monitor the progress of investigations published about SCs collected from deciduous teeth so they can educate parents about the collection, cultivation, preservation and potential uses of these cells.

## CONCLUSIONS

The technique for collecting and cultivating SCs obtained from deciduous teeth proved to be relatively simple and fast. Although very promising, the use of these cells is not yet suitable for everyday clinical use. Therefore, further *in vitro* and *in vivo* experiments and clinical studies are necessary to confirm SCs safety and viability. Nevertheless, this finding should be disseminated among dentists in order to make them aware of the potential of cell therapy and allow them to inform their patients and/or legal guardians about this source of stem cells, since tissue from deciduous teeth is only available for a short period of time.

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