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Comparison between canine and molar swine tooth: tissue and stem cell view

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ABSTRACT

Dental tissues are an abundant and rich source for easily and continually obtaining of mesenchymal stem cells (MSC), which are able to differentiate in vitro into several types of tissues, such as fat, cartilage, bone, among others. In swine, canine teeth display continuous growth, suggesting it could represent a different niche of stem cells. In this study, we compare dental pulp mesenchymal stem cell (sDPSC) niches from canine and molar teeth in swine. Tooth tissues were obtained and characterized by histological, microscopy and cellular analyses. Tissues were submitted to immunohistochemistry analysis and showed expression for mesenchymal stem cells markers, such as CD73, CD90, CD105 and for pluripotent markers (Oct-4, Nanog and Sox-2). Molar and canine sDPSC were also cultured and characterized according to MSC properties, such as plastic adherence capability, fibroblast-like morphology and cell surface antigen profile. sDPSC displayed an exponential growth pattern by MTT assay and increased in-vitro differentiation potential for adipogenic and osteogenic lineages. Tumorigenic test indicated these cells were unable to generate tumor in nude mice. Thus far, stem cells derived from canine and molar teeth in swine did not expose significant differences related to cell or plasticity markers and they indicate to be safe for animal cellular therapy use since they are devoid of tumorigenic disposition.

KEY WORDS

Mesenchymal stem cell; MSC; Dental tissue; DPSC; Swine.

INTRODUCTION

Currently, stem cell research is extensively investigated, leading to an expansion in different applications and approaches in regenerative medicine. Most of the expertise in stem cells is applied to human stem cells. However, information on animal stem cells is of extreme relevance for veterinary research for use in cell therapy [1, 2]. Mesenchymal stem cells (MSC) have been greatly studied due to their regenerative potential, immunomodulatory signaling properties and cellular plasticity [3, 4, 5].

MSC-like populations derived from dental tissue are one of the 5 different types of stem cells found in specialized tissues already isolated and characterized, being the postnatal dental pulp stem cells' (DPSC), exfoliated deciduous teeth stem cells (SHED) ,apical papilla (SCAP), periodontal ligament stem cells (PDLSC) and dental follicle precursor cells' (DFPC) [6,7, 8]. Regenerative potential of dental pulp stem cells has been associated to dentin formation [9]. Several studies in different niches of human dental pulp stem cells have reported relevant features like multipotentiality, clonogenicity, proliferation and cell therapy potential. In addition, therapeutic application of these cells was previously reported for dental tissue regeneration [10]. Application for other diseases is still under investigation. On the other hand, few information is available concerning dental cells from animal species, being worth to

investigate if they retain the same potential described for human dental cells.

In swine, teeth development is specific according with the region of the mouth. Swine teeth are classified like incisors, canines, premolars and molars. Shape and number of teeth are variable between breeds of swine, which is called heterodontia (Greek "heteros", different, and "odont" tooth [11]. Dental from swine are easy to obtain, since they have low or no commercial value and are often discarded. Swine DPSC (sDPSC) have been previously isolated and reported to display MSC-like characteristics, as well as regenerative potential [9,12,13]. Interestingly, canine teeth in swine display continuous growth. Here we take advantage of cell culture technology to compare and characterize MSC phenotype of cells from molar and canine teeth from swine to investigate if canine teeth could be a niche of stem cells with particular features.

MATERIALS AND METHODS

Animals and Ethical Statement

This study was approved by the Ethics Committee in the use of animal of the School of Veterinary Medicine and Animal Science - University of São Paulo - protocol # 2387/2011. Five (05) jaws from males of Sus domesticus were donated by Suzano Refrigerator. The animals were approximately four (04) months old.

Teeth Collection

All five jaws were disarticulated from Sus domesticus righ after slaughter. They were immediately rinsed with water and cleaned with benzalkonium chloride (5%). After being washed with PBS, they were placed on ice in a thermal box containing PBS and 5% antibiotics (500U/mL penicillin and 500µg/mL streptomycin - Invitrogen, CA, USA) for transportation. Canine and molar teeth were removed using Sierra-tape (model HSF 3200/Seg - Hobart, São Paulo, Brazil) and placed in PBS containing 5% antibiotics (500U/mL penicillin and 500µg/mL streptomycin - Invitrogen). Samples were splited for histological analysis and cell isolation.

Histological characterization

For histological characterization, two molars and two canines were fixed in 10% formalin (Sigma-Aldrich, St. Louis, USA) for Hematoxylin-Eosin (Synth, São Paulo, Brazil) and Masson's Trichrome stainings (Synth).

Scanning Electron Microscopy

Canine and molar teeth were fixed in 4% glutaraldehyde (Merck) for 48 hours at room temperature and the fragments were post-fixed in osmium tetroxide 1% (Labsynth – São Paulo - Brazil) and dehydrated in graded series of ethanol. The material was dried in a critical point apparatus using CO2 (PCD Union Balzers, Liechtenstein). The samples received metal coating by sputtering gold (K-550, Emitech, Kent, England) and were analyzed in a scanning electron microscope (Leo 435 VP - Zeiss, Oberkochen, Germany).

Immunohistochemistry assay

For immunohistochemistry, molar and canine dental pulp tissues were fixed in 4% paraformaldehyde (Sigma-Aldrich St. Louis, MO) and submitted to histological procedures; antigenic sites were unmasked in microwave in 10% citrate buffer diluted in PBS (pH 6.0) 3 times of 5 min or until boiling. The immunological assay was performed using anti-CD73, anti-CD105, anti-CD34, anti-CD45, anti-Oct4, anti-Nanog, anti-Sox-2, anti-PCNA3 and anti-Vimentin (Santa Cruz Biotechnology, CA, USA – 200mg/ml – 1:50) antibodies. Material visualization was done by Advanced Link HRP system (Dako, CA, USA). After reaction with DAB (diaminobenzine 3,3'), samples were analyzed using Nikon Eclipse 80i Microscope and NIS-Elements program-version3.22 (Nikon, Tókio, Japan).

Cell culture

After collection, dental pulp tissues from canine and molar teeth were isolated and precessed using an adapted protocol described before [6]. The tissues were extensively washed with buffered saline containing 5% antibiotic-antimycotic solution (penicillin/streptomycin/amphotericin B - final concentration 100U/mL, 0.1mg/mL, 0.25mg/mL, respectively - Sigma-Aldrich), and then minced into small pieces and digested using collagenase type I (3mg/mL-Invitrogen) for 1 hour at 37ºC, being shaken every 10 minutes an adapted protocol by [6]. Next, the collagenase was inactivated and cells were centrifuged. The pellet was suspended in a-MEM (LGC Biotecnologia, SP, Brazil), supplemented with 20% of inactivated fetal bovine serum (Invitrogen), 1% L-glutamine, 1% MEM NEAA and 1% antibiotic-antimycotic solution (penicillin/streptomycin/amphotericin B- Sigma-Aldrich) and kept in incubator with temperature of 37ºC and a 5% CO2 atmosphere.

Cryopreservation

Cell morphology was observed by inverted light microscopy (Nikon Eclipse TS100) and photographed daily. sDPSC from canine and molar teeth were submitted to cryopreservation process for cryoviability analysis. Cryopreservation solution was composed of 90% FBS (Life Technologies) and 10% DMSO (Life Technologies) and cells were gradually submitted to low temperature conditions. After 30 days in liquid Nitrogen, cells were thawed and observed and photographed in bright field.

MTT assay

MTT assay was performed as described before [14, 15]. 1x103 cells were plated in quadruplicate into 96 well/plate. MTT (Thiazolyl Blue Tetrazolium –Sigma-Aldrich) was added 2, 4, 7 and 9 days after plating and following 3h of incubation at 37ºC, MTT product was precipitated and solubilized using DMSO. The product originated, formazan, was measured in a spectrophotometer (m-Quant Plate Reader, BioTek Instruments Inc., Winooski, VT, USA) at 550 nm. Growth rates were plotted in a graphic using Graph Pad program (GraphPad Software, CA, USA).

Real time PCR

Total cellular RNA was extracted from ~2x106 cells using Trizol Reagent (Life Technologies, CA) and reverse transcriptase

was performed using SuperScript III (Invitrogen) according to manufacturer's suggestions. Primers used for characterization were CD34, CD45 and CD105. β-actin was used as housekeeping gene (Table 1). cDNA amplification was performed using SYBR Green Dye (Applied Biosystems) in a GeneAmp 7300 Sequence Detection System (Applied Biosystems) according to manufacturer's instructions under the following conditions: 50ºC for 2 min, 95ºC for 10 min, 40 rounds of 95º C for 15 s and 60º C for 1 min. The mRNA relative expression was calculated by 2(-ΔΔCT).

Table 1: Primers used in this study.

Gene (Primer)	Sense	Anti-sense
CD34	TTCTGTCCAGCCTCAGACCT	GCTACCTGGGGTAGGAGGAG
CD45	GGACATGTGACCTGGAAACC	CCATTACGCTCTGCTTTTCC
CD105	TGCTCCTGATCCTCAGTGTG	GCTCAGCAGCAGAGATGATG
β-Actin	CTGGGGCCTAACGTTCTCAC	GTCCTTTCTTCCCCGATGTT

DPSC differentiation

For adipogenic differentiation induction in vitro, 5x103 cells were plated in a 24-well plate containing glass coverslips and cultured in α-MEM supplemented with fetal bovine serum (7.5%), dexamethasone (10μM - Schering-Plough, SP, Brasil), insulin (10μg/mL Sigma-Aldrich), indomethacin (5μM-Sigma-Aldrich) and rosiglitazone (5μM - Sigma-Aldrich). For osteogenic differentiation induction in vitro, the same amount of cells were cultivated in α-MEM containing FBS (7.5% - Invitrogen), dexamethasone (0.1μM- Schering-Plough), ascorbic acid (100 μM - Sigma-Aldrich) and β-glycerolphosphate (10mM - Sigma-Aldrich). Controls in adipogenic and osteogenic differentiation were maintained in α-MEM medium containing FBS (7.5%Invitrogen). After 14 days, cells were submitted to oil red staining for adipogenic differentiation, and after 21 days, to alizarin red staining for osteogenic differentiation, in order to observe lipid-laden and calcium deposits, respectively.

Tumorigenic Potential Test

Approximately 1x106 stem cells from canine and molar teeth were suspended in PBS and injected subcutaneous in the cervical region of four nude mice. The animals were observed twice a week, for 8 weeks. After this period, they were euthanized according to the ethical committee recommendation. Organs were carefully excised, fixed in 4% paraformaldehyde (Merck) and processed for histopathological evaluation.

RESULTS

Teeth share a common basic architecture and are divided into three parts: crown, neck and root (Figure 1A). The large, curved canine tooth, also known as fang, are the most striking feature in swine dentition, displaying continuous growth throughout life. In the figure 1, it is possible to visualize the crown has a cover called mineralized enamel, with roots coated by cementum. Both coatings are found in dental cervix. Dentin consists of a mineralized tissue filling around the pulp cavity, which is formed by innervated and vascularized loose connective tissue richly composed by fibroblasts, odontoblasts, thin collagen fibrils and amorphous substance full of glycosaminoglycans. Right below it, there is an access foramen for blood and lymph vessels and nerves. Representative images of canine and molar teeth were used in this work (Figure 1B).

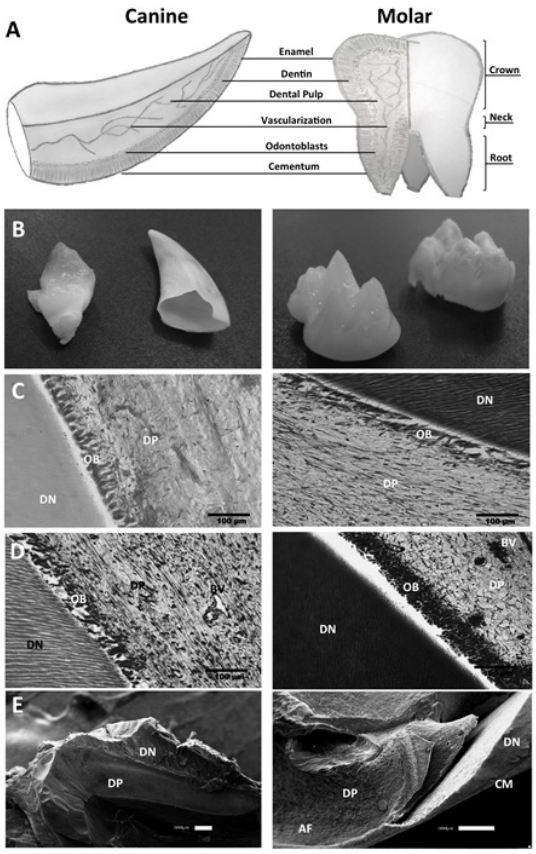

Figure 1: The Teeth

A) Schematic illustration of a swine canine and molar teeth and structures.

B) Photography of canine and molar tooth after separation of the pulp from the crown.

C) Histological analysis of swine canine and molar dental pulp tissue by HE staining. DN (Dentin), DP (Dental Pulp), OB (Odontoblast). Scale bar 100μm.

D) Histological analysis of swine canine and molar dental pulp tissue by Masson's Trichrome staining. DN (Dentin), DP (Dental Pulp), OB (Odontoblast), BV (Blood vessels). Scale bar 100μm.

E) Scanning Electronic Microscopy from canine and molar dental pulp tissue. DN (Dentin), DP (Dental Pulp) CM (Cementum), AF (Apical Foramen). Scale bar 300μm.

HE staining showed that the dental unit is composed of different tissues, such as dentin (DN), dental pulp (DP) and odontoblast (OB) (Figure 1C). Masson trichrome staining showed an

innervated and vascularized (blood vessels - BV) dental pulp layer (DP), as dentin (DN) and odontoblast (OB), observed in HE staining as well. However, nerve extensions are portions of odontoblasts that intertwine through microtubules located in DN. The odontoblast layer was more pronounced on the dental pulp of molar teeth than on canine. (Figure 1D).

Scanning electronic microscopy revealed at the dental base a convergence of the dental pulp (DP) tissue, dentin (DN) and cementum (CM) into the apical foramen (AF). Periodontal ligaments in lower dental lap curvature can be observed, securing the tooth. This curvature is more pronounced in molar teeth and very discrete in canine teeth (Figure 1E).

In order to characterize the dental pulp tissue as a stem cell niche, immunological study was performed using antibodies against mesenchymal and pluripotent markers. CD73 and CD105 were expressed in both, canine and molar, as well as CD105 that showed a perinuclear labeling more evident in samples derived from molar teeth than canine-derived cells. CD34 and CD45 hematopoietic and endothelial markers, respectively, were not expressed. Oct4, Sox2 and Nanog - pluripotency markers showed positive reactions to these antibodies in canine and molar tissues. PCNA3 and vimentin, mitotic and cytoskeleton marker, were expressed in both canine and molar tissues (Figure 2).

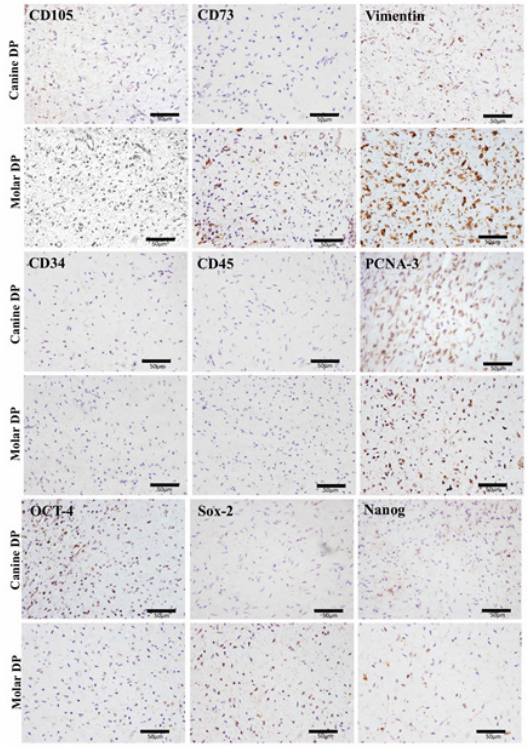

Figure 2: Analysis of canine and molar dental pulp tissue by immunohistochemistry using an antibody panel for mesenchymal stem cells characterization. Scale bar 50µm.

Cultured cells from canine and molar teeth exhibited similar morphology as mesenchymal stem cells, such as plastic adherence capability and fibroblast-like morphology (Figure 3A). sDPSC from canine and molar teeth were viable after 30 days of freezing and apparently kept their ability to proliferate and preserved their cell morphology (Figure 3B).

After 9 days, sDPSC from canine and molar teeth displayed a constant and exponential growth, but without differences between then (Figure 3C). Molecular analysis revealed that both canine and molar derived cells expressed CD105 and failed to express CD34 and CD45 markers (Figure 3D).

Adipogenic differentiation of sDPSC from canine and molar teeth was successful as observed by small lipid deposits in cytoplasm, as well as osteogenic differentiation, observed by irregular arrangements of calcium deposits (Figure 3E), suggesting cell plasticity and MSC profile. sDPSC derived from canine and molar teeth showed no evidence of tumor formation in nude mice (Figure 3F).

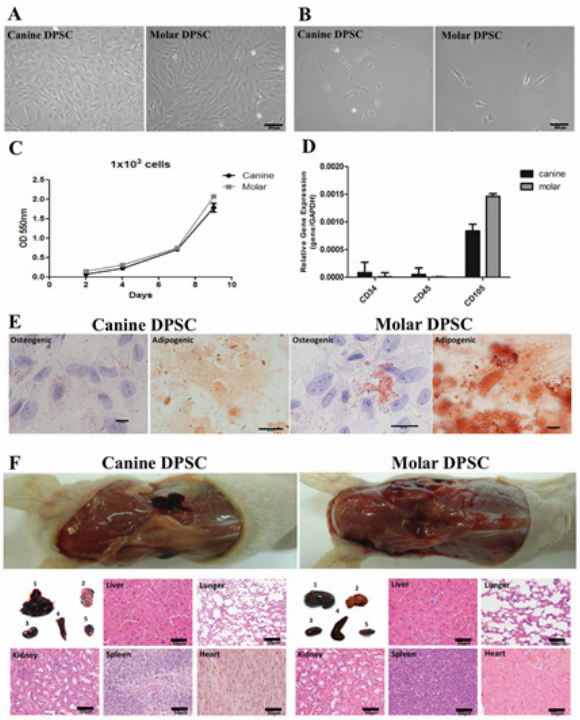

Figure 3: Swine canine and molar dental pulp stem cells characterization.

A) Morphological analysis. Scale bar 100µm.

B) sDPSC from canine and molar teeth showed viability after cryopreservation process. Scale bar 100µm.

C) sDPSC from canine and molar teeth showed a constant and exponential growth by MTT colorimetric assay.

D) Real time PCR using swine specific primers.

E) Adipogenic and osteogenic differentiation ability. Scale bar 100x.

F) Tumor formation assay. sDPSC derived from canine and molar teeth showed no evidence of tumor formation in nude mice. 1-Liver, 2-Lunger, 3-Kidney, 4-Spleen, 5-Heart. Scale bar 50µm.

DISCUSSION

Little information is available on the processes of teeth development in swine [16]. In addition, swine teeth retain MSC in the pulp, even after birth. Interestingly, canine teeth present continuous growth during the whole life of the swine, which

is an interesting subject to investigate in animal kingdom. The main goal of this work was compare the MSC niche found in the pulp of canine and molar teeth from swine, in order to verify if these cells present a different mesenchymal profile.

Studies isolating and characterizing dental pulp stem cells from swine have already been performed, but focusing in self-renewal potential, differentiation into odontoblasts and modulation of T lymphocytes proliferation [9, 12, 13]. Moreover, dental pulp cells from human teeth have demonstrated dentin repair and regeneration potential, highly important for dentistry and endodontic fields, making these cells an attractive source to obtain and study [9, 12, 13]. Regarding dental pulp stem cell in swine, to our knowledge, this work is the first that compares canine and molar teeth from swine.

Cultured dental pulp cells from canine and molar teeth were able to adhere in plastic and were positive for mesenchymal stem cells markers, as well as were able to differentiate into adipocytes and osteocytes. Based on these results, both canine and molar teeth present mesenchymal stem cells in their pulps, as defined before as by the Mesenchymal and Tissue Stem Cell Committee (Dominici et al. 2006 [3].

MTT assay revealed an exponential growth cell profile in both canine and molar teeth, which was surprisingly considering the continuous growth found in canine teeth and which could not be used to explain this characteristic. Moreover, dental tissue from canine and molar teeth exhibited pluripotency markers. However, gene expression of pluripotency cell markers were not found in cultured cells, suggesting that the expression of these pluripotency markers could be significantly decreased during cultivation process, changing cell profile in vitro (data not shown).

sDPSC from canine teeth displayed low expression for CD34 and CD45 markers. Same observation was made to stem cells obtained from adipose tissue. Recent studies suggest that adipose stem cells (ASC) express CD34 because they have a niche in the vasculature of adipose tissue [17-20]. According to this study, a vascularization in the dental pulp tissue could explain the CD34 expression.

Stem cells derived from dental pulp of canine and molar teeth in swine showed no evidence of tumor formation, as characteristic of MSC [3]. In this work we showed that MSC from dental pulp of swine could be used as potential cells for cellular therapy protocols, regarding tissue engineering or as immunosuppressive devices for cell therapy in veterinary medicine [20,21]. However, in this work, based on MSC characterization, we couldn't explain the continuous growth profile of canine teeth.

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