Osteogenic Differentiation Potential of Equine Dental Pulp vs. Periodontal Ligament Stem Cells: A Comparative *In Vitro* Study

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Abstract

Equine mesenchymal stem cells (MSCs) are promising for bone tissue engineering (BTE) because of their capabilities of differentiating into osteoblasts. Cell therapy using equine MSCs has been introduced. Recently, dental-derived MSCs have gained significant attention due to their capabilities and ease of collection with minimally invasive collection methods. Dental stem cells show high plasticity, accessibility, and applicability for regenerative medicine and are thus considered alternative sources of MSCs. This study evaluated the characterization, osteogenic differentiation potential, and migration assay of equine dental pulp stem cells (eDPSCs) as compared with equine periodontal ligament stem cells (ePDLSCs). Equine dental stem cells from eDPSCs and ePDLSCs (n = 4) were isolated and expanded to passage 3. The morphology, colony-forming capability, cell proliferation assay, stemness and surface markers, trilineage differentiation potential, and migration assay were investigated *in vitro*. Both eDPSCs and ePDLSCs exhibited a fibroblast-like morphology; showed a colony-forming capability; were able to proliferate based on the results of the cell proliferation assay; expressed stemness and surface markers (*NANOG*, CD29, CD44, CD90, CD18); maintained the ability to differentiate into osteocytes, chondrocytes, and adipocytes and demonstrated migration capacity based on the migration assay. Surprisingly, ePDLSCs showed significant differences in matrix mineralization, quantification of Alizarin Red staining by cetylpyridinium chloride, and mRNA expression of the osteogenic marker *RUNX2*. ePDLSCs and eDPSCs may be better alternative MSCs than dental stem cells for the further design of therapeutic regimens for BTE and wound-healing therapy.

Keywords: bone tissue engineering, equine dental pulp stem cells, equine mesenchymal stem cells, equine periodontal ligament stem cells, osteogenic differentiation potential

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INTRODUCTION

Cell therapy is considered an innovative treatment for the restoration, repair, and regeneration of the normal function of cells and tissues affected by aging, injury, or diseases. Over the past decade, stem cells have been extensively studied and are considered promising for restoring, repairing, and regenerating damaged tissues. Mesenchymal stem cells (MSCs) are commonly obtained from the bone marrow, umbilical cord, placenta, and adipose tissue. However, dental stem cells obtained from the oral cavity have garnered significant interest as alternative MSCs due to their capabilities and the minimally invasive techniques used in their collection (Bundgaard *et al*., 2018; Egusa *et al*., 2012; Ishikawa *et al*., 2017). Researchers have characterized dental stem cells as dental pulp stem cells (DPSCs), stem cells from exfoliated deciduous teeth, periodontal ligament stem cells (PDLSCs), and gingival mesenchymal stem cells. These dental stem cells have the same ability as MSCs obtained from sources such as bone marrow, umbilical cord, and adipose tissue (Purbantoro *et al*., 2024; Purwaningrum *et al*., 2023a; Purwaningrum *et al*., 2021) for the treatment of degenerative diseases and also bone

defects that often occur in horses. Equines frequently sustain injuries to joints, muscles, and bone, and MSC therapy shows promise as a therapeutic option for tissue and bone engineering (TE and BTE) (Carvalho *et al*., 2013). MSCs derived from dental stem cells have advantages, including their ease of being obtained because they are unused waste and their ability to differentiate into various lineages, such as ectoderm, mesoderm, and endoderm (Rodas-Junco *et al*., 2017; Gugjoo and Sharma, 2019; Kawamura *et al*., 2019).

In this study, we aimed to isolate, characterize, and evaluate the osteogenic differentiation potential of equine DPSCs (eDPSCs) and equine PDLSCs (ePDLSCs). To date, there have been no adequate comparisons between eDPSCs and ePDLSCs for treatment consisting of autologous, allogenic, and exogenic TE and BTE (Kornicka *et al*., 2019) to repair cells and damaged tissues. Autologous and allogeneic MSC therapy is expected to become the gold standard for treating equine bone defects.

MATERIALS AND METHODS

Ethical Approval

The current study protocol was approved by the Ethics Committee, Faculty of Veterinary Medicine, Universitas Gadjah Mada (approval code: 078/EC-FKH/Int./2024).

Study Period and Location

This research was conducted from May to November 2024 at the Stem Cells and Molecular Biology Integrated Laboratory, Faculty of Veterinary Medicine, and the Biochemistry and Molecular Biology Laboratory, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta.

Cell Isolation, Culture, and Expansion

We collected eDPSCs and ePDLSCs from stallions and mares aged 8 to 15 years. The isolation protocol, adapted from previous studies, involved the tissue explant technique (n = 4) (Banyatworakul *et al*., 2021; Nowwarote *et al*., 2018; Purbantoro *et al*., 2022; Purwaningrum *et al*., 2023b; Tantilertanant *et al*., 2019). Briefly, we collected eDPSCs and ePDLSCs from the tooth cavity and the middle-third tooth root, respectively. The eDPSCs and ePDLSCs were grown in a 35-mm cell culture plate containing culture medium. Once the cells reached 80% confluence, they were subcultured to three new 35-mm cell culture plates (TPP®, Switzerland) in Dulbecco's modified Eagle's medium (Gibco™, USA) supplemented with 15% fetal bovine serum (Gibco™), 2% antibiotic–antimycotic (Gibco™), and 1% Glutamax (Gibco™) at 37°C and 5% CO_2. The culture medium was refreshed every 48 hours. We evaluated the stemness-related marker (*NANOG*) and surface markers (CD29, CD44, CD90, and CD18) using reverse transcription quantitative polymerase chain reaction (RT-qPCR). Cells in passages 3 to 5 were used in all experiments.

Colony-Forming Assay

Following an earlier published protocol, 500 cells of eDPSCs and ePDLSCs were grown for 2 weeks in a 60-mm culture dish (TPP®) (Nowwarote *et al*., 2020; Purbantoro *et al*., 2022; Purwaningrum *et al*., 2023a). After washing with phosphate-buffered saline (PBS), we fixed the cells in cold methanol (Sigma Aldrich Corporation, USA) for a further 20 minutes at 4°C, followed by 5 minutes of staining with crystal violet (Sigma Aldrich Corporation), as outlined (Nantavisai *et al*., 2020; Purbantoro *et al*., 2022; Purwaningrum *et al*., 2023a) (n = 4). At least 50 aggregated cells were counted as a colony.

Proliferation Assays

We assessed cell proliferation after incubating the cells with 5% alamarBlue™ (Sigma Aldrich Corporation) in culture medium for 3 hours (n = 4). The absorbance of each sample was measured at 570 nm using spectrophotometry. To measure cell viability quantitatively, the reduction percentage was computed according to the manufacturer's guidelines.

Trilineage Differentiation

The isolated eDPSCs and ePDLSCs (n = 4) were differentiated to the osteogenic lineage, and the cells were grown in a 24-well plate (TPP®) at 2×10^4 cells/well. After 1 day, the cells were cultured in an osteogenic medium containing 25 mg/mL ascorbic acid (Sigma Aldrich Corporation), 50 nM dexamethasone (Sigma Aldrich Corporation), and 5 mM β-glycerophosphate (Sigma Aldrich Corporation) until day 21. We examined the mineralization of the extracellular matrix using 2% Alizarin Red (Sigma Aldrich Corporation) staining and *RUNX2* osteogenic mRNA expression by RT-qPCR using undifferentiated cells as the control group.

For chondrogenic induction, the cells were grown at 3×10^4 cells/well in chondrogenic induction medium containing L-glutamine 1% (Gibco™), 50 mg/mL ascorbic acid (Sigma Aldrich Corporation), 40 mg/mL L-proline (Sigma Aldrich Corporation), 0.1 μM dexamethasone (Sigma Aldrich Corporation), insulin-transferrin-selenium 1% (Sigma Aldrich Corporation), and 10 ng/mL TGFβ3 (Sigma Aldrich Corporation) until day 21 in a 24-well plate (NUNC Sigma Aldrich Corporation) 1 day after plating. To assess the presence of glycosaminoglycans, we evaluated chondrogenic induction using Alcian Blue staining.

For adipogenic induction, the cells were cultured at 5×10^4 cells/well in adipogenic induction medium supplemented with 1 μM dexamethasone (Sigma Aldrich Corporation), 0.1 mM indomethacin (Sigma Aldrich Corporation), 1 mM 3-isobutyl-1-methylxanthine (Sigma Aldrich Corporation), and 0.1 mg/mL insulin (Sigma Aldrich Corporation) 1 day after seeding in a 24-well plate (NUNC Sigma Aldrich Corporation) over 3 days, followed by adipogenic maintenance medium supplemented with 0.1 mg/mL insulin (Sigma Aldrich Corporation) for 1 day. The adipogenic induction cycle was repeated four times over 28 days. The cells were stained with Oil Red O solution (Sigma Aldrich Corporation) for 1 hour to visualize the intracellular lipid droplets under a microscope.

Mineralization Assay

In accordance with previous studies, we assessed the mineral deposit using Alizarin Red staining at day 21 of osteogenic induction (n = 4) (Nantavisai *et al*., 2020; Rodprasert *et al*., 2021). The eDPSCs and ePDLSCs were rinsed with PBS and fixed in cold methanol at 4°C for 15 minutes. After washing three times with deionized water (pH 4.2), we stained the cells for 5 minutes at 37°C with Alizarin Red solution 2% (Sigma Aldrich Corporation). The cells were subsequently rinsed with deionized water (pH 4.2) three times to eliminate any excess stain. Calcium deposition, indicating mineralization, appeared as a red color under the inverted microscope. Cetylpyridinium chloride (CPC) (Sigma Aldrich Corporation) 10% was used to elute each well to quantify Alizarin Red staining, and the absorbance was recorded at 550 nm using μDrop™ DUO (Thermo Fisher Scientific, USA).

RT-qPCR

We extracted total eDPSCs and ePDLSCs RNA using TRIzol® reagent (Invitrogen Corporation, Carlsbad, CA, USA) (n = 4) and Direct-zol™ RNA Miniprep kit (ZymoResearch, USA), followed by quantification with NanoDrop™ (Thermo Fisher Scientific). We performed RT-PCR to synthesize complementary DNA from 500 ng of mRNA using the ImProm-II™ Reverse Transcription System kit (Promega). RT-qPCR was carried out on Rotor-Gene Q (Qiagen) using SYBR™ Green (Thermo Fisher Scientific); the primer sequences are provided in Table 1. The mRNA expression levels were normalized to the GAPDH (housekeeping gene) using the formula $2^{-\Delta\Delta Ct}$, in which $\Delta\Delta Ct = [Ct_{\text{gene of interest}} - Ct_{GAPDH}]$ group of treatment $-[Ct_{\text{gene of interest}} - Ct_{GAPDH}]$ group of control to determine the relative mRNA expression.

Migration Assay

We evaluated the migration of eDPSCs and ePDLSCs based on the number of confluent cells after 48 hours of scratching on a 60-mm culture dish as the parameter.

Data Analysis

We analyzed the results using SPSS version 28.0 for Mac (IBM Corporation, Armonk, NY, USA). The two independent groups were compared using the Mann–Whitney *U* test. We set the statistically significant difference at a probability (*p*) value of <0.05 (n = 4). The graphics were generated using Prism9.

Table 1. List of primer sequences

Gene	Accession Number	Forward and Reverse Primer sequence	Tm°C	GC%	Length (bp)
GAPDH	NM_0011	5’-CCACCCCTAACGTGTCAGTC-3’	60.04	60	215
	63856.1	5’-AAAGTGGTCGTTGAGGGCAA-3’	60.11	50	
NANOG	XM_0236	5’-TCCTGCCTTTTTGCATGAGTG-3’	59.39	47.62	186
	43093.1	5’-TGGAGGAAGGCAGAGGAGAGA-3’	61.76	57.14	
CD29	NM_0013	5’-TGCCAAATGGGACACGCAAG-3’	61.80	55	179
	01217.1	5’-TTTGCACAGCGAGTGCTCAT-3’	60.89	50	
CD44	NM_0010	5’-TCCACACCCACCTTCCAACC-3’	60	60	177
	85435.2	5’-TGCTTGCCCCACCTTCTTGA-3’	62.01	50	
CD90	XM_0702	5’-CCGCCACACCCATCCAGTAT-3’	61.98	60	182
	73787.1	5’-GCATGTGTAGAGCCCCTCGT-3’	61.96	60	
CD18	NM_0011	5’-GGAACCTCAGGACTGGGCAT-3’	61.57	60	194
	14177.4	5’-CACACCCTAAGCACGGGTCT-3’	61.83	60	
RUNX2	XM_0236	5’-GGAGTGGACGAGGCAAGAGT-3’	61.54	60	127
	24253.1	5’-TGCCTGGGGTTTCTGGGTT-3’	61.40	57.89	

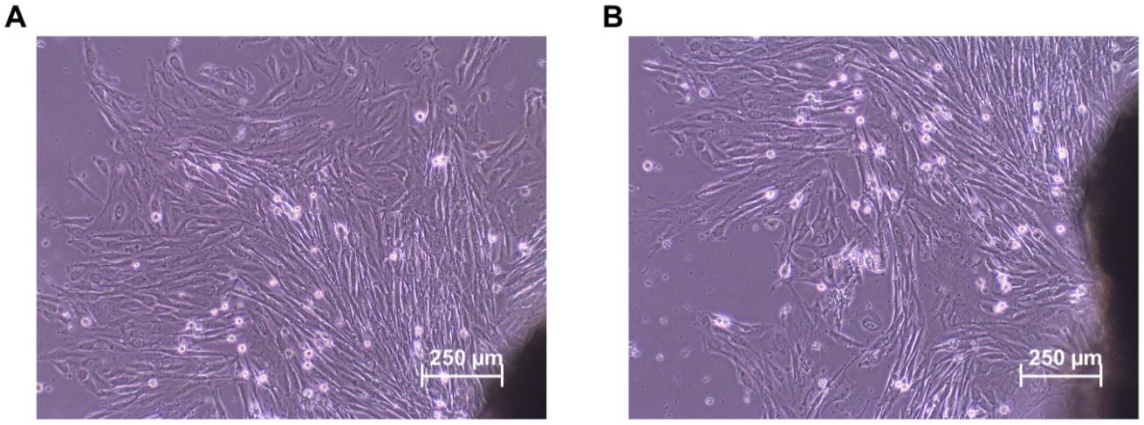

Figure 1. Morphological appearance of the isolated (A) eDPSCs and (B) ePDLSCs.

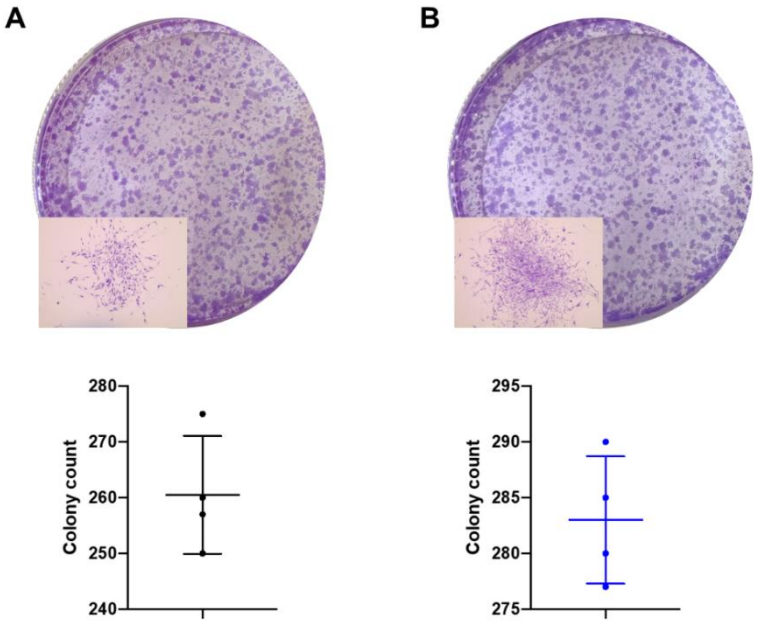

Figure 2. Colony and total colony count for (A) eDPSCs and (B) ePDLSCs.

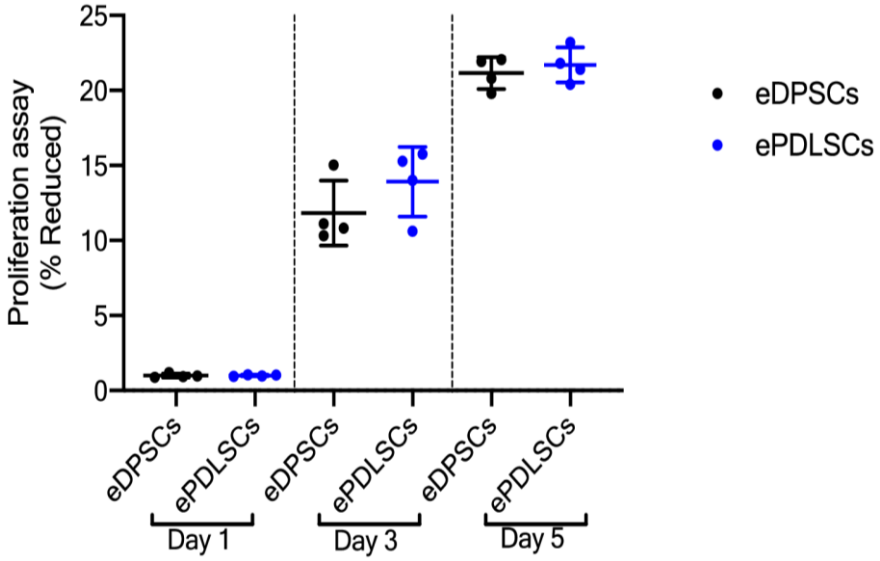

Figure 3. Proliferation days 1, 3, and 5, as determined by alamarBlue™.

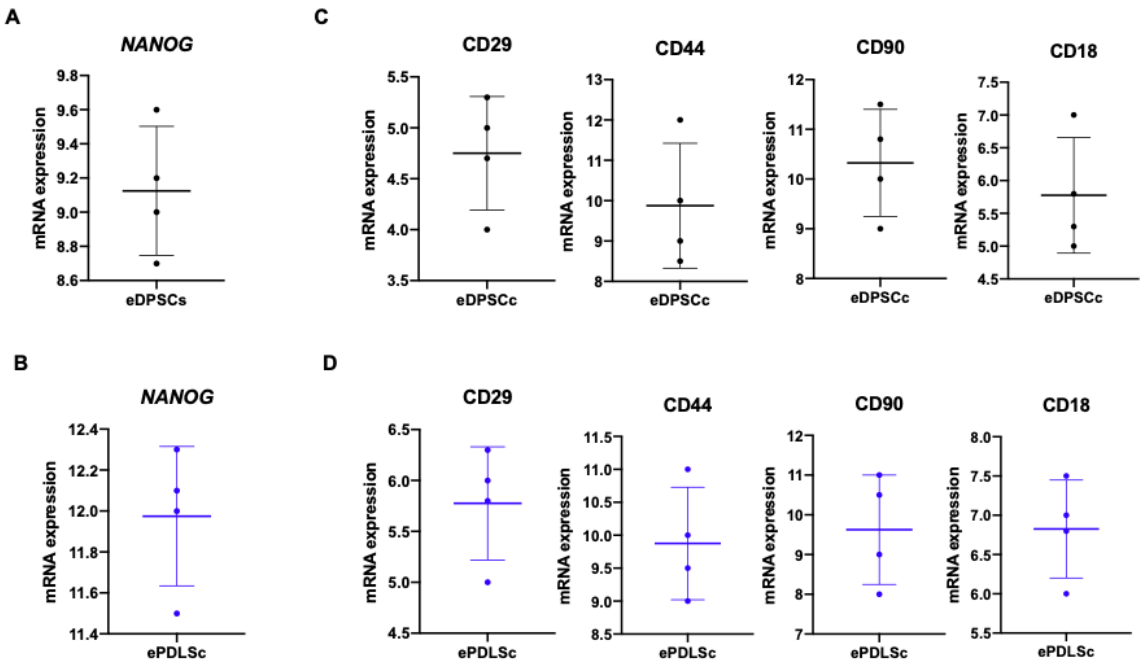

Figure 4. Stemness-related markers: (A) eDPSCs, (B) ePDLSCs and surface markers, (C) eDPSCs, and (D) ePDLSCs.

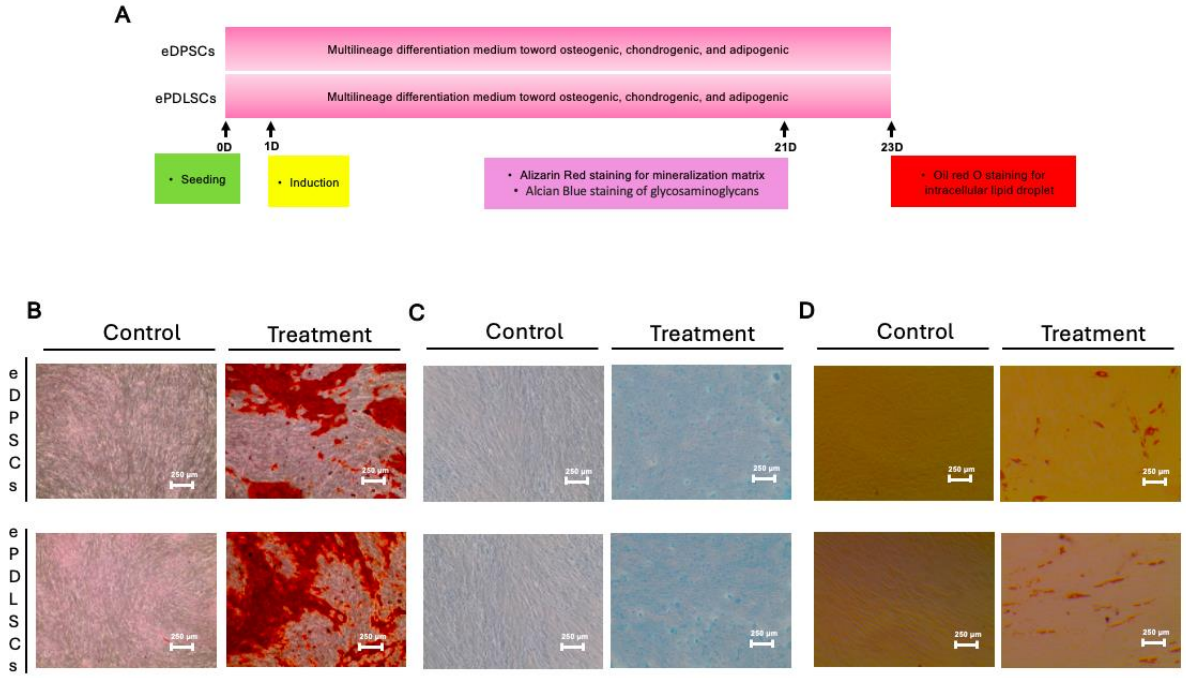

Figure 5. (A) Experimental schema. (B) Trilineage differentiation potential toward osteogenic. (C) Chondrogenic. (D) Adipogenic.

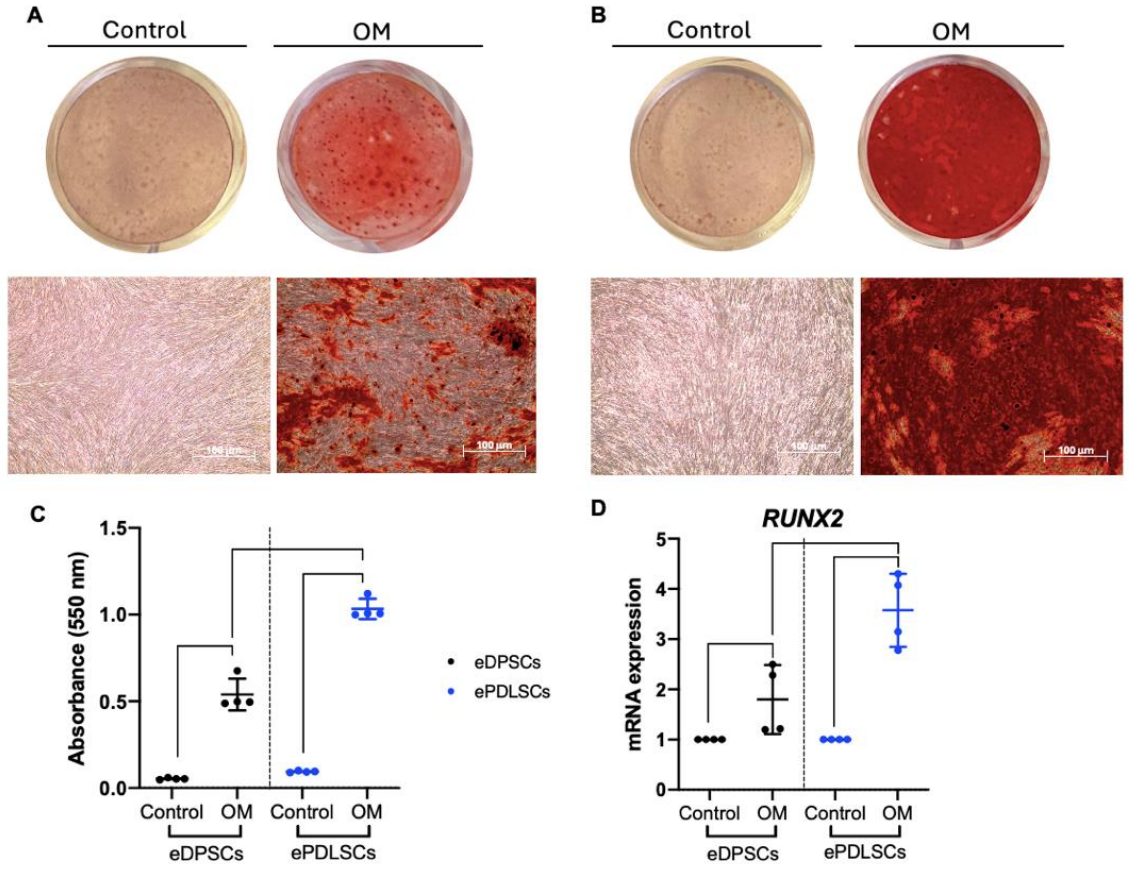

Figure 6. Osteogenic differentiation potential *in vitro* for (A) eDPSCs and (B) ePDLSCs. (C) Quantification of Alizarin Red staining (D) of the osteogenic markers. Bars on Alizarin Red staining quantification results and mRNA expression indicate statistically significant differences ($p < 0.05$).

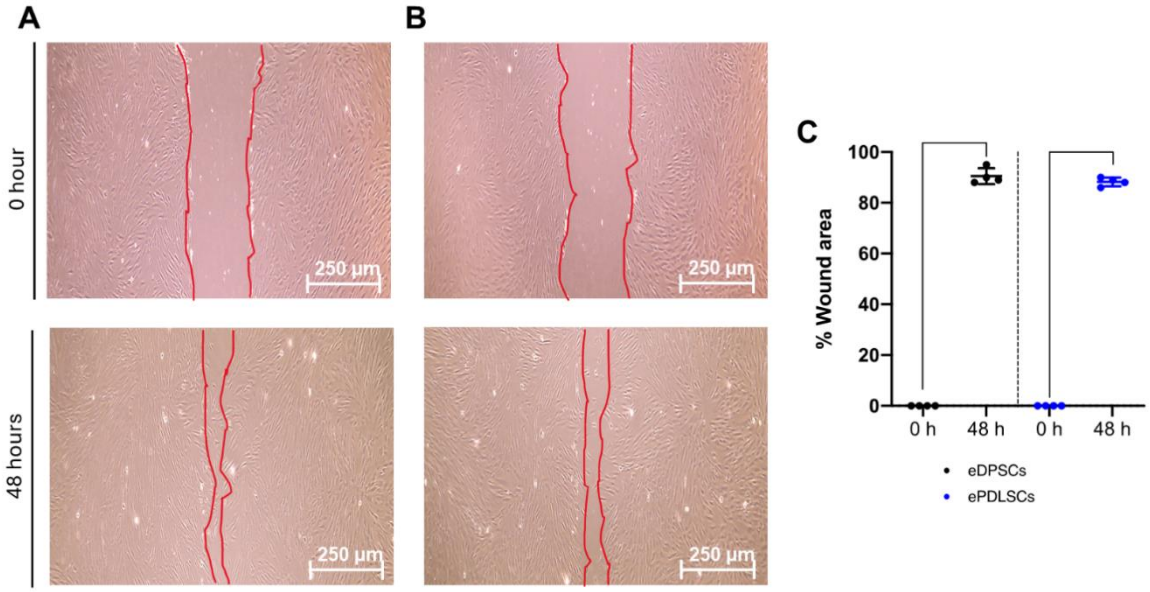

Figure 7. Migration assay for (A) eDPSCs and (B) ePDLSCs. (C) Percentage wound closure at the time points during the scratch wound assay. Bars indicate the statistically significant difference between the groups ($p < 0.05$).

RESULTS AND DISCUSSION

The isolated eDPSCs and ePDLSCs attached to the plastic surface and exhibited a fibroblast like morphology within 3 days. The adherent cells proliferated after 5 days (Figure 1). We observed the colony-forming capability of eDPSCs and ePDLSCs. We observed round colonies in several areas of the culture dish (Figure 2). We determined the growth curve of cells in passage 3 by alamarBlue for 5 continuous days. After reseeding, both eDPSCs and ePDLSCs were in the adaptive phase for three time points on days 1, 3, and 5 (Figure 3). The relative mRNA expression of the stemness marker (*NANOG*) and surface markers (CD29, CD44, CD90, CD18) was detected by RT-qPCR (Figure 4), and the trilineage differentiation is represented in Figure 5.

We performed osteogenic differentiation by maintaining the eDPSCs and ePDLSCs in the osteogenic medium. After 2 weeks of osteogenic induction, the phenotypic results of the extracellular calcium deposit of the mineral were detected by Alizarin Red staining (Figure 6A, B) and then eluted to quantify Alizarin Red staining by 10% CPC (Figure 6C). We determined the genotypic results of mRNA marker expression using RT-qPCR (Figure 6D). In addition, these results demonstrated the *in vitro* differentiation potential of eDPSCs and ePDLSCs toward the osteogenic lineage. After 48 hours of scratching, the wound area was almost completely covered by eDPSCs and ePDLSCs (Figure 7A–C).

This is the first *in vitro* comparison of the osteogenic induction potential of eDPSCs and ePDLSCs. In this study, we successfully isolated, characterized, and evaluated the osteogenic differentiation potential of both eDPSCs and ePDLSCs. Moreover, eDPSCs and ePDLSCs showed proliferative capacity, clonogenic capability, expression of stemness and surface-related markers (*NANOG*, CD29, CD44, CD90, CD18), migration ability, and *in vitro* differentiation potential toward osteogenic, chondrogenic, and adipogenic lineages. These results align with previous findings on eDPSCs, which have been described as fibroblast-like in shape, proliferative, clonogenic, and expressing CD11a/CD18, CD29, CD44, CD90, CD105, MHC class I and II, *in vitro* differentiation into osteogenic, adipogenic, and chondrogenic lineages (Ishikawa *et al*., 2017; Radcliffe *et al*., 2010). An additional report of foal DPSCs found that they can differentiate into angiogenic and epidermal stem cells (Srionrod *et al*., 2016). Furthermore, a previous study reported that ePDLSCs were characterized as expressing *Ki67* (Mukhtar and Alqutub, 2020; Pöschke *et al*., 2018; Warhonowicz *et al*., 2006) and differentiated into chondrogenic cells, adipogenic cells, and tenocytes; similar results regarding their osteogenic differentiation potential were also reported (Mensing *et al*., 2011; Pöschke *et al*., 2018; Safitri *et al*., 2024). Collectively, these findings highlight MSC characteristics similar to those of non-dental stem cells, including bone marrow–derived mesenchymal stem cells that exhibit fibroblast-like shaped, proliferative, clonogenic, express *OCT4*, *SOX2*, and *NANOG*, multilineage differentiation into osteogenic, chondrogenic, adipogenic, and tenocytes (Violini *et al*., 2009); adipocyte-derived mesenchymal stem cells that showed fibroblast-like shaped, proliferative, clonogenic, express CD14, CD29, CD44, CD73, CD90, CD105, CD140b, and CD164, Oct4, TRA1-60, and TRA1-81 (Alipour *et al*., 2015; Braun *et al*., 2010; Delaiah *et al*., 2024), lack of CD34, differentiation into osteogenic, adipogenic, and chondrogenic lineage (Alipour *et al*., 2015; Braun *et al*., 2010; Vidal *et al*., 2007); and umbilical cord– employed for chondrogenic and hepatogenic lineage (Reed and Johnson, 2008).

This study is the first to report and validate the finding that ePDLSCs might have a better capability of differentiating toward an osteogenic lineage than eDPSCs from the mineral deposit and to quantify Alizarin Red S staining by CPC as well as *RUNX2* expression. The results of the present study confirmed that the teeth are attached to the alveolar bone by the periodontal ligament (Men *et al*., 2020). The periodontal ligament, which is a specialized connective tissue located between the cementum and alveolar bone, is a promising cell source for stem cell therapy due to

its high osteogenic differentiation ability and its need to support and remodel the alveolar bone and cementum for repairing bone defects (Han, 2021; Wu and Gong, 2024).

CONCLUSION

In this study, we successfully isolated, characterized, and evaluated the osteogenic differentiation potential of both eDPSCs and ePDLSCs. Collectively, these results highlight the MSC-like properties of eDPSCs and ePDLSCs. These findings provide evidence of the proliferation capability, clonogenic capacity, and migration ability of eDPSCS and ePDLSCs. However, as compared with eDPSCs, ePDLSCs showed superior osteogenic differentiation potential from the mineral deposit as well as better mRNA expression of bone markers This finding is important for identifying the most suitable dental MSC source, which may enable the design of improved therapeutic regimens for allogenic and/or exogenic TE and BTE.

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AUTHORS' CONTRIBUTIONS

MP was responsible for conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, writing – original draft, visualization, project administration, and funding acquisition. MP and CS contributed to software development, while MP, AH, CS, and YK contributed to writing – review and editing. Supervision was carried out by MP, AH, and CS. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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