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Original Article

Exploring the impact of culture techniques and patient demographics on the success rate of primary culture of human periodontal ligament stem cells

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KEYWORDS
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Abstract *Background/purpose:* Periodontal ligament stem cells (PDLSCs) have the potential for regenerating periodontal tissue. The study aims to investigate the impact of demographics (ages, gender, disease) and culture techniques (shipping storage time and culture method) on the success of primary culture.
Materials and methods: PDLSCs were collected from 51 teeth of 26 patients and cultured via outgrowth (OG) and enzymatic digestion (ED) methods. Cells characteristics were confirmed by flow cytometry, MTT, and ARS. The primary culture success rate was evaluated with a serial chi-square test to determine the relationship with culture technique (ED/OG and $\leq$4 h/prolonged culture) and patient demographics (Young/Old, Female/Male, and Health/Periodontitis).

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Results: The overall success rate of Health group (69.7%) was higher than Periodontitis (38.9%). Culturing within 4 h possessed a higher success rate (71.8%) than prolonged group (16.7%) regardless of patient demographics, and using OG method (81.5%) revealed more promising. Subgroup analysis of 39 cases (culture within 4 h) found that the success rate of OG was higher than ED in the Old group (87.5%–25.0%) and in the Periodontitis group (83.3%–25.0%).
Conclusion: Primary culturing of PDLSCs within 4 h and using the outgrowth method led to higher success rates regardless of patient demographics. It can achieve successful PDLSCs culture of older patients or patients with periodontal disease by appropriate culture technique.
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Introduction

Periodontitis is a prevalent disease in the oral region that causes the destruction of the tooth supporting tissues, including alveolar bone, periodontal ligament (PDL), and root cementum. Several approaches were suggested in the treatment of periodontal disease, including guided tissue regeneration (GTR) or guided bone regeneration (GBR), application of enamel matrix derivative, and various growth factors.[1–3] However, the presence of healthy periodontal tissue around the tooth or implant is the prerequisite for GTR or GBR, and there are currently insufficient techniques to regenerate complete periodontal tissue, including alveolar bone, cementum, and well-oriented collagen fibers.[4,5]

Stem cell research has become a promising field for tissue regeneration and application of regenerative medicine. Mesenchymal stem cells (MSCs) generate from bone marrow, and comparable MSCs populations derived from other tissues have been characterized. The presence of adult stem cells in dental tissues revealed as multipotent stem cells with capability of self-renewal and differentiation into numerous cell types.[6–8] Periodontal ligament stem cells (PDLSCs), isolation of adult stem cells from human PDL tissues, has become new opportunities for dental tissue engineering.[4] And cell-based approaches have beneficial effects on periodontal tissue regeneration, such as positive effects on new bone, cementum, and PDL formation in periodontal defects.[9]

The effectiveness of cell therapy depends on the different conditions of the cell donor, cell acquisition, processing, and shipping storage conditions.[10–13] The storage solution and time from specimen acquisition until the laboratory begins to carry out primary culture affect cell survival rate;[4,14] however, there are few literatures on the effect of shipping storage time on the viability and success rate of primary cultured cells.

Culture methods also affect cell differentiation, outgrowth (OG) method and enzymatic digestion (ED) method are commonly used to obtain primary cultured cells from tooth tissue;[3,15–17] however, the results were different among the research teams. Tanaka et al. reported that the PDLSCs cultured by ED method showed a higher proliferation rate and mesenchymal stem cell-like properties than cultured by OG method.[17] But Tran et al. pointed out both ED and OG method did not influence the cells immunophenotypic profiles and the proliferation rate are similar in the two different cultural methods.[16] The conflicting results may be due to their different culture media and related culture conditions.[18,19] Therefore, establishment of efficient protocols for primary culture of human PDLSCs experimental research is essential.

The aims of the study were to explore the effect of factors including demographics (ages, gender, disease) and culture techniques (shipping storage time and culture method) on the success rate of primary periodontal cell culture by analyzing 51 teeth from 26 patients. By improving the success rate of primary periodontal cell culture, the application of personal cell banking in the future can be realized, providing effective benefits for regenerative medicine, and establishing clinically applicable solutions for future periodontal tissue reconstruction.

Materials and methods

Patient selection and demographics

All participants were outpatients without pregnancy, smoking habit, significant systemic disease, or immune defects. Patients were requested to sign an informed consent. This study was performed in accordance with the guidelines of the Ethics Committee of the Institutional Review Board (approval no. CMUH102-REC3-134) at China Medical University Hospital, Taichung, Taiwan.

51 teeth extracted from 26 patients as shown in Fig. 1A. The criteria for extracting teeth with severe periodontitis included pocket depth over 7 mm, bone loss over 65%, grade 3 mobility, and Class III furcation involvement.[20–22] There was no significant age difference between genders or between culture methods (Table 1). However, patients with periodontitis are inherently older than healthy patients (see Table 2).

Teeth preparation

Human teeth were extracted and immersed into culture medium: Dulbecco's modified eagle medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA) with 10% PSA (1000 IU/mL penicillin, 1000 μg/mL of streptomycin and 2.5 μg/mL amphotericin B) (Antibiotic-Antimycotic, Gibco). The dental specimen was stored at 4 °C and brought to the laboratory for culture after the end of dental clinic. The

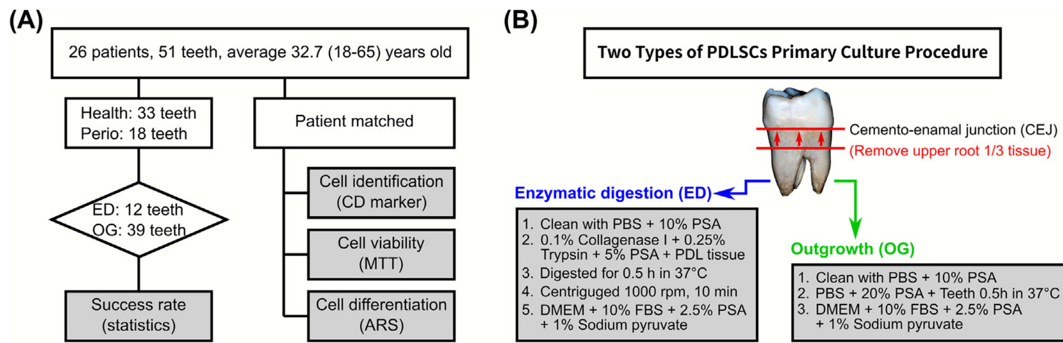

Figure 1 Experimental scheme of human periodontal ligament stem cells (PDLSCs) primary culture with Enzymatic digestion (ED) and outgrowth method (OG). (A) Human PDLSCs were primary cultured with Enzymatic digestion and outgrowth methods. 51 teeth, of which 33 were healthy teeth (Health) and 18 were extracted from teeth with periodontitis (Perio). Cell viability, cell differentiation and cell identification were performed for patient matched sample. (B) ED method: root surface was scraped with surgical blade, cut into small pieces, and treated with collagenase. OG method: whole root was explanted without collagenase. CD marker: cluster of differentiation; MTT: MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay; ARS: Alizarin Red S, an anthraquinone dye, to evaluate calcium deposits in differentiated cells.

time of period from specimen storage to lab culture usually takes 1—20 h, which was then divided into two groups: within 4 h and prolonged (5—20 h). Usually, a clinical visit lasts approximately 4 h. Thus most samples were taken to the laboratory for initial cultivation after the clinical visit, which usually over 5 h. Some of the samples were transfer to the laboratory immediately during the break period (within 4 h). The records were all kept for further analysis.

The upper third portion of the root soft tissue was scraped using #15 scalpel. The middle and lower third portion of the root soft tissue were left for experiments. For removal of microorganisms during operation, the root-containing solution was triple times vibrated with vortex mixture for 30 s and then changed medium with DMEM (Gibco) containing 10% FBS (HyClone) and 2.5% PSA (Gibco) at 37 °C in 5% CO_2 incubator for 8 h (Fig. 1B).

Table 1 Demographic data of study population (n = 51). Patients were catalogued according to their genders (male/female), health condition (health/periodontitis), and primary culture methods (Enzymatic digestion/Outgrowth).

Patient characteristics	Patient numbers in different age interval	Ages (year) Mean ± SD (Median)	Statistics significances
Genders: Male	18∼23 y: 7 24∼40 y: 3 41∼65 y: 7 Total (%): 17 (33.3%)	37.5 ± 17.6 (28)	$P = 0.48979$
Female	18∼23 y: 13 24∼40 y: 13 41∼65 y: 8 Total (%): 34 (66.7%)	30.4 ± 12.0 (26)	
Diseases: Health	18∼23 y: 20 24∼40 y: 9 41∼65 y: 4 Total (%): 33 (64.7%)	26.3 ± 10.0 (23)	***$P = 0.00073$
Periodontitis (Perio)	18∼23 y: 0 24∼40 y: 7 41∼65 y: 11 Total (%): 18 (35.3%)	45.0 ± 13.7 (48)	
Methods: Enzymatic digestion (ED)	18∼23 y: 6 24∼40 y: 2 41∼65 y: 4 Total (%): 12 (23.5%)	34.1 ± 18.4 (24.5)	$P = 0.57148$
Outgrowth (OG)	18∼23 y: 14 24∼40 y: 14 41∼65 y: 11 Total (%): 39 (76.5%)	32.5 ± 13.3 (27)	

Table 2 Comparison of studies on human derived primary culture PDL cells/PDLSCs.

Author	Age and p't number[a]	Cell type[b]	Culture method	Specimen management	Digested agent	Medium	Confluence time and results
Park, Kim, Jung, Kim, Choi, Cho and Kim[36]	16∼29 yrs 6p't's premolars	PDLSCs	Enzymatic digestion	PDL tissues minced into the smallest pieces	3 mg/mL Collagenase (I) + 4 mg/mL Dispase	Alpha MEM+ 15%FBS	No mentioned. PDLSCs showed more mineralization nodules than p-PDLSCs
	42∼57 ys, 6p't's premolars	p-PDLSCs	Enzymatic digestion	Inflammatory granulation tissue	3 mg/mL Collagenase (I) + 4 mg/mL Dispase	Alpha MEM+ 15%FBS	No mentioned. p-PDLSCs showed more migratory capacity than PDLSCs.
Tran, Doan, Le and Ngo[15]	Not provide ortho ext.	PDLCs	Outgrowth method	Small fragments of PDL were placed	None	DMEM + F12	3 wks (35 mm dish), then 5–7 days after subculturing. Easy, although a little long in beginning.
			Enzymatic digestion	PDL tissues minced into the smallest pieces	3 mg/mL Collagenase (I)+ 4 mg/mL Dispase	DMEM + F12	2 wks (35 mm dish), then 5–7 days after subculturing. Difficulty of technique sensitivity.
Tanaka, Iwasaki, Feghali, Komaki, Ishikawa and Izumi[16]	16∼40 yrs, 19p't's, 3rd molars	PDLCs	Outgrowth method, cut into small pieces	Middle root scraped with dental scaler	None	DMEM+ 10%FBS	3 colonies (Per dish). Fibroblasts were the prominent cell type in outgrowth PDL cells.
			Enzymatic digestion	Minced using #15 surgical scalpel	0.25% Trypsin+ 0.1% EDTA (60 min, 37 °C)	DMEM+ 10%FBS	160 colonies (Per dish). Higher growth rate, colony-forming activity, and capacity to differentiate.
This study	26p't (Health 16, Perio 10), 51 teeth, 18–65yrs, ortho ext. or Perio ext.	PDLSCs (25) p-PDLSCs (14) GMSCs (3) p-GMSCs (3)	Outgrowth method	Whole tooth explant, only remove gingival tissue with #15 scalpel	None	DMEM+ 10%FBS	3–4 wks (100 mm dish), then 5–7 days after subculturing. Easy, even though a little long in the beginning.
		PDLSCs (8) p-PDLSCs (4)	Enzymatic digestion	Root surface scraped with surgical blade, cut into small pieces	0.1% Collagenase (I) +0.25% Trypsin (60 min, 37 °C)	DMEM+ 10%FBS	2–3 wks (100 mm dish), then 5–7 days after subculturing. Difficulty of technique sensitivity.

[a] p't: patient, ortho ext.: orthodontic extraction, Perio ext.: extraction due to periodontal disease.
[b] PDLSCs: periodontal ligament stem cells, GMSCs: gingival mesenchymal stem cells, p-PDLSCs/p-GMSCs means PDLSCs/GMSCs cultured from patients with periodontitis.

Outgrowth (OG) and enzymatic digestion (ED) methods of primary culture

The conditioned culture medium used in this study consisted of DMEM containing 4.5 mg/mL glucose (Gibco), 1% sodium pyruvate (Gibco), 10% FBS (HyClone), and 1% PSA (Gibco).[3,15—17] For collection of OG-PDLSCs, the prepared whole root was immersed in conditioned culture medium and incubated at 37 °C in 5% CO_2.

For the ED method, the soft tissue of prepared teeth was minced into 1×1 mm^2 pieces with #15 scalpel. Then, we immersed these pieces into a solution of 0.25% trypsin—0.1% EDTA (Gibco) along with 0.1 U/mL collagenase (Gibco). The solution was maintained at a temperature of 37 °C for 60 min. During the incubation, the tissue-containing solution was mixed using vortex mixture 30 s per 10 min. After tissue digested with enzymes, 4 mL of culture medium was added and passed through a cell strainer (70 μm pore size) to remove the aggregated cells and tissues.[15—17] The pass-through was then centrifuged at 1000 rpm for 10 min. The resulting cell pellets were resuspended in 2 mL of culture medium and seeded in 10 cm diameter dishes.

For both the OG and ED methods, the conditioned culture medium was replaced every 3 days. Once the cells reached 80% sub-confluency, they were passaged using a 0.25% trypsin-0.1% EDTA (Gibco) solution. After 2nd passage (P2) reached to 80% sub-confluent, the 10% DMSO cryoprotectant was added. 1×10^6 cells/mL were placed in 1.8 mL cryovials and placed in a cell freezing container (CoolCell LX, Corning Incorporated, Corning, NY, USA), then stored in a freezer at −80 °C for 8 h before being transferred to liquid nitrogen at −196 °C for long term storage. For subsequent experiments, P3 cells that had been thawed are primarily used for culture, while P5 is the generation suitable for clinical use.

Definition of the primary culture success rate

The success of cell subculture is defined as obtaining pollution-free primary cells (P0) from ED or OG that can cover 80% of a 10 cm culture dish. The primary culture success rate is calculated as the percentage of specimens with successful cell subculture divided by the total number of specimens. All experiments used cell passages no higher than P7. To characterize PDLSCs, we performed cell viability analysis (MTT) (Sigma—Aldrich), cell differentiation (ARS, Alizarin red S) (Sigma—Aldrich), and cell marker confirmation (Flow cytometry) (BD Biosciences). And keratinized gingiva (as comparison) was prepared from patient matched specimen using the same medium to obtain gingival mesenchymal stem cells (GMSCs) by outgrowth method.

Grouping for analysis

To analyze the success rates of primary cell cultures, 51 teeth were divided into 2 subgroups based on 5 different classifications: age, gender, disease, method, and storage time. The age categories included young adults (18—40 years old) and older adults (41—65 years old), i.e., Age (Young/Old), while the gender categories were female and male, i.e., Gender (Female/Male). The disease categories were healthy teeth and teeth with periodontitis, i.e., Disease (Health/Perio). Two different methods of culture techniques were used: Method (ED/OG). The storage time was divided into two groups: those stored for within 4 h and those stored for a prolonged time (5—20 h), i.e., Time ($\leq$4 h/$\geq$ 5 h).

Cell viability (MTT)

In this experiment, a patient matched experiment was used to culture the 3rd passage (P3) of PDLSCs and GMSCs from the extracted teeth and surrounding keratinized gingiva of the same orthodontic treatment patient. Cultured 1×10^5 cells in a 6-well culture dish, add 2 mL of culture medium; removed the culture medium after 48 h, washed once with PBS, and then added cells containing 0.5 mg/mL MTT (Sigma—Aldrich) culture medium for 2—4 h of incubation. When the reaction time was over, absorbed the culture medium dry, added isopropanol containing low concentration of hydrochloric acid to elute the purple crystals deposited on the bottom of the culture dish. The results were interpreted with a spectrophotometer (Bio-Rad, Hercules, CA, USA) with a detection wavelength of 570 nm.[23]

Cell differentiation (ARS staining assay)

Cells (1×10^5, P3) were cultured in a 10 cm culture dish with a volume of 10 mL of culture medium. After 24 h of culture, replace with the culture medium with osteoinductive supplements (DMEM/H, 10% FBS, 1% Sodium pyruvate, 1% PSA, 10 nM Dexamethasone, 100 mg/mL L-Ascorbic acid, 10 mM Glycerol 2-phosphate disodium salt hydrate), the osteoinductive cell culture medium was changed every 3 days. After being cultured in osteoinductive cell culture medium for 14 days, washed twice with PBS, fix with paraformaldehyde at room temperature for 30 min, washed once with PBS and add 2% alizarin red solution for 30 min after the PBS was blotted dry. Drain the Alizarin Red dye, washed it twice with deionized water until the background value faded, and observed the results under a microscope.[24] After dissolving the precipitation in a 0.1 N NaOH solution, complete dissolution can be detected at a wavelength of 548 nm, which allows for quantitative detection of mineralization ability.

Cell identification (flow cytometry)

The 5th passage (P5) of PDLSCs and GMSCs were used for this experiment. After filtering through a cell strainer 70 μm sieve, washed with PBS and blocking buffer (2% FBS in PBS), counted 1×10^6 cells, and then added 5 μL CD34 (BD Biosciences, Catalog No: 555822), CD49d (BD Biosciences, Catalog No: 555503), CD90 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD106 (BD Biosciences, Catalog No: 565417) and CD166 (BD Biosciences, Catalog No: 565461), incubated at room temperature for 30 min in the dark, and washed the cells twice with PBS after 30 min to remove non-specific antibody binding. In addition, the

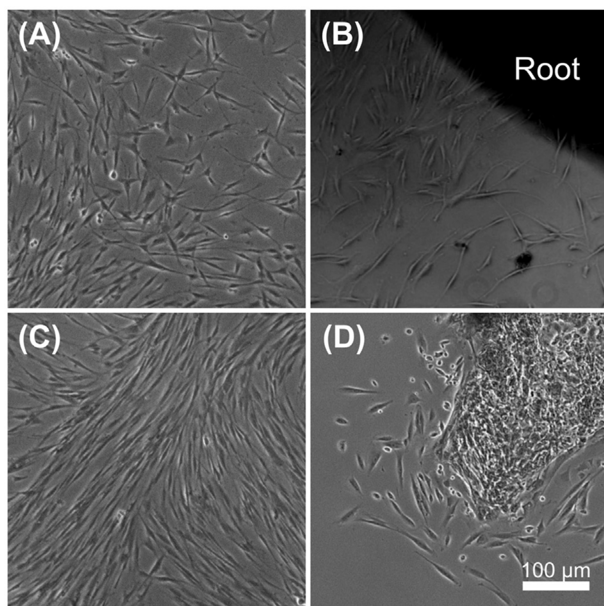

Figure 2 Microscopic observation in human primary cells of periodontal ligament cells (PDLCs) and gingival fibroblasts (GFs) were cultured with enzymatic digestion or outgrowth culture method. (A) Enzymatic digestion of PDLCs were cultured for 48 h. (B) Outgrowth of PDLCs migrated from the tooth root ("Root" in the upright corner) after 48 h. (C) Outgrowth of PDLCs were shown obviously high proliferation status after 48 h. (D) Outgrowth of GFs from the gingival tissue were cultured for 48 h.

cell suspension only added with PBS was used as the experimental control group. Samples were analyzed with flow cytometer BD FACSCanto™ Clinical software. The data results were analyzed and drawn with BD FACSDiva™ Software (BD Biosciences).

Statistical analysis

The data are presented as mean ± S.E.M. To detect the differences among groups, data were analyzed using SPSS 29.0 (IBM-SPSS), and one-way ANOVA with Tukey's HSD post hoc comparison was applied. For nominal variables, the chi-squared test was applied, completed by the Spearman's Rho test[25,26] and the Ordinal Somers' D test[19,27] when both variables were ordinal. Statistical significance was set at $P < 0.05$.

Results

Cell morphology of primary cultured cell

The ED primary periodontal ligament cells (PDLCs) were polygonal in shape and adhered to the culture dish scattered (Fig. 2A). In contrast, the OG primary PDLCs appeared spindle-shaped and were evenly distributed, having crawled out from the root surface (Fig. 2B). After 48 h, OG primary PDLCs displayed a flowy sheet-like growth pattern, indicating that the cells were in excellent proliferation state (Fig. 2C). For comparison, we obtained keratinized gingival tissue from the vicinity of the teeth and cultured it using the OG method for 48 h. The primary gingival fibroblasts (GFs) migrated outward from the gingival tissue and presented a spindle shape (Fig. 2D).

Overall success rate of primary cell culture (51 cases)

The success rates of the five categories were summarized in Fig. 3 The success rates between the subgroups in Age, Gender, and Methods categories shown no significant difference, while the success rates between the subgroups were different in Disease and Time categories (Fig. 3A–E). In the Disease category, the success rate of Health (69.7%) was higher than that of Perio (38.9%). In the Time category, the success rate of ≤4 h (71.8%) was higher than that of ≥5 h (16.7%). The analysis of success rates in the Health and Perio subgroups in the Time category revealed that the ≤4 h subgroup outperformed the ≥5 h subgroup in both subgroups (Fig. 3F).

Success rate of subgroup cultured within 4 h (39 cases)

Considering the shipping storage time significantly affected the success rate, we therefore analysis the success rate in 39 cases of ≤4 h by patient demographics (Young/Old, Female/Male, and Health/Perio) and culture methods (OG/ED). Interestingly, patient demographics did not affect the success rates, as there was no significant difference observed between Young/Old, Female/Male, and Health/Perio (Fig. 3G–I). The success rate of OG (81.5%) was higher than ED (50.0%) (Fig. 3J). In the Method category, OG had a higher success rate than ED, with 87.5% success in the elder population and 83.3% success in periodontal disease cases. However, in the Health and Young subgroup, there was no significant difference in success rates between the two methods (Fig. 3K–M).

Comparing cell viability by groups and methods (P3 cells)

In this patient-matching experiment, both in the Health and Perio groups, the cell viability of OG obtained by primary culture method was higher than that of ED ($P < 0.01$, $P < 0.001$). MTT assay showed that the OG method was more active than ED method (Fig. 4A). Therefore, all subsequent experiments used the OG method culture cells.

ARS staining of cell mineralization in healthy PDLSCs and GMSCs by OG method (P3 cells)

In this patient-matching experiment, the teeth of healthy patients and the surrounding keratinized gingival tissue were removed and cultured by OG method. The keratinized gingival tissue was cultured into GMSCs and tooth roots surrounding tissue was cultured into PDLSCs. There was a significant change in mineralized red granules in PDLSCs from day 2 to day 14, but there was no significant change in

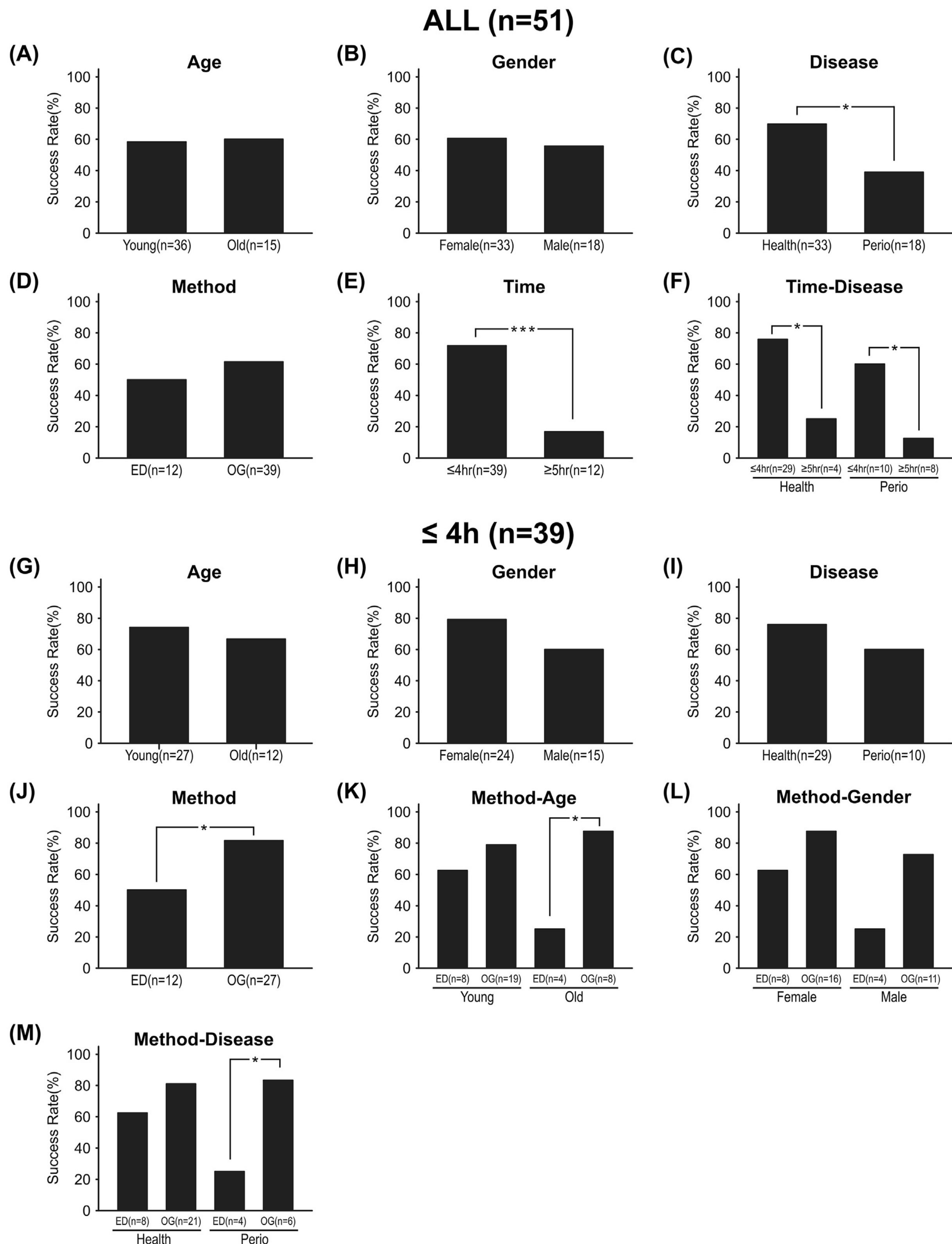

Figure 3 Summarized the success rate in different patient groups and cultural method groups. Success rate in Health and in periodontitis (Perio) periodontal ligament stem cells (PDLSCs) with enzymatic digestion (ED) and outgrowth (OG) primary culture methods. (*$P < 0.05$, ***$P < 0.001$).

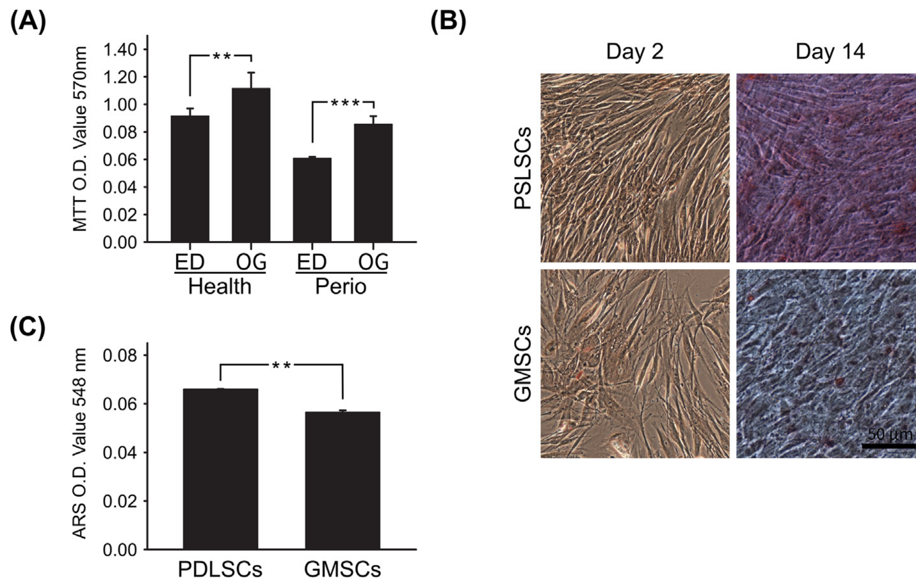

Figure 4 The results of cell viability and differentiation were according to patient matched data. (A) Periodontal ligament stem cells (PDLSCs) viability of MTT assay was tested on ED and OG primary culture methods in Health and Perio groups. (B) Alizarin red S staining of cell mineralization was tested in healthy PDLSCs and gingival mesenchymal stem cells (GMSCs) at day 2 and day 14. (C) The optical density (O.D.) at 548 nm represents mineral ability in cultured PDLSCs and GMSCs. (**$P < 0.01$, ***$P < 0.001$).

GMSCs (Fig. 4C). PDLSCs showed higher cell differentiation ability than GMSCs (Fig. 4B).

Identification of PDLSCs and GMSCs (P5 cells)

Monoclonal antibodies against CD34, CD49d, CD90, CD106, and CD166 were used to stain isolated PDLSCs and GMSCs, which were then analyzed by flow cytometry. Results showed that both PDLSCs and GMSCs were negative for CD34, while CD90, CD106, and CD166 were all positive. Notably, the percentage of CD49d-positive cells was significantly higher in GMSCs (78.5%) than in PDLSCs (13.8%) (Fig. 5). This finding is particularly useful in distinguishing between PDLSCs and GMSCs.

Discussion

In this experiment, females had more cases, and the age of males and females was similar. The OG group had more cases than that of ED, but the age of the two groups was similar. Healthy individuals had more cases and were younger, while periodontal patients were older ($P < 0.001$), which probably because periodontal disease occurs more frequently in older patients (Table 1). However, the overall success rates were similar for both young and old populations as shown in Fig. 3A. The success rate of primary culture for the Periodontitis group was significantly lower than that of the Health group (Fig. 3C, $P < 0.05$). This could be attributed to the destruction caused by periodontal disease, leading to a decrease in the number of residual PDL cells on the root surface. While some studies have suggested that younger patients have better cell proliferation and differentiation,[12] our experiment highlights that the impact of the disease on the success rate is much greater than that of age. Our results also showed that the gender factor has little influence on the success rate.

PDL cells have a survival time of less than 1 h in a dry environment.[28] Additionally, the storage time of the specimens is also affected by the type of culture medium used.[11,14] In this study, we found that the optimal time for the experiment was within 4 h (Fig. 3E, $P < 0.001$). When

Cell Type	CD34	CD49d	CD90	CD106	CD166
PDLSCs	0.1%	13.8%	98.8%	5.1%	100.0%
GMSCs	0.1%	78.5%	98.8%	16.0%	99.9%

Figure 5 The isolated periodontal ligament stem cells (PDLSCs) and gingival mesenchymal stem cells (GMSCs) were stained with monoclonal antibodies against to CD 34, CD49d, CD90, CD106 and CD166 followed by flow cytometric analysis. Both PDLSCs and GMSCs CD34 are negative; CD90, CD106 and CD166 are positive. GMSCs is higher in CD49d than PDLSCs.

using the OG method, the success rate was higher than the ED method (Fig. 3J, $P < 0.05$). Moazami et al. found that the use of DMEM, HBSS, powdered milk and soymilk can prolong the survival of cells to 8 h,[14] but they use trypan blue staining assay which cannot reveal the cell viability. On the other hand, MTT was used in this experiment to detect the oxidation-reduction ability of mitochondria, which can better represent the true cell viability.

Our results indicate that the OG method outperforms the ED method. Different culture methods may lead to varying levels of cell viability. Scholars have used either collagenase[29] or trypsin[17] for the ED method, resulting in different characteristics of primary cultured cells and varying results. In this experiment, the ED method involved physically scraping the tooth root tissue before chemical enzymatic digestion, which could be more harmful to the cells. While there was little difference observed in the healthy group, the impact on periodontal damage specimens with less residual tissue may differ. However, the OG method demonstrated little difference, and its experimental steps were relatively simple, with high reproducibility. Even in periodontitis patient, cell viability of OG was better than ED.

Although Health-PDLSCs are known to possess superior osteogenic potential compared to Perio-PDLSCs,[30] recent research by Tang et al. highlighted the importance of cell proliferation and migration abilities of Perio-PDLSCs.[10] These findings suggest that Perio-PDLSCs also have significant potential for use in periodontal regeneration. By utilizing the OG culture method, it is possible to culture Perio-PDLSCs for tissue engineering applications. Alizarin Red S is an acidic dye that forms red nodules with mineralized substances like calcium and magnesium in tissues or cells.[31] In this experiment, red mineralized granules appeared in PDLSCs after 2 weeks of osteogenic medium, but not in GMSCs. However, GMSCs reveal mineralized particles after 3 weeks of culture,[32] implying that the mineralization time of GMSCs is slower than that of PDLSCs. Future research on GMSCs will help to understand the physiological characteristics of keratinized and non-keratinized gingiva and contribute to the clinical application of regenerative medicine in periodontal disease.

Periodontal tissue includes gingiva, periodontal ligament, cementum, alveolar bone. The gingiva and PDL are located close to each other, but their functions are different. In addition to obtaining PDL and gingival tissue according to different anatomical locations, we also used CD markers to identify the PDLSCs and GMSCs. PDLSCs and GMSCs were positive for stromal cell markers extracellular matrix protein CD90[15,16,33]; adhesion molecules CD49d,[34–36] CD106[29,34,35] and CD166[29,34,35]; and were negative for the hematopoietic stem cell marker CD34,[10,16,29] which coincided with the expression pattern of BM-MSCs. The expression of CD49d in GMSCs (78.5%) was significantly higher than that in PDLSCs (13.8%) (Fig. 5), and the cell sources were determined to be different. CD49d is an adhesion molecule that regulates cell–cell and cell-extracellular matrix interactions. When functioning as a cellular receptor, the CD49d antigen binds to vascular cell adhesion molecule-1 (VCAM-1)/CD106 and plays an important role in stabilizing lymphocyte adhesion to endothelial cells.[35,36] When PDLSCs and GMSCs were cultured in the primary culture, the ossification ability of PDLSCs was significantly greater than that of GMSCs after being cultured in osteogenic medium.[37–40]

This study, using an outgrowth culture method for the primary culture of PDLSCs, identifies factors that influence the success of primary cell cultures and increases access to periodontal reconstruction for those who need it most, such as those with periodontal disease and the elderly. This high success rate combined with advances in cell cryopreservation increases the feasibility of individual cell banking. Additionally, previous studies started the primary culture technique with an 1 h sample collection, whereas this experiment extended the time to 4 h. This not only allows time for clinical examination, but also increases opportunities for patient recruitment and sampling. However, the number of patients in this study was insufficient to truly represent the current state of the population. Further research will help gain a deeper understanding.

The fundamental concept of tissue engineering is to combine a scaffold with living cells or biologically active molecules to create a "tissue-engineering construct" that promotes tissue repair or regeneration when supplied with adequate blood flow. In periodontal tissue engineering, the focus has been on using progenitor (stem) cells to promote new attachment of periodontal tissues. Promising results have been obtained using periodontal ligament stem cells and other mesenchymal stem cells. Therefore, the ability to obtain periodontal ligament cells from primary cultures and establish personalized cell banking is crucial for tissue engineering in dentistry.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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