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# The effect of aging on the pluripotential capacity and regenerative potential of human periodontal ligament stem cells

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ABSTRACT

Multipotent postnatal stem cells can be isolated from human periodontal ligaments (PDLs) and have the potential for large-scale expansion, offering a reliable cell source for clinical use in periodontal regenerative therapies. However, the effects of aging on the mesenchymal stem cell (MSC) properties of these cells remain undefined. The aims of this study were to isolate and characterize the periodontal ligament stem cells (PDLSCs) derived from human impacted third molars of donors of different ages and to compare their pluripotential capacity and regenerative potential. PDL tissues were obtained from 90 surgically extracted third molars and divided into four groups according to the donor's age. For each group, the colony-forming ability, proliferative capacity, migratory potential, cell surface antigens, differentiation ability, alkaline phosphatase activity, and gene expression of the PDLSCs were contrastively evaluated and quantified for statistical analysis. The *in vivo* tissue regenerative potential of PDLSCs was assessed by an *in vivo* ectopic transplantation model. It was found that human PDLSCs were successfully isolated and characterized as MSCs in all 90 teeth. PDLSCs derived from donors of different ages were successfully differentiated under an osteogenic and adipogenic microenvironment. The proliferative and migratory potential and the differentiation capacity of PDLSCs decreased as age increased ($p < 0.05$). PDLSCs derived from donors whose age is $62.6 \pm 6.8$ have a statistically significant decrease in pluripotential capacity compared with those derived from relatively young donors ($p < 0.01$). There is no identified cementum and PDL-like tissue formation *in vivo* among the two aging groups. We conclude that human PDLSCs could be successfully isolated from PDL tissue derived from donors of different ages, but the age-related changes of the MSC properties should be taken into account whenever they are intended for use in research or cytotherapy.



## 1. Introduction

Periodontal-tissue breakdown around natural teeth due to periodontitis, a worldwide medical concern and a leading cause for tooth loss, poses a critical challenge for clinical regenerative dentistry [1]. The reestablishment of damaged tooth-supporting apparatus is an extremely complex process because of the involvement of the regeneration of three unique tissue types, namely cementum, periodontal ligament (PDL), and alveolar bone, and the need to rebuild their intricate structures [2]. It is obvious that the current treatment strategies for periodontitis fail to completely and reliably reconstitute all tissues and connections damaged through periodontal disease [3]. In light of this, cell-based tissue engineering paradigm is considered as an alternative approach to the current treatments and may help to alleviate the shortcomings of more conventional therapeutic options by regenerating living and functional periodontal structures [4–8]. Among the three essential components required for tissue engineering design, stem cells are the most fascinating area of research today and have been used in the field of pre-clinical and clinical regenerative medicine with the aim to treat many degradative diseases including periodontitis [9–11]. Of note, there are substantial data that confirm the presence of various stem cells in dental tissues, such as PDL, dental pulp, follicle, and papilla, in and around a tooth; these dental stem cells

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have received extensive attention in the field of periodontal regenerative medicine due to their accessibility and multilineage differentiation capacity [8,12]. The accumulating knowledge on the cell biology of dental stem cells, together with recent advances in materials science and developmental science, have opened new avenues that aim to use those easily available stem cells in clinical reconstructive dentistry for the treatment of periodontal disease [4,6–8]. It is remarkable that, following several feasible/pilot studies [13–15], several clinical trials (e.g., NCT01357785 ) involving the transplantation of stem cells (i.e. autologous PDLSCs) into periodontal defects have already begun or are in preparation.

Before the identification of stem cell populations in the PDL tissue, there has been mounting evidence that PDL cells exhibit osteoblastic characteristics because they are capable of producing mineral-like nodules [16] and express bone-associated genes, osteocalcin (OCN), osteopontin (OPN), bone sialoprotein (BSP), bone morphogenic protein (BMP)-2, BMP-4, and alkaline phosphatase (ALP) [17–20]. In 2004, a pioneer study demonstrated that the PDL contains stem cells, generally termed PDL stem cells or PDLSCs, that have the potential to differentiate into cementoblast-like cells, adipocytes, and collagen-forming cells *in vitro* and to generate new cementum/PDL-like compartments *in vivo* [21]. Further research demonstrated that they exhibit a fibroblast-like morphology and express CD90, CD29, CD44, CD166, CD105, and CD13 antigens that have also been identified as stromal precursors of bone marrow [22]. Interestingly, recent findings suggest that PDLSCs could be isolated from both healthy and inflamed young PDL tissue (16–29 years of age); the inflamed PDLSCs retain their regenerative potential for cementum and related PDL tissues, suggesting an alternative rich source (i.e., teeth extracted for periodontitis reasons) of mesenchymal stem cells (MSCs) for cytotherapeutic use due to the large number of periodontitis patients involved [23]. Clearly, the use of PDLSCs involves less religious and ethical concerns than using MSCs derived from bone marrow because they are easily obtainable from medical waste, i.e., the teeth extracted for orthodontic, impaction, or irreversible periodontic reasons [8]. Therefore, PDLSCs represent a unique population of MSCs that may facilitate translational research and have future clinical application in, but not limited to, periodontal regenerative medicine [8,24].

The ability to identify and manipulate human PDLSCs has been a significant advancement in regenerative dentistry and has contributed to a significant development in the progress toward predictable periodontal regeneration [21–24]. In general, cell-based periodontal therapy involves the delivery of *ex vivo* expanded cell populations to the periodontal defect [8] or the mobilization of endogenous progenitor cells capable of proliferating and differentiating into the required tissues [2]. The latter strategy that relies on the stimulation of the intrinsic regenerative potential of host tissues unfortunately does not offer a universal regenerative solution. Innate powers of regeneration may be restricted by an age-related decline in progenitor populations that can be mobilized or by the intrinsically low regenerative potential of certain tissues [25–27]. In these cases or when the original tissue has been completely destroyed, biomaterial interventions that include an external source of stem cells may be required [8]. Today, the methods to isolate, purify, and characterize multipotent stem cells in human PDL have been well delineated [21–24], and the large-scale expansion of stem cells to cater to the need for clinical quantity without using animal sera as nutritional supplements is becoming possible [28,29]. Nevertheless, considering the complexity of the PDL attachment apparatus and the heterogeneity of its cell populations, the standardization of the appropriate cell populations from PDL tissues necessary for complete regeneration remains one of the key factors in implementing optimal approaches to periodontal regeneration [6–8].

Despite the presence of a feasible study that uses autologous PDLSCs in clinical periodontal therapy [14], the influences of aging on the MSC properties of PDLSCs remain largely unexplored. These influences may have significant effects on final clinical outcomes because tissue regenerative potential may be negatively regulated by aging. Understanding such effects is particularly crucial for autologous therapy development in older subjects whom degenerative diseases typically afflict. In fact, the age-related effects of stem cells have been noted in different tissue sources [30–36]. Furthermore, the loss of the proliferation and differentiation capacity of PDLSCs was also found in six aged female donors with a mean age of 54 ± 3.2 years compared with the capacities of eight young female donors aged 15 ± 2.4 years [37]. The identification of the effects of aging on PDLSCs needs further investigation that involves looking at different groups of people of different ages, each group including a relative narrow age range of subjects. The aims of this study were to isolate and characterize the PDLSCs derived from a wider range of donor ages (from less than 20 years to more than 60 years) and to compare their pluripotential capacity and regenerative potential in a relatively large amount of samples.

## 2. Materials and methods

### 2.1. Study design

Human third molars without caries, inflammation, or periodontitis were extracted for impaction reasons from 54 systemically healthy donors between 16 and 75 years of age at the Fourth Military Medical University Dental Hospital, Xi'an, China. For the aged donors whose third impacted molars were not removed in the correct stage, the extraction of teeth was based on complete oral health examinations showing that the corresponding teeth were harmful to the donors' oral health and must be removed. The age and sex of the donors are shown in Table 1 and their extracted teeth were divided into A, B, C, and D groups solely based on the donors' age. If one donor had more than two teeth that could be extracted, two teeth were randomly included in the present investigation. If a tooth was destroyed or contaminated during or after the surgery, it was automatically excluded from the study. However, if the cells failed to grow from tissue samples for nontechnical reasons, the donor information was included into final statistical analysis. Each tooth was studied independently, even if two were from the same donor.

### 2.2. Isolation of PDLSCs

Human PDLSCs were freshly isolated and cultured according to previously reported protocols with slight modification [24,37]. Briefly, each tooth was rinsed and then the PDL tissues were separated from the mid-third of the root surface. PDL tissues were further washed several times and then minced into small tissue cubes (approximately 1 $mm^3$). Subsequently, the tissue cubes were digested with a solution of 3 mg/mL collagenase (type I) with 4 mg/mL dispase (both from Sigma–Aldrich, St. Louis, MO, USA) in α-minimum essential medium (α-MEM, Hyclone, Road Logan, UT, USA) for 15 min at 37 °C with vigorous shaking. The tissue explants were then plated into six-well culture dishes (Costar, Cambridge, MA, USA) containing α-MEM supplemented with 10% fetal bovine serum (FBS; Hyclone), 0.292 mg/mL glutamine (Hyclone), 100 units/mL penicillin streptomycin (Hyclone), and 100 μM/L ascorbic acid (Sigma–Aldrich) and incubated at 37 °C in a humidified atmosphere containing 5% $CO_2$. Single cell-derived colony cultures were obtained using the limiting dilution technique and passage 0 (P0) cells were cultured. To avoid changes in cell behaviors that are associated with prolonged culture, the cells at passages P3–P5 were used for contrastive investigation in the present study.

**Table 1**
Isolation of human periodontal ligament stem cells from impacted third molars of different aged donors.

| Groups | Age range (year) | Average age (year) | Donors (*n*) | Sex | Teeth (*n*) |
|---|---|---|---|---|---|
| A | 16–30 | 22.3 ± 7.7 | 15 | M = 7; F = 8 | 24 |
| B | 31–40 | 35.6 ± 4.3 | 14 | M = 7; F = 7 | 23 |
| C | 41–55 | 48.2 ± 6.1 | 12 | M = 6; F = 6 | 21 |
| D | 56–75 | 62.6 ± 6.8 | 13 | M = 6; F = 7 | 22 |

A total of 90 healthy teeth from 54 donors were involved in the present study and divided into A, B, C, and D groups according to the age of the donors. Periodontal ligament tissues from each tooth were independently collected for cell culture and subsequent investigations. M, male; F, female.