

Cultivation and expansion of mesenchymal stem cells from human gingival tissue and periodontal ligament in different culture media

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ABSTRACT

Background: Gingival tissue and periodontal ligament act as sources of mesenchymal stem cells (MSCs) that play a vital role in periodontal regeneration, but they both have limitations for cell availability. MSCs cultivated and expanded in various media formulations could be used as a basis for the development of cell therapy protocols. **Purpose:** This study aimed to determine the optimum culture media formulation for cultivation and expansion of human gingival-derived mesenchymal stem cells (hGMSCs) and human periodontal ligament stem cells (hPDLSCs). **Methods:** The hGMSCs and hPDLSCs were obtained from gingival tissue and periodontal ligament specimens from an adult patient. The two different culture media formulations used were: 1) α -minimum essential media (α -MEM) supplemented with 10% FBS, 100 U/mL penicillin, 100mg/mL streptomycin and 2.5 μ g/mL amphotericin B; and 2) Dulbecco's minimum essential media-Low Glucose (DMEM-LG) supplemented with 10% FBS, 2 mMol/L L-glutamine, 100 U/mL penicillin, 100mg/mL streptomycin and 2.5 μ g/mL amphotericin B. The minced-gingival tissue and periodontal ligament samples were seeded in 3 cm tissue culture dishes with one of two experimental culture media, and incubated at 37°C in a humidified atmosphere of 5% CO₂. **Results:** Cell morphology was observed on days two and five of the third passage. The gingival tissue and periodontal ligament primary cells exhibited fibroblast-like morphology, long processes and were spindle-shaped. The hPDLSCs grown in α -MEM exhibited a significant increase in cell viability and proliferation rate compared to the hPDLSCs grown in DMEM-LG. However, hGMSCs displayed similar cell viability and proliferation rate on both types of experimental media. Both the hGMSCs and hPDLSCs expressed MSC markers, including CD105, CD146, and CD90, but did not express CD45. **Conclusion:** Culture media formulations of α -MEM and DMEM-LG can be used for the cultivation and expansion of both hGMSCs and hPDLSCs.

Keywords: cultivation; expansion; gingival-derived mesenchymal stem cells; periodontal ligament stem cells; proliferation rate.

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INTRODUCTION

Human dental tissue is a rich source of easily obtainable adult stem cells, and has a multipotent feature appropriate for use in tissue engineering and regenerative medicine. Periodontal ligament is an effective cell source that encourages bone regeneration, while gingival tissue is an easily available source of mesenchymal stem cells (MSCs) that have excellent proliferation abilities.¹⁻⁴ Both these cell types exhibit high differentiation capability and can be

used to heal various types of periodontal disease. There are however, some major similarities and differences between gingival-derived mesenchymal stem cells (GMSCs) and periodontal ligament stem cells (PDLSCs). For example, CD146, CD105 and CD90 are markers frequently used to determine cells with multi-lineage differentiation potential, and are expressed in MSCs from human dental tissue and bone marrow.² MSCs have to fulfil three criteria, namely: 1) cells are capable of attaching to the cell culture matrix, 2) cells can differentiate into osteocytes, chondrocytes, and

adipocytes,^{5,6} and 3) cells capable of expressing surface markers CD146, CD105 and CD90, but may not express surface markers CD34 and CD45 at the same time.⁷

Culture media is one component that maintains the biological properties of MSCs. Dulbecco's minimum essential media (DMEM) and α -minimum essential media (α -MEM) are widely used to grow mesenchymal stem cells, because they contain L-glutamine and L-ascorbic acid-2-phosphate, so they can preserve stem cell phenotypes, such as expressing CD146, Stro-1, CD44, and CD105, until the eighth passage.⁸ However, there is no consensus on the most effective culture media to optimise the culture conditions of human GMSCs (hGMSCs) and human PDLSCs (hPDLSCs). Optimising cell quality and standardising the manufacturing method is very important for the development and application of periodontal tissue regenerative therapy. Therefore, the determination of the most suitable media in which to cultivate and expand hGMSCs and hPDLSCs is undoubtedly very necessary. This study aimed to analyse the stemness maintenance of hGMSCs and hPDLSCs originating from the same patient using α -MEM media formulation compared to using DMEM-Low Glucose (DMEM-LG) media formulation.

MATERIALS AND METHODS

Tooth extraction was performed in the Department of Oral Surgery at *Persaudaraan Djama'ah Haji Indonesia* (PDHI)-Islamic Hospital, Yogyakarta, Indonesia, and all the participants involved in this study had given their informed consent in writing prior to the study. The experimental protocol was approved by the Ethics Committee of Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta, Indonesia, with document number of 001607/KKEP/FGK-UGM/EC/2018. The hGMSCs and hPDLSCs were isolated from extracted third molar teeth of a healthy patient (34-year-old female) with no history of periodontal disease. The culture media consisted of two experimental media: 1) α -MEM supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin and 2.5 μ g/mL amphotericin B; and 2) DMEM-LG supplemented with 10% FBS, 2 mMol/L L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 2.5 μ g/mL amphotericin B.

Gingival tissue samples attached to the cervical teeth were collected and minced, whereas PDL tissue samples were obtained from one-third to apical root of the third molar, and then minced. For the human gingival and periodontal ligament primary cells, the minced-tissue samples were plated in 3 cm tissue culture dishes with one of two experimental culture media, and incubated at 37°C in a humidified atmosphere of 5% CO₂. The culture media was freshened up twice a week throughout the 14-day incubation period. Before harvesting, the human gingival and periodontal ligament primary cells were visually examined with an inverted microscope.

Next, the subculture of human gingival and periodontal ligament primary cells was carried out on day 14 of incubation. The cells in two experimental culture media attained up to 80% confluency, and they were then harvested with a 0.25% trypsin-EDTA solution. For the consecutive experiments, human gingival and periodontal ligament primary cells were cultured using one of two experimental culture media until passage two (P2). Culture media was refreshed every three days. Then, the following P3 was evaluated for cell morphology, cell proliferation and immunophenotype.

Both human gingival and periodontal ligament primary cells were regularly observed under the inverted microscope to check for any morphological changes. The cell morphology was identified by cell shape into round, branched and spindle cell. Images were recorded at a magnification of $\times 100$ and were taken of five fields in randomly selected dishes from each group. For morphological analysis of hGMSCs and hPDLSCs, they were categorised as round-shape (RS), spindle-shaped (SS), and flat-shape (FS).

The proliferation rate and cell viability of hGMSCs and hPDLSCs were determined by MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide].⁹ Briefly, the cells were harvested from a 25 cm² flask, reseeded at 5×10^3 cells/cm² into a 96-well plate, and incubated at 37°C in a CO₂ incubator for periods of 24, 48, 72 and 96 h. The media were removed, and 20 μ l of MTT dye (5 mg/ml of phosphate buffered saline (pH 7.2) was added into all wells. The plate was incubated at 37°C in a CO₂ incubator for 4 h, and 150 μ l of dimethylsulfoxide (DMSO) was added to dissolve the formazan crystals. The optical density (OD) was measured at 570 nm of wavelength using a microplate reader. The experiments were conducted in triplicate wells. Proliferation rate (PR) was calculated from the formula PR = OD of cells at Tn/OD of cells at T0.

Surface markers for hGMSCs and hPDLSCs were analysed using flow cytometry incorporating four fluorochrome-conjugated antibodies (Human Mesenchymal Stem Cell: Multi-Color Flow Cytometry Kit. Catalog No. FMC020. R & D System, MN-USA). Confluent cells were detached from their place by 0.25% trypsin-EDTA solution, adjusted to 1×10^6 cells in 100 μ L, washed with 2 mL of staining buffer, and centrifuged at 300 \times g for five minutes. Fc receptor blocking reagents were added, then blocking and staining took place using 1 μ g of pre-immune IgG. Cells were incubated along with mouse monoclonal antibodies (10 μ L) specific for human CD105, CD146, CD90, and CD45, or isotype-matched control immunoglobulin Gs for 30–45 minutes at room temperature in darkness. At the end of the incubation period, the cells were washed with 2 mL of staining buffer, and then the cell pellet was resuspended in 200–400 μ L of staining buffer for flow cytometric analysis.

Data for proliferation rate and cell viability were stated as mean \pm standard deviation (SD). Statistical analysis was carried out using one-way analysis of variance, followed by

Tukey's post hoc multiple-comparison tests to determine differences between the groups. The degree of significance was considered to be $p < 0.05$.

RESULTS

The hGMSCs and hPDLSCs were maintained in an adherent culture, and three morphologically distinct cell types were observed: rounded-shape (RS) cells, spindle-shaped (SS) cells and flatten-shape (FS) cells (Figure 1). Cell morphology was observed on day two and day five of P3. The gingival and periodontal ligament primary cells showed fibroblast-like morphology, long processes and were spindle-shaped. Cells exhibited differences in cell morphology using both experimental media. The hGMSCs (G1 group) and hPDLSCs (PL1 group) had predominantly

SS cells on DMEM-LG supplemented with 10% FBS, 2 mMol/L L-glutamine, 100 U/mL penicillin, 100mg/mL streptomycin and 2.5 μ g/mL amphotericin B. However, RS cells were found abundantly from hGMSCs (G2 group) and from hPDLSCs (PL2 group) that had been grown on α -MEM supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin and 2.5 μ g/mL amphotericin B. Besides, few cells were determined to be binucleated-cells and FS cells on both experimental media.

The effects of culture media formulations were analysed on the hGMSCs and hPDLSCs viability (Table 1 and Figure 2), and proliferation rate (Figure 3). The comparison of the cell viability demonstrated that the hPDLSCs grown in α -MEM presented a significant increase in cell viability and proliferation rate compared to the hPDLSCs grown in DMEM-LG. The growth curve of PDLSCs showed that the media formulation of α -MEM was more able to induce cell

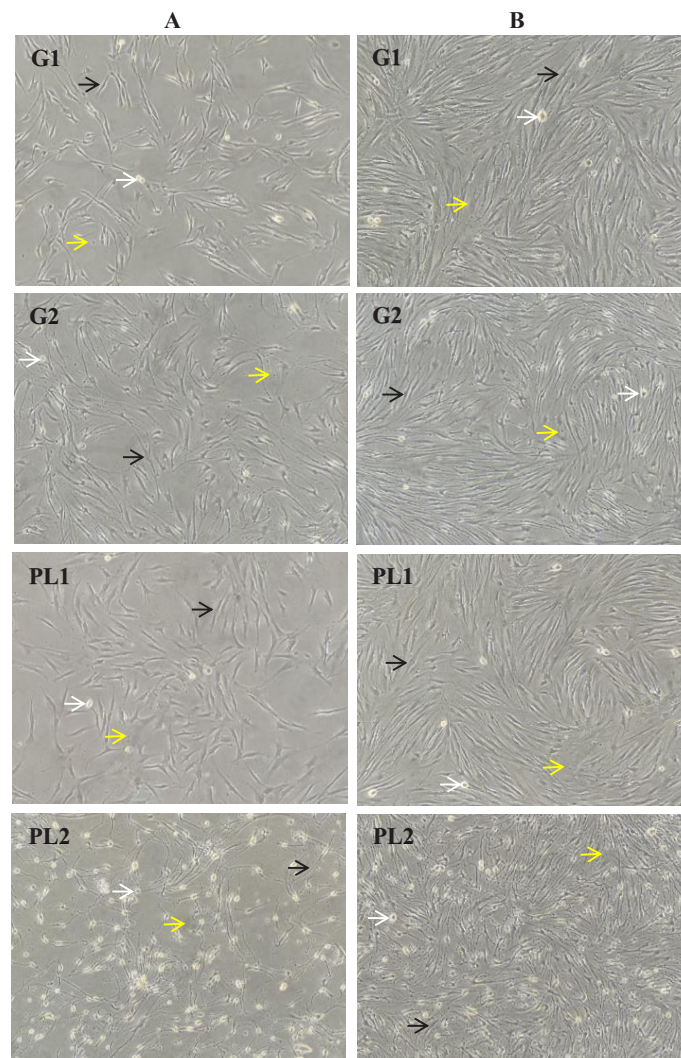


Figure 1. Cell morphology of gingival stem cells in DMEM-LG (G1) and α -MEM (G2), and periodontal ligament stem cells in DMEM-LG (PL1) and α -MEM (PL2), observed on day 2 (A) and day 5 (B) on the third passage, phase-contrast inverted microscopy, index 100 μ m. All cells were tightly adherent, spread apart, and displayed spindle-shaped fibroblastic-like cells. The hGMSCs and hPDLSCs cultured in DMEM-LG formulation grew faster with earlier confluence. Shape indicators: spindle-shape (black arrow), rounded-shape (white arrow) and flat-shape (yellow arrow).

proliferation. Besides, the cell viability and proliferation rate were similarly recorded at hGMSCs on both types of experimental media.

The hGMSCs and hPDLSCs expressed the positive MSC surface markers CD105, CD146, and CD90, and expressed the negative hematopoietic cell marker CD45

(Table 2, Figure 4 and Figure 5). The expression levels of CD146 in all groups were very low, whereas the surface marker expressions of CD105 and CD90 were high in all groups. The hGMSCs and hPDLSCs showed an absence of CD45 expression. These results indicated that hGMSCs and hPDLSCs had similar MSC-like potential.

Table 1. Cell viability of gingival stem cells and periodontal ligament stem cells on experimental media of DMEM-LG and α -MEM for 0, 24, 48, 72 and 96 hours incubation

Duration (h)	Cell Viability			
	GMSCs		PLSCs	
	DMEM-LG	α -MEM	DMEM-LG	α -MEM
0	84.67 \pm 6.13	118.50 \pm 8.31	68.12 \pm 17.60	154.03 \pm 42.39*
24	98.03 \pm 10.56	102.77 \pm 10.50	76.01 \pm 7.66	132.42 \pm 12.89 [†]
48	88.60 \pm 9.20	113.65 \pm 11.34	79.09 \pm 4.33	126.70 \pm 6.95 [§]
72	93.90 \pm 1.93	106.52 \pm 2.20	78.47 \pm 2.63	127.54 \pm 4.25 [‡]
96	100.62 \pm 15.74	100.98 \pm 15.41	70.87 \pm 10.67	143.13 \pm 20.34 [#]

*†§‡# Significant difference in relation between groups

Table 2. Flow-cytometric analysis of mesenchymal stem cell markers

Cells	CD105	CD146	CD90	CD45
Gingival primary cells + DMEM (G1)	79.16	4.03	75.95	1.81
Gingival primary cells + α -MEM (G2)	67.35	3.21	75.00	2.61
Periodontal ligament primary cells + DMEM (PL1)	89.98	13.29	79.42	4.34
Periodontal ligament primary cells + α -MEM (PL2)	77.20	9.94	71.27	9.37

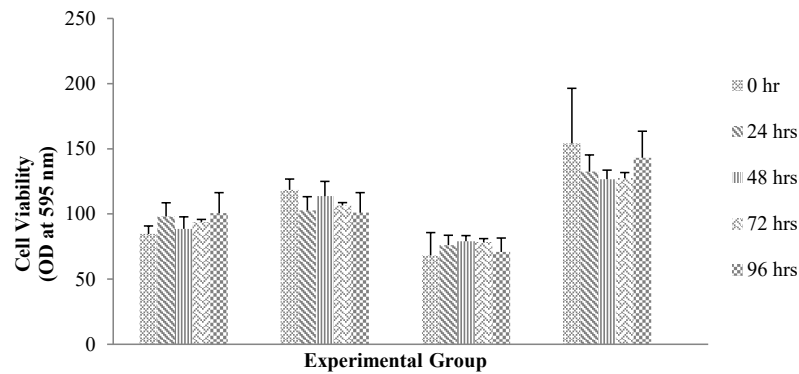


Figure 2. Cell viability of gingival stem cells in DMEM-LG (G1) and α -MEM (G2), and periodontal ligament stem cells in DMEM-LG (PL1) and α -MEM (PL2) for 0, 24, 48, 72 and 96 hours incubation.

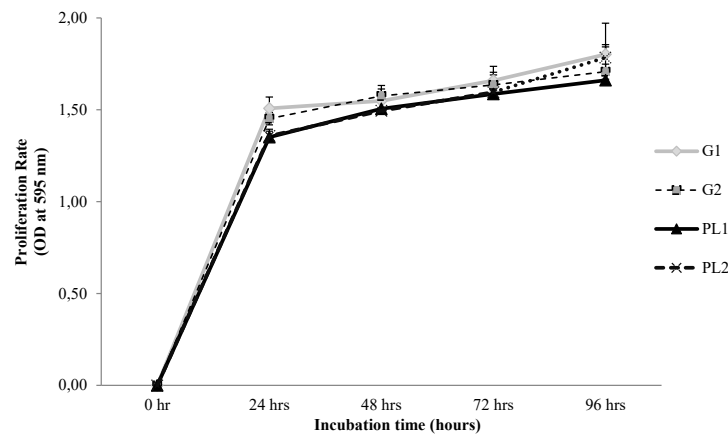


Figure 3. Proliferation rate of gingival stem cells in DMEM-LG (G1) and α -MEM (G2), and periodontal ligament stem cells in DMEM-LG (PL1) and α -MEM (PL2) for 0, 24, 48, 72 and 96 hours incubation.

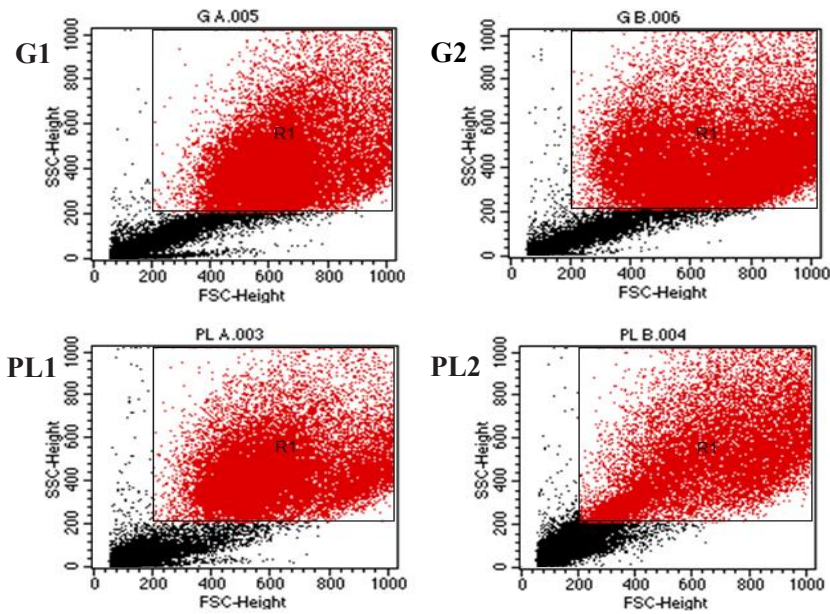


Figure 4. The dot plot of gingival stem cells in DMEM-LG (G1) and α -MEM (G2), and periodontal ligament stem cells in DMEM-LG (PL1) and α -MEM (PL2).

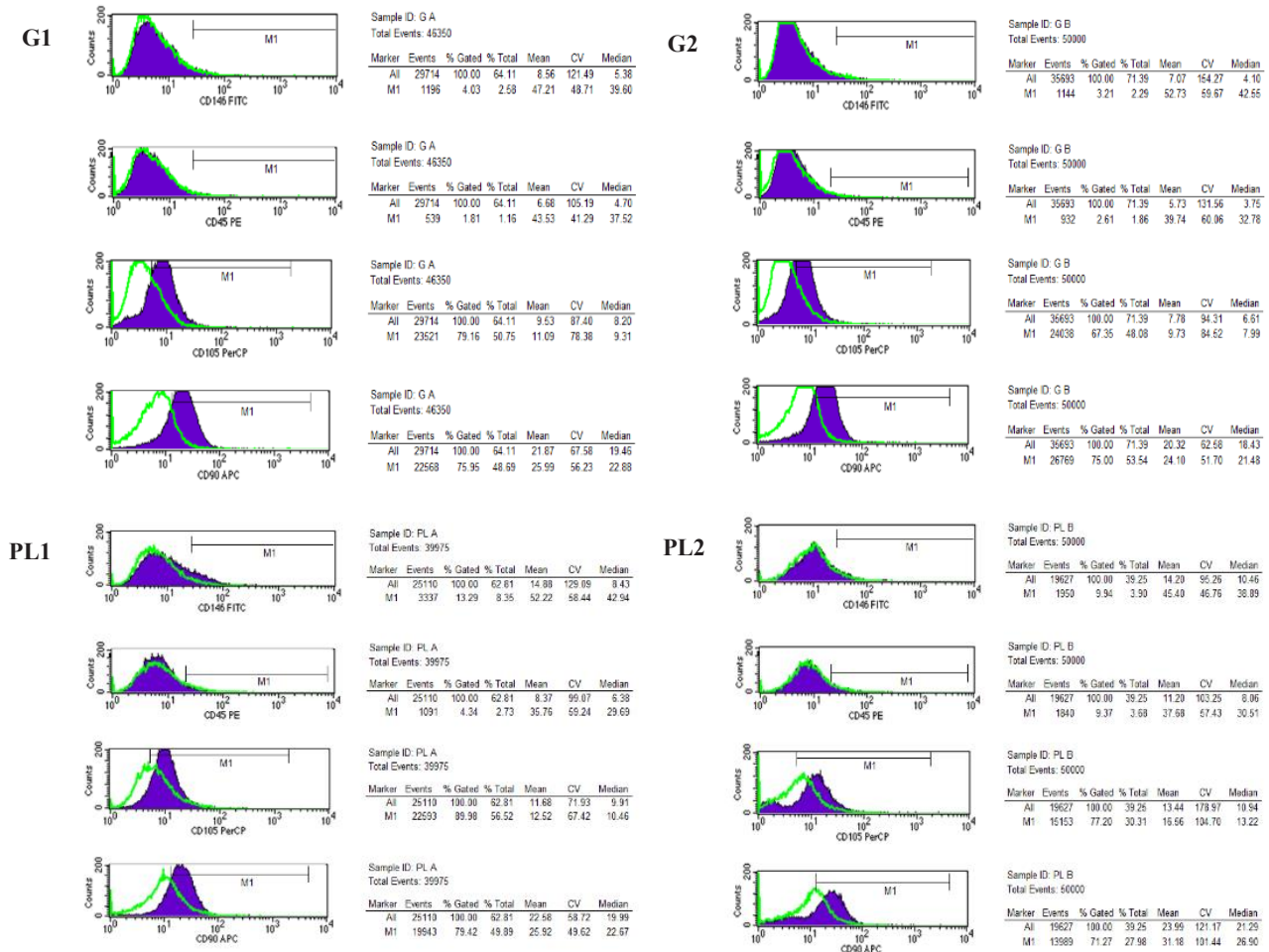


Figure 5. Flow-cytometric analyses for gingival stem cells in DMEM-LG (G1) and α -MEM (G2), and periodontal ligament stem cells in DMEM-LG (PL1) and α -MEM (PL2) have similar surface molecule phenotype. There was a lack of CD146 and CD45, but a positive showing for CD105 and CD90.

DISCUSSION

GMSCs and PDLSCs play a pivotal role in periodontal tissue regeneration.¹⁰ Functionally, GMSCs and PDLSCs can maintain their biological properties *in vitro*, and can rejuvenate periodontal tissue *in vivo*.¹¹ However, the number of GMSCs and PDLSCs that can be obtained from a single sample are very limited.^{2,12} Consequently, GMSCs and PDLSCs require expansion before application.

This study of the third passage of hGMSCs and hPDLSCs, examined the morphology, viability and proliferative ability of the cells in two culture media formulations of α -MEM and DMEM-LG. On the second and fifth day of expansion, the predominantly SS cells were found from hGMSCs (G1 group) and hPDLSCs (PL1 group) in the culture media formulated with DMEM-LG. The hGMSCs demonstrated no statistically significant difference in viability or proliferation rate when they were grown in DMEM-LG and α -MEM formulation, whereas hPDLSCs exhibited a significant increase in viability and proliferation rate in α -MEM formulation compared to DMEM-LG formulation. This study suggested that culture media strongly influences the biological properties of hGMSCs and hPDLSCs to maintain the cell stemness. This study reported that DMEM-LG served as a suitable culture medium for cultivating hGMSC. Lower glucose concentrations may be most appropriate for large-scale hGMSC culture, but α -MEM formulation does not serve as a proper media for hGMSCs, although α -MEM has the lower concentration of glucose. Otherwise, hPDLSCs cultivated in α -MEM formulation had greater proliferation rates than hPDLSCs cultivated in DMEM-LG formulation. This maybe due to the fact that α -MEM has a higher concentration of amino acids, vitamins, and nucleotides compared to DMEM.^{8,13} In addition, glucose and glutamine used in culture media are essential for providing energy (ATP), which is necessary for cell growth and cell maintenance.^{14,15} However, L-glutamine can be degraded to ammonium ions, which are harmful to cell viability under culture conditions.

Based on the immunophenotypic profile of the hGMSCs and hPDLSCs, the data exhibited that more than 70% of the cells expressed CD105 and CD90, low expression of CD146 and negative expression of CD45 (Table 2, Figure 4 and Figure 5). Generally, MSCs have to express CD73, CD105 and/or CD90, and do not express CD14 or CD11b, CD34, CD45, HLA class II, CD79a or CD19.^{16,17} Other study also reported that approximately 60% of the hDPSCs expressed CD105, and that they did not express CD14 or CD45.¹⁸ Furthermore, PDLSCs express CD105, CD73, CD166, CD90, and they show a negative expression for CD14, CD34, CD31, CD45, CD40, CD79a, CD54, HLA-DR, CD80 and CD86,^{11,18} whereas GMSCs were positive for CD29, CD73, CD90, CD105, CD146, and STRO-1, and they show a negative expression for CD45 and CD34.^{19,20} More specifically, this study also found that hPDLSCs-expanded with DMEM-LG formulation expressed CD146.

This study demonstrated that hPDLSCs have pericyte-like characteristics, which may originate from pericytes located in the perivascular wall of the periodontal ligament. In this case, PDLSCs have similarities to pericytes in morphology, phenotype, differentiation potential, and that they have the ability to form capillary structures *in vitro*.^{21,22}

This study concluded that α -MEM and DMEM-LG culture media maintained cell morphology, proliferation rate and immunophenotypes of hGMSCs and hPDLSCs. Both culture media formulations were suitable media culture for the cultivation and expansion of hGMSCs and hPDLSCs. More importantly, these findings favour the pursuit of standardised protocols in cultivation and expansion of hGMSCs and hPDLSCs. Nevertheless, more studies are required to define the effects of culture media formulation on the osteogenic, chondrogenic and adipogenic potential of hGMSCs and hPDLSCs.

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