Carious deciduous teeth are a potential source for dental pulp stem cells

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Abstract

Objective The objectives of this study are to isolate, cultivate, and characterize stem cells from the pulp of carious deciduous teeth (SCCD) and compare them to those retrieved from sound deciduous teeth (SHED—stem cells from human exfoliated deciduous teeth).

Material and methods Cells were obtained of dental pulp collected from sound ($n=10$) and carious ($n=10$) deciduous human teeth. Rate of isolation, proliferation assay (0, 1, 3, 5, and 7 days), STRO-1, mesenchymal (CD29, CD73, and CD90) and hematopoietic surface marker expression (CD14, CD34, CD45, HLA-DR), and differentiation capacity were evaluated.

Results Isolation success rates were 70 and 80 % from the carious and sound groups, respectively. SCCD and SHED presented similar proliferation rate. There were no statistical differences between the groups for the tested surface markers. The cells from sound and carious deciduous teeth were positive for CD29, CD73, and CD90 and negative for CD14, CD34, CD45, and HLA-DR and were capable of differentiating into osteogenic, chondrogenic, and adipogenic lineages.

Conclusion SCCD demonstrated a similar pattern of proliferation, immunophenotypical characteristics, and differentiation ability as those obtained from sound deciduous teeth. These SCCD represent a feasible source of stem cells.

Clinical relevance Decayed deciduous teeth have been usually discarded once the pulp tissue could be damaged and the activity of stem cells compromised. These findings show that stem cells from carious deciduous teeth can be applicable source for cell-based therapies in tissue regeneration.

Keywords Dental pulp stem cells · Deciduous tooth · Caries · Proliferation · Differentiation

Introduction

Populations of mesenchymal human stem cells have been isolated from the pulp of permanent (dental pulp stem cells—DPSCs) [1] and deciduous sound teeth (stem cells from human exfoliated deciduous teeth—SHED) [2]. Although these groups of cells share similar characteristics, SHED shows higher proliferation rates and better differentiation potential than DPSCs [3]. Moreover, deciduous teeth are more attractive because they physiologically exfoliate and pulp tissue is usually discarded [4, 5]. Thus, sound deciduous teeth, especially those in advanced exfoliation process [6], are considered a feasible stem cell source.

The use of dental pulp stem cells from sound teeth is successfully reported in several strategies of regeneration in animal models, like treatment of muscular dystrophy [7], corneal reconstruction [8], and repair of the central nervous system [9]. Moreover, DPSC and SHED are focus of dental researches and have showed promising results for pulp regeneration [10, 11] and bone repair in animal [12] and human [13] models.

Recent studies have shown conflicting results regarding the differentiation and proliferation potentials of stem cells obtained from the pulp of permanent teeth with deep carious lesions, which can be associated with the pulp inflammation stage [14–17]. The questions remains whether stem cells are modified

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by the carious process in deciduous teeth, since under the carious process, there is an increase of cytokine expression [18] resulting from the inflammatory response to the bacteria and their products. Moreover, the stem cells present in the pulp, after death of odontoblast layer, proliferate up to a sufficient number to repair the defect and then initiate a cascade of differentiation into odontoblast-like cells, responsible for the secretion of dentin matrix and producing a reparative barrier [18, 19].

Despite the decreasing prevalence of dental caries worldwide, epidemiological studies have shown that in developing countries, such as Brazil, it represents a public health problem, affecting mainly young children. At 5 years old, 53.4 % of children are affected by caries, with a mean of 2.4 of decayed, missing, or filled tooth (DMFT) [20, 21]. Therefore, when deciduous teeth undergo exfoliation, the likelihood of becoming decayed is high.

The current knowledge about the behavior of SHED comes from studies using cells obtained from sound teeth. Considering carious deciduous teeth as a source of stem cells, alteration on proliferation and differentiation potentials and immunophenotype characteristics need to be determined. This study aimed to evaluate the effects of active carious lesion in dentin on the characteristics of stem cells from the pulp of deciduous teeth.

Materials and methods

Subjects

Incisors, canines, or molar deciduous teeth with at least 1/3 of physiologic root resorption [6] were selected for the carious ($n=10$) and the sound groups ($n=10$) from 17 donors, aged 8 to 12 years. The inclusion criteria for carious group were as follows: teeth with active caries lesions (internal half of dentin), no history of spontaneous pain, absence of fistula, no edema, no abscess, no increased mobility, no radiolucency at the furcation or periapical region, no increase in the periodontal space, and an absence of internal resorption or sign suggesting irreversible pulpitis or pulp necrosis.

Dentists not related to this research planned the extractions as part of the orthodontic treatment for each patient. Before the extractions, the teeth were cleaned with 0.12 % chlorhexidine solution.

Isolation and cell culture

Immediately after the extraction, the teeth were placed into a Falcon tube containing 2 mL of cool culture medium—Dulbecco's modified Eagle medium (DMEM) supplemented by 10 % fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), 100 U/mL penicillin, 100 mg/mL streptomycin, and 0.45 mg/mL gentamicin (Gibco, Grand Island, NY). In a laminar flow, the dental pulp tissue was separated from the dentinal walls by using endodontic files, and the cells were retrieved by

enzymatic digestion. These procedures were performed up to 3 h after tooth extraction. Briefly, the dental pulp was placed into a 0.2 % type I collagenase solution (Gibco, Grand Island, NY) for 60 min in a 37 °C bath and centrifuged (4 °C, $800\times g/10$ min). The supernatant was discarded and the remaining pellet was resuspended and seeded onto a 12-well plate. The culture medium was changed after 3 days and every 3 or 4 days after the first medium change. Passages were performed when the cultures reached 90 % confluence. In each passage, the cells were stained with trypan blue, counted in a hemocytometer, and the number of cells was registered. Cell suspensions were seeded onto new plates, respecting the proportion of 5,000 cells/cm^2. When the cells were not present after 30 days, the well was inactivated and the culture registered as a failure. All the tests were carried out with the cells in the fifth passage (P5). During this whole period, the cells were incubated in a 37 °C and 5 % CO_2 environment.

Proliferation assay

A water-soluble tetrazolium monosodium salt Cell Counting Kit-8 (WST-8, Sigma-Aldrich, St. Louis, MO) was used for colorimetric evaluation of cellular metabolic activity through the experimentation course. A total of 2.5×10^3 cells/well of each sample ($n=6$) were seeded a 24-well plate and incubated with 1 mL of culture medium. Tests were performed according to the manufacturer's instructions, in triplicate. The analysis of absorbance was performed with a wavelength of 450 nm on days 1, 3, 5, and 7 after seeding. Three wells filled with culture medium added with WST-8 without cells were used as background. The absorbance of the test was subtracted from the absorbance of the control, resulting in the final absorbance. The calibration curve (cell number×absorbance values) was performed with six measurement points, between 500 and 25×10^3 cells, for both groups. Proliferation was also evaluated by using the mean time (days) between the cell isolation and the fifth passage.

Flow cytometric analysis

A total of 10^5 cells of each sample ($n=5$) were incubated with the following types of conjugated antibodies: CD14/FITC, CD29/PE, CD34/PE, CD45/FITC, CD73/PE, CD90/FITC, HLA-DR/FITC, and STRO-1/PE. Unstained cells and cells labeled with mouse IgG1 isotype control conjugated with PE and FITC were used as controls. The 7-aminoactinomycin D (7AAD) is a nuclear stain for apoptosis, used to separate dead cells from living cells. Therefore, only living cells were analyzed by excluding dead cells that were positive for 7AAD (Invitrogen). Two-cell surface markers were evaluated simultaneously using the monoclonal antibodies against the antigens. Data was acquired using the FACS Aria flow cytometer (BD Bioscience) and 10,000 events were analyzed (FACSDiva 6.1.3—BD Bioscience).

In vitro multipotent assay

Cells from both groups (1×10^4) ($n=5$) were seeded onto 12-well plates and cultivated in a regular medium until confluence reached 70 %. The medium was then replaced by one of three differentiation-inducing mediums, as follows:

– Osteogenic differentiation: DMEM/4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) supplemented with 10 FBS; 0.1 % dexamethasone, 10–5 mol/L; 10 % β-glycerophosphate, 10 nmol/L; and ascorbic acid 2-phosphate, 5 mg/mL.
– Chondrogenic differentiation: DMEM/HEPES supplemented with 6.25 mg/mL bovine insulin, 50 nmol/L ascorbic acid 2-phosphate, and 10 ng/mL transforming growth factor beta 1 (Millipore, Tokyo, Japan).
– Adipogenic differentiation: Iscove's Modified Dulbecco's Medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10 % FBS, 1 μL/mL; bovine insulin, 5 μM/L; rosiglitazone, 10–7 μM/L; dexamethasone, 5 μM/L; and indomethacin, 1/10 mL.

The cell cultures were maintained in differentiation-inducing mediums up to 28 days and then washed with deionized water and fixed in 4 % paraformaldehyde for 20 min (osteogenic and chondrogenic) or 1 h (adipogenic). After the paraformaldehyde was removed, deionized water was used to wash the culture. Alizarin red was used to stain calcium depositions in osteogenic differentiation. Alcian blue was used to stain extracellular matrix in chondrogenic differentiation and Oil-Red-O to stain lipid vacuoles in adipogenic differentiation. The cultures were stained for 5 min and then washed with deionized water until the excess dye was removed. The cells cultured in a regular culture medium were used as control. The cultures were observed microscopically.

At least three independent experiments for each sample were performed to verify reproducibility of results.

Statistical analysis

The means for each surface marker in the flow cytometry analysis were submitted to two-way ANOVA. Time-course proliferation assay was analyzed using repeated-measure ANOVA followed by Bonferroni's test, for the comparison within the groups. Student's t test was used to compare the mean time between isolation and the fifth passage.

The statistical analysis was performed using the Statistical Package for Social Sciences version 20.0 (SPSS Inc., Chicago, IL, USA, 2011) at 5 % significance level.

Results

Establishment of cultures

The cell culture was considered a successful outcome when the cells were seen adhering at the bottom of the well within 30 days after isolation and had reached confluence for the passage. Both groups showed a homogenous layer of spindle-shaped, fibroblast-like cells.

The carious and sound group showed 70 (7/10) and 80 % (8/10) of culture success, respectively (Table 1). There was no contamination during cell culture for both groups.

Proliferation assay

The increase of cell number resulted in the increase of absorbance values. During the time of the evaluation, a similar and exponential growth for both groups was observed. There was no significant difference in all the periods evaluated (0, 1, 3, 5, and 7 days) between the groups ($p>0.05$). The carious group showed a significantly higher proliferation in the later periods, 5 ($p=0.035$) and 7 days ($p=0.014$) (Fig. 1). The mean time (days) between isolation and the fifth passage was 63 (±20.2) for sound and 68 (±21.4) for carious groups ($p=0.71$).

Flow cytometry analysis

For the sound and carious groups, more than 98 % of the cells were positive for CD29, CD73 and CD90 and more than 97 % were negative for hematopoietic markers (CD14, CD34, CD45 and HLA-DR). The expression of STRO-1 was similar in groups, 0.7 % (0.6) for sound and 0.8 % (1.3) for carious teeth. No statistical

Table 1 Sample description for both sound and carious groups

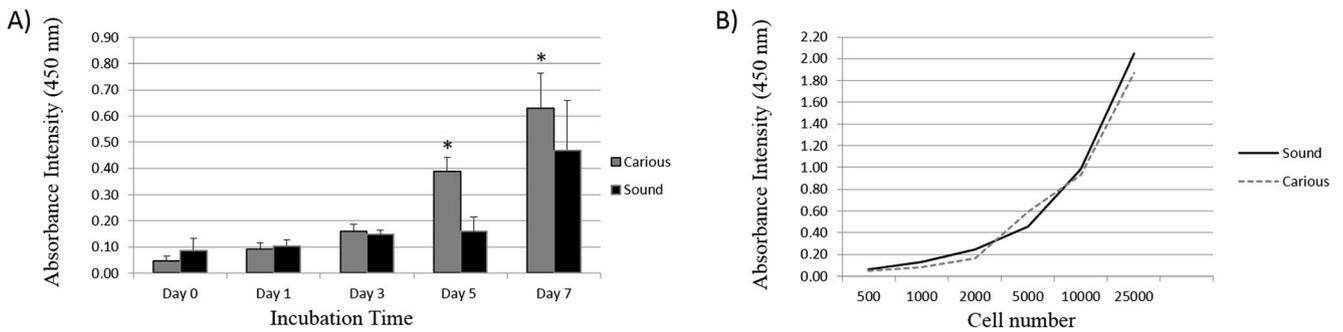| Groups	Donor age (mean DP)	Teeth (isolated/culture success)				Days until first passage
		Completely resorbed root	1/3 residual root	Incisor or canine	Molar	
Sound	10.2 (1.8)	7/6	3/2	7/6	3/2	26.6 (6.2)
Carious	9.1 (1.5)	8/7	2/0	2/0	8/7	25.4 (4.4)

Fig. 1 Proliferation assay analyzed by WST-8. **a** Means and SEM (standard error of the mean) of absorbance for proliferation assay on days 0, 1, 3, 5, and 7. The *asterisks* indicate statistically significant differences within the carious group on day 5 (*p*=0.035) and 7 (*p*= 0.014). There is no difference between groups. **b** Calibration curve. The absorbance values indicate the increase of metabolic activity, related to living cell number

difference (*p*>0.05) was observed between the groups for the tested surface markers (Fig. 2).

In vitro multipotent assay

Cultures from both groups grown in the presence of an osteo-inductive medium showed the ability to form alizarin red-positive condensed nodules of calcium (14 days).

Twenty-eight days were necessary for the cells of both groups to show the formation of an alcian blue-stained extracellular matrix (chondrogenic differentiation). The adipogenic potential was also seen in the cultures of oil red-positive lipid clusters (21 days). The negative control, consisting of the cells of both groups cultured in a regular culture medium, showed no differentiation (Fig. 3).
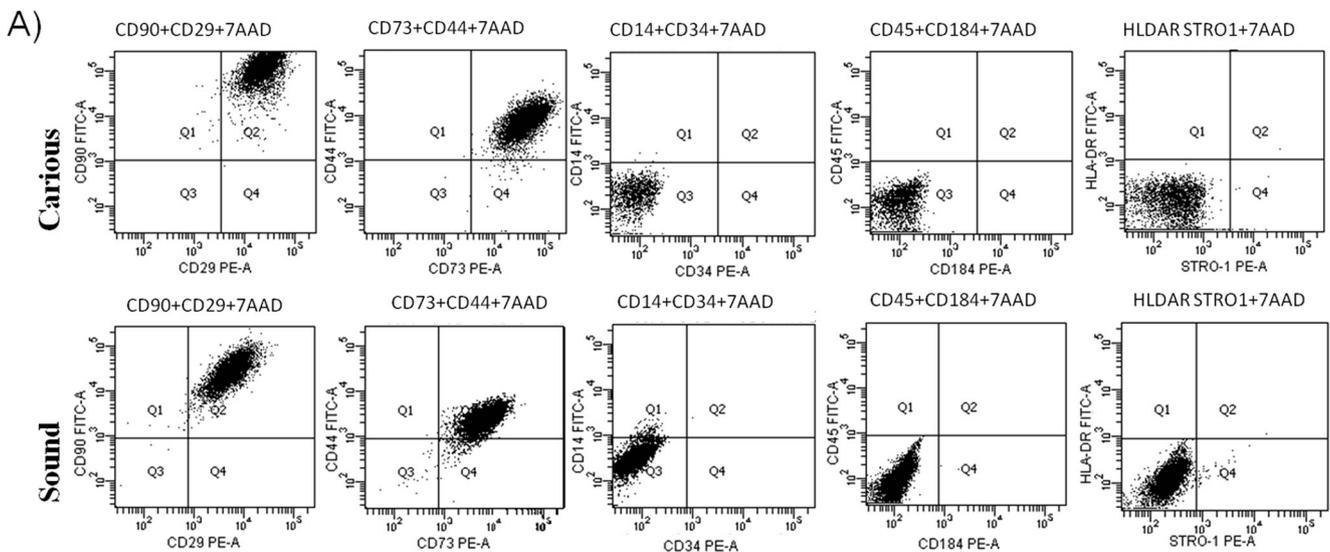
GROUP	Mesenchymal markers			STRO-1	Hematopoietic markers			
	CD29	CD73	CD90		CD14	CD34	CD45	HLA-DR
Carious	99.0 (0.7)	99.0 (2.2)	99.7 (0.5)	0.8 (1.3)	2.1 (1.5)	0.0 (0.0)	0.0 (0.0)	0.1 (0.1)
Sound	99.4 (2.4)	98.7 (1.2)	99.7 (0.3)	0.7 (0.6)	1.8 (4.5)	0.0 (0.0)	0.0 (0.1)	0.1 (0.1)

Fig. 2 Surface marker expression. **a** Surface marker expression profile (flow cytometry) for cells obtained from carious and sound group, confirming that they are populations of mesenchymal stem cells. **b** Surface marker expression (%) for cells from carious and sound teeth. All values are expressed by means (standard deviation). No statistical difference (*p*>0.05) was observed between the groups
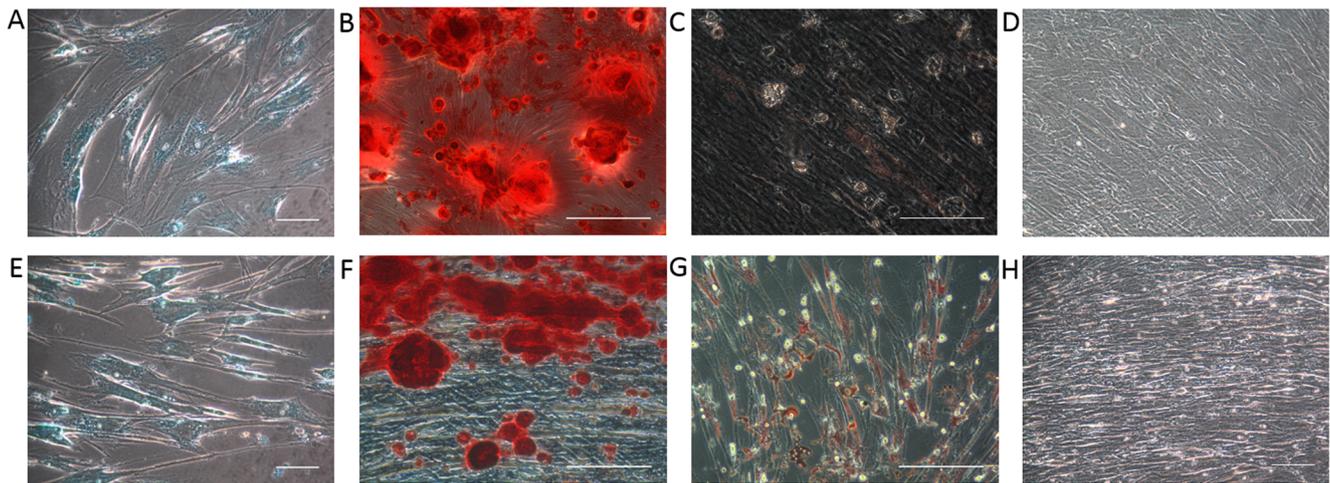
Fig. 3 In vitro multipotent assays. Chondrogenic differentiation visualized by alcian blue staining of the glycosaminoglycans matrix deposits in the sound (**a**) and carious (**e**) groups. Osteogenic differentiation showing calcium wide sheets stained with alizarin red in the sound (**b**) and carious (**f**) teeth. Adipogenic differentiation shown by oil red staining of lipid vacuoles in the sound (**c**) and carious (**g**) groups. Pulp cell culture without induction medium for negative control for the sound (**d**) and carious (**h**) groups. No visible differences were found in the groups. Scale bar=100 μm)

Discussion

The overall result of the present study demonstrates that pulp tissue from carious deciduous teeth constitute another source of stem cells, similarly to the pulp tissue from sound deciduous teeth. This result is promising since, until now, carious deciduous teeth have been discarded in the belief that the pulp tissue could be damaged and the activity of the stem cells would be compromised. These findings show that stem cells from pulp of carious deciduous teeth can be successfully cultivated in vitro, becoming one more source for mesenchymal stem cells.

Stem cells from carious deciduous teeth (SCCD) showed isolation rate similar to the control cultures (sound group), corroborating with other studies from sound deciduous [6] and permanent teeth [17]. Pereira and colleagues found a lower isolation success rate for permanent teeth with irreversible pulpitis, compared to the sound control, associating this lower rate to the lower pulp volume obtained in the test group. This rate increased when pulp with higher volume was selected. However, in our study, the sample was obtained from deciduous (incisors, canines, or molars) teeth with root resorption, where the amount of pulp tissue is really small compared to permanent teeth, and even then, the isolation rates observed were above 70 %. Another important observation should be addressed about the inflammatory profile of the pulp tissue that usually is used as a clinical parameter to indicate a more conservative or radical pulp treatment. It is expected to find in dental pulps with irreversible pulpitis a compromised response capacity, when compared to a pulp tissue with a transitory or reversible inflammation.

The cultured samples proliferated similarly in both groups, corroborating a previous study that compared stem cells from deciduous teeth with inflamed and normal pulp [22]. However, Ma and colleagues found a higher proliferation potential for cells from permanent teeth with deep caries lesions, a clinical condition (inclusion criteria) similar to the present study. Despite no differences between the groups in the proliferation assay, for intra-group analysis, the carious teeth presented a higher number of cells in the last days of analysis.

There is no specific surface marker characterizing DPSC, but typical markers of mesenchymal stem cells were established, such as CD73, CD90, and CD29. CD34, CD45, and HLA-DR are the markers usually used as negative control [23]. It has been reported that inflammation of pulp enhances the expression of CD90 and STRO-1 markers [24]. The results of the present study did not show differences of mean expression of these surface markers between the groups. The mean of CD90 expression was the same for both groups (99.7), while STRO-1 expression was small and there were no presented differences between groups, corroborating the findings from previous studies with stem cell obtained from permanent teeth with irreversible pulpits [15, 17]. However, the findings from Ma and Alongi and their colleagues reinforce a higher expression of STRO-1 in permanent teeth with reversible and irreversible pulpits, respectively.

Studies have shown that the function of odontoblasts participates in the activation and modulation of immune cells and they are involved in the production of antimicrobial peptides. It has been suggested that, at first, the odontoblast layer, through the regulation of its gene expression, attenuates the inflammatory process of carious lesions, maintaining homeostasis of the pulp tissue. It has been shown that the odontoblast layer possesses a distinctive gene expression when compared with other areas of the pulp tissue, suggesting a compartmentalized response in answer to the aggression to the pulp [18].

Thus, the low rate of STRO-1 expression found in this study, for both groups, is probably due to the inflammatory process arising from physiologic root resorption. What strengthens this hypothesis is that the decayed teeth selected for this study, according to the inclusion criteria, presented normal pulp or reversible pulpitis.

The differentiation in three lineages, osteogenic, adipogenic, and chondrogenic, is proposed as criteria for defining multipotent mesenchymal stem cells [23]. This cell capacity was similar in both groups, suggesting that the carious process did not impair the capacity of the stem cells to differentiate into different cell lineages. The differentiation potential provides the basis for the use of stem cells from the pulp of deciduous teeth in tissue engineering researches of pulp regeneration [10, 25–27] and even for the recovery of systemic disorders and diseases [7–9].

In conclusion, the stem cells from the pulp tissue from carious deciduous teeth exhibit similar properties to those cells obtained from the pulp of sound deciduous teeth. It was shown that SCCD could also be used as a source of stem cells.

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