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In vitro investigation of canine periodontal ligament-derived mesenchymal stem cells: A possibility of promising tool for periodontal regeneration

Hamideh Salari Sedigh [a,1], Anna Saffarpour [c,1], Shahram Jamshidi [b,**], Mahdi Ashouri [d], Seyed Mahdi Nassiri [e], Mohammad Mehdi Dehghan [f], Esmail Ranjbar [g], Reyhaneh Shafieian [g,h,*]

[a] Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
[b] Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
[c] Department of Periodontology, Tehran University of Medical Sciences, International Campus, Tehran, Iran
[d] Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, Shahed University of Medical Sciences, Tehran, Iran
[e] Department of Clinical Pathology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
[f] Department of Surgery & Radiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
[g] Department of Anatomy and Cell Biology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[h] Stem Cell and Regenerative Medicine Center, Mashhad University of Medical Sciences, Mashhad, Iran

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ABSTRACT

Objectives: Recent investigations indicate that canine periodontal ligament-derived stem cells (cPDLSCs) may reveal a reliable strategy for repair of periodontal tissues via cell-based tissue engineering approaches. Due to limited research, this study aimed to demonstrate the phenotypic characterization of cPDLSc in comparison with canine bone marrow-derived mesenchymal stem cells (cBMSCs) *in vitro*.

Methods: Mesenchymal stem cells (MSCs) were obtained from PDL and BM of five male adult Mongrel dogs. *In vitro* isolation and expansion as well as biologic characterization including colony unit formation (CFU), osteogenic and adipogenic differentiation, flow cytometric analysis of CD34 and CD44, and RT-PCR of alkaline phosphatase (ALP), osteocalcin (OCN), periostin (POSTN) and S100A4 were performed. Furthermore, electron microscopy analysis was done to complement the comparative research.

Results: CFU assay revealed that colonies of cPDLSCs presented 70% confluency with a more finite lifespan than BM-MSCs, showing a significant increase in cPDLSCs. Both types of MSCs showed osteogenic and adipogenic phenotypic characterized with clusters of mineralized depositions and lipid vacuoles, respectively. Both types of MSCs expressed CD44 with limited expression of CD34. RT-PCR of cPDLSCs revealed that expression of ALP, POSTN, OCN and S100A4 genes were significantly higher than those of BMSCs. In addition, comparison of SEM and revealed that cPDLSCs expressed more extracellular collagen fibers.

Conclusions: The current study indicated that cPDLSCs show potency as a novel cellular therapy for periodontal regeneration a large animal model.

1. Introduction

In recent years, both in human and veterinary medicine, plenteous interest in comparative characterization and potential therapeutic application of mesenchymal stem cells (MSCs) has risen making great accomplishments in the emerging field of stem cell research.[1] Indeed, veterinary medicine seems as an un-separable piece of regenerative therapies which makes scientists practical promises in stem cell application for regenerative medicine and tissue engineering purposes in human medicine.[2] Thus, extensive studies by clinical and veterinary scientists are going on examining characteristics and potential therapeutic application of various stem cell sources both *in vitro* and *in vivo*.[3]

* Corresponding author. School of Medicine, Department of Anatomy and Cell Biology, VakilAbad Blv, Azadi Sq., Mashhad University of Medical Sciences, PO 91779-48564, Mashhad, Iran.
** Corresponding author. Faculty of Veterinary Medicine, Department of Clinical Sciences, 16th Azar St., Enghelab Sq., University of Tehran, PO 1417466191, Tehran, Iran.
E-mail addresses: shjamshidi@ut.ac.ir (S. Jamshidi), shafieianr@mums.ac.ir (R. Shafieian).
[1] These authors contributed eqully in this research paper.

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Dogs have been considered an interesting animal model for establishment of various regenerative treatment protocols due to their translational potential to human disorders besides the conventionally profitable share of affected canines.[4] Although a great fraction of MSC studies have aimed at the investigation of MSCs efficacy in rehabilitation of different diseases in canine animal models,[5–7] a few studies examined the comparative characteristics of canine MSCs.[8]

For many years of stem cell research and study, bone marrow-derived MSCs (BMSCs) were considered as the gold standard in stem cell-based regenerative therapy and the superlative choice of therapeutic explorations; however, in current days, the pioneering place of BMSCs is receiving challenging drawbacks including donor site morbidity, aggressive harvesting procedure and low cell yield rate.[9] The resulting scarcity of BMSCs puts off some aspects of basic and clinical tissue regenerative investigations. Consequently, other MSC sources has received plentiful attention for their alternative potency. The periodontal ligament (PDL) is a fibrous and highly vascularized tissue, responsible for connecting the alveolar bone to the adjacent teeth. It has been widely demonstrated that PDL tissue, derived from human,[10] rat[11] and dog,[12] contains a population of heterogeneous progenitor cells, called periodontal ligament stem cells (PDLSCs), with the differentiation ability into chondrocytes, osteoblasts and adipocytes *in vitro*.[13] PDLSCs are considered as one of those easy-to-obtain substitutes that along with the property that can be derived with minimally aggressive cell isolation processes, show noticeable capacity in regeneration of various damaged tissues including periodontium[14,15] and bone tissue.[16,17]

Canine PDLSCs (cPDLSCs) have been characterized as colonogenic MSCs with multi-lineage differentiation capability under specific conditions.[12] Yet, *in vitro* comparative investigation of phenotypic characteristics of cPDLSCs with BMSCs, considered as the gold standard of stem cell sources for regeneration medicine, is lacking in the literature and need to more explorations still persist in canine medicine. One research, performed by Tsumanuma et al., has compared cPDLSCs with cBMSCs and canine alveolar periosteal cells (cAPCs) *in vivo*, proposing a potential capacity of cPDLSCs in combination with TCP/collagen scaffold for periodontal regeneration in one-wall intrabony defects.[18] Therefore, a potential capacity for periodontal disease regeneration in clinical treatment is assumed for PDLSCs. However, the hypothetical efficacy should be evaluated regarding gold standard source of BMSCs *in vitro* for designing coming steps of *in vivo* experimental procedures. Hence, we aimed to conduct an *in vitro* investigation comparing phenotypic characterizations of cPDLSCs and cBMSCs. Based on our literature search, this issue has not been fully reported yet and thus, this is the first study in this field.

2. Materials and methods

2.1. Animal selection

All the animal experiments performed in this study were in accordance with the National Institutes of Health (NIH) animal care guidelines for the care and use of laboratory animals and approved by the Animal Care Committee of Veterinary College, University of Tehran.

Five young (8–10 months old), male mongrel dogs, with intact dentition and healthy periodontium, weighting around 20 ± 5 Kg, were selected for this study. The animals had the opportunity for adaptation to the housing and diet for 2 weeks prior to the operation. In this period, vaccination and deworming treatments were carried out. All the dogs received polyvalent and rabies vaccines (Canvac, Canada) as well as antiparasitic treatment (Ivermectin SC, Alfasan and Caniverm tablets, Bioveta). During the whole experiment, all animals were kept individually in cages, fed once a day with nutripet dry food for dogs (Behintash Company, Karaj, Iran) and monitored for general appearance, activity, exertion and feeding.

All the animal experiments were carried out under general anesthesia using acepromazine maleate (Alfasan; 0.1 mg/kg; IM) and ketamine-diazepam (Alfasan, 10 mg/kg and 0.2 ml/kg; IV). After induction, anesthesia was provided with isoflurane (1.5–2.0%) in oxygen. Pre- and post-operative analgesic maintenance was induced via administration of meloxicam (0.2 mg/kg, SC), which endured twice daily for 3 consecutive days. In addition, ampicillin (25 mg/kg) was administered orally twice daily during the next 7 days after surgery to limit possible infection.

2.2. Isolation and culture of stem cells

2.2.1. Isolation and culture of cBMSCs

Briefly, iliac crests of 3 animals were drawn for bone marrow aspirates (5 ml each) into using a sterilized 13-gauge Jamshidi needle (Cardinal Health). The pool of aspirates was collectively transferred to a 15 ml syringe containing heparinized (3250 units/ml) saline solution and underwent dissociation and resuspension with a pipette. After a round of centrifuge at 300×*g* for 5 min, the acquired pellet containing marrow cells was collected and then, resuspended in 10 ml PBS (Phosphate Buffered Saline 10; Gibco) supplemented with 10% FBS (Fetal bovine Serum, Gibco). Next, the suspension was carefully layered onto 5 ml of Lymphoprep. (Axis-Shield) and underwent centrifugation at 800×*g* for 30 min. Then, the MSC-enriched nucleated cells were collected from the interface, washed in PBS and seeded in T-75 cell culture flasks containing 15 ml of growth medium, that is DMEM (Dulbecco Modified Eagle Medium, Gibco) supplemented with 10% FBS, 100 μg/ml penicillin-streptomycin (Gibco) and 0.3 μg/ml fungizone (Gibco). Cells, seeded at a density of 5×10^4 cells/plate, were incubated in a humidified 5% CO2 incubator atmosphere at 37 °C. After 48–72 h, non-adherent round cells, presumably mature leukocytes and hematopoietic stem/progenitor cells, were removed by a series of irrigation in PBS and subsequent medium replacement. Cultures were exchanged every 3 days until confluency of 70–80%. Dissociation of attached cells was carried out by exposure to 0.05% trypsin/EDTA (Gibco) for 3 min, following with replating in 1:2 ratios, considered as passage 1 (P1). A sufficient number of cells was acquired upon P3.

2.2.2. Isolation and culture of cPDLSCs

Maxillary premolar teeth of the two remaining animals were extracted for PDLSCs isolation. After a gentle wipe with sterile gauze soaked in chlorhexidine, PDL tissues were mildly separated from the middle third of the root surface using forceps and then, incubated with a solution of collagenase type I (3 mg/ml; Sigma) plus dispase (4 mg/ml; Sigma) for 1 h at 37 °C for enzymatic digestion. A single-cell suspension of PDL pool was obtained via using a 70-μm cell strainer (Falcon, BD Labware) and then, seeded in T-25 culture flasks containing DMEM supplemented with 15% FBS and 2 mM glutamine plus a solution of 100 IU/ml penicillin and 100 μg/ml streptomycin (all from Sigma). Seeding was performed at a density of 1×10^4 cells/plate, incubated at 37 °C in a humidified atmosphere of 5% Co_2).[19,20] At 70–80% confluency, trypsinization was caried out with 0.05% trypsin/EDTA (Gibco) for 3 min and then, additional passages were performed, as described earlier.

2.3. Colony-forming unit assay

To identify the capacity of cells isolated from pdl and BM to generate colonogenic clusters of adherent fibroblastic-like cells, single-cell suspensions of were seeded in 25 cm^2 culture flasks under specific conditions and incubated therein for 7 days. There-after, appeared cultures were fixed with 10% buffered formalin (Sigma) for 1 h and stained with 0.3% crystal violet (BD Biosciences) for 5 min. Collections of 50 or more cells were considered as colonies (P1).[12]

2.4. In vitro differentiation assay

The multilineage differentiation capacity of cBMSCs and cPDLSCs, including osteogenic and adipogenic differentiations, was assessed on

cell cultures at P3, as described elsewhere.[21]

2.4.1. Osteogenic induction

Osteogenic induction was accessed via plating P3 cultured cells on 6-well plates at a density of 5×10^3 cells/cm^2, provided with osteogenic medium consisting of growth medium supplemented with ascorbic 2-phosphate (50 μg/ml, Sigma), dexamethasone (10 nM, Sigma) and ß-glycerol phosphate (10 mM, Sigma) for a period of 21 days. The medium was changed twice a week. Alizarin red S (AZR S; Sigma) staining was used to observe the mineralized deposits under an inverted microscope (Olympus).

2.4.2. Adipogenic induction

To induce adipogenic differentiation, another 6-well plate was used for sub-culture of P3 cells at a density of 8×10^3 cells/cm^2, treated with an adipo-inductive medium containing growth medium plus 250 nM dexamethasone, 66 nM insulin, and 0.2 mM indomethacin (Sigma) for the next 14 days. The medium was changed every 3 days. Oil Red O (Sigma) staining was done to analyse adipogenic differentiation under an inverted microscope (Olympus).

2.5. Flow cytometric analysis of cell surface markers

Flowcytometry assay was employed to evaluate the expression of specific cell-surface markers including CD34 and CD44, as described anywhere.[21] Briefly, expanded cBMSCs and PDLSCs at P3, at a density of 1×10^6 cells/tube, were washed with special FACS buffer and exposed to two monoclonal FITC antibodies including mouse anti-dog CD_{34} (Cat No. MCA2411F, Serotec) and mouse anti-dog CD_{44} (Cat No. ab95138, Abcam). After 1 h incubation with specific or isotype control antibodies in 100 ml of 3% BSA (bovine serum albumin; Sigma), cells underwent thorough irrigation with FACS buffer and then, incubated with anti-mouse IgG secondary antibody labeled with FITC for another 1 h. After fixation with 1% PFA (paraformaldehyde; Sigma), Cell fluorescence was assessed by flow cytometry in a FACSCalibur instrument (BD Biosciences) and data was analyzed with Flomax software (Partec).

2.6. mRNA expression analysis using quantitative real-time PCR (qRT-PCR)

RT-PCR analysis was employed in this study to evaluate the mRNA expression levels of alkaline phosphatase (ALP), osteocalcin (OCN), periostin (POSTN) and S100A4. Total RNA was isolated from BMSCs and PDLSCs at P2 via Qiazol RNA Extraction Kit (Qiagen) according to the manufacturer's protocol and then, measured by spectrophotometry. There-after, total RNA (1 μg) was reverse-transcribed into cDNA with QuantiTect Reverse Transcription Kit (Qiagen), following instructions provided by the constructor. Real-time PCR analysis was carried out by means of gene-specific primers, designed based on canine mRNA sequence in the GeneBank database (Table 1). All experiments, performed in triplicate to validate acquired data, were caried out on the Corbett Life Science (QIAGEN) using SYBR Premix EX Tag (TAKARA), based on the instructions recommended by the manufacturer. Relative expression of the target genes was determined via the delta delta cycle threshold (2-ΔΔCt) approach, as described elsewhere.[22] GAPDH mRNA, used as internal reference, was amplified as a housekeeping gene to normalize data.

Table 1
Sequence of primer pairs.

Gene	Sequence	NCBI Reference
GAPDH	F- 5′ TTCCACGGCACAGTCAAG 3′ R-5′ CTCAGCACCAGCATCACC 3′	NM_001003142.1
Osteocalcin	F- 5′ TGCTCACAGACCCAGACAG 3′ R- 5′ ACCTTTGCGGGATTCAG 3′	XM_547536.2
Periostin	F- 5′ TTACTAACATTCTGAAGACCACTC 3′ R- 5′ AGGTGTGTCTGCTGGGTA 3′	XM_534490.2
ALP	F- 5′ TGGACTACGCTCACAACAAC 3′ R- 5′ GCCGCATAAGCCATCAC 3′	NM_001197137.1
S100A4	F- 5′ TCCTTTTCCCCAAGAAGC 3′ R- 5′ CTCTGGATGTGATGGTGTCTAC 3′	NM_001003161.1

2.7. Scanning electron microscopy (SEM) assessment

Ex vivo-expanded cPDLSCs and cBMSCs were qualitatively assessed through SEM observation. Firstly, cells were fixed with 3% glutarldehyde in sodium cacodylate buffer (0.1 mol/L; pH 7.2) for 2 h at 4 °C. Following a thorough wash with sodium dimethyl arsenate (SDS) buffer, the cells were then post-fixed in osmium tetraoxide, dehydrated with ascending concentrations of ethanol, and incubated with isoamyl acetate. After being totally dried, the specimens were sputter-coated with gold to examine under SEM (TESCAN VEGA). Untreated cells served as controls.

2.8. Transmission electron microscopy (TEM)

Sub-cultured cPDLSCs or cBMSCs in 6-well plates were fixed in 3% glutaraldehyde at 4 °C for 1 h, washed in PBS, and post-fixed in 1% osmium tetroxide. The samples were then dehydrated through a graded ethanol series and embedded in epoxy resin. Thin sections (70 nm), mounted on copper grids, were double-stained with uranyl-acetate (10 min) and lead citrate (4 min) to be imaged ultrastructurally under TEM (Zeiss). Untreated cells served as controls.

3. Results

3.1. Colonogenic capability of cBMSCs and cPDLSCs

Within 3–5 days, adherent cells from plated cells isolated from both BM and PDL became visible on the plastic surface of culture flasks. After 7 days of initial plating, numerous colonies containing fibroblast-like cells with short-shaped (Fig. 1(A)), large flattened (Fig. 1(B)), and long spindle-shaped (Fig. 1(C)) appearances were observed in those flasks sub-cultured from BM. After 10 days of initial plating, plastic-adherent cells with morphological characteristics similar to those isolated from BM were also described in those flasks sub-cultured from PDL. After another 7 days, colonies presented 70% confluency with a more finite lifespan than BM-MSCs (Fig. 1(D)–(F)). Exhibiting colony-forming capability, both cell types formed colony-forming unit-fibroblasts (CFU-Fs) (Fig. 1(G) and (H)). The number of CFU-Fs per 500 cells of cBMSCs and cPDLSCs was (30.3 ± 2.2) and (52.4 ± 7.6), respectively; showing a significant increase in cPDLSCs.

3.2. In vitro differentiation assays

In this study, osteogenic and adipogenic differentiation were verified histochemically via AZR S and Oil Red O staining assays, respectively (Fig. 2(A) and (B)).

After incubation in osteogenic induction medium for 3 weeks, both MSCs isolated from canine BM and PDL showed osteogenic phenotype characterized with clusters of mineralized depositions (Fig. 2(C) and (D)). Alongside, lipid vacuoles appeared on day 14 of culture in adipogenic induction culture (Fig. 2(E), (F)).

3.3. Flow cytometric analysis of cell surface markers

Isolated cells from canine PDL and BM were examined for expression of MSC-related surface antigen phenotyping via flow cytometry, which revealed that BMSCs and PDLSCs presented a similar expression pattern of cell surface markers including CD44 (leukocyte marker) and CD34 (hematopoietic progenitor cells/endothelium marker). Both types of cells expressed receptor molecule protein CD44 (BMSCs, 98.41 ±

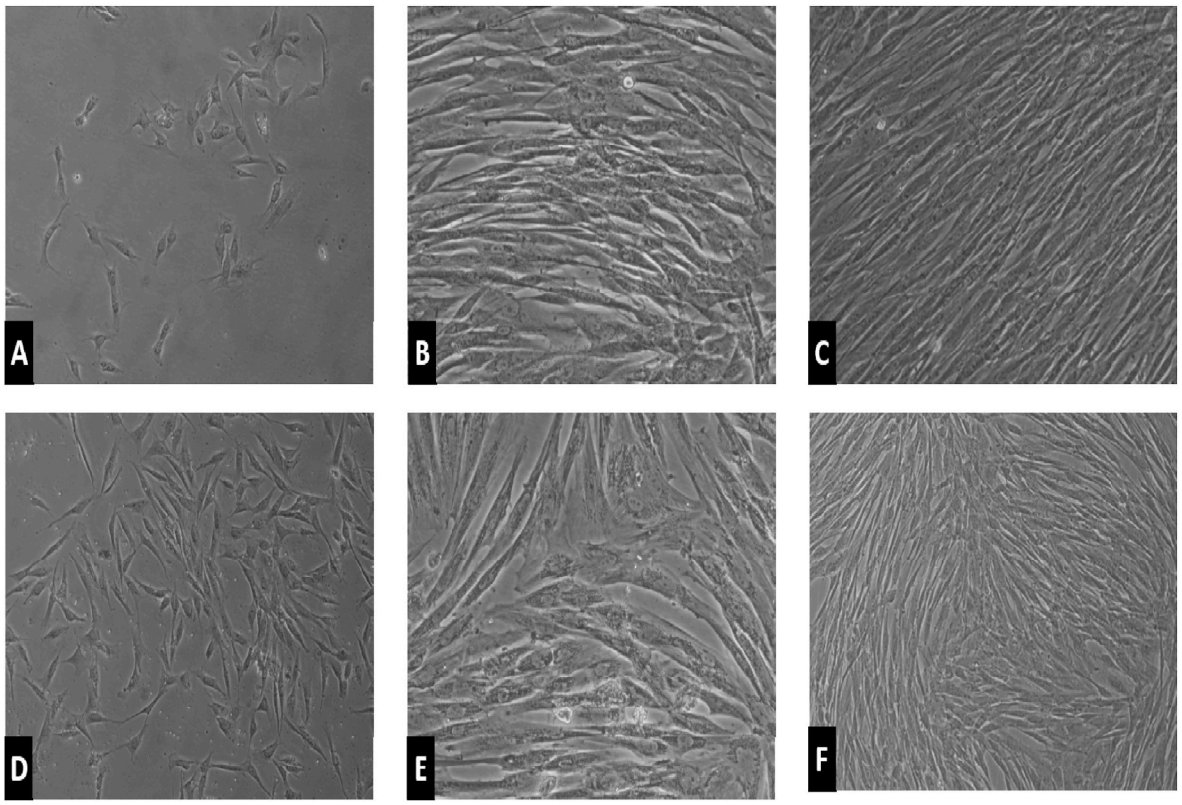

Fig. 1. Canine Bone marrow and Periodontal cell culture. Morphological features of *in vitro* expanded adherent of cBM-MSCs and cPDL-MSCs on the first passage by Phase-contrast microscope. cPDL-MSCs morphology: A- Short-spindle shaped cells, B- Large flattened cells, C- Long-spindle shaped cells. cBM-MSCs stem cells Morphology: D- Short-spindle shaped cells, E- Large flattened cells, F- Long-spindle shaped cells. Original magnification, × 10.

0.53%; %; PDLSCs, 97.85 ± 0.94%) (Fig. 3(A) and (B)), with limited expression of surface antigen CD34 (BMSCs, 0.0081 ± 0.0081%; PDLSCs, 0.038 ± 0.038%) (Fig. 3(C) and (D)).

3.4. mRNA expression analysis using quantitative real-time PCR (qRT-PCR)

RT-PCR revealed that these PDL and BM-derived cells expressed PDL and BM-specific markers of alkaline phosphatase, osteocalcin, periostin and S100A4 (Figs. 4 and 5). Quantitative real-time analysis showed that in undifferentiated cells, expression of alkaline phosphatase and Periostin in cPDLSCs is lower than those of cBM-MScs, however, expression of S100A4 is higher than those of cBM-MSCs and expression of Osteocalcin in both undifferentiated cells is the same. A noticeable increased expression of all genes in PDL relative to BM is seen in differentiated cells (Fig. 6).

3.5. SEM

Secreted extracellular matrix, which has a critical role in regeneration of damaged tissues, was discernible by scanning electron microscopy. It seems that the extracellular matrix (ECM) produced by cBM-MSCs is a little thicker and more prominent (Fig. 7(A and B)).

3.6. TEM

cPDLSCs and cBM-MSCs were readily distinguishable by electron microscopy. cPDLSCs contained numerous secretary granules and relatively abundant mitochondria within the cytoplasm (Fig. 7(C)), whereas the cytoplasm of cBM-MSCs was full of vacuoles of variable size, some of which were very large (Fig. 7(D)). cPDLSCs were characterized by developed rough endoplasmic reticulum (RER) and free ribosomes (Fig. 6 (d)). Granule exocytosis was also seen in some cPDLSCs (Fig. 7 (E)). Moreover, extracellular collagen fibers were discernible by close ultrastructural scrutiny of cPDLSCs (Fig. 7(F)). Nuclear outline was completely irregular in MSCs, but cPDLSCs had round nuclei with regular outlines. A single, large nucleolus was present in the nuclei of both types of cells (Fig. 7 (G)).

4. Discussion

It seems that investigations about PDLSCs in humans and animal models have become more extensive, but no standard protocol for PDLSCs culture and identification is available; which leads to non-comparable studies.[23] In addition, a consensus on isolating, culturing, identifying, and using PDLSCs is needed.[23] The present study explained the isolation and characterization processes of canine PDLSCs as well as investigation of whether canine PDLSCs derived from the ectomesenchyme are similar to MSCs derived from bone marrow *in vitro*. Interestingly, the results of the present study showed that isolation and characterization of undifferentiated mesenchymal stem cells from PDL in dogs was successful and satisfying. Yet there are few published data on isolation and identification of PDLSCs in dogs[12,24,25] and there is also several publication addressing the isolation and identification of BMSCs in dogs.[26–28] None of these publications stated a specific report comparing of BM-MSCs and PDLSCs in dogs. In the study purified cBMSCs were applied as positive control because of their well-characterized stem cell properties.

In human, MSCs are characterized by minimally 3 criteria according to the International Society for Cellular Therapy[1]: plastic adherence when maintained in standard culture conditions,[2] expression of a specific cell surface antigen markers[3] at least two of tri-lineage (osteo-, chondroand, adipogenic) differentiation potential.[29,30]

The present study revealed that cPDLSCs and cBMSCs showed a cell-

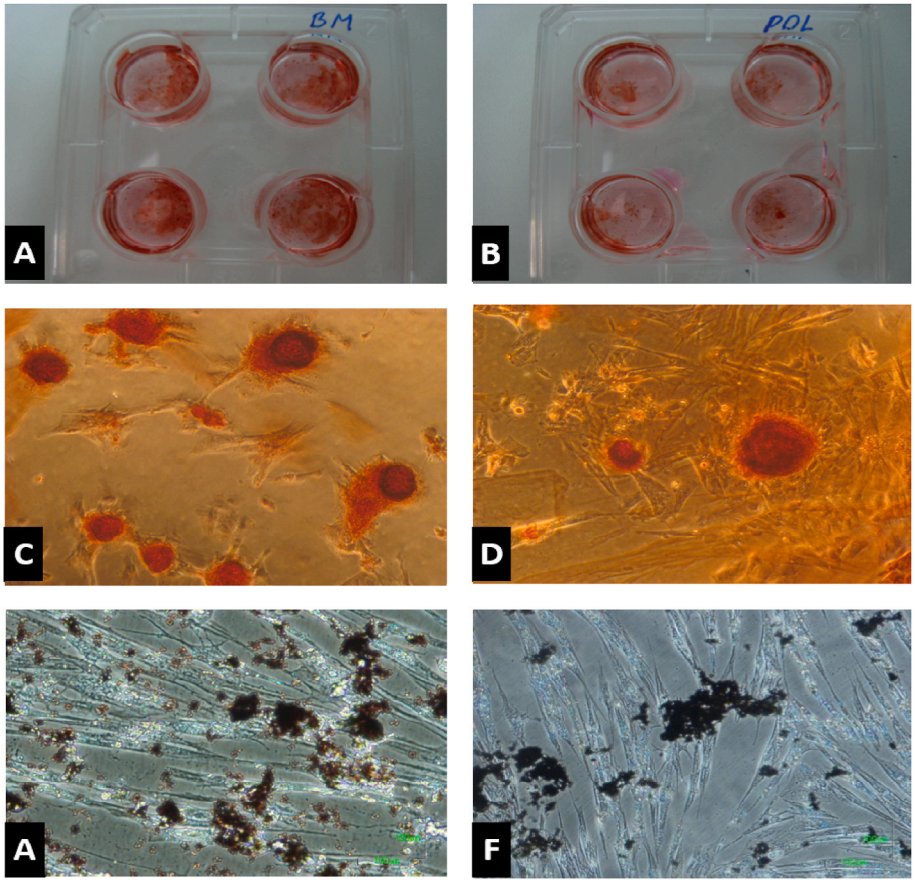

Fig. 2. Canine BM-MSCs and PDL-MSCs differentiation potential: Both of mesenchymal Stem cells were able to differentiate into osteoblasts and adipocytes. A, B- osteogenic and adipogenic differentiation were verified histochemically via AZR S and Oil Red O staining assays, respectively. C, D- Differentiation of BM-MSCs and PDL-MSCs into osteoblasts, these were evident in alizarin red staining. E, F-Differentiation of BM-MSCs and PDL-MSCs into adipocytes, these were evident in oil red staining.

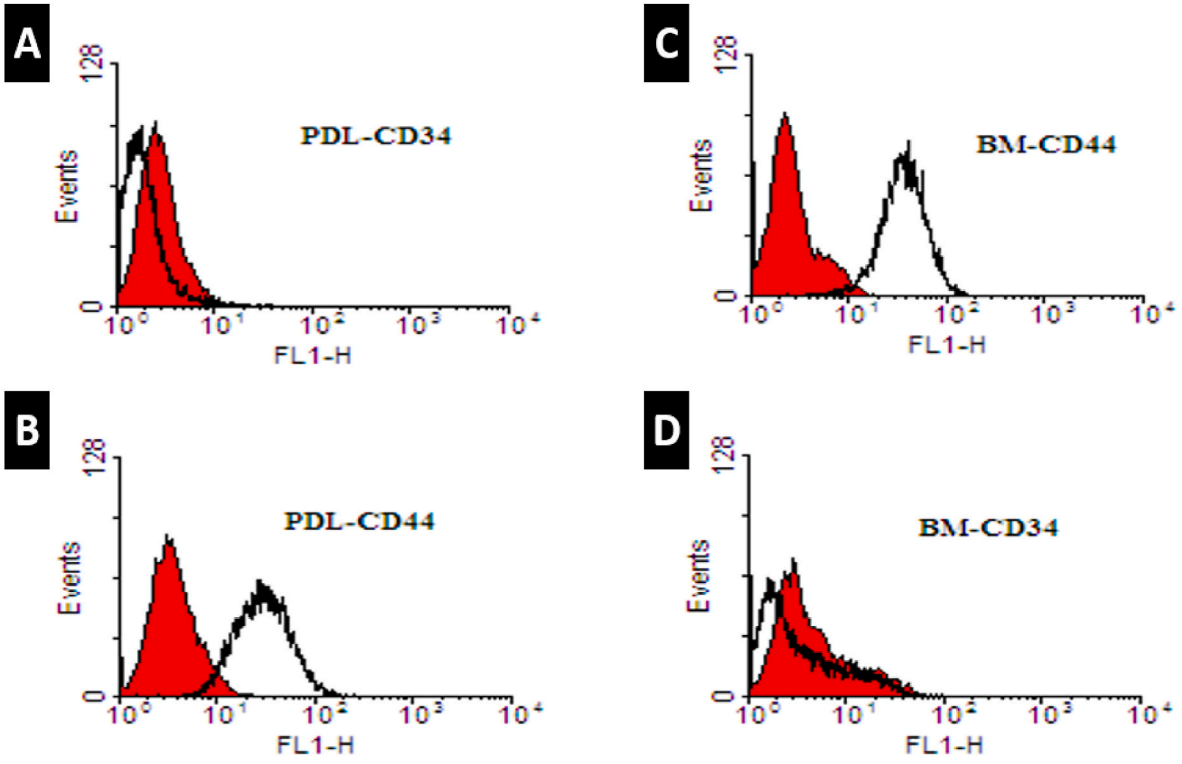

Fig. 3. Cell surface markers and differentiation capacity of Canine PDL-MSCs and BM-MSCs. Diagram analysis of cell surface markers (CD44 and CD34) in PDL-derived stem cells (left) and BM-derived mesenchymal stem cells (right).

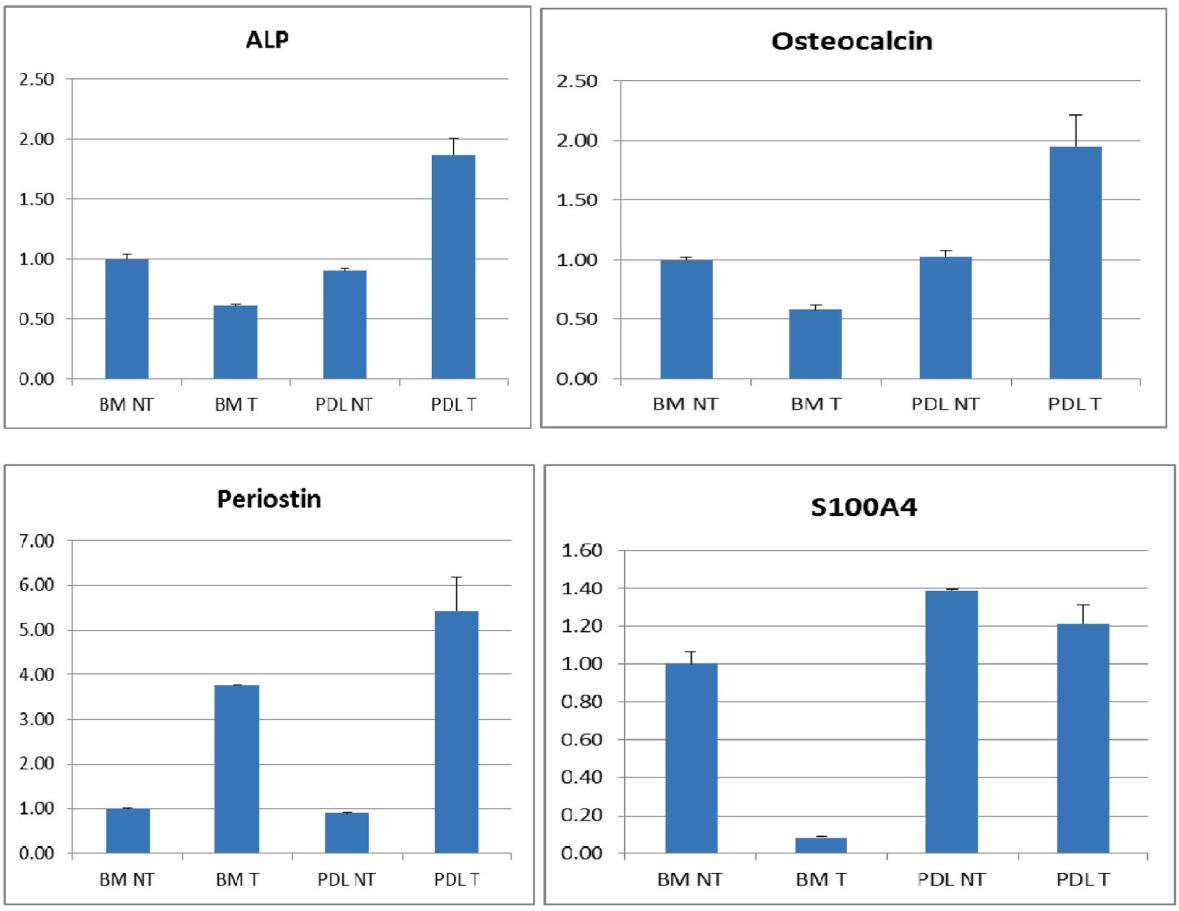

Fig. 4. mRNA expression of Alkaline Phosphatase (a), Osteocalcin (b), Periostin (c) and S100A4(d) in culture of canine PDL-MSCs and BM-MSCs. Quantitative Real-time PCR analysis, effect of osteoinductive supplement on the gene expression in cPDL-MSCs relative to cBM-MSCs. NT: Non-Treatment, T: Treatment.

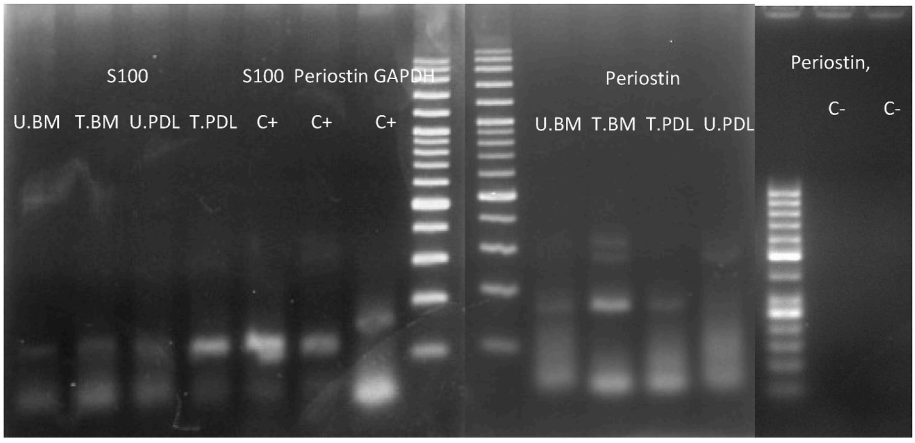

Fig. 5. The results from expression of special genes by RT-PCR for Periostin and S100A4 in undifferentiated and 5th day after differentiated into osteoblast in canine bone marrow and periodontal cells.

surface mesenchymal antigen phenotype positive for CD_{44} (a glycoprotein mesenchymal stromal cell marker), but not for CD_{34} (primitive hematopoietic progenitor marker).[31,32] Proteoglycans are extracellular and cell surface-associated macromolecules that regulate cell adhesion, cell growth, matrix formation and bind growth factors.[33] CD_{44} as a proteoglycan is present in connective tissue of gingiva and periodontal ligament as non-collagenous ECM proteins and was localized on fibroblast cell surfaces.[33,34] In addition, the majority of cPDLSCs showed little expression of CD_{34}.[35,36] These results indicate that the mentioned isolated cells were kind of MSCs, and not undifferentiated cells of hematopoietic origin37. In addition to expression of specific surface antigens, cPDLSCs were plastic-adherent; which agreed with those of other studies.[23,38]

Two types of cells showed a typical spindle-shaped fibroblastic morphology; cell morphologies of the cultured cPDL cells were considerably visible in each well plate. CPF is formed from elongated cells with oval-shaped nuclei and an extended cytoplasm that grow long cell processes, in the study the morphology showed no difference between

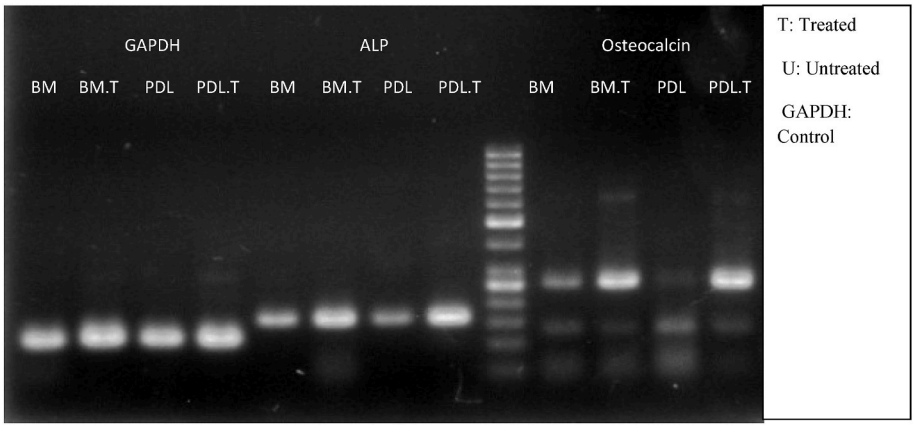

Fig. 6. The results from expression of genes by Real-time quantitative RT-PCR for Alkaline phosphatase and Osteocalcin in undifferentiated and 5th day after differentiated into osteoblast in canine bone marrow and periodontal cells. Two negative controls are difference for two PCR; for comparing genes with each other, and because of every big tray has 17 wells and this study's samples were 4 in each, 16 wells were designed for them and one well for ladder,[17] that's why negative controls individually were run.

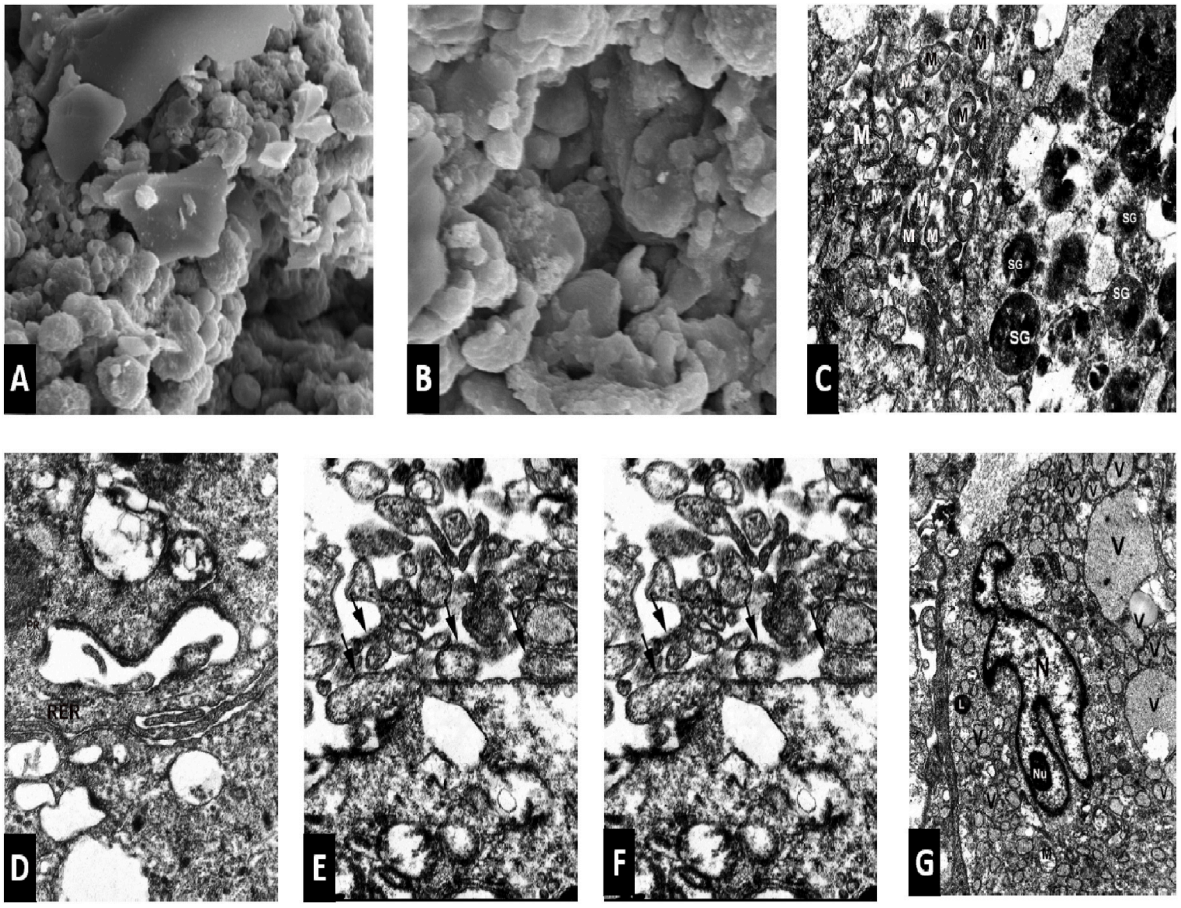

Fig. 7. Scanning and Transmission electron microscope assessment. **(A,B)** scanning electron microscope, clusters of BM and PDL cells, respectively, with secreted ECM. **(C–G)** Transmission electron microscope; **(c)** A PDL cell with numerous intrascytoplasmic secretory granules (SG) and many mitochondria (M). **(d)** Rough endoplasmic reticulum (RER), polyribosomes (PR), along with numerous free ribosomes is seen in the cytoplasm of PDL-MSCs. **(e)** Granule exocytosis in PDL cells. **(f)** Synthetics collagen fiber organizing in the extracellular space. **(g)** Variable-sized vacuoles (V) in the cytoplasm of MSCs. A single mitochondrium (M) and a lipid droplet (L) are seen in the cytoplasm. Note the irregularity of the nuclear outline.

cPDLSCs and cBM-MSCs. One of the most prominent properties of MSCs can be cultured at low densities with duplicitous potential and form colonies. This suggests the ability and potential of PDL cells to produce fibroblastic-like colonies from a single cell.[12,39,40] CFU-F assays have been used to evaluate self-renewal ability, which is a characteristic of mesenchymal stem cells.[41] In accordance with some previous studies, we found that the cPDLSCs exhibited a greater number of CFU-Fs than cBM-MSCs.[42–44] Moreover, we confirmed cPDLSCc and cBM-MSc phenotype by inducing their passage-3 confluent cultured to differentiate along with the classical mesenchymal pathways, osteoblastic and adipocytic lineage, which revealed PDLSC multipotential capability *in vitro*. Furthermore, osteoblastic cell differentiation was characterized by matching the results obtained using various techniques such as isolation of mRNA, PCR, quantitative real-time PCR and cell or matrix staining. The duration time for mineralization was 17 days after exposure to differentiating media which is different from other published data.[45]

Different experimental conditions or species are responsible for this difference requires further investigation.

We attempted to isolate pure cPDLSCs and then maintain high purity in their expansion. In this study, mRNA expression of both osteoblastic/cementoblastic markers (ALP and OCN) and periodontal markers (S100A4 and POSTN) was upregulated by osteoinductive medium at passage-3 culture. mRNA expression of periodontal marker genes were higher in cPDLSCs than cBM-MSCs, there are few published facts in accordance these results in human but there is no data in dogs so far.[46–48] S100A4 is an intracellular calcium-binding protein which expressed by osteoblastic cells. Duarte et al. indicated that S100A4 is a novel negative regulator of *in vitro* in mineralization process and osteoblast differentiation.[49] Although cPDLSCs also exhibited a higher level of S100A4 marker than cBM-MSCs, little information exists in the literature and further investigation is needed. Furthermore, literature proposes little information on ultrastructural changes of PDLSCs and most of the reported changes are based on light microscope observations.

However, there were some limitations related to criteria's characterization for MSCs from animal origin in general and from canine origin in specific.[29,30,50,51] In the current study unavailability of cell surface markers for dogs was a limitation.[12] In addition, little inquiry of comparative viability potential of these two stem cell sources *in vivo* was another major limitation of this study, which is needed to be more explored in future investigations.

Although dogs are frequently used as a large-animal model for study of periodontal disease progression, tissue regeneration, and dental implants, but little consideration has been paid to the identification of the cells involved in this species. This study showed that cPDLSCs have many characteristics similar to previously reported studies on humans,[12,13] ovine,[52] equine,[53] porcine PDLSCs,[54] canine,[12,20] other BM-MSCs[55,56] and dental pulp derived mesenchymal stem cells.[57,58] Moreover, findings of this study clearly demonstrated that canine PDL includes a population of multipotent postnatal stem cells at different stages of differentiation and lineage commitment.[12,23,39] Based on our knowledge, this is the first report in this field. Interestingly, there were significant differences between PDLSCs and BMSCs.

Our results suggests PDL as a unique reservoir of stem cells for regenerative procedures without the necessity of invasive procedures and expensive facilities needed for stem cell isolation from bone marrow. This issue alleviates the complexities of clinical procedures and makes new roads to become cell therapy more straightforward bench-side.[30,59,60]

Since most of these factors are still unknown about canine MSC, critical basic knowledge is urgently required to motivate and correctly translate the potential therapeutic applications of these stem cells in both dog and human.

5. Conclusions

In conclusion, this study successfully established that canine PDL contains a subpopulation of cells with the phenotypic characteristics of MSCs that appear to be equivalent in minimal criteria to similar populations isolated from canine bone marrow. These results indicate that PDLSCs may serve as a promising tool for periodontal regeneration.

Declaration of competing interest

Authors declare no conflict of interest.

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