

Isolation and characterization of dental pulp stem cells from permanent third molars

Catiane Telles
Alessandra Dutra da Silva
André Wiltgen
Márcia Kijner
Melissa Camassola
Paulo de Borba

ABSTRACT

Objective: To characterize pulp stem cells and evaluate their capacity for expansion and differentiation *in vitro*.

Methods: Pulp tissue was collected from permanent third molars and digested and then the cells were seeded onto plates containing HDMEM medium. Expansion of the cells was performed and then differentiation tests were conducted with osteogenic, chondrogenic and adipogenic induction media; followed by determination of immunophenotypical profiles using specific antibodies with assessment by flow cytometry.

Results: It was observed that the pulp stem cells exhibited the capacity to adhere to plastic and a high rate of expansion and, after detection with specific stains, it was shown that the cells were capable of differentiation into osteoblasts and chondroblasts, but not into adipocytes. Analysis of the cellular phenotype showed that the cells were negative for CD45, CD69, CD117 and HLA-DR, and positive for CD13, CD44, CD73, CD90 and CD105.

Conclusions: The cells isolated from dental pulp exhibited characteristics compatible with those expected for mesenchymal stem cells (MSCs) and are good candidates for cell therapy applications and tissue bioengineering.

Keywords: Stem Cells; Pulp; Characterization; Differentiation.

Isolamento e caracterização de células-tronco pulpareas derivadas de terceiros molares permanentes

RESUMO

Objetivo: Caracterizar as células-tronco pulpareas e avaliar sua capacidade de expansão e diferenciação *in vitro*.

Métodos: O tecido pulpar de terceiros molares permanentes foi retirado, digerido e as células plaqueadas em placas contendo meio HDMEM. Foi feita a expansão das células obtidas;

Catiane Telles is dental surgeon from Universidade Luterana do Brasil (ULBRA), Canoas, RS, Brazil.

Paulo de Borba, Alessandra Dutra da Silva, Márcia Kijner and André Wiltgen are PhD and adjunct professors at ULBRA, Canoas, RS, Brazil.

Melissa Camassola is professor and researcher at the Graduate Program in Molecular Genetic Diagnosis at ULBRA, Canoas, RS, Brazil.

The authors have no conflicts of interest to declare concerning the publication of this manuscript.

Correspondence: Alessandra Dutra da Silva, Curso de Odontologia, Rua Farroupilha, 8001, CEP 92425-900, Canoas, RS, Brazil. Tel.: +55 (51) 3477.4000. E-mail: dra.alessandradutra@gmail.com

Stomatos	Canoas	Vol. 22	Nº 42	p.32-41	Jan./Jun. 2016
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foram realizados ensaios de diferenciação com meios indutores osteogênicos, condrogênicos e adipogênico; e caracterizado o perfil imunofenotípico com anticorpos específicos e avaliado por citometria de fluxo.

Resultados: Observou-se que as células-tronco pulpares apresentaram capacidade de aderência ao plástico e uma alta taxa de expansão e, após revelação com corantes específicos, se verificou que as células foram capazes de se diferenciarem em osteoblastos e condroblastos, porém não em adipócitos. A análise do fenótipo celular mostrou que as células foram negativas para CD45, CD69, CD117 e HLA-DR, e positivas para CD13, CD44, CD73, CD90 e CD105.

Conclusão: As células isoladas de polpa dentária apresentaram características compatíveis com as esperadas para células tronco-mesenquimais (CTMs), sendo boas candidatas para trabalhos de terapia celular e bioengenharia de tecidos.

Palavras-chave: Células-Tronco; Polpa; Caracterização; Diferenciação.

INTRODUCTION

Stem cells are cells with a low degree of differentiation that have the capacity to reproduce and can generate differentiated cells of several different types of tissues (1).

Tissue bioengineering has the capacity to accelerate regeneration and repair of damaged tissues using patients' natural curative potential and it is facilitated by several types of biomaterials (2).

Many studies have demonstrated tissue bioengineering's potential and its applications for dentistry and regenerative medicine (3-5). Pulp stem cells (PSCs) are able to form ectopic dentin *in vitro* and *in vivo* and also to generate a dentine-pulp complex composed of a mineralized matrix, with dentinal tubules that are aligned and filled with odontoblastic prolongations, containing vascularized pulp tissue, in a similar arrangement to that observed in natural dental structures (3,6).

The objective of this study is to isolate and characterize stem cells from the pulp of permanent third molars and analyze the cells obtained in terms of their morphology and chondrogenic, osteogenic and adipogenic cell differentiation.

METHODOLOGY

This study was approved by the Human and Animal Research Ethics Committee at ULBRA (CEP-ULBRA 2009-361H) and all participants signed a free and informed consent form.

Ten healthy third molars were extracted at the orthodontist's recommendation and were immediately disinfected with 0.2% chlorhexidine digluconate followed by sectioning at the cemento-enamel junction and removal of the pulp with a curette. The pulp tissue was then placed in a graduated tube containing HEPES buffered Dulbecco's modified eagle medium (HDMEM) (Figure 1) for transportation to the ULBRA Stem Cell and Gene Therapy Laboratory.

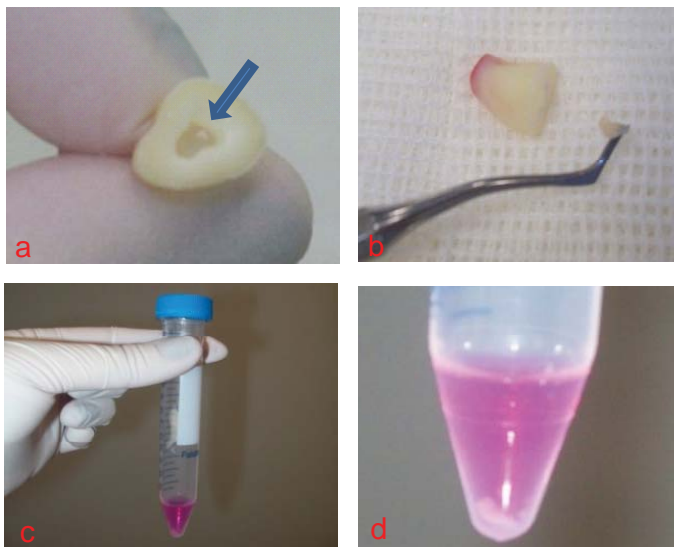


FIGURE 1 – Removal of pulp tissue. a) Sectioned tooth with the pulp visible within the pulp chamber (arrowed). b) Total removal of the pulp (coronal and radicular) with a curette. c) Pulp in the graduated tube containing HDMEM medium. d) Close-up of the pulp tissue obtained from one third molar.

The pulp was later placed in the HDMEM medium, centrifuged and incubated at 37°C. The cells were seeded onto six-well plates in DMEM medium containing 2.5 g/l of HEPES and supplemented with 10% fetal bovine serum, and the medium was changed after 72 hours to remove cells that had not adhered, while the cells that had adhered were transferred to a graduated flask for storage and culturing.

When the culture exhibited confluence, the cells were passaged, using trypsin, expanded and counted in a Neubauer counting chamber, to monitor cellular kinetics.

During the fifth passage, the cultures were exposed to a series of cell differentiation inducers. Using a 12-well plate, three differentiation mediums were added to one three-well column each and one column was used to conduct a negative control with complete HDMEM medium only. The cultures were kept in the plate for 30 days, with medium changes every 4 days.

Osteogenic differentiation was induced using a culture medium containing complete HDMEM, Beta-glycerol phosphate, ascorbic acid (ASAp) and Dexamethasone. For chondrogenic differentiation, the cell cultures were placed in a medium containing complete HDMEM, insulin, TGF-Beta and ASAp. For adipogenic differentiation, Iscove's medium with dexamethasone, insulin, indomethacin and rosiglitazone was used.

After one month, the cultures were cured using alizarin for osteogenic differentiation and alcian blue for chondrogenic differentiation, while the adipogenic differentiation culture was stained with *oil red*. Each of the stains were used separately in the negative controls.

Finally, an immunophenotyping technique was used with the following specific antibodies: CD105, CD73, CD90, CD45, CD34, CD14/CD11b, CD79a/CD19, and HLA-DR.

RESULTS

Kinetics and cell morphology

Isolation of stem cells was only successful for four of the ten pulp samples collected. In these four cases it was observed that the cells had the capacity to adhere to the plastic of the culture flasks. PSCs have a similar morphological appearance to fibroblasts, with a polygonal shape and visible cytoplasmic prolongations (Figure 2).

On average, the stem cells remained in culture for 17 passages and maintained a high rate of expansion up to the most advanced passages, with a mean of 7.61×10^5 cells/ml.

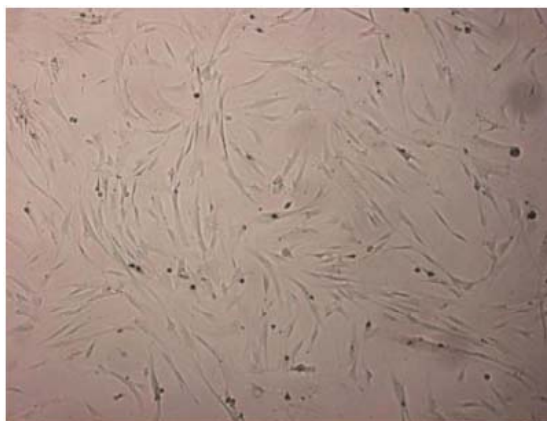


FIGURE 2 – Culture of pulp stem cells.

It was observed that during the initial passages, the mean time taken to achieve confluence was 2 days, while the mean for the final passages was 4 days.

Cytometry and immunophenotyping

Analysis of the surface markers showed that the cells were negative for CD45, CD69, CD117 and HLA-DR, which are markers of hematopoietic lineages, and positive for CD13, CD44, CD73, CD90 and CD105, as can be observed in Figure 3.

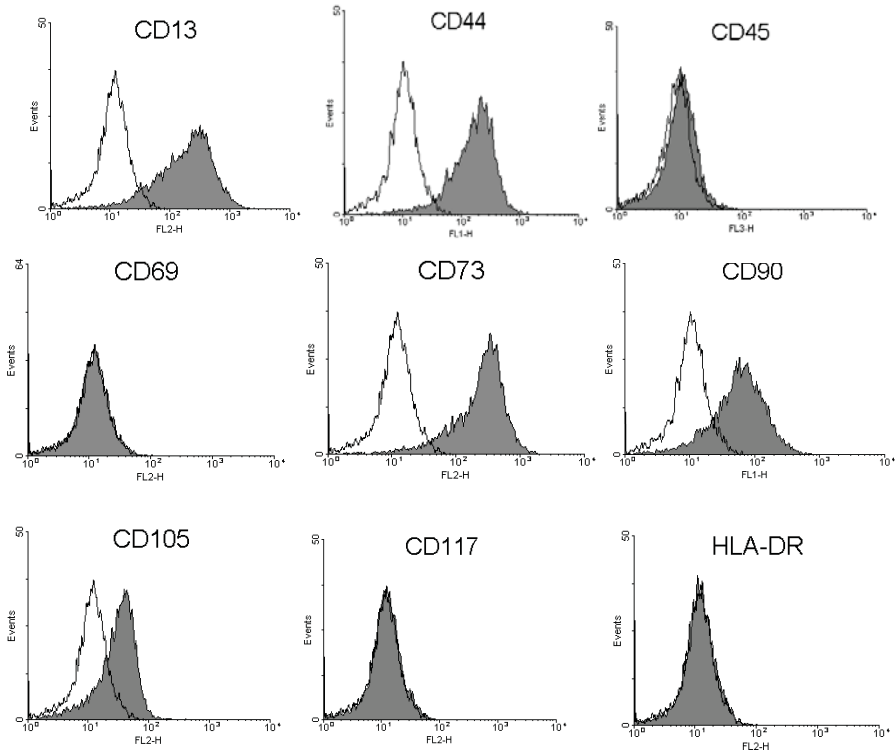


FIGURE 3 – Analysis of the immunophenotypical profile: CD45-, CD69-, CD117- and HLA-DR-, CD13+, CD44+, CD73+, CD90+ and CD105+. Markers for which both histograms are superimposed did not mark the cells.

Cell differentiation

It was possible to observe PSC differentiation into osteoblasts in cultures kept in induction medium specific for bone differentiation (Figure 4). Staining with alizarin, which has an affinity for calcium, revealed deposition of an extracellular matrix rich in calcium over the culture.

It was possible to observe PSC differentiation into chondroblasts in cultures kept in chondrogenic differentiation medium (Figure 5), which was confirmed by alcian blue staining, which stained them blue, demonstrating presence of chondroitin sulfate, one of the principal structural components of cartilage.

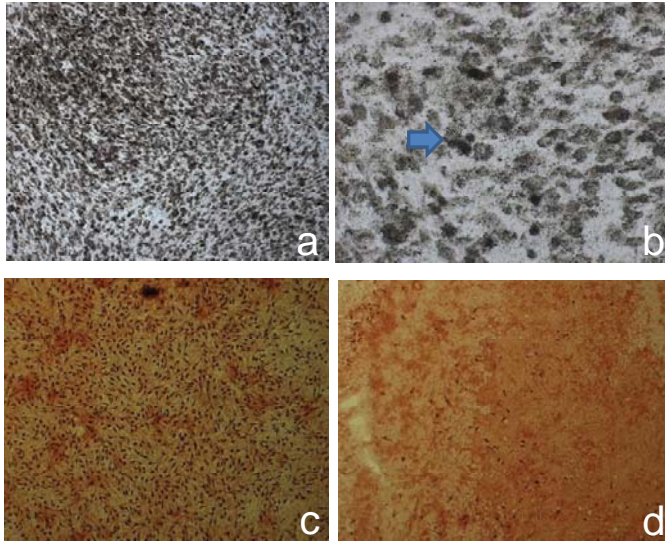


FIGURE 4 – Osteogenic differentiation. Culture shown at 50X magnification (a) and culture at 100X magnification (b) showing calcium deposits (arrowed). c, d) Osteogenic Differentiation stained with alizarin, at 50X and 100X respectively.

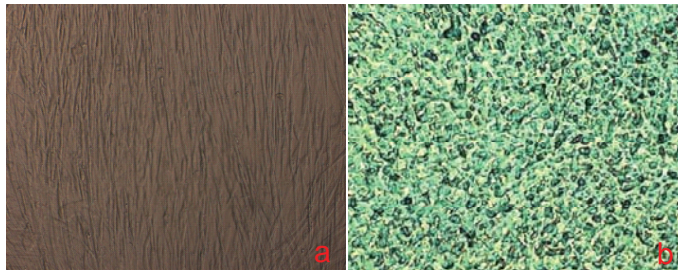


FIGURE 5 – Chondrogenic differentiation. a) Cell culture in chondrogenic induction medium; b) staining with alcian blue, 100x magnification.

The negative control (Figure 6) was maintained in HDMEM culture medium only and in this case, even without osteogenic induction medium, it was possible to observe differentiation of the cells into osteoblasts. Cultures containing adipogenic induction medium did not differentiate.

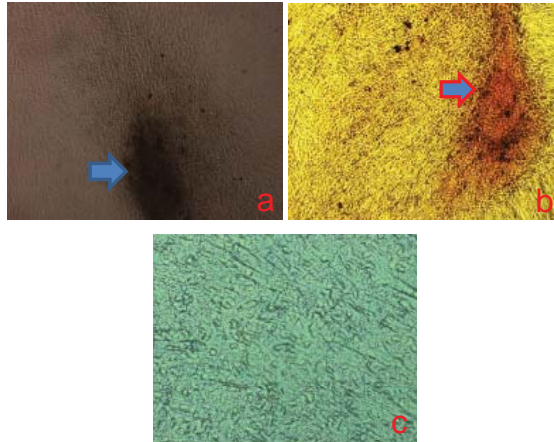


FIGURE 6 – Negative control. a) Cell culture with HDMEN medium at 50x magnification. Intense calcium deposits can be observed (arrowed). b) Staining with alizarin at 50x magnification. Intense calcium deposits can be observed (arrowed). c) Staining with alcian blue at 100x magnification.

DISCUSSION

There are several different protocols proposing methods for effective isolation of stem cells. The majority of them are based on the method proposed by Gronthos et al. (3,7) and Gronthos et al. (7), which is the method that was used in this study, with certain modifications. The protocol for isolation and culturing of the PSCs was very effective and the cells exhibited good proliferation results.

The stem cells exhibited an elongated and irregular shape with cytoplasmic prolongations (Figure 2), which are characteristics that can also be observed in fibroblasts. Although they are morphologically similar to fibroblasts, PSCs differ because fibroblasts do not have the capacity for self-renewal or transdifferentiation, whereas both of these properties are characteristic of the mesenchymal stem cells (MSCs) found in pulp. Other authors have also conducted studies in which cells morphologically similar to fibroblasts were observed (8-10).

The immunophenotypic profile exhibited by the PSCs demonstrated that they were negative for CD45, CD69, CD117 and HLA-DR, which are markers of hematopoietic lineages, and positive for CD13, CD44, CD73, CD90 and CD105. These findings have also been described by several other authors (3,11-13).

There is not yet consensus on a surface marker that is specific for stem cells, which is why a set of heterogeneous markers associated with the activity of undifferentiated mesenchymal cells is used (14). According to the International Society for Cellular Therapy, it is consensus that identification of the markers CD105, CD73 and CD90 and absence of expression of hematopoietic markers is sufficient for immunophenotyping of

these cells, as long as this characterization is combined with demonstration of cellular adhesion for long periods in culture and differentiation into at least two distinct cell lineages (15,16).

It proved possible to induce the PSCs to differentiate into osteoblasts and chondroblasts, but not into adipocytes. In osteogenic differentiation treatments an intense deposition of calcium over the culture was observed (Figure 4), which was more easily seen when alizarin staining was applied (6,17). Gronthos et al. (3) described how after 6 weeks of osteogenic induction it was possible to observe nodules with high calcium content located sporadically throughout the adherent layer in the form of single mineralized zones that were positive for alizarin. The same result was observed in this study.

In chondrogenic differentiation treatments (Figure 5), formation of chondroblasts was observed and, by staining with alcian blue, formation of cartilage-like tissue with many collagen fibers could be observed, as described in many studies as type II collagen (17-19).

The negative control, which was only cultured in HDMEM medium enriched with fetal bovine serum, spontaneously differentiated into bone matrix-producing cells (osteoblasts), even in the absence of osteogenic induction medium (Figure 6b), and when stained with alcian blue it was also observed that the negative control exhibited a slight tendency towards chondrogenic differentiation (Figure 6c), also spontaneous, but with a considerably weaker expression than its osteogenic differentiation.

CONCLUSIONS

The results observed allow for the conclusion that the cells isolated from the pulp of permanent third molars were compatible with MSCs, on the basis of the following principal characteristics: the capacity to adhere to plastic, a strong proliferative potential, clonogenic potential and their multi-lineage precursor potential, confirmed by osteogenic and chondrogenic differentiation, in addition to the phenotypical analysis results which showed that the PSCs were negative for hematopoietic markers, but positive for CD13, CD44, CD73, CD90 and CD105, which is a profile compatible with that expected of MSCs.

The possible applications for PSCs in odontology are highly diverse, since these PSCs can differentiate into several types of tissue. It was demonstrated that they can differentiate into odontoblasts, so they could be used to repair dental tissue lost to caries, and into osteoblasts, allowing patients to recuperate bone tissue, facilitating treatment of periodontitis and traumas, grafts for dental implants and treatment for bone pathologies. It was also shown that they are capable of chondrogenic differentiation, which could be an option for treatment of temporomandibular dysfunctions.

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