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New considerations in selecting donors for dental pulp stem cells: a pilot study

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Abstract

Background/purpose: Tissue engineering based on stem cell therapy necessitates a substantial quantity of high-quality stem cells. However, current sources face limitations, including narrow donor pools, compromised biological properties due to cryopreservation, and cellular senescence resulting from in vitro passaging and expansion. This study examines the impact of mild periodontitis on the biological performance of dental pulp stem cells (DPSCs) to explore the potential of broadening the donor pool for these cells.

Materials and methods: The experiment included two variables: age and the presence of periodontitis. DPSCs were isolated from six healthy subjects and six patients with mild periodontitis. Healthy subjects were categorized into Groups A (28–32 years) and B (52–54 years), and patients with mild periodontitis were categorized into Groups C (31–33 years) and D (50–53 years). The analyses included cell morphology, proliferation rate, multilineage differentiation capacity, apoptosis, and surface marker expression.

Result: No significant differences in cell morphology, pluripotency, or senescence were observed between healthy controls and periodontitis patients across age groups. Additionally, data on proliferation, pluripotency, and senescence were not significantly different. In healthy subjects, increased age was correlated with more elongated, flattened, and broader cells, alongside greater heterogeneity and intercellular granules. The proliferation and differentiation capacities decreased, whereas the degree of apoptosis increased. Similar trends were noted in patients with periodontitis.

Conclusion: The biological properties of DPSCs remain unchanged in teeth with mild periodontitis, providing valuable insights for addressing the shortage of DPSCs in tissue engineering. Teeth with mild periodontitis have the potential to be pulp stem cell donors.

Keywords: Periodontitis, Dental pulp stem cells (DPSCs), Regenerative medicine, Tissue engineering

Introduction

Tissue engineering, also known as regenerative medicine, involves repairing or reconstructing tissues and organs via the use of bioactive substances through in vitro culture or construction methods [1–3]. Stem cells, a unique type of cell with multidifferentiation potential,

play crucial roles in tissue engineering and are the focus of extensive research and application [4]. Stem cells possess diverse applications in tissue engineering and hold significant potential for treating challenging diseases, including diabetes, various cancers, neurodegenerative disorders, and cardiovascular illnesses [5]. Therefore, tissue engineering demands a substantial quantity of stem cells for both research and clinical applications [6]. Stem cells are primarily sourced from a variety of tissues and organs, including bone marrow, adipose tissue, peripheral blood, umbilical cord, placenta and amniotic fluid [6]. Numerous ethical concerns [7] accompany the extraction of large quantities of stem cells, making it essential to rely on a limited number of sources for extensive in vitro expansion and culture to fulfill the requirements of commercial applications [8]. The storage and expansion of stem cells also present challenges. During cryopreservation, stored cells are subjected to stressful conditions that can cause irreversible damage or cell death, a phenomenon known as cryoinjury [9]. Stem cells undergo cellular senescence after multiple in vitro passages, and the biological properties of the cells are also affected [10]. The shortage of stem cells poses a barrier to developing tissue engineering [2]. Dental pulp stem cells (DPSCs), which are found within the pulp cavity of teeth, are adult stem cells known for their high proliferative capacity and multidifferentiation potential. Additionally, DPSCs offer advantages in terms of low immunogenicity, accessibility, and low tumorigenicity, outperforming other stem cell types in terms of biological performance [11]. They have become a new source for tissue engineering. Pulp stem cells are primarily derived from healthy pulp tissue obtained from extracted teeth. His range of sources is comparatively limited. One significant yet often overlooked advantage of harvesting stem cells from teeth is the ability to obtain them from inflamed dental tissues [12]. Periodontitis, an increasingly prevalent inflammatory disease, is among the most common oral health issues [13]. Periodontitis affects periodontal tissues, influencing tooth stability [14]. Typically, if untreated, it can lead to loose teeth and eventual tooth loss, a primary cause of adult tooth loss [15, 16]. The utilization of teeth that cannot be retained due to periodontal disease could significantly advance tissue engineering and help address the shortage of DPSCs. Periodontitis induces an inflammatory environment around the tooth root, and because the pulp is connected to the periodontium through structures such as the apical foramen, dentin tubules, and lateral root canals, the condition of the pulp is inevitably affected [17–19]. Research indicates that the proliferation and differentiation of pulp stem cells extracted from teeth with aggressive periodontitis are diminished to varying extents [20]. This experiment investigated the biological properties of pulp stem cells in teeth with mild periodontitis (stage I or II, class A or B). We will examine donor age as a factor to determine whether there is a difference in resistance to periodontitis between teeth from middle-aged and elderly patients. This study aims to expand the knowledge base related to stem cells, evaluate the impact of mild periodontitis on the biological properties of extracted dental pulp stem cells (DPSCs), and propose a solution to address the shortage of tissue-engineered DPSCs.

Results

Morphological characteristics of DPSCs

Microscopic observations of Groups A, B, C, and D were performed (Fig. 1a). The DPSCs in Groups A and C were spindle-shaped, resembling fibroblasts, with homogeneous morphology, full cytosol, and fibrous, colony-like growth. No significant differences

were observed between Groups A and C. In Groups B and D, a slight increase in cell volume was noted. The cell morphology became narrower, flatter, and more irregular, with increased heterogeneity, cellular debris and inclusions. No significant differences were observed between Groups B and D. These results indicate that mild periodontitis has a minimal impact on cell morphology in middle-aged or elderly individuals. Age-related changes were more pronounced.

Assessment of the proliferation capacity of DPSCs

After 5 days of continuous culture, no statistically significant difference in proliferation capacity was found between Groups A and C ($P > 0.05$), or between Groups B and D ($P > 0.05$). However, Groups B and D presented significantly slower cell growth rates and reduced proliferation capacity than Groups A and C did, and these differences were statistically significant ($P < 0.05$) (Fig. 1b). These results suggest that chronic periodontitis minimally affects the proliferative capacity of DPSCs, whereas donor age has a significant effect.

Analysis of DPSC surface antigens

No statistically significant differences in the expression of surface antigens were observed among the DPSCs from different groups ($P > 0.05$). The negative markers CD34 and CD45 were expressed at low levels, whereas the positive markers CD73, CD90, and CD105 were highly expressed. Notably, CD105 expression was related to donor age ($P < 0.05$) but not to periodontal health status ($P > 0.05$). CD105 expression tended to decrease with increasing donor age (Fig. 2, Table 1). CD105 serves as a surface epitope marker for dental pulp stem cells, and the reduced expression of these markers in older individuals compared to middle-aged ones further underscores the impact of aging on dental pulp stem cells. This reduction in surface marker expression corresponds with diminished biological properties. These results indicate that isolated DPSCs conform to the phenotype of mesenchymal stem cells (MSCs), and that chronic periodontitis does not affect surface antigen expression. However, the expression of surface antigens is related to donor age.

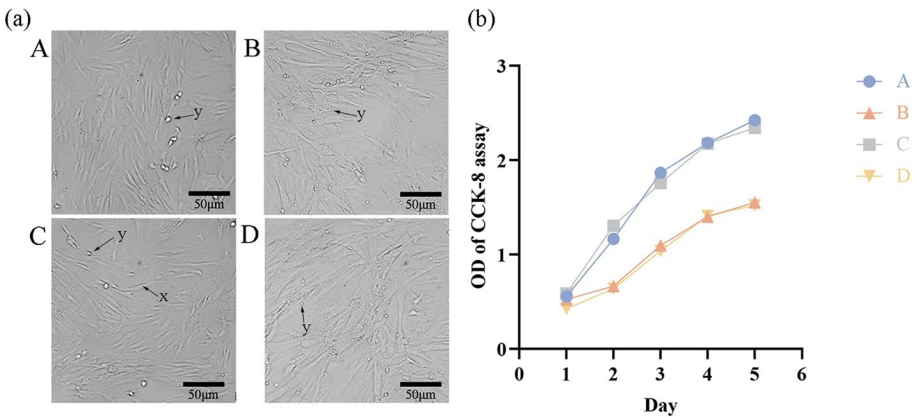

Fig. 1 Microscopic observations and proliferative capacity of the groups. **a** Microscopic observations of DPSCs from Groups A, B, C, and D; x: cytoplasm; y: cellular debris and inclusions. **b** Proliferation changes of DPSCs from Groups A, B, C, and D

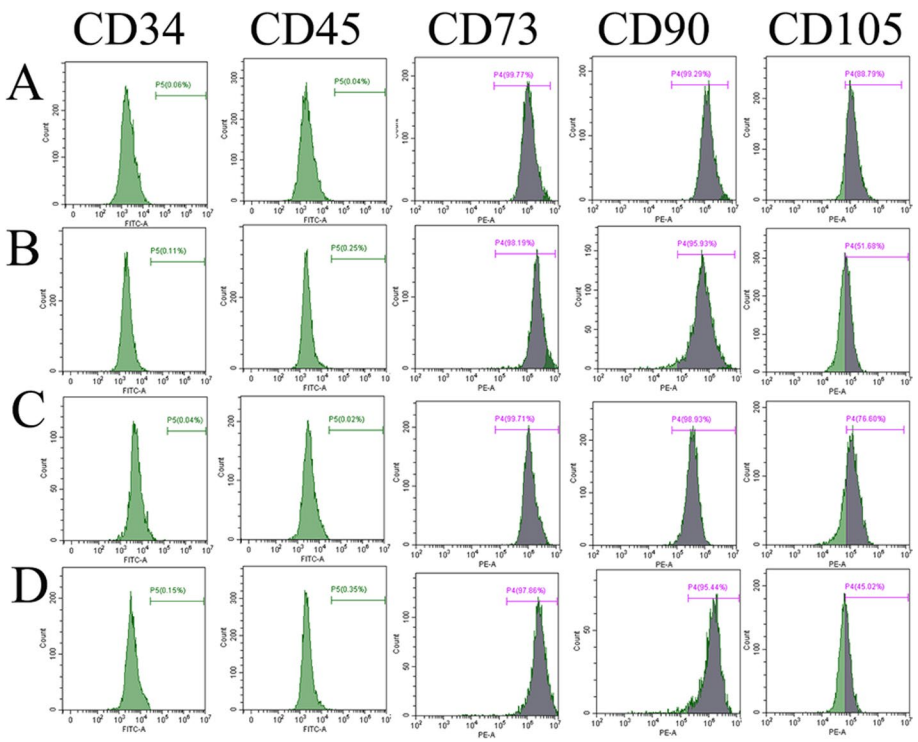

Fig. 2 Expression results of surface markers on DPSCs from Groups A, B, C, and D

Table 1 Statistical table of surface marker expression on DPSCs ($n = 12$, mean ± SD)

Marker	A	B	C	D	*F*	*P*
CD34	0.10 ± 0.09	0.15 ± 0.09	0.07 ± 0.04	0.08 ± 0.06	0.68	0.59
CD45	0.05 ± 0.03	0.27 ± 0.21	0.07 ± 0.07	0.21 ± 0.14	1.93	0.20
CD73	99.81 ± 0.15	95.36 ± 6.13	99.54 ± 0.54	94.95 ± 6.68	1.00	0.44
CD90	97.85 ± 2.80	92.12 ± 9.77	99.49 ± 0.51	91.65 ± 10.09	0.92	0.47
CD105	93.58 ± 5.59	57.41 ± 16.11	80.77 ± 6.70	49.83 ± 4.62	13.86	0.0016

There were no significant differences in the expression of CD34, CD45, CD73 and CD90 in DPSCs at different passages ($P > 0.05$); the expression of CD105 showed a decreasing trend with the increasing donor age ($P < 0.05$)

Multipotent differentiation potential of DPSCs

Oil Red O staining of DPSCs from Groups A, B, C, and D (Fig. 3a) revealed the formation of red lipid droplets in all groups after 21 days of adipogenic induction. Compared with Groups A and C, Groups B and D presented more lipid droplets, with no significant differences between Groups A and C or between Groups B and D.

Alizarin Red staining of DPSCs from Groups A, B, C, and D (Fig. 3b) revealed extensive red mineralized nodule formation after 21 days of osteogenic induction. Compared with Groups A and C, Groups B and D presented more mineralized nodules, with no significant differences between Groups A and C or between Groups B and D.

Quantitative analysis of the staining results (Fig. 3c, d) revealed a shift in agreement with the qualitative results. Statistically significant differences were found between Groups A and B ($P < 0.05$), and between Groups C and D ($P < 0.05$). No significant differences were observed between Groups A and C or between Groups B and D ($P > 0.05$).

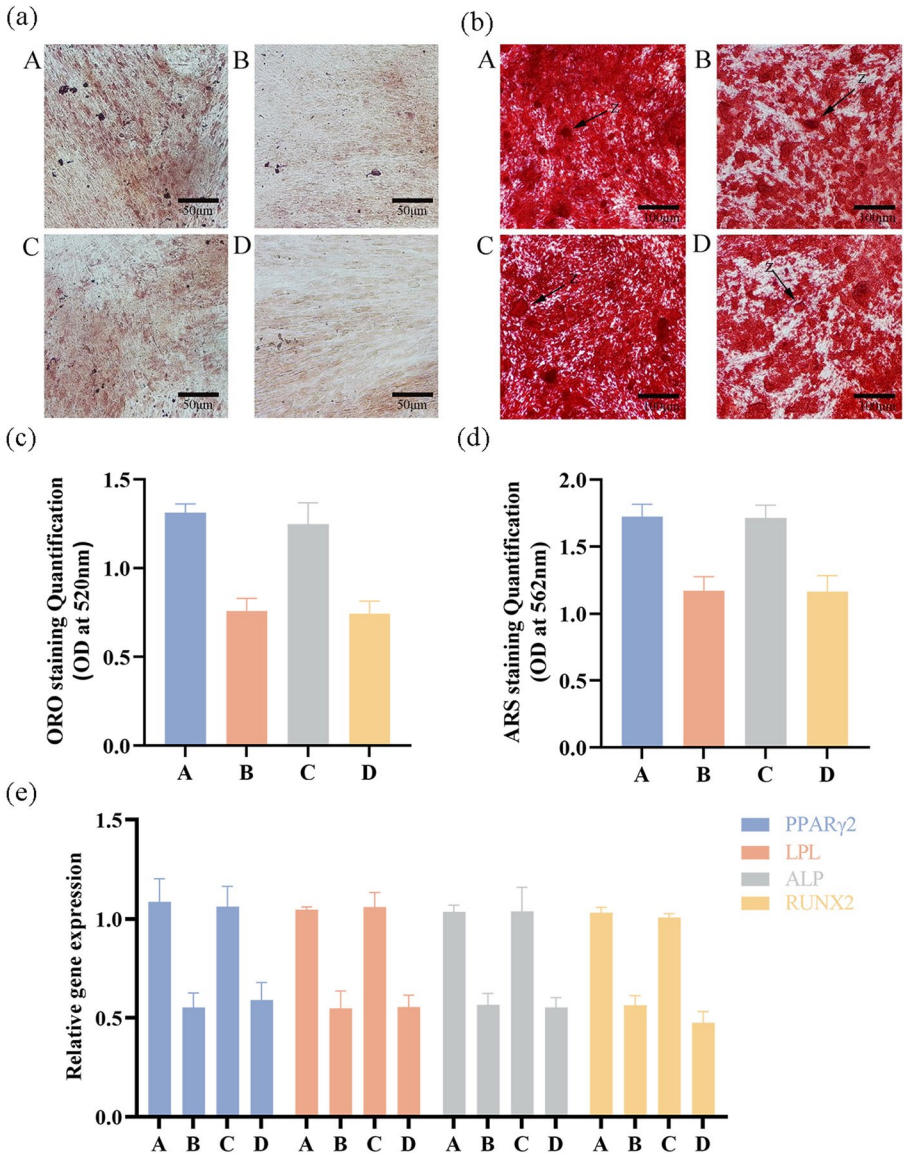

Fig. 3 The multidifferentiation capacity of DPSCs. **a** ORO staining for lipogenic diferentiation: numerous lipid droplets form and are colored red. **b** ARS staining for osteogenic differentiation: numerous calcium nodules have formed and are deeply stained. z: calcium nodule. **c** Quantitative analysis of ORO staining. **d** Quantitative analysis of ARS staining. **e** Real-time fluorescent quantitative PCR results for adipogenic and osteogenic gene expression. Expression of ALP and RUNX2, markers associated with osteogenic differentiation, and PPARγ2 and LPL, markers associated with adipogenic differentiation

The real-time quantitative PCR results (Fig. 3c) indicated that, compared with Groups A and C, Groups B and D highly expressed adipocyte marker genes (PPARγ2, LPL) and osteoblast marker genes (ALP, Runx2), with statistically significant differences ($P < 0.05$). The expression of osteogenic and lipogenic genes decreased in the older donor group. There was no significant difference between groups A and C ($P > 0.05$) or between groups B and D ($P > 0.05$), indicating that gene expression was not associated with chronic periodontitis.

Senescence cell detection results

The expression of SA-β-gal in DPSCs from each group was assessed to determine cell senescence. Under microscopic observation, cells with deep blue cytoplasm were identified as SA-β-gal positive, indicating senescence (Fig. 4a). The SA-β-gal positivity rate was 2.33 ± 0.67% in Group A, 7.02 ± 1.7% in Group B, 2.15 ± 0.55% in Group C, and 7.83 ± 2.23% in Group D. Statistically significant differences were found between Groups A and B ($P < 0.05$), and between Groups C and D ($P < 0.05$). No significant differences were observed between Groups A and C or between Groups B and D ($P > 0.05$) (Fig. 4b). These results indicate that DPSC senescence is related to donor age but not to the presence of periodontitis.

Cell apoptosis detection

Flow cytometry analysis revealed that the percentage of cells in early and late stages of apoptosis was 6.31 ± 0.85% for Group A, 17.8 ± 0.39% for Group B, 6.6 ± 0.91% for Group C, and 17.77 ± 0.75% for Group D (Fig. 5a). Statistically significant differences were detected between Groups A and B ($P < 0.05$), and between Groups C and D ($P < 0.05$). No significant differences were found between Groups A and C ($P > 0.05$), or between Groups B and D ($P > 0.05$) (Fig. 5b). These results indicate that DPSC apoptosis is related to donor age but not to the presence of periodontitis in teeth.

Discussion

Owing to their unique properties and multipotent differentiation potential, mesenchymal stem cells (MSCs) have become promising tools in regenerative medicine [21, 22]. Stem cell applications in tissue engineering have the potential to cure various diseases currently managed with palliative or symptomatic relief, or by preventing their occurrence and progression [23]. As a research focus in tissue regeneration, MSCs are renowned for their accessibility, multipotent differentiation potential, and high proliferative capacity [24]. They play a significant role in tissue regeneration, maintaining homeostasis, and treating diseases [6].

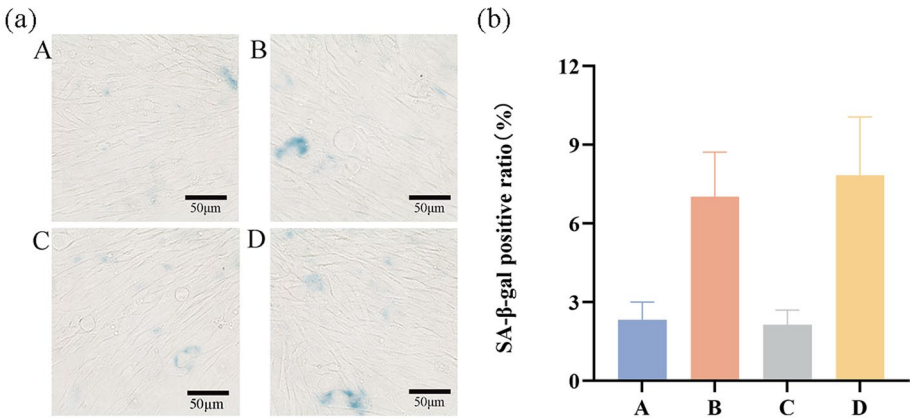

Fig. 4 Senescence of DPSCs. **a** β-Galactosidase staining: cells colored blue are senescent cells. **b** The percentage of positive stained DPSC

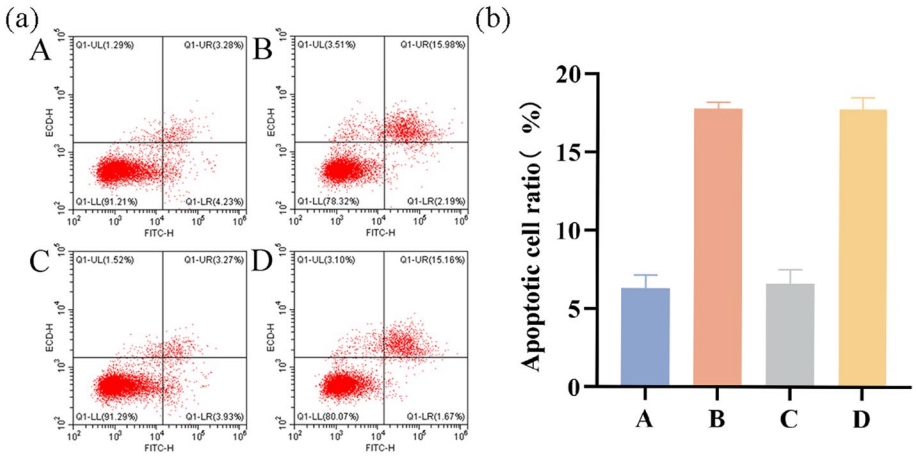

Fig. 5 Degree of apoptosis in dental pulp stem cells. **a** Apoptosis detection for DPSCs from Groups A, B, C, and D. Early and late apoptosis are distinctly compartmentalized from living cells. **b** Quantitative analysis of apoptosis detection

DPSCs are a subset of MSCs that are capable of differentiating into various facial tissues [25]. As an easily accessible source of adult stem cells, DPSCs are emerging as a key type of seed cell in regenerative medicine. They can differentiate into osteoblasts, fibroblasts, cementoblasts, neuronal cells, adipocytes, epithelial cells, etc. [26]. In addition to dental regeneration, DPSCs have potential clinical applications in neurotissue [27], bone [28], retinal [29], and lung tissue regeneration [30, 31] and vascular regeneration [32]. DPSCs are considered to have the best prospects for tissue engineering because of their ease of collection, expansion, preservation, high activity, and low tumorigenicity [11]. However, as with many stem cell applications, challenges exist in the extraction, storage, and culture of dental pulp stem cells. Extracting large quantities of cells raises ethical concerns [7]. Furthermore, low-temperature storage may diminish the biological performance of the cells [9]. Mass expansion in vitro can induce cell senescence [33, 34]. Consequently, DPSCs are scarce and often insufficient to meet the clinical and scientific demands of tissue engineering.

This study explores the expansion of the donor pool to expand the sources of dental pulp stem cells.

The extraction of stem cells from inflammatory tissues offers a promising avenue of exploration. Understanding the impact of inflammation on stem cells is crucial for advancing tissue engineering. Periodontitis is one of the most prevalent oral health issues among adults [13]. Teeth extracted from individuals with periodontitis could serve as a potential donor source. The local effects of periodontitis include gingival inflammation, bleeding on probing, loss of attachment, periodontal pockets, alveolar bone resorption, and tooth loss [35]. Periodontitis is multifactorial, and results from the interplay of bacterial biofilms, host immune responses, genetic predispositions, and environmental elements. It is initiated by microbial dysbiosis and influenced by environmental and genetic factors, along with the virulence of the microbiome, which affects the host immune response [36–38]. Clinical manifestations arise from direct mechanisms (microbial virulence factors) and indirect mechanisms (inflammation

triggered by microorganisms). The inflammatory environment of periodontitis negatively affects PDLSCs. Studies indicate accelerated cellular senescence under periodontitis conditions [39]. Inflammatory cytokines such as TNF-α [40, 41] and IL-1β [42, 43] alter stem cell proliferation, differentiation, migration, and immune regulatory properties. A close connection exists between the periodontium and the dental pulp [17]. The pulp cavity is connected to periodontal tissues via the apical foramen, dentin tubules, and lateral branches of the root canals [19]. Virulence factors and inflammatory molecules produced by microorganisms can enter the pulp cavity and adversely affect its activity [44]. Furthermore, severe periodontal lesions may lead to pulp necrosis [45]. Research indicates that in aggressive periodontitis, the biological properties of DPSCs may be compromised; however, DPSCs retain their defining stem cell characteristics [20]. Aggressive periodontitis, classified as Stage III or IV, Grade C, involves rapid tissue destruction and often results in premature tooth loss in healthy subjects [46]. These characteristics may influence the properties of extracted DPSCs. This study examined mild periodontitis, classified as Stage I or II, Grade A or B. DPSCs were extracted from both healthy subjects and chronic periodontitis patients and cultured. We observed no significant differences in cell morphology, surface markers, proliferation rates, multilineage differentiation potential, or apoptotic rates. Periodontitis does not influence the expression of surface markers, which aligns with previous studies, suggesting that MSC-like stem cells can be isolated even under inflammatory conditions. However, our observations of proliferation and differentiation differ from those of previous studies. Previous studies have suggested that stem cells are influenced by various cytokines in the inflammatory environment, which play crucial roles in several cellular processes that subsequently affect cell proliferation and differentiation [12].

Our findings indicate that slower-progressing, less severe periodontitis does not negatively affect DPSC extraction or viability. Our analysis suggested that the pulp–dentin complex possesses defensive functions, with the pulp and surrounding dentin forming a structural unity against external stimuli [47, 48]. Furthermore, varying concentrations of inflammatory mediators may differentially affect the pulp [47, 48]. The pulp is also capable of generating both innate and adaptive immune responses to inactivate and counter incoming bacteria and their components [47, 48]. A study using gene sequencing and bioinformatics revealed differences in the subgingival flora between patients with aggressive periodontitis and those with chronic periodontitis [49]. These microbial differences may contribute to the varying effects on dental pulp and the characteristics of extracted DPSCs. The experimental outcomes may reflect the defensive mechanisms of the pulp, the mild nature of chronic periodontitis, and complex microbial interactions. Variations in sample sizes and individual differences might also account for these results. The experiments incorporated age as a variable and found that periodontitis did not affect pulp stem cells at any age. This indicates that the age of the donor is not related to the impact of mild periodontitis on the biological properties of DPSCs. This provides strong evidence that individuals with mild periodontitis can serve as donors for pulp stem cells, regardless of their age. However, younger donors generally provide stem cells with superior cellular status compared to those obtained from older donors. These results enhance the understanding of the

impact of inflammation on stem cells, providing a theoretical basis for addressing the shortage of dental pulp stem cells by identifying suitable donor sources.

During the experiment, several challenges were encountered. The clinical acquisition of dental pulp stem cell (DPSCS) donor samples required strict adherence to the ethical review process, resulting in a prolonged sample collection period that could potentially impact the initial experimental progress. To address this, we actively collaborated with the hospital's ethical review board and optimized the sample extraction procedure [50], significantly improving extraction efficiency. Additionally, some batches of DPSCS experienced microbial contamination during the amplification process, compromising experimental reproducibility. To mitigate this, we implemented a more rigorous cell identification protocol and refined the cell culture technique [51], reducing contamination rates in subsequent batches to less than 3%. Furthermore, individual variability in DPSCS proliferation rates and phenotypic expression across different donor sources posed challenges to result consistency. Standardized media formulations [52] were employed to minimize these variations. Ultimately, the experiment was successfully completed.

However, the experiment has certain limitations, and further research should explore the effects of other periodontal disease subtypes on pulp stem cell status. Additionally, constructing a knowledge network based on different stages and grades is necessary to gain a more comprehensive understanding of how periodontitis affects the biological properties of pulp stem cells. In addition, the sample size should be increased to enhance the accuracy of the experimental results. There is still considerable progress to be made in identifying patients with mild periodontitis for the extraction of DPSCs as reserve cells for tissue engineering. It would be more advantageous to address the shortage of pulp stem cells by utilizing teeth with mild periodontal lesions, rather than dismissing their potential utility.

Conclusion

The biological properties of dental pulp stem cells (DPSCs) remain unchanged in teeth with mild periodontitis, offering valuable insights for addressing the shortage of DPSCs in tissue engineering. Such teeth have the potential to serve as sources for pulp stem cell donors.

Materials and methods

Experimental reagents and equipment

Main reagents

α-MEM (Procell, China); penicillin–streptomycin, double antibiotic (Procell, China); phosphate-buffered saline (PBS) (Procell, China); fetal bovine serum (FBS) (Procell, China); 0.25% trypsin (Procell, China); CCK-8 assay kit (Beyotime, China); β-galactosidase detection kit (Beyotime, China); mesenchymal stem cell (MSC) osteogenic induction medium (Procell, China); MSC adipogenic induction medium (Procell, China); CD34, CD45, CD73, CD90, and CD105 antibodies (Elabscience, China); Oil Red O (Procell, China); Alizarin Red (Procell, China); RIPA lysis buffer (Beyotime, China); trizol reagent (Procell, China); type I collagenase (Solarbio, China); cell apoptosis assay kit (Abbkine, China); RT-PCR reverse transcription kit (Elabscience, China).

Main instruments

Cell Culture Incubator (WIGGENS, Germany); Microplate Reader (Bio-TEX, USA); Low-Speed Centrifuge (Zhongke, China); Electron Microscope (Hitachi, Japan); Laminar Flow Hood (Jinghua, China); High-Speed Centrifuge (Xiangyi, China); Ultracentrifuge (Himac, Japan); Flow Cytometer (DxFLEX, USA); Real-Time Quantitative PCR Machine (Bio-Rad, USA).

Experimental methods

Isolation and culture of DPSCs

Teeth were collected from 12 patients (informed consent was signed) visiting the Department of Oral and Maxillofacial Surgery at Qingdao Stomatology Hospital, and informed consent obtained (ethical approval number: 2023KQYX060, 2 May 2023–1 September 2024). Patients were divided into 4 groups, each consisting of 3 individuals. Healthy subjects were assigned to Group A (ages 29–33) and Group B (ages 52–54), whereas periodontitis patients (clinically diagnosed with Stage I or II, Grade A or B) were categorized into Group C (ages 31–33) and Group D (ages 51–53). The sample selected for this experiment consisted of six males and six females: one male and two females in Group A; two males and one female in Group B; one male and two females in Group C; and two males and one female in Group D. The third molars of the selected teeth were removed for treatment. Groups A and B were free of caries, pulpitis, apical periodontitis, and other dental or periodontal diseases. Groups C and D had only mild periodontitis (clinically diagnosed as Stage I or II, Class A or B) and no other dental or periodontal diseases. No systemic diseases in any group.

Teeth from each group were subjected to pulp extraction, and the modified tissue block method was used to isolate primary cells [50, 53]. The tissue was cut into blocks approximately 1.0 mm^3 in size, digested with collagenase type I (3 mg/ml) for 15 min, and used for cell extraction. Monoclonal cells were selected through limited dilution to purify DPSCs. Once the cells reached approximately 80% confluence, they were passaged and cultured in α-MEM supplemented with an appropriate concentration of fetal bovine serum and double antibiotics. All the cells were stored under constant humidity at 37 °C and 5% CO_2. The morphological characteristics of dental pulp stem cells were qualitatively analyzed through microscopic observation, focusing on cell shape, size, and cellular debris and inclusions.

Cell proliferation assay

DPSCs were seeded at a density of 1×10^4 cells per well in a 96-well culture plate and incubated for 5 days. At each designated time point, the complete medium was replaced with fresh α-MEM basal medium containing 10% CCK-8 solution, and the mixture was incubated in the dark at 37 °C for 2 h. The absorbance at 450 nm was measured via a microplate reader to assess cell proliferation capacity.

Surface antigen analysis of DPSCs

The DPSCs were digested, centrifuged, and resuspended in PBS to adjust the cell density to 1×10^7 cells/mL. Then, 100 μL of the cell suspension was added to a centrifuge

tube, and fluorescently labeled antibodies against CD34, CD45, CD73, CD90, and CD105 were added at a ratio of 50:1 to the cell suspension. The control group was incubated with PBS. The mixture was incubated in the dark at 4 °C for 2 h, and then centrifuged and the supernatant was discarded. The cells were washed twice with PBS and resuspended them in 200 μL of PBS. The cells were analyzed via a flow cytometer.

Multipotent differentiation potential of DPSCs

The DPSCs were digested, centrifuged, and resuspended in complete culture medium. The cells were plated at a density of 2×10^5 cells per well in a 6-well cell culture plate. Once the cells reached the appropriate density, the medium was changed according to the differentiation induction kit instructions, and 1.5 mL per well was used. After 21 days of induction, the culture medium was discarded, the cells were washed twice with PBS, and the cells were fixed with cell fixation solution for 30 min. The cells were washed with PBS again, stained with Oil Red O and Alizarin Red, and observed under a light microscope. After observation, the stained plates for adipogenesis and osteogenesis were dissolved using isopropanol and 10% cetylpyridinium chloride solution, respectively. The decolorized solutions were then collected, and their absorbance was measured at 520 nm and 562 nm using a microplate reader.

Detection of osteogenic and adipogenic-related genes

At the end of the multidifferentiation induction, the culture medium was discarded, and the cells were washed twice with PBS. Total RNA was extracted from each cell group according to the reagent kit instructions, cDNA was synthesized, and the samples were stored at (− 20 °C) for later use. GAPDH was used as the internal reference. The sequences of primers used were as follows:

- ALP:
  - Forward: 5′-CCCAAGAATAAAACTGATGTG-3′
  - Reverse: 5′-CTTCCAGGTGTCAACGAG-3′
- RunX2:
  - Forward: 5′-GAATGCCTCTGCTGTTATG-3′
  - Reverse: 5′-ACTCTTGCCTCGTCCACT-3′
- PPARγ2:
  - Forward: 5′-GGTTGACACAGAGATGCC-3′
  - Reverse: 5′-TGGAGTAGAAATGCTGGAGA-3′
- LPL:
  - Forward: 5′-ATCCCATTCACTCTGCCT-3′
  - Reverse: 5′-GTCTCTCCTGCTTTTACTCTG-3′

- GAPDH:
  - Forward: 5′-CAAGGCTGAGAACGGGAAGC-3′
  - Reverse: 5′-AGGGGGCAGAGATGATGACC-3′.

Follow the instructions in the reagent manual and use a real-time quantitative PCR machine to determine the expression levels of these genes. Analyze the data using the $2^{-\Delta\Delta CT}$ method.

Cellular senescence assay

The DPSCs were digested, centrifuged, and resuspended in complete culture medium. The cells were plated at a density of 2×10^5 cells per well in a 6-well cell culture plate. Assess the activity of SA-β-gal. The presence of a deep blue color under the microscope indicates positive staining. Six fields of view were randomly selected for observation and counting, and the percentage of positively stained cells among the total cell population was analyzed.

Cell apoptosis assay

The DPSCs were digested, centrifuged, and resuspended in PBS. A total of 2×10^5 cells were resuspended in 100 μL of 1×Annexin V binding buffer, and 5 μL of Annexin V-AbFlourTM488 and 2 μL of PI were added to each 100 μL of cell suspension. The mixture was incubated in the dark on ice for 15 min. After incubation, 400 μL of 1×Annexin V binding buffer was added, the mixture was incubated on ice, and the samples were analyzed via a flow cytometer.

Statistical analysis

Data analysis was performed via GraphPad Prism 10. Differences between two groups were compared via an independent samples *t*-test (LSD-t), and differences among multiple groups were analyzed via one-way ANOVA. $P<0.05$ was considered statistically significant.

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Author contributions
A and G conceptualized the study. A and B designed the method. A and C performed the data analysis. E and D provided resources and interpreted the data. A, E and F acquired the data. A wrote the original draft preparation, and G reviewed and edited the manuscript. G supervised the study. All the authors have read and agreed to the published version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Qingdao Stomatological Hospital (2023KQYX060, June 2023). Informed consent was obtained from all the subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

Competing interests
The authors declare no competing interests.

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