



Isolation and characterization of equine dental pulp stem cells derived from Thoroughbred wolf teeth

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ABSTRACT. Mesenchymal stem cells (MSCs) are adult multipotent stem cells that are capable of self-renewal and differentiation into multiple cell lineages. Methods for cell therapy using MSCs have been developed in equine medicine. Recently, human dental pulp stem cells (DPSCs) have drawn much attention owing to their trophic factor producing ability and minimally invasive collection methods. However, there have been no reports on equine dental pulp-derived cells (eDPCs). Therefore, the aim of this study was to isolate and characterize the eDPCs from discarded wolf teeth. Plastic-adherent spindle-shaped cells were isolated from wolf teeth. The doubling time of the isolated eDPCs was approximately 1 day. Differentiation assays using induction medium eDPCs differentiated into osteogenic, chondrogenic and adipogenic lineages. The eDPCs expressed mesenchymal makers (CD11a/18, CD44, CD90 CD105 and MHC class I and II), but did not express hematopoietic markers (CD34 and CD45). Taken together, the results show that eDPCs can be isolated from discarded wolf teeth, and they satisfy the minimal criteria for MSCs. Thus, these eDPCs can be referred to as equine DPSCs (eDPSCs). These eDPSCs may become a new source for cell therapy.

KEY WORDS: cell therapy, dental pulp, horse, mesenchymal stem cells, wolf teeth

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Mesenchymal stromal cells (MSCs) are adult multipotent stem cells that are capable of self-renewal and differentiation into cells of mesenchymal and non-mesenchymal lineages, such as osteoblasts, chondrocytes, adipocytes and neurocytes [4, 10–12]. Since MSCs can be greatly expanded in culture and can secrete cytokines, chemokines and growth factors that could potentially repair several injuries, MSCs hold promise for use in cell therapy [13, 14]. In equine medicine, MSCs have been considered for the treatment of various diseases, such as tendon and bone cartilage diseases [2, 17]. To date, equine MSCs have been isolated from the bone marrow (BM-MSCs), adipose tissue (AT-MSCs), umbilical cord blood, peripheral blood (PB-MSCs) and synovial fluid (SF-MSCs) [2, 9, 15, 16]. Although the MSCs derived from these diverse sources have many similarities, recent studies have revealed that equine MSCs also have characteristics unique to each source [3, 15]. For example, plate cultures of SF-MSCs in chondrogenic induction medium form a gelatinous sheet, while BM-MSCs and AT-MSCs do not [15]. Thus, SF-MSCs might be a superior source of MSCs for cartilage regeneration. These differences in the characteristics of equine MSCs from different sources have practical implications for MSC selection in cell therapy.

Human adult dental pulp stem cells (DPSCs) possess self-renewal capability, multi-lineage differentiation, and express early markers of MSCs; thus, they are thought to be MSCs [7, 8]. DPSCs secrete more trophic factors that promote cell survival, proliferation, differentiation and migration than other MSCs [14]. In addition, since extracted adult wisdom teeth are typically discarded, DPSCs can be obtained without adverse health effects. Thus, DPSCs are a unique source of MSCs for cell therapy.

Equine vestigial first premolars, so-called “wolf teeth”, typically cause biting problems, and their removal in performance horses is widely accepted [6]. Extracted wolf teeth are discarded as medical waste. Therefore, if MSCs-like cells can be isolated from wolf teeth, they could become a new non-invasive sources for cell therapy. However, there have been no reports of equine dental-pulp derived cells (eDPCs) from wolf teeth. Thus, the aim of this study was to isolate self-renewal-capable cells from equine wolf teeth and to analyze these cells for MSCs-like characteristics.

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MATERIALS AND METHODS

Collection and transport of extracted wolf teeth

Extracted wolf teeth were obtained from 3 1-year-old female Thoroughbred horses at the Japan Racing Association Miyazaki Training Farm that were discarded as medical waste. The extracted teeth were washed with tap water and placed into a 15-ml sterile centrifuge tube containing 7 ml of α -minimal essential medium (α -MEM; Wako Pure Chemical Industries, Osaka, Japan). During the 48-hr transport to the laboratory, the teeth were kept at 4°C.

Isolation and culture of eDPCs

The surface of the teeth was cleaned with 70% ethanol. The cemento-enamel junction was cut with a sterilized diamond cutter machine. Pulp tissue was separated from the crown and root, and cut into small pieces. A small piece of dental pulp was isolated and digested in a solution of 2 mg/ml collagenase type I (Wako) and 2 mg/ml dispase (DOJINDO, Kumamoto, Japan) for 1 hr at 37°C. Single-cell suspensions were centrifuged at 300 \times g for 5 min at room temperature. The cell pellet was resuspended in 1 ml of proliferation medium (PM) containing α -MEM, 10% fetal bovine serum (FBS; Japan Bioserum, Hiroshima, Japan) and 1% penicillin-streptomycin-amphotericin B (Wako). Single-cell suspensions of dental-pulp derived cells were seeded into 100 mm culture dishes. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂, and the medium was changed twice weekly. When the primary cultures reached 70–80% confluence, the attached cells were passaged by treatment with 0.25% trypsin and 1 mM EDTA (Wako) and replated at a density of 6×10^3 cells/cm².

Growth curves and doubling time

To determine the proliferation capacity of the eDPCs, the cells from the teeth of each horse at passage 3 were seeded in 12-well plates at a density of 10,000 cells per well. After 24 hr, the cells in 3 wells were trypsinized, counted and averaged on days 1–7 to analyze cell growth. The population doubling time (PDT) was calculated as follows: $(T - T_0) \log 2 / (\log N_t - \log N_0)$, where T_0 = culture start time, T = culture end time, N_t = cell number at the end of culture and N_0 = cell number at the beginning of culture.

In vitro differentiation

For osteogenic differentiation, passage 3 eDPCs were seeded in 6-well culture plates at a density of 2.5×10^3 cells/cm², and after incubation in PM for 24 hr, the medium was changed to osteogenic induction medium (Differentiation Basal Medium-Osteogenic; Lonza, Basel, Switzerland) supplemented with 100 μ M ascorbic acid, 10 mM β -glycerophosphate and 1 μ M dexamethasone. The medium was changed 3 times per week. At 3 weeks, alizarin red staining was performed to analyze osteogenic differentiation.

For chondrogenic differentiation, passage 3 eDPCs were seeded in 6-well culture plates at a density of 2.5×10^3 cells/cm², and after incubation in PM for 24 hr, the medium was changed to chondrogenic induction medium (Differentiation Basal Medium-Chondrogenic, Lonza) supplemented with 4.5 g/l D-glucose, 350 μ M L-proline, 100 nM dexamethasone and 0.02 g/l transforming growth factor (TGF)- β 3. The medium was changed 3 times per week. At 3 weeks, alcian blue staining was performed to analyze chondrogenic differentiation.

For adipogenic differentiation, passage 3 eDPCs were seeded in 6-well culture plates at a density of 1.5×10^4 cells/cm², and after incubation in PM for 24 hr, the medium was changed to adipogenic induction medium, (Dulbecco's Modified Eagle's Medium [DMEM; Life Technologies, Carlsbad, CA, U.S.A.] supplemented with 4.5 g/l D-glucose, 100 μ M indomethacin, 10 μ g/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μ M dexamethasone and 5% rabbit serum). At 4 days, oil red O staining was performed to analyze adipogenic differentiation.

Flow cytometry

Passage 3 eDPCs (2×10^5 cells) were resuspended in 500 μ l of FACS buffer (PBS containing 1% sodium azide and 0.5% BSA, pH 7.2). The cells were incubated with various antibodies, including CD11a/CD18 (Clone CZ3.2, 117, 2E11, B10; Gifts from Dr. Douglas Antczak, Cornell University, Ithaca, NY, U.S.A.), CD34-FITC (Clone CA 581/CD34; BD Biosciences, San Jose, CA, U.S.A.), CD44 (Clone CVS18; AbD Serotec, Raleigh, NC, U.S.A.), CD45-FITC (Clone 2D1; BD), CD90-FITC (Clone 5E10; BD), CD105-FITC (Clone SN6; Serotec), MHC Class I (Clone CZ3, 117, 1B12, C11; Gifts) and MHC Class II (Clone CZ11, 130, 8E8, D9; Gifts) at 4°C for 30 min. All clones have been previously used in equine MSC experiments [15–18]. The cells were washed twice with FACS buffer and resuspended in 500 μ l of FACS buffer. The cells incubated with the CD11a/CD18, CD44 and MHC class I and II antibodies were incubated with anti-mouse IgG secondary antibodies labeled with FITC (Rockland, Gilbertsville, PA, U.S.A.) at 4°C for 30 min. Nonspecific FITC mouse immunoglobulin G1 κ was used as a negative control. Cells were then washed twice with FACS buffer and resuspended in 500 μ l of FACS buffer. Cell fluorescence was evaluated by flow cytometry with a FACSCalibur instrument (BD Biosciences). Data were analyzed using CellQuest Pro software (BD Biosciences).

RESULTS

Cell isolation and culture

Adherent spindle-shaped cells were observed at the bottom of culture flasks within 2 days (Fig. 1a). After 5–7 days, the adherent cells proliferated, and round colonies developed in several areas of culture (Fig. 1b). The cells were uniformly distributed immediately after passage and formed a homogeneous-appearing population of fibroblast-like cells at the end of second passage (Fig. 1c).

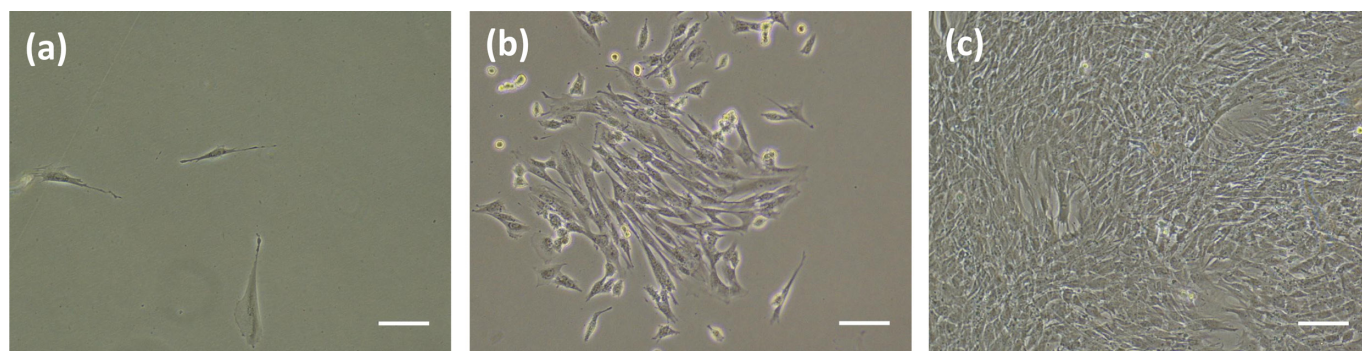


Fig. 1. Representative morphology of equine dental pulp-derived cells (eDPCs) after 2 days (a), 7 days (b) and at the end of the second passage (c). Scale bar, 100 μ m.

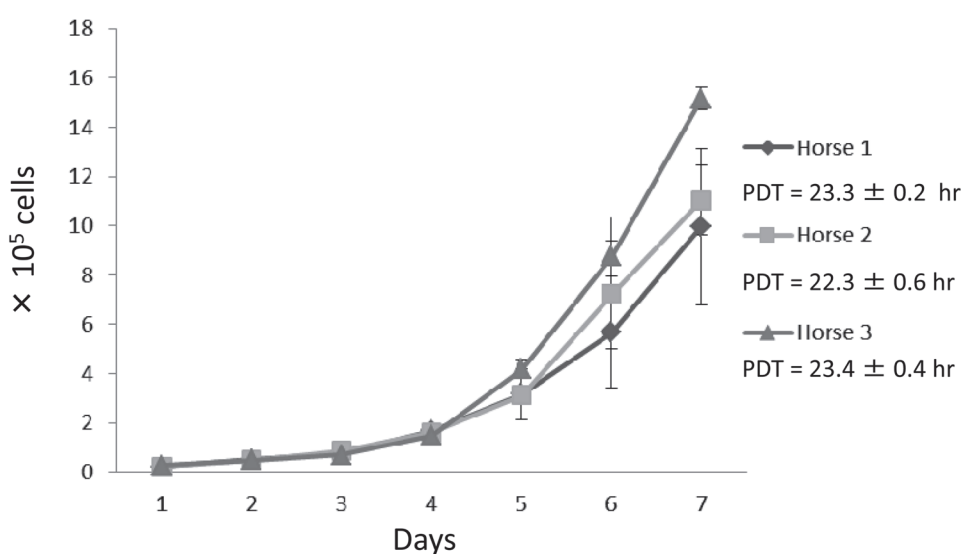


Fig. 2. Growth curve and population doubling time (PDT) of passage 3 eDPCs from 3 different horses. Results are presented as mean \pm SD from triplicate experiments.

Growth curve analysis

The growth curve of cells in passage 3 was determined by counting viable cells for 7 continuous days (Fig. 2). After re-seeding, the eDPCs were in adaptive phase for 2–3 days. Then, the eDPCs began to expand rapidly and move into the logarithmic phase of growth. Based on the growth curve, the PDT of the cells was between 22–24 hr.

In vitro differentiation

In vitro differentiation was performed by incubating the eDPCs in induction medium (Fig. 3). After 3 weeks of osteogenic induction, extracellular calcium deposit was detected by alizarin Red staining. After 3 weeks of chondrogenic induction, proteoglycans were detected by alcian blue staining. After 4 days of adipogenic induction, lipid droplets were detected by oil red O staining.

Characterization of surface makers

Cell surface antigen phenotyping was performed by flow cytometry (Fig. 4). The eDPCs expressed high levels of CD44, CD90 and MHC Class I, and low levels of CD11a/CD18, CD105 and MHC Class II. In contrast, CD34 and CD45 were not expressed.

DISCUSSION

In this study, we successfully isolated eDPCs from equine wolf teeth. Morphologically, these cells were spindle-needle shaped. Under standard culture conditions, they adhered to plastic and proliferated while forming colonies, which are reported characteristics of equine MSCs [17]. Since the kinetics of cell proliferation for all 3 samples was nearly identical, we believe that the isolation method is highly reproducible. In addition, since the eDPCs maintained the same morphology and proliferation rate

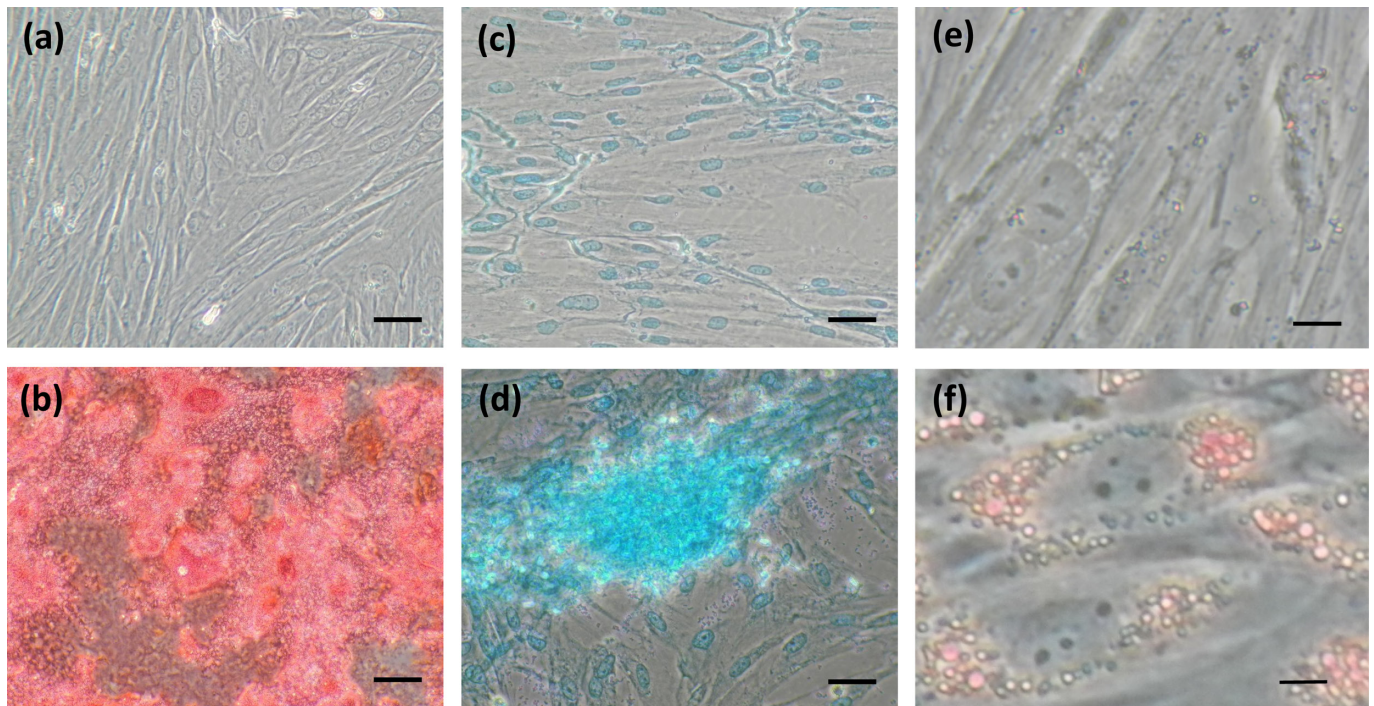


Fig. 3. Representative images of *in vitro* differentiated eDPCs. Alizarin red staining of control (a) and osteogenic induction (b) cultures. Alcian blue staining of control (c) and chondrogenic induction (d) cultures. Scale Bar, 50 μ m (a–d). Oil red O staining of control (e) and adipogenic induction (f) cultures. Scale Bar, 5 μ m (e, f).

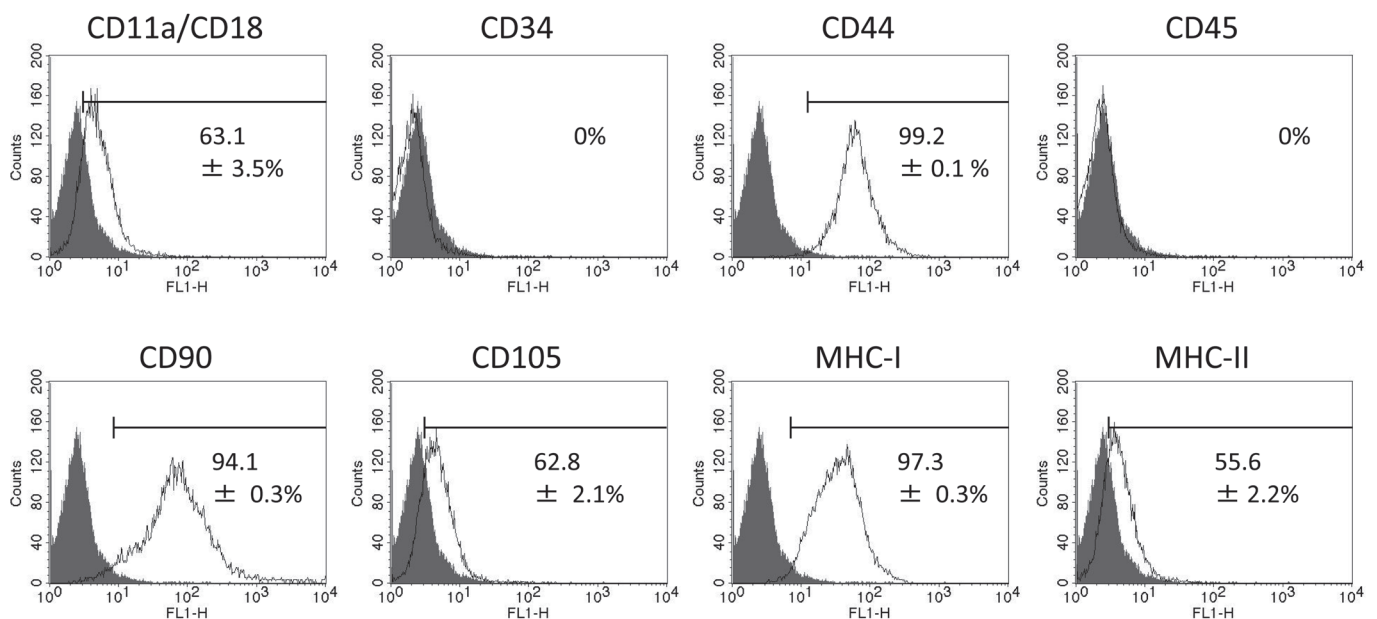


Fig. 4. Flow cytometry analysis of eDPCs. Solid histograms show nonspecific isotype control staining, and open histograms show specific staining for the indicated marker. Three different donor eDPC populations were analyzed, and representative samples are shown. The horizontal line in the individual histograms indicates the population of positive cells, which was drawn based on the nonspecific isotype control staining. The signal intensity of the indicated antigen is presented as the mean \pm SD from triplicate experiments.

over 10 passages (data not shown), they may have the capacity for self-renewal.

The PDT of passage 3 eDPCs was approximately 1 day for all 3 samples. Since it was reported that the PDT of both BT-MSCs and AT-MSCs was nearly 2 days [1, 19], the proliferation rate of dental pulp-derived cells may be faster than that of other MSCs. This fast proliferation rate may be advantageous for cell therapy.

Cellular differentiation occurs in response to antigen exposure. One of the defining properties of stem cells is multi-differentiation potential. Based on the results of the differentiation assays using induction medium, eDPCs could differentiate into osteogenic, chondrogenic and adipogenic lineages. Since the eDPCs possess self-renewal and multi-differentiative potential, they are considered stem cells and can be referred to as eDPSCs.

The eDPSCs population expressed the mesenchymal markers LFA-1 (CD11a/18), receptor molecule protein CD44, thy-1 (CD90), endoglin (CD105), and MHC Class I and II. The intensity of the positive signals was similar to that reported from previous equine MSCs studies [15, 19]. In contrast, the eDPSCs did not express hepatopoietic markers, adhesion molecule protein (CD34) and LCA (CD45). Based on the above findings, the pattern of surface molecule on the eDPSCs fulfills the criteria for MSCs. In addition, the expression of MHC antigens indicates that the eDPSCs possess immunogenic potential. Further studies are needed to determine whether they would be suitable for allotransplantation.

In conclusion, we successfully isolated eDPSCs from Thoroughbred wolf teeth that possess self-renewal potential, can differentiate into osteogenic, chondrogenic and adipogenic lineages, and express the surface markers of MSCs. Since these 3 characteristics are minimal criteria for confirmation as MSCs [5], we believe that these eDPSCs can be called MSC-like cells. We expected further research will promote the application of eDPSCs in cell therapy.

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