ORIGINAL ARTICLE

Proliferative Capacities and Differentiation Potentials of Human Periodontal Ligament Stem Cells after Slow-freezing Cryopreservation

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ABSTRACT

Introduction: The cryopreservation of periodontal ligament stem cells (PDLSCs) required a good combination of CPA composition as a step in the preparation of PDLSCs. This study aimed to analyze the proliferative capacities and differentiation potentials of PDLSCs after slow-freezing cryopreservation with CPA in different combinations. **Methods:** The fourth passage of the primary PDL cells were examined their fibroblast-like morphology and colony forming unit-fibroblast (CFU-F), and characterized by surface markers for mesenchymal stem cells using flow cytometry. PDLSCs were divided into two groups of freshly-PDLSCs (fPDLSCs) and cryopreserved-PDLSCs (cPDLSCs). The PDLSCs were cryopreserved using slow freezing method with CPA in different combinations: 1) 90%FBS+10%DMEM (FD-group), 2) 90%DMEM+10%DMSO (DDs-group), 3) 90%FBS+10%DMSO (FDs-group), and 4) 100% Cell Banker (CB-group) as positive control. The proliferation of fPDLSCs and cPDLSCs were evaluated by trypan blue dye exclusion method. The multipotency of cells was assessed by Oil Red O, Alizarin Red, and Alcian Blue staining. **Results:** The primary PDL cells had fibroblast-like morphology and CFU-F ability. They expressed more than 95% positive MSC surface markers of CD90, CD73, CD150, and CD44, but showed less than 2% hematopoietic cell markers of CD11b/CD19/CD34/CD45 and HLA-DR. The cPDLSCs viability of FDs-group was 81.5% and 80% in -80°C and LN2, respectively. The fPDLSCs and cPDLSCs proliferation and doubling time were no statistically significant difference ($p>0.05$). They could differentiate into adipogenic, osteogenic, and chondrogenic differentiation. **Conclusion:** The cPDLSCs could maintain their proliferative capacities and differentiation potentials after slow-freezing cryopreservation with 90%FBS+10%DMSO in -80°C.

Keywords: Cell proliferation, Cryoprotectant agents, Multipotency, Periodontal ligament stem cells, Slow-freezing cryopreservation

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INTRODUCTION

Periodontal ligament stem cells (PDLSCs) are mesenchymal stem cells (MSCs) subpopulation from dental tissue, which promised for periodontal tissue regeneration (1). The accessibility and availability of PDLSCs in cell bank required a good cryopreservation procedure to preserve cell survival without interference of their self-renewal. Cryopreservation methods and cryoprotectant agents (CPA) are successfully factors to determine the cryopreservation of cells. These factors are associated with the cryopreservation to cease all biological functions of cells by maintaining a high degree of their functional integrity and viability (2).

Cryopreservation methods have been used to maintain dental stem cells such as dental pulp stem cells (3,4), PDLSCs (2), and stem cells from apical papilla (5). Various methods of cryopreservation that have been developed still have the risk of cryo-injury. Slow freezing is preferable method for preserving cells because it has a low potential contamination by pathogenic agents but has a higher risk of cryo-injury, so it is necessary to develop a method with CPA that is able to reduce the risk of ice crystal formation (6). CPA is compound to protect the cells from osmotic pressure during freezing. The lucid CPA with slow freezing cryopreservation causes fewer injuries (7). Determination of the most suitable CPAs to cryopreserve human PDLSC is very necessary.

Dimethyl sulfoxide (DMSO) is a chemical compound to penetrate cell membranes because it has a small molecule weight (8). The concentration of 5-15% of DMSO protects the cells from any mechanical injuries (7). However, DMSO is not sufficiently considered for preserving the biological properties of cells, so it is necessary to combine with other CPA. Fetal bovine

serum (FBS) is a transport protein to carry lipid that can be used as CPA to maintain the viability and proliferation of the cells after cryopreservation (5,9). Further, cell protection is required by providing a good combination of CPA composition as a step in the preparation of PDLSCs. Therefore, our study aimed to analyze the proliferative capacities and differentiation potentials of PDLSCs in different combinations of CPAs by slow-freezing cryopreservation in -80°C and LN_2.

MATERIALS AND METHODS

Isolation and culture of human PDLSCs

Tooth extraction was obtained from Dental Clinic, Jember, East Java, Indonesia. A patient had given their consent before this study was performed. The experimental protocol was approved by the Ethics Committee of Faculty of Dentistry, Gadjah Mada University, Yogyakarta, Central Java, Indonesia (No. 001607/KKEP/FKG-UGM/EC/2018). This study was conducted at Molecular Medicine Laboratory, Center for Development of Advanced Science and Technology (CDAST), University of Jember, Jember, East Java, Indonesia. Human PDL was isolated from the first premolars of a healthy subject (15 years old) without a history of periodontal disease. The first premolars were extracted for orthodontic reasons. PDL was obtained by scraping from the third part of the root surface. PDL was digested in a solution of 4 mg/mL dispase (Sigma-Aldrich, USA) and 3 mg/mL collagenase type I (Sigma-Aldrich, USA). The primary PDL cells were seeded in 3 cm of tissue culture dish (TCD) with Dulbecco's Modified Eagle's medium (D-MEM) (Gibco, Canada) supplemented by 10% fetal bovine serum (Gibco, Brazil), 2 mM L-Glutamine, 110 mg/L Sodium Pyruvate, 100 U/mL penicillin, 100 µg/ml streptomycin (Gibco, Germany) and 2.5 µg/ml amphotericin B (Gibco, USA), and incubated at 37°C in a humidified atmosphere of 5% CO2. The culture medium was replaced every three days for two weeks. Then, the cells were cultured until the fourth passage in a 10 cm TCD.

Cell morphology

The primary PDL cells, fPDLSCs, and cPDLSCs were observed under the inverted-phase contrast microscope (Evos Cell Imaging Systems, Thermo Fisher Scientific, US) at 100x magnification to determine the cell morphology. The cell morphology was categorized as rounded-shaped (RS), spindle-shaped (SS), and flatten-shaped (FS) (10).

Colony forming unit-fibroblast (CFU-F) assay

The primary PDL cells were seeded with a concentration of 500 cells/cm^2 in 2 ml DMEM and 15% FBS into six well-plate, and incubated at 37°C in a 5% CO2 humidified atmosphere for nine days (11). Furthermore, the cells were washed twice with phosphate-buffered saline (PBS) (Gibco Invitrogen, NY, USA), fixed for 10 minutes at room temperature with 1% crystal violet (Sigma Chemical, St. Louis, USA), and dissolved with methanol. The cell colonies were rinsed three times with distilled water. CFU-F was visualized under the inverted-phase contrast microscope at 40x magnification after the plates were then allowed to air dry. CFU-F colonies were observed ranging in diameter from 1 to 8 mm (12).

Flow cytometry analysis

The surface markers were analyzed with four fluorochrome-conjugated antibodies (Human MSC Analysis Kit, BD StemflowTM, 562245, USA). The fourth passage of primary PDL cells were detached after 80% confluent with 0.25% Trypsin-EDTA solution (Gibco, Germany). The cells were adjusted to $1x10^6$ cells and rinsed with 2 ml of BD PharmingenTM Stain Buffer (Cat. No.554656). Cell suspension was centrifuged at 300 x g for 5 min at 25°C. The pellet was added with 10 µl for MSC markers of CD90, CD105, CD73, and CD44 or isotype control antibodies CD34/CD11b/CD19/CD45/HLA-DR, and incubated in the dark at room temperature for 30 minutes. Furthermore, the cells were rinsed twice with BD PharmingenTM Stain Buffer and re-suspended in 400 µL in BD PharmingenTM Stain Buffer. The surface markers were analyzed by AttuneTM NxT Flow Cytometer (Thermo Fisher Scientific).

Cryopreservation of PDLSCs

The PDLSCs were prepared with cell concentration of 7x105 cells/mL) into cryovial (TrueLine, Mexico) in each CPAs-group solution. We used CPAs-group including of: 1) 100% Cell Banker (CB-group) (Nippon Zenyaku Kogyo Co., LTD, Japan), 2) 90% FBS + 10% DMEM (FD-group), 3) 90% DMEM + 10% DMSO (Sigma Aldrich, USA) (DDs-group), and 4) 90% FBS + 10% DMSO (FDs-group). Furthermore, the cells were kept in Freezing container, Nalgene® Mr. Frosty C1562 (Sigma-Aldrich, Germany) with a decrease of 1°C/minute in -80°C for 24 hours. After 24 hours, cryovials were transferred from -80°C to LN_2. The cells were cryopreserved for seven days.

Cell viability

Cryovials aseptically were thawed after seven days of cryopreservation. The cryovial lid was turned a quarter turn to reduce the pressure and then re-tightened. Cryovials was warmed into a water bath at 37°C for 1 minute to melt cell suspension. The viability of cPDLSCs was calculated using the trypan blue dye exclusion method. The cell viability (CV) was calculated by the formula as below:

$$CV = (LC / NC) \times 100\%$$

LC is the number of living cells, and NC is the number of cells (12).

Cell proliferation

The fPDLSCs and cPDLSCs were seeded $2x10^3$ cells/cm^2 into 24 wells plate and incubated at 0, 24, 48, 72, 96, 120 and 144 hours. After the incubation period, the

cells were harvested with 0.25% trypsin-EDTA solution. The cell suspensions were centrifuged at 1000 x g for 5 min at 2°C. The pellet was dissolved in 50 µl of culture media, and calculated with the trypan blue dye exclusion method. Doubling time (DT) was determined by the formula as below:

$$DT = t \times \log 2 / (\log Nt / \log N0)$$

There were three replications, incubation time in hours (t), the number of cells at the end of the incubation time (Nt) and the number of cells at 0 hour (N0) (13).

Multipotency

Multipotency was used to determine the differentiation of fPDLSCs and cPDLSCs to specific lineages of adipogenic, osteogenic, and chondrogenic. The protocol was represented by OriCellTM Mesenchymal Stem Cell Adipogenic (Cat. No. GUXMX-90031), Osteogenic (Cat. No. GUXMX-90021, and Chondrogenic (Cat. No. GUXMX-90041) Differentiation Medium (Cyagen, USA).

Adipogenic induction (Cat. No. GUXMX-03031) supplemented by 10% FBS (Cat. No. GUXMX-05001), 2% penicillin-streptomycin, glutamine, 10 µM insulin, 500 µM isobutylmethylxanthine (IBMX), rosiglitazone, 100 nM dexamethasone and maintenance medium (Cat. No. GUXMX-03032) supplemented with 10% FBS, 2% penicillin-streptomycin, glutamine, 10 µM insulin were prepared. The PDLSCs were plated at $2x10^4$ cells/cm^2 in 24 well plate, added in induction medium for three days, and further cultured in maintenance medium. After 24 hours, maintenance medium was replaced back to induction medium. The cycle of induction and maintenance was repeated five times. Thereafter, the cells were grown in maintenance medium for seven days until a large droplet is obtained. During this period, the medium was replaced every three days. The cells were fixed with 4% formaldehyde solution for 30 minutes and stained with 250 µl Oil Red O solution for 30 minutes.

Osteogenic differentiation medium was supplemented with 10% FBS, 2% penicillin-streptomycin, glutamine, ascorbate, β-Glycerophosphate, and 100 nM dexamethasone. The PDLSCs were seeded at 2x104 cells/cm^2 in 24 well plate pre-coated with 0.1% gelatin. After 70% confluent, the cells were fed every three days for 28 days in osteogenic differentiation medium. The cells were fixed for 30 minutes with 4% formaldehyde solution, and stained with Alizarin Red S for 5 minutes.

Incomplete and complete chondrogenic medium were prepared. Incomplete medium was augmented with chondrogenic basal medium (Cat. No. GUXMX-03041), 100 nM dexamethasone, 50 ng/mL ascorbate, 50 mg/mL ITS+supplement, 100 µg/mL sodium pyruvate, 40 µg/mL proline. Complete medium was composed by 10 µL TGF-β3 and dissolved in 1 ml of incomplete chondrogenic medium. Aliquot 0.5 mL ($2.5x10^5$ cells) of the PDLSCs suspension was pelleted by centrifugation at 150 x g for 5 mins 25°C in incomplete medium. After 24 hours, the pellets were cultured in complete medium and were fed every three days for 28 days in culture. After 28 days, the pellets were fixed by formalin, embedded paraffin, and stained with Alcian Blue.

Data analysis

CFU-F, cell viability, cell proliferation and doubling time were analyzed as mean ± standard deviation (SD) with Microsoft Excel version 16.14.1 (Redmont, WA). Statistical analysis was performed by using one-way analysis of variance followed by Tukey's post hoc multiple-comparison test to determine differences between groups (SPSS 22.0). The degree of significance was considered when p-value was ($p<0.05$).

RESULTS

Morphology of the primary PDL cells and PDLSCs

The primary PDL cells and PDLSCs were found to adhere in TCD. The cells were homogeneous monolayer fibroblastic-like cells. The primary PDL cells and PDLSCs had the cell morphology of RS, SS, and FS. The group of PDLSCs before cryopreservation (fPDLSCs) and after cryopreservation (cPDLSCs) also had similar morphology of RS, SS, and FS (Fig. 1).

CFU-F of the primary PDL cells

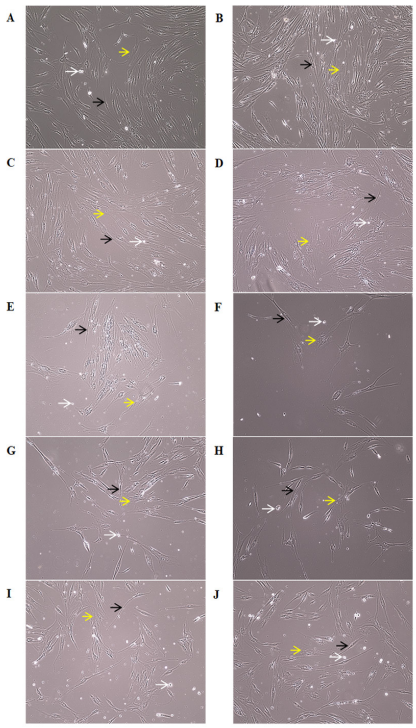

Figure 1: The morphology of primary PDL cells, fPDLSCs and cPDLSCs was identified on five days in incubation. All groups showed cell morphology of rounded-shaped (white arrow), spindle-shaped (black arrow), flatten-shaped (yellow arrow). (A, B) The primary PDL cells and fPDLSCs were in passage 4. (C, D) The cPDLSCs were cryopreserved in Cell Banker-1 (C, D); 90% FBS + 10% DMEM (E, F); 90% DMEM + 10% DMSO (G, H); 90% FBS + 10% DMSO (I, J). The cPDLSCs were cryopreserved at -80°C (C, E, G, I), and at LN_2 (D, F, H, J). The magnification: x100; scale bar: 200 µm.

The primary PDL cells formed the number of colonies in diameter ranging from 1 to 8 mm were evaluated in every three days for nine days. The number of colonies in three days, six days, and nine days were 536.5 ± 13.4, 1083.5 ± 2.1, 1476 ± 17.7, respectively (Fig. 2). The cell colonies were statistically significant difference ($p<0.05$).

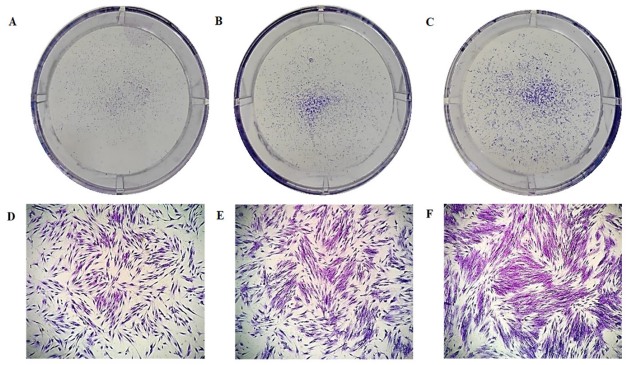

Figure 2: The primary PDL cell colonies were performed by absorbing crystal violet. The cell colonies were in the middle of 6-well tissue culture plate surface area (A-C). The number of cell colonies in three days and six days (A, B) were less than that of the nine days of incubation (C). (D-F) The cell colonies were also visualized under an inverted microscope (magnification: x40; scale bar: 200 µm).

Characterization of PDLSCs

The PDLSCs did not express the negative hematopoietic cell markers of CD19 / CD45 / HLA-DR/ CD34 / CD11b, but expressed the positive MSC surface markers of CD105, CD73, CD44, and CD90 (Fig. 3). The expression levels of all positive markers were more than 95%, while the expression of negative markers was less than 1%.

Viability of cPDLSCs

The cPDLSCs viability showed the effect of CPAs during cryopreservation in each group. The FDs-group could maintain their cell viability of 81.5% and 80% in -80°C and LN_2, respectively. The cell viability of the FDs-group was slightly lower than that of the CB-group. The CB-group had cell viability of 90% and 88% in -80°C and LN_2, respectively. However, the FD-group and the DDs-group were less to maintain their cell viability, both in -80°C and LN_2. The FD-group had cell viability of 52.5% and 48% in -80°C and LN_2, respectively. The DDs-group had cell viability of 68.5% and 63% in -80°C and LN_2, respectively.

Proliferation of fPDLSCs and cPDLSCs

The fPDLSC and cPDLSC showed the similar proliferative capacities during incubation from 0 to 144 hours (Fig. 4). According to our results, the culture of both fPDLSC and cPDLSC had almost no lag time. The doubling time indicated the time of cell population to double. The doubling time was 15.62±0.07 hours to 29.19±0.14 hours for fPDLSCs and 15.54±0.07 hours to 29.15±0.03 hours for cPDLSCs. The difference between the two groups was not significant ($p>0.05$) (Fig. 4).

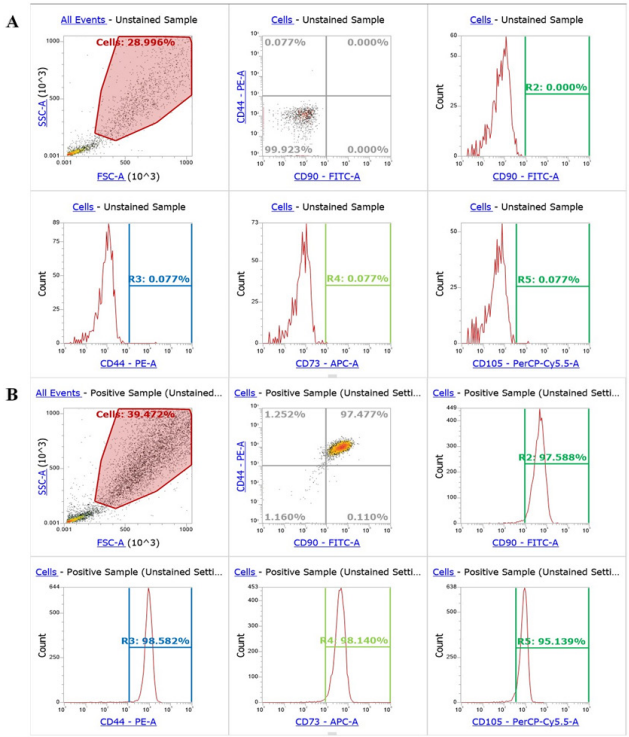

Figure 3: Characterization of PDLSCs was analyzed by flow cytometry. The negative markers expressed less than 2% of CD45/ CD34/ CD11b/ CD19/ HLA-DR (A). The MSC surface markers expressed more than 95% of CD90, CD105, CD73, and CD44 (B).

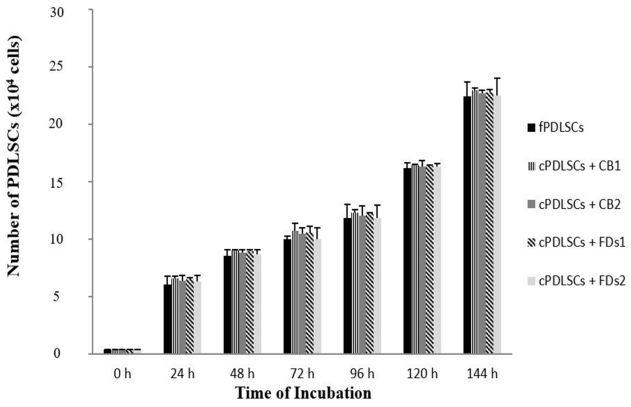

Figure 4: The proliferation activities of fPDLSC and cPDLSCs. The fPDLSC and cPDLSCs showed the similar cell proliferation during incubation from 0 to 144 hours. The doubling time of fPDLSCs was 15.62±0.07 hours to 29.19±0.14 hours and 15.54±0.07 hours to 29.15±0.03 hours for cPDLSCs. The difference between the two groups was not significant ($p>0.05$). The cPDLSCs were cryopreserved in Cell Banker at -80°C (CB1), Cell Banker at LN_2 (CB2), 90% FBS + 10% DMSO at -80°C (FDs1), 90% FBS + 10% DMSO at LN_2 (FDs2).

Multipotency of fPDLSCs and cPDLSCs

The adipogenic differentiation of fPDLSC and cPDLSCs was determined by the formation of lipid droplets over 27 days of incubation (Fig. 5). After 28 days incubation for osteogenic differentiation, the fPDLSCs had a greater capacity for osteogenic differentiation than cPDLSCs (Fig. 6). For chondrogenic differentiation, the fPDLSC and cPDLSCs had similar chondrogenic differentiation over 28 days of incubation. The fPDLSCs and cPDLSCs

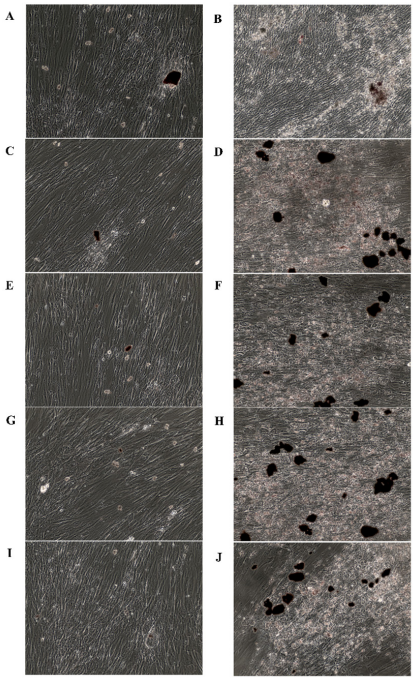

Figure 5: Oil Red O staining was performed to determine the adipogenic differentiation of fPDLSCs and cPDLSCs. The fPDLSCs and cPDLSCs had similar adipogenic differentiation. The cells without the induction of adipogenic differentiation medium had a low adipogenic differentiation capacity. The fPDLSCs were grown with culture medium (A), and adipogenic induction medium (B). The cPDLSCs were grown with culture medium (C, E, G, I), and adipogenic induction medium (D, F, H, J). The cPDLSCs were cryopreserved in Cell Banker (C-F), 90% FBS + 10% DMSO (G-J). The cPDLSCs were cryopreserved at -80°C (C, D, G, H), and LN2 (E, F, I, J). The magnification: x200; scale bar: 200 µm.

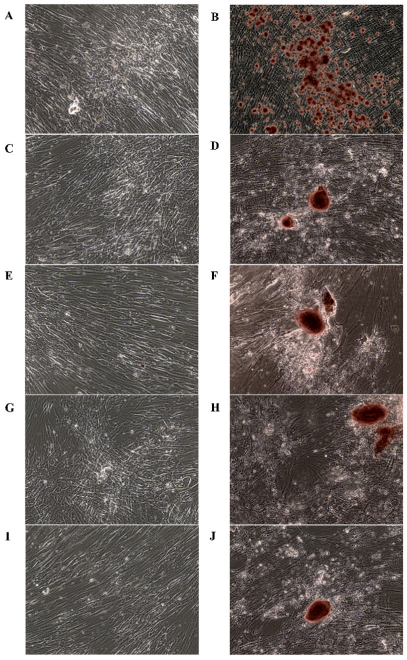

Figure 6: Osteogenic differentiation of the fPDLSCs and cPDLSCs was indicated by the presence of calcium deposits. The osteogenic differentiation of fPDLSCs had a greater potency than cPDLSCs. The fPDLSCs were grown with culture medium (A), and osteogenic induction medium (B). The cPDLSCs were grown with culture medium (C, E, G, I), and osteogenic induction medium (D, F, H, J). The cPDLSCs were cropreserved in Cell Banker (C-F), 90% FBS + 10% DMSO (G-J). The cPDLSCs were cryopreserved at -80°C (C, D, G, H), and LN_2 (E, F, I, J). The magnification: x200; scale bar: 200 µm.

had large and round pellets compared to cells without induction of chondrogenic differentiation media (Fig. 7).

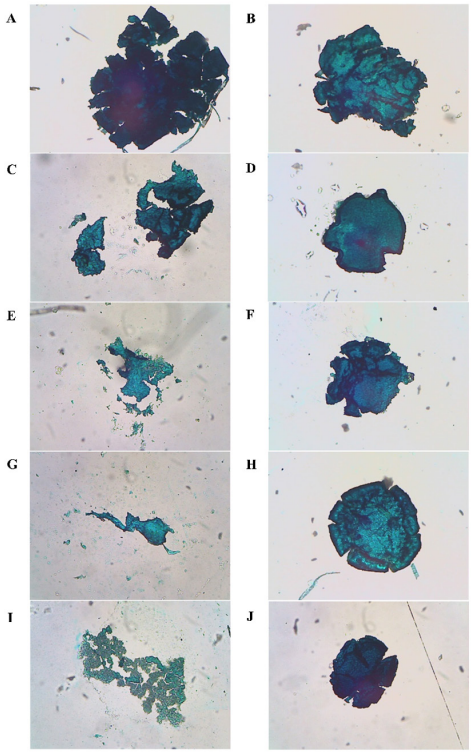

Figure 7: The absorption of Alcian Blue staining in the pellets showed the proteoglycan synthesis of fPDLSCs and cPDLSCs. The chondrogenic differentiation of fPDLSCs and cPDLSCs had similar round and big pellets. The fPDLSCs were grown with culture medium (A), and chondrogenic induction medium (B). The cPDLSCs were grown with culture medium (C, E, G, I), and chondrogenic induction medium (D, F, H, J). The cPDLSCs were cropreserved in Cell Banker (C-F), 90% FBS + 10% DMSO (G-J). The cPDLSCs were cryopreserved at -80°C (C, D, G, H), and LN_2 (E, F, I, J). The magnification: x100; scale bar: 200 µm.

DISCUSSION

The primary PDL cell cultures are fibroblasts characteristic from original tissue for maintaining periodontal tissue regeneration (14). The potential of primary PDL cells can be characterized to be PDLSCs. Indeed, PDLSCs are a promising cell source for MSCs of the dental tissue which can differentiate into various cells, and are effective in self-renewal so they promote tissue regeneration as well as cell therapy for clinical applications (15). However, the numbers of PDLSCs that can be obtained by progenitor cells are limited (16). Consequently, the accessibility and availability of PDLSCs in cell bank required expansion procedure before clinical applications. Moreover, the specific cryopreservation procedure that preserves cell survival without damaging its biological properties of PDLSCs is needed. In our study, the cryopreserved PDLSCs (cPDLSCs) were able to maintain morphology, proliferation, doubling time and differentiation which are no different from pre-cryopreserved PDLSCs (fPDLSCs).

The fourth passage of the primary PDL cells were

examined their morphology, CFU-F, and characteristics of the cells. On the five days of expansion, the primary PDL cells and PDLSCs had less RS and FS cells, but they predominantly had SS cells (Fig. 1). The fPDLSCs and cPDLSCs had also similar cell morphology. The cPDLSCs could maintain the morphology of fibroblast-like cells. We suggested that culture conditions strongly influence the efficacy of the primary PDL cells and PDLSCs to maintain the stemness of cells. Our study showed that primary PDL cells represented colony-forming ability. The number of CFU-F in our primary PDL cells increased two-fold from the third day to the ninth day of incubation (Fig. 2). We expected that the number of CFU-F from the primary PDL cells was influenced by FBS concentration in cell culture media and the concentration of inoculation. Culture media supplemented with FBS (10-15%) could form cell colonies with a diameter of 2-5 mm, while the higher density of cells showed a decrease in multi-potential capacity and self-renewal cells (11,12,17). In this case, we used culture media supplemented with 15% FBS and low number of cell density (500 cells /cm^2).

According to the International Society for Cellular Therapy (ISCT), expression of MSC markers have been confirmed more than 95% of CD90, CD105, CD73, and CD166 expression, but less than 2% on the hematopoietic markers of CD45 / CD79α / CD34 / CD14, endothelial (CD31), and immune antigens (CD86, CD80, CD54, CD40, HLA-DR) (18,19). Our PDLSCs also weakly expressed the negative hematopoietic markers and strongly expressed positive MSC markers (Fig. 3). Other study indicated that flow cytometric analysis of human PDLSCs confirmed a positive presence of STRO-1, CD146, CD90, and CD44, but did not express negative markers of CD19 and CD14 (20).

For clinical applications, a good protocol of cryopreservation is effectively required to preserve MSCs. It has to maintain the survival rate and the functional properties of MSCs such as differentiation ability and immunomodulatory. Besides, the protocol of cryopreservation such as concentrations and types of CPA reduce the possibility of harmful effects on MSCs that should be optimized (6). In clinics and research laboratories, the slow freezing is preferable method for the cryopreservation of MSC due to easier handling and the low risk of contamination. The number of MSCs in one vial can be easily cryopreserved at low concentrations of CPA (<1.5 M) with a freezing rate of 1°C/min. During the freezing process, non-sterile liquid nitrogen and the cells are no direct interaction to decrease the possible risk of contamination. The cryopreserved-MSCs with CPAs were frozen using ''Mr. Frosty'' with a decrease of 1°C/min or a non-programmable time freezing protocol in -20°C (6,21,22).

We designed the CPA for slow freezing cryopreservation to maintain cell survival without interference of their self-renewal. The CPA played an important role for cPDLSCs protection during the freezing process. In addition, the concentration of 10% or 1.28 M of DMSO can maintain the cell viability of cPDLSCs. Functionally, DMSO can penetrate cell membranes to prevent intracellular crystal formation with water molecules and hydrogen bonds (23,24). However, DMSO can be toxic if its concentration is more than 10% (8). Other study demonstrated that the concentration of 10% DMSO or 1-1.5 M was ideal CPA to maintain post-thaw viability for dental pulp-derived stem cells (25). Besides DMSO, the composition of CPA in our study also used FBS concentration of 90%. The FBS with concentration of 90% also maintained the biological properties of cPDLSCs during the freezing process. Naturally, this serum is a transport protein to carry hormones, minerals, lipids, and amino acids (26). We successfully implemented the combination of 90% FBS and 10% DMSO in FDs-group. The highest cell viability was obtained from cPDLSCs in FDs-group that was stored at -80°C for seven days. The combination of 90% FBS and 10% DMSO can prevent a decrease viability of cPDLSCs and may protect the risk of cell dehydration. Previous study described that the complex cryopreservation media with efficient protocol helped to maintain stabilization of the osmotic and pH buffer, to protect the free radicals effect, and to provide substrate energy (27).

The proliferative capacities of fPDLSCs and cPDLSCs were assessed by trypan blue dye exclusion method to compare their proliferation rate at 0 to 144 hours of incubation. The fPDLSCs and cPDLSCs demonstrated the similar high capacities for proliferation (Fig. 4). The culture of both fPDLSCs and cPDLSCs showed almost no lag time indicated that cells were extremely adaptable to culture conditions and began to proliferate immediately after plating. The cPDLSCs had a slightly higher cell proliferation and had a slightly lower cell doubling time than fPDLSCs, suggesting that the CPA and cryopreservation environment did not affect the cPDLSCs proliferation. Cell density can affect the rate of cell division (28). The proliferation rate of mesenchymal stem cells is also influenced by various factors such as cell density, cell source, quality and composition of culture media such as supplements (FBS, platelet lysate), presence of hypoxia, glutamine and glucose concentration, plastic quality, plate size, and addition of various growth factors (29).

The multipotency profile of the fPDLSCs and cPDLSCs successful differentiated into adipogenic, osteogenic, and chondrogenic. The cPDLSCs and fPDLSCs had similar adipogenic differentiation potentials. They showed lipid droplet formation by absorbing the Oil Red O solution (Fig. 5). The osteogenic differentiation of fPDLSCs and cPDLSCs were characterized by the presence of calcium deposits. The fPDLSCs had a greater potency than cPDLSCs (Fig. 6). For the chondrogenic differentiation, the fPDLSCs and

cPDLSCs had similar round and big pellets. It could be determined by Alcian Blue absorption, which indicated the proteoglycan synthesis by chondrocytes (Fig. 7). These results indicated that the cPDLSCs could retain the tri-lineage differentiation potentials, but cPDLSCs had slightly lower osteogenic differentiation compared to fPDLSCs. However, previous study described that the cryopreservation of adipose-derived stem cells (ASCs) increased seven-fold for calcium deposits and did not alter the phenotype characteristics. These ASCs were cryopreserved in freezing solution of LG-DMEM containing 20% FBS and 10% Me2SO (30). This difference in osteogenic differentiation might be due to differences in CPA composition and cell types. The slow-freezing cryopreservation of PDLSCs with the combination of 90% FBS and 10% DMSO in -80°C and LN_2 could maintain the morphology, proliferation capacities, doubling time activities, and differentiation potentials. The cells were stored at -80°C and LN_2, did not have metabolic demands and could protect from biological variation or infection due to genetic drift (31). The genetic instability of stem cell in culture must be assessed to prevent a substantial immunogenic or tumorigenic risk before preclinical applications (32,33). Therefore, a method of cell storage is required for the self-availability to reach the cell numbers, and maintained cell functional properties before clinical applications (34,35).

CONCLUSION

We concluded that the cPDLSCs could maintain their proliferative capacities and differentiation potentials after slow-freezing cryopreservation with 90% FBS + 10% DMSO. Our findings support the development of a standard cryopreservation protocol for human PDLSCs.

ACKNOWLEDGEMENT

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