IRANIAN JOURNAL OF VETERINARY SURGERY (IJVS)
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Canine Periodontal Stem Cells: Isolation, Differentiation Potential and Electronic Microscopic Characterization

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Abstract

Objective- Investigating of the isolation, culture, differentiation potential and electronic microscopic characterization of canine periodontal ligament stem cells (PDLSCs).
Design- Experimental in vitro study
Animals- Four intact, male, mongrel dogs, 8-10 months-old were selected to collect PDLSCs from their teeth.
Procedures- The dogs were anesthetized and the first maxillary and mandibular premolars were extracted for the isolation of periodontal ligament cells (PDLCs). Canine PDLCs were plated and culture expanded through 3 successive subcultures. The resultant cells were then plated at differentiating conditions into bone and adipose cell lineages in order to confirm their mesenchymal stem cell character. Furthermore for the first time we

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studied PDLSCs ultrastructural morphology by scanning and transmission electron microscopy (SEM and TEM).
Results- based to our observations, isolated canine PDLSCs exhibited mesenchymal stem cells (MSCs) characteristics such as fibroblastic appearance, clonogenicity, high proliferation and easily differentiation into osteocyte and adipocyte. This study determined morphologically by electron microscopy a secreted extracellular matrix (ECM) around the PDLSCs and also they contained abundant organelles, such as mitochondria, ribosome, rough endoplasm and collagen fiber as assessed by transmission electron microscopy.
Conclusion- Fibroblastic cells from canine PDLCs are capable to be expanded and proliferated in vitro and have differentiation potential into two skeletal lineages; hence they are MSCs that are suitable for cell therapy experimentations.
Key Words- Stem cell, Periodontal ligament, canine.

Introduction

Periodontitis is an inflammatory disease of the periodontium that consist of the supporting structures of the teeth comprising periodontal ligament, cementum, alveolar bone and gingiva[1]. Several diseases are caused the destruction of the connective tissue matrix and cells, the loss of periodontal ligament attachment and resorption of alveolar bone which resulting in tooth loss.[2,3] In recent years, a number of protocols for periodontal treatment including grafting, root surface conditioning, application of various growth factors as well as bone and enamel matrix proteins [4,5] and guided tissue regeneration have been applied. Among these methods, guided tissue regeneration is currently the most popular procedure, however it has some problems consisting variable results, limited clinical improvement, and susceptibility to infection.[6] There is a high demand to achieve more predictable clinical procedures in periodontal regeneration which has long been a challenge. To date, cell-based therapies have progressed rapidly[7]. The defining characteristics of stem cells (SCs) are clonogenic self-renewal cells which have the capability of production and differentiation of mature progeny cells. Embryonic SCs and adult SCs are the two major categories of SCs which have been described.[8]
The periodontal ligament (PDL), dental papilla, dental pulp and follicle in human are identified as easily accessible sources of adult SCs.[9] The PDL is a specialized, vascular, and highly fibrous tissue that connects cementum to inner wall of alveolar bone to maintain tooth in situ. This highly supports tooth function, maintenance of homeostasis and tissue healing in response to any periodontal insults or mechanical trauma.[10,11] This indicates that progenitor cells and possibly stem cells, exist within the PDL cell population.[12] It has been demonstrated that human PDL which contains a heterogeneous cell populations [13], has in vitro capability of differentiation into chondrocytes, osteoblasts and adipocytes.[14-17] In a recent study, human PDL-derived mesenchymal stem cells as periodontal ligament stem cells were initially identified and subsequently PDLSCs were characterized.[18] The objectives of this study were primarily to isolate and characterize for the first time canine PDL-derived mesenchymal stem cells.

Materials and Methods

Animals

Four intact, male, mongrel dogs, 8-10 months-old and weighting 18 to 24 Kg were selected for this study. The animals had intact dentition with apparently healthy periodontium in clinical exam. Dogs were adapted to environment 2 weeks prior to the study. In this period

vaccination and deworming were carried out. The animal research protocol was approved by the institutional Animal Care and use committee of Tehran, University of Medical Sciences, and it was according to the standards of Association for Assessment and Accreditation of Laboratory Animal Care. Throughout the experiments, the animals were kept individually in the cages, fed with Nutripet (Behintash Company, Karaj, Iran) and monitored for general appearance, activity, exertion and weight.

Isolation and culture of periodontal stem cells

Under general anesthesia using acepromazine (0.1 mg/kg, IM) and ketamine-diazepam (10 mg/kg, IV), the teeth were wiped with gauze soaked in chlorhexidine.[19] The PDL cells were gently separated from the root surface of the middle third of the extracted maxillary premolars in each dog. After separation, PDLCs culture was performed in accordance with the technique described by Seo et al..[18] The obtained PDL cells was enzymetically digested for 1 hour at 37°C in a solution of 3mg/ml Collagenase type I (Gibco, UK) and 4 mg/ml Dispase (Gibco, Uk). Single-cell suspensions were obtained by filtering it through a 70 µm cell strainer[1]. Single-cell suspension (1×10^5 cells) were seeded into 25-cm^2 culture flasks in Dulbecco Modified Eagle Medium (DMEM) (Gibco, Uk) supplemented with 15% fetal bovine serum (FBS) (Gibco, Uk) and 100 IU/ml penicillin G and 100 µg/ml streptomycin solution (Gibco, Uk) and incubated in a humidified atmosphere (37°C, 5% Co2)[19,21]. Canine PDLSCs were harvested with 0.25% trypsin/EDTA (Gibco, Uk) at 37°C for 3 minutes.[20]

Differentiation

Osteogenic induction

To investigate the potential of PDLSCs to differentiate into osteoblasts, confluent passage-3 cells were provided with DMEM supplemented with 50µg/ml ascorbic2-phosphate (Gibco, Uk), 10 nM dexamethasone (Sigma, USA) and 10 mM ß-glycerol phosphate (Sigma) for 3 weeks. At the end of this period, alizarin red staining was used to show the deposition of mineralization matrix. For staining, the cultures were first fixed by methanol for 10 minutes and then subjected to alizarin red solution 2 g in 100 ml water for 2 minutes.

Adipogenic induction

To induce adipose differentiation, passage-3 confluent culture was treated with the medium containing 100 nM dexamethasone (Sigma) and 50 mg/ml indomethasine (Sigma, USA). After 3 weeks, the cells were fixed with 4% paraformaldehyde at room temperature for 30 min, washed with 70% ethanol and stained by oil red solution (0.5 g in in 99% isopropanol) for 15 minutes.

Scanning Electron Microscopy

Ex vivo-expanded PDLSCs grown for 7 days were fixed using 2.5% glutarldehyde in 0.1 mol/l sodium cacodylate buffer (PH=7.2) for 2 hours at 4°C. After being washed with sodium dimethyl Arsenate buffer, the cells were post-fixed in 1% Osmium tetroxide in distilled water at room temperature for 20 minutes, dehydrated with gradient alcohol, and then incubated

with isoamyl acetate. After gold coating, the samples were examined using a TESCAN VEGA scanning electron microscopy (TESCAN VEGA, USA, www.Tescan-usa.com).

Transmission Electron Microscopy

After washing with phosphate-buffered saline (PBS), the cells were fixed in 3% glutaraldehyde in 0.1 mol/L PBS at 4 °C for 1 h and post-fixed in 1% osmium tetroxide for 1 h. The samples were then dehydrated through a graded ethanol series and embedded in epoxy resin. The thin sections were mounted on copper grids and double-stained with uranyl acetate for 10 min and lead citrate for 4 min as described previously[22], then ultrastructurally scoped using an electron microscopy (Zeiss, Germany).

Results

Cell culture

To identify the capacity to generate clonogenic clusters of adherent fibroblastic-like cells, single cell suspension of canine PDL cells was plated in 25-cm^2 culture flasks under specific culture conditions. After 7 days numerous colonies were formed, in fact the ability of canine PDL-derived cells to form adherent clonogenic cell clusters of fibroblast-like cells, similar to those recorded for human PDLSCs, was shown by the formation of approximately 21 single colonies generated per 10^5 single cells cultured at low density and the cells were spindle-shaped (Fig. 1), morphological characteristic of the plastic-adherent colony-forming canine PDLSCs were similar to other MSCs [23] including large flattened cells (Fig. 1A), long spindle-shaped cells (Fig. 1B), and short-shaped cells (Fig. 1C) and colonies had a high proliferation rate and a more finite lifespan (data not show).

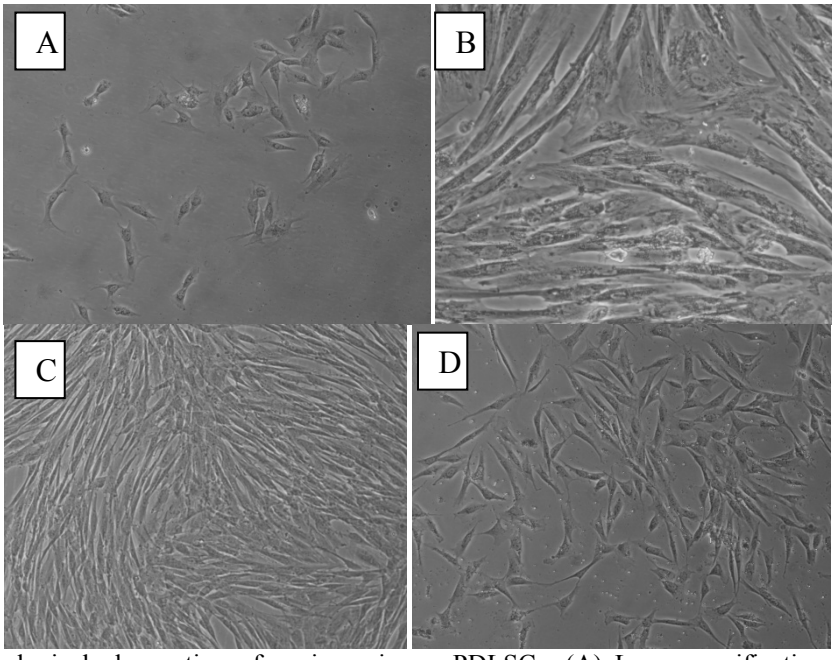

Figure 1. Morphological observation of canine primary PDLSCs. (A) Low magnification view of primary culture on day 7. Primary PDLSCs grew in colonies with various cell morphologies comprising each colony. Small rounded cells were occasionally observed on top of adherent PDLSCs (arrow). There were at least three major types of adherent cells as observed under higher magnification views on day 14; (B) large flattened cells, (C) long spindle-shaped cells, and (D) short spindle-shaped cells. Scale bar=100 μM.

Differentiation assays

We assessed in vitro whether PDL cells, like the other mesenchymal stem cells, had similar capacity to differentiate into other cell lineages such as osteocytes and adipocytes. Mineral deposition: Alizan red staining — After 17 days of induction in mineralization medium, alizarin red staining of the PDL stem cell cultures (Fig. 2A,B) was associated with distribution of mineralized deposits throughout the tissue culture wells as well as cell clusters in both of them. Adipogenic differentiation: Oil red O staining — After 14 days of induction in adipogenic media, lipid droplets were detected in the cytoplasm of differentiated PDLSCs (Fig. 2C).

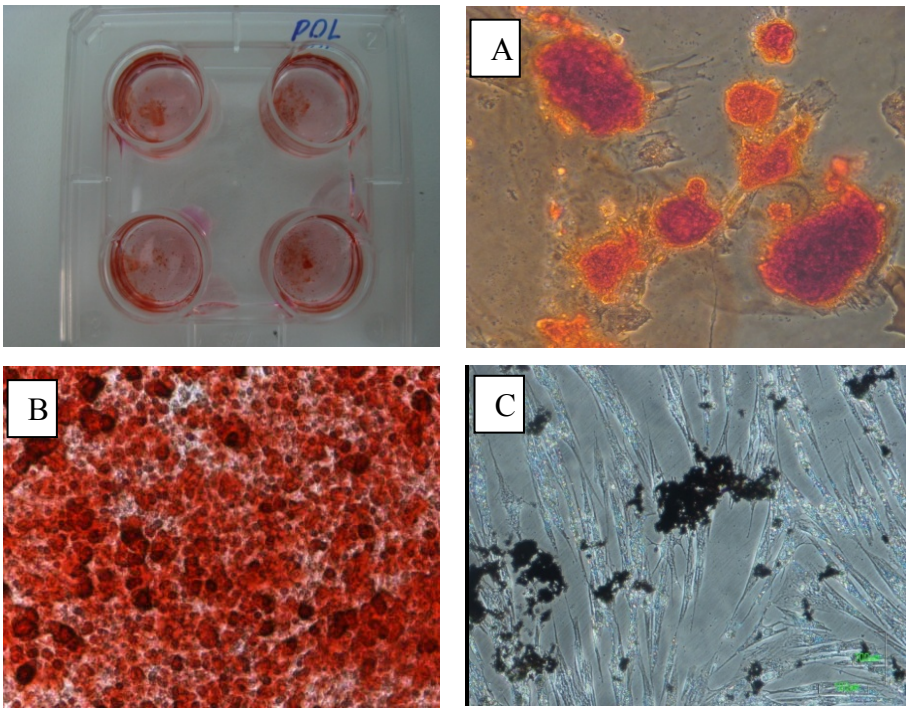

Figure 2. Canine differentiation potential: The cells were able to differentiate into osteblasts and adipocytes. A, B- Differentiation into osteocytic, these were evident in alizarin red staining. C- Differentiation into adipocytes, this was evident in oil red staining.

SEM

Secreted ECM, which has a critical role in regeneration of damaged tissues, was discernible by scanning electron microscopy. It seems the ECM produced by PDLSCs is comparable in thickness and prominence for the same mesenchymal stem cells from bone marrow source (Fig. 3a).

TEM

PDLSCs were readily distinguishable by electron microscopy. PDLSCs contained numerous secretary granules and relatively abundant mitochondria within the cytoplasm (Fig. 3b-f). PDLSCs were characterized by developed rough endoplasmic reticulum (RER) and free ribosomes. Granule exocytosis was also seen in some PDLSCs. Moreover, extracellular

collagen fibers were discernible by close ultrastructural scrutiny of PDLSCs and had round nuclei with regular outlines, a single, large nucleolus was present in the nuclei of these types cells.

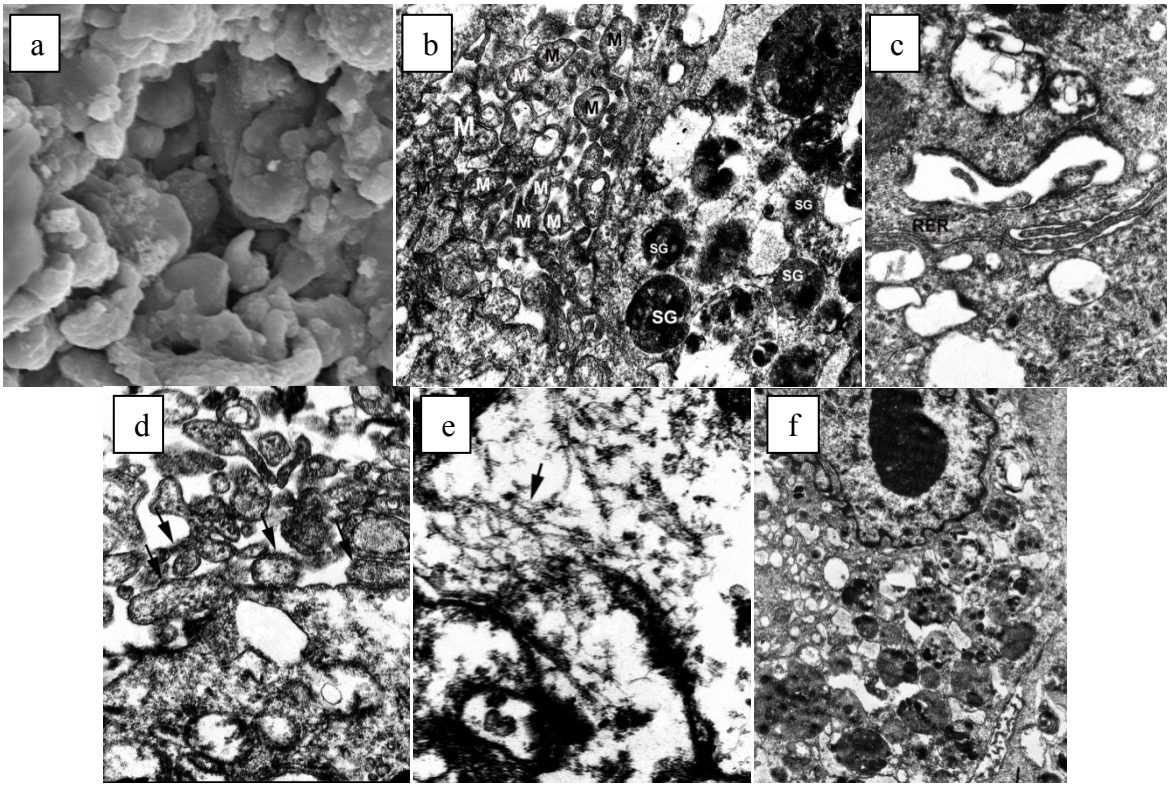

Figure 3. Scanning and Transmission electron microscopy analyses of PDLSCs. (a): Scanning electron microscopy, clusters of PDL cells, with secreted ECM. (b-f): Transmission electron microscopy; (b) A PDL cell with numerous intracytoplasmic secretory granules (SG) and many mitochondria (M). (c) Rough endoplasmic reticulum (RER), polyribosomes (PR), along with numerous free ribosomes is seen in the cytoplasm of PDL cells. (d) Granule exocytosis in PDL cells. (e) Synthesized collagen fibers organizing in the extracellular space around PDL cells. (f) PDLSCs had round nuclei with regular outlines; a single, large nucleolus was present in the nuclei of these types of cells.

Discussion

In the present study, we successfully isolated and studied morphology, proliferation capacity, differentiation ability, ultrastructural characteristics multipotent mesenchymal stem cells from canine PDL, which can serve as a unique reservoir of stem cells to apply to regenerative procedures without the necessity of more invasive procedures. PDLSCs in this report showed many characteristics similar to those already reported for human[24], ovine [25], equine [26], porcine PDLSCs [27] and canine [28] and other mesenchymal stem cells [29-31] and dental pulp derived stem cells.[32-35] Our findings also clearly demonstrated that canine PDL includes a population of multipotent postnatal stem cells at different stages of differentiation and lineage commitment, Nagatomo and et al have founded the similar results for human PDL stem cells.[36] As researches have earlier demonstrated, PDL cells can be cultured at low densities with duplicitous potential indicating the capacity of PDL cells to proliferate and produce colonies from a single cell.[36] We observed heterogenicity in cell morphology for colony-forming unit-fibroblastic (CFU-F) derived from PDL in primary passages. Two distinct morphologies were

detected to grow concomitantly: solitary canine periodontal fibroblast (CPF) was elongated cells with oval-shaped nuclei and an extended cytoplasm that grew long cell processes; canine dental cementoblast (CDC) is round cells with a small cytoplasm and intensive nuclei. CPF in the vicinity of the CDF, became bipolar and spindle-shape.[26] In addition, we induced cell differentiation along the two classical mesenchymal pathways, osteocytic and adipocytic lineages, and we confirmed differentiation PDLSCs capability for the production of mineralized deposits and lipid droplets as previously has been shown with other mesenchymal cell populations. In this study, we attempted to isolate PDLSCs purely and then maintained high purity in the expansion of them in third passage, thus we did not interfere of differentiated cell types such as fibroblasts with mineralization process.[37] Morphologically, PDLSCs displayed many short and long branching cytoplasmic processes under a scanning electron microscopy, surrounded by secreted extracellular matrix the cells contained abundant organelles, such as mitochondria, ribosome, and rough endoplasm, and secreted extracellular matrix, as assessed by transmission electron microscopy. The collagen fibrils produced by PDLSCs were large and thick.

Consequently, canine model in stem cell investigations are a great importance since they are genetically more close to humans in contrast to rodents. Hence preclinical therapeutic procedures such as cell and gene therapies require an intermediate step between rodent or in vitro study and clinical trials due to safety and investigation of efficacy. For this reason, results from canine models have been much more predictive with regard to human clinical trials.

Conclusion

Recent finding reveal that periodontal ligament contains a population of multipotent postnatal stem cells that can be isolated and expanded in vitro, providing a unique source of stem cells. We hope this present study preliminary demonstrates that canine PDLSC may hold promise as the utility of using an autologous PDLSCs therapeutic approach to treat periodontitis in dogs as preclinical model.

Acknowledgment

The authors gratefully acknowledge the tremendous contribution and financially supported by Research Centre of Dentistry Faculty, Hamadan University of Medical Sciences, and we would like to thank Dr. Kajbafzadeh, Dr. Mohseni and Dr. Rouhi from Department of Urology, Children's Hospital Medical Center, Tehran University of Medical Sciences for supporting the study.

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چکیده

سلولهای بنیادی مزانشیمی لیگامان پریودنتال سگی: جداسازی، کشت، پتانسیل تمایز و مشخصات میکروسکوپ الکترونی

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هدف- جداسازی، کشت، پتانسیل تمایز و خصوصیات سلولهای بنیادی مزانشیمی لیگامان پریودنتال

طرح مطالعه- مطالعه تجربی- آزمایشگاهی

حیوانات- ۴ قلاده سگ نژاد ایرانی، نر، اخته نشده ۱۰-۸ ماهه

روش کار- در این مطالعه ابتدا تحت بیهوشی کامل دندان پرمولار فک بالا و پایین سگها برای جداسازی سلولهای پریودنتال لیگامنت کشیده شدند. سلولهای لیگامان پریودنتال سگی کشت و طی سه پاساژ متوالی تکثیر گردیدند. سلولهای حاصل در شرایط تمایز به استخوان و چربی قرار گرفتند تا ماهیت بنیادی مزانشیمی آنها تأیید شود. علاوه براین برای اولین بار از نظر مورفولوژیکی سلولهای بنیادی مزانشیمی لیگامان پریودنتال سگی توسط میکروسکوپ الکترونی مورد بررسی قرار گرفتند.

نتایج- بر اساس مشاهدات ما، سلولهای بنیادی مزانشیمی لیگامان پریودنتال سگی مشخصات مخصوص سلولهای بنیادی مزانشیمی مانند: شکل فیبروبلاستی، میل به کلونیزه شدن، تکثیر سریع و تمایز راحت به سلولهای استئوسیت و آدیپوسیت را نشان دادند. در بررسی مورفولوژیکی توسط میکروسکوپ الکترونی یک ماتریکس خارج سلولی در اطراف سلولهای بنیادی مزانشیمی لیگامان پریودنتال سگی مشخص گردید و همچنین در بررسی با میکروسکوپ الکترونی ترانسمیشن این سلولها حاوی ارگانل‌های فراوانی از جمله: میتوکندری، ریبوزوم، اندوپلاسم خشن و فیبرهای کلاژنی بودند.

نتیجه‌گیری و کاربرد بالینی- سلولهای فیبروبلاستی جدا شده از سلولهای لیگامان پریودنتال قدرت کشت و تکثیر در محیط کشت و پتانسیل تمایز به دو رده اسکلتی را دارا هستند. از این رو آنها به عنوان سلولهای بنیادی مزانشیمی برای سلول درمانی در کارهای تجربی مناسب می‌باشند.

کلید واژگان- سلول بنیادی، لیگامان پریودنتال، سگ.