ORIGINAL ARTICLE

Slow Freezing Cryopreservation in Combination With Cryoprotectants Preserve Gingival Mesenchymal Stem Cells

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ABSTRACT

Introduction: Development of gingival mesenchymal stem cells (GMSCs) cryopreservation procedures is needed to conserve GMSC in stem cell-based therapy. This study aimed to evaluate slow freezing cryopreservation and cryoprotectants for GMSCs. **Methods:** Gingival tissue from extracted human third molars was separated from teeth. The minced-gingival tissues were plated in tissue culture dishes, added culture media, and incubated at 37°C in 5% CO_2. Morphology and flowcytometry analysis were determined on the fourth passage of gingival cells. GMSCs were separated into two groups of noncryopreserved-GMSCs (ncGMSCs) and cryopreserved-GMSCs (cGMSCs). The GMSCs were frozen by slow freezing with CPA in the following combinations: 1) 100% Cell Banker (CB-group), 2) 90% FBS+10% DMSO (FDs-group), 3) 90% FBS+10% DMEM (FD-group), and 4) 90% DMEM+10% DMSO (DDs-group). Trypan blue dye exclusion was used to assess the proliferation of ncGMSCs and cGMSCs. The Oil Red O, Alizarin Red, and Alcian Blue staining were used to determine their multipotencies. **Results:** Gingival cells and GMSCs showed fibroblastic-like morphology. They did not express hematopoietic cell markers of CD11b/CD19/CD34/CD45 and HLA-DR, but expressed more than 90% positive MSC surface markers of CD90, CD73, CD150, and CD44. The cGMSCs viability of FDs-group was 81% and 80% in -80°C and LN_2, respectively. There was no statistically significant difference ($p>0.05$) in proliferation and doubling time between ncGMSCs and cGMSCs. They had ability to develop into chondrogenic, osteogenic, and adipogenic differentiation. **Conclusion:** Slow freezing cryopreservation in combination with 90% FBS+10% DMSO retain the biological properties of GMSCs, and it can be developed to GMSCs banking.

Keywords: Cell proliferation; Cryopreservation; Cryoprotectants; Gingival mesenchymal stem cells; Multipotency

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INTRODUCTION

Gingival mesenchymal stem cells (GMSCs) are ideal candidates for cell-based therapy and regenerative medicine (1), as they have strong immunoregulatory functions and accelerated healing compared to periodontal ligament stem cells (PDLSCs) (2). When compared to bone marrow mesenchymal stem cells (BMSCs), GMSCs provide a source of stem cells that are more stable in their ability to proliferate, differentiate into specific cell types such as adipose, bone or cartilage, and maintain morphology in long-term culture, which is necessary for clinical applications, and support the use of GMSCs in allogeneic transplantation (2-4). In addition, GMSCs can be isolated from gingival tissue, which tends to be disposed of as waste from surgical procedures (3).

For clinical application, large numbers of GMSCs are required, so they need to be expanded in culture, or they have to be collected from many donors. In both cases, a cryopreservation method for the long-term preservation and collection of GMSCs is required for the availability of cells ready for use in clinical applications. After cryopreservation, GMSCs should be able to retain their immunomodulatory properties and multilineage differentiation capability. If cryopreservation is not performed, cells must continue to be subcultured, resulting in accumulation of genetic alterations, heterogeneity or tumorigenicity (5-7).

Cryopreservation of MSCs has been developed by slow freezing and vitrification methods, but still has limitations because preserved MSCs are at risk of

cryo-injury even in combination with cryoprotective agents (CPAs) as cell protectors (8). On the other hand, CPAs such as dimethyl sulfoxide (DMSO) are able to induce undesired differentiation of MSCs into neuron-like cells (9). It is imperative to develop good cryopreservation procedures to effectively conserve GMSCs for stem cell-based therapeutic applications. The procedure must be able to maintain the biological properties and viability of GMSCs. Many challenges need to be solved related to the cryopreservation procedure of GMSCs, including cryopreservation procedures must be optimized to reduce the risk of harmful effects on GMSCs, and GMSCs must be preserved without direct contact with liquid nitrogen to reduce the risk of pathogen contamination. Based on those mentioned above, this study aimed to evaluate the proliferation and differentiation of noncryopreserved-GMSCs (ncGMSCs) and cryopreservedGMSCs (cGMSCs) after slow freezing cryopreservation with various compositions of CPA.

MATERIALS AND METHODS

Human subject

This research protocol was approved by the Ethics Committee of the Dentistry Faculty, Universitas Gadjah Mada, Yogyakarta, Indonesia (No. 001607/KKEP/FKG-UGM/EC/2018). Patients were recruited during their dental visit for orthodontic reasons. Informed consent was required and was performed prior to gingival tissue collection. Human gingival tissue isolated from extracted third molars due to impaction in a healthy 24-year-old patient.

Human gingival cells isolation and culture

Gingival tissue was minced into small pieces. The minced-tissue samples were plated in 3-cm tissue culture dishes, added culture media, and incubated at 37°C in a humidified atmosphere of 5% CO_2. The culture media was changed every three days for two weeks. After reaching 80% confluence, the gingival cells were subcultured until the third passage. The culture media consisted of Dulbecco's Modified Eagle's medium (D-MEM) (Gibco, Canada), 10% fetal bovine serum (Gibco, Brazil), 100 U/mL penicillin, 100 g/ml streptomycin (Gibco, Germany), and 0.5% Amphotericin B.

Characterization of GMSCs

Four fluorochrome-conjugated antibodies were used to examine the surface markers (Human MSC Analysis Kit, BD StemflowTM, 562245, USA). After 80% confluence, the fourth passage of gingival cells was detached with 0.25% Trypsin-EDTA solution (Gibco, Germany). Concentration of $1x10^6$ cells were adjusted and washed with 2 ml of BD PharmingenTM Stain Buffer (2 mL) (Cat. No.554656). For five minutes, the primary gingival cells suspension was centrifuged at 300 x g at 25°C. The primary gingival cells was incubated in the dark at room temperature for 30 minutes with 10 µl of MSC markers CD90, CD105, CD73, and CD44 or isotype control antibodies CD34/CD11b/CD19/CD45/HLA-DR. Next, the primary gingival cells were washed twice with BD PharmingenTM Stain Buffer, and were resuspended with BD PharmingenTM Stain Buffer in 400 µL. A flowcytometer (FACS Aria II; BD Biosciences) was used to perform flowcytometric analysis.

Cryopreservation of GMSCs

GMSCs were prepared in cryotube (TrueLine, Mexico) with a $7x10^5$ cells/mL of cell concentration. The CPA combination-groups consisted of 1) 100% Cell Banker (CB-group), 2) 90% FBS + 10% DMSO (FDs-group), 3) 90% FBS + 10% DMEM (FD-group), and 4) 90% DMEM + 10% DMSO (DDs-group). For 24 hours, GMSCs were stored in a freezing container, Nalgene® Mr. Frosty C1562 (Sigma-Aldrich, Germany) with 1°C/minute reduction at -80°C. Next, cryotubes were transported from -80°C to LN_2. GMSCs were cryopreserved for seven days in -80oC and LN_2.

Viability of GMSCs

After seven days in -80oC and LN^2, cryopreserved-GMSCs (cGMSCs) were aseptically thawed. The cryotube cap was rotated a quarter turn and then tightened to relieve the pressure. To thaw GMSCs, cryotubes were put in a water bath at 37°C for 1 minute. The trypan blue dye exclusion was used to determine the viability of GMSCs. The following formula was used to determine cell viability (CV):

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

The number of live cells is LC, whereas the number of cells is NC (10).

Morphology of GMSCs

The morphology of primary gingival cells, noncryopreserved-GMSCs (ncGMSCs) and cGMSCs was observed on five days incubation, and determined using an inverted-phase contrast microscope (Evos Cell Imaging Systems, Thermo Fisher Scientific, US) at 100x magnification. Their morphology was divided into three categories: rounded-shaped (RS), spindle-shaped (SS), and flatten-shaped (FS) (3).

Proliferation of GMSCs

The proliferation of ncGMSCs and cGMSCs was observed for 0, 24, 48, 72, 96, 120, and 144 hours. The cell density of 2x103 cells/cm2 was seeded into 24-wells plate. The cells were collected with 0.25% trypsin-EDTA solution after the incubation period. The cell suspension was centrifuged for 5 minutes at 25°C with 1000x g. The pellet was

dissolved in 50 µl of culture medium and the trypan blue dye exclusion was used to quantify the number of cell. Then, doubling time (DT) was determined as follows:

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

The number of cells at the end of incubation period is Nt, the number of cells at the beginning of incubation time is N0, and incubation time in hours is t (11). This assay used three replications.

Multipotencies of GMSCs

The multipotencies of ncGMSCs and cGMSCs were observed to determine chondrogenic, osteogenic, and adipogenic differentiation. OriCell™ Mesenchymal Stem Cell Adipogenic Differentiation Medium (Cat. No. GUXMX-90031, Cyagen, USA), OriCell™ Mesenchymal Stem Cell Osteogenic Differentiation Medium (Cat. No. GUXMX-90021, Cyagen, USA), and OriCell™ Mesenchymal Stem Cell Chondrogenic Differentiation Medium (Cat. No. GUXMX-90041, Cyagen, USA) were used in this study.

To induce adipogenic differentiation, the ncGMSCs and cGMSCs were seeded $2x10^4$ cells/cm^2 into 24-wells plate and incubated for three days in induction medium and maintenance medium. The induction and maintenance cycles were performed five times. The cells were then cultivated for seven days in maintenance medium until a big droplet was formed. The medium was changed every three days throughout this time. The cells were fixed for 30 minutes in a 4% formaldehyde solution and stained for 30 minutes in 250 µl Oil Red O solution.

Osteogenic medium consisted of 10 % FBS, 2% penicillin-streptomycin, glutamine, ascorbate, ß-Glycerophosphate, and 100nM dexamethasone. The ncGMSCs and cGMSCs were seeded $2x10^4$ cells/cm^2 into 24-wells plate pre-coated with 0.1 percent gelatin. The cells were induced with osteogenic differentiation media every three days for 28 days. The cells were fixed in a 4% formaldehyde solution for 30 minutes solution and stained for 5 minutes with Alizarin Red S.

The ncGMSCs ($2.5x10^5$ cells) or cGMSCs ($2.5x10^5$ cells) were needed to form one chondrogenic pellet. The ncGMSCs and cGMSCs suspension in incomplete medium was pelleted by centrifugation at 25°C, 150 x g for five minutes. The pellets were grown in complete medium after 24 hours and fed every three days in culture for 28 days. The pellets were formalin fixed, embedded in paraffin, and dyed with Alcian Blue.

Data analysis

Microsoft Excel version 16.14.1 was used to analyze cell viability, and cell proliferation. The data were calculated as mean standard deviation (SD). One-way analysis of variance followed by Tukey's post hoc multiple-comparison test (SPSS 22.0) was used to analyze the differences between groups. When the p-value was ($p<0.05$), the degree of significance was considered.

RESULTS

Characterization and morphology of GMSCs

GMSCs did not express the negative MSC surface markers of CD19/CD45/HLA-DR/CD34/CD11b, but they expressed the positive MSC surface markers of CD105, CD73, CD44, and CD90. Positive markers were expressed more than 90%, whereas negative markers were expressed less than 1% (Figure 1). The primary gingival cells, ncGMSCs and cGMSCs showed fibroblastic-like morphology, and formed homogenous monolayer culture. The ncGMSCs and cGMSCs displayed identical RS, SS, and FS morphologies (Figure 2).

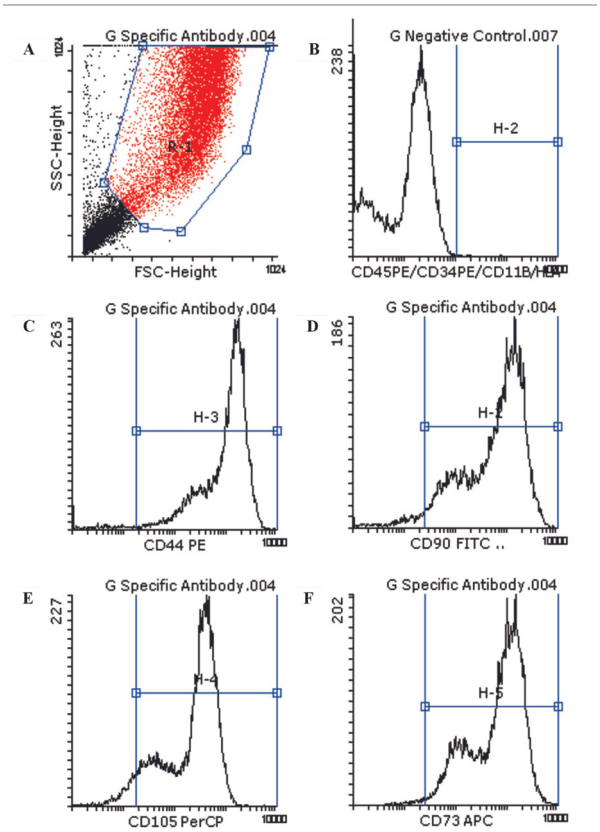

Figure 1 : Characterization of GMSCs. Negative MSC surface markers of CD19/CD45/HLA-DR/CD34/CD11b were expressed less than 1% (B). Positive MSC surface markers of CD105, CD73, CD44, and CD90 were expressed more than 90% (C-F).

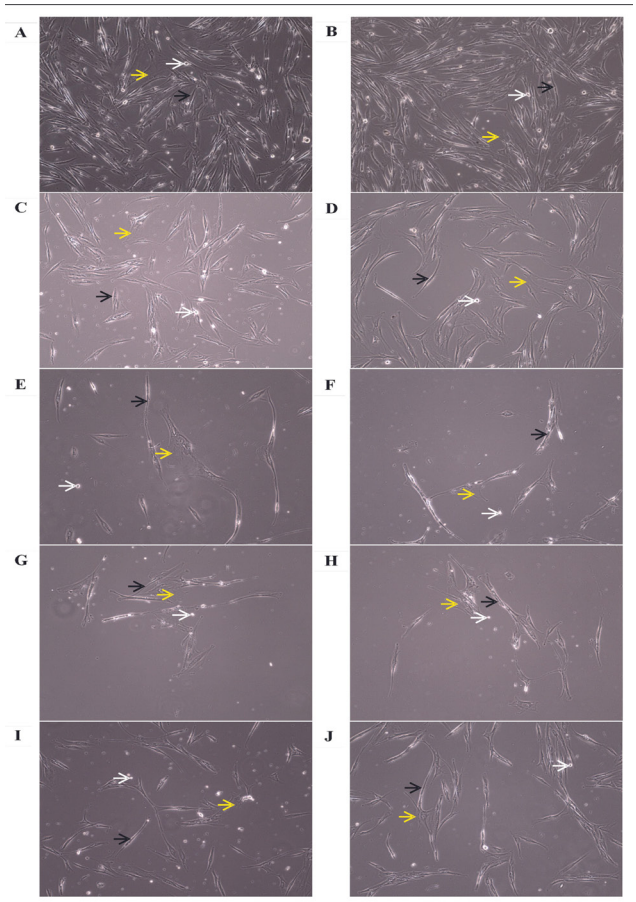

Figure 2 : Morphology of GMSCs. Primary gingival cells (A,B), ncGMSCs (C,D), and cGMSCs (E-J) showed fibroblastic-like morphology, formed homogenous monolayer culture, and displayed cell morphology of rounded-shaped (white arrow), spindle-shaped (black arrow), and flatten-shaped (yellow arrow). Magnification: x100; scale bar: 200 µm.

Viability of cGMSCs

The viability of cGMSCs in each group revealed the influence of CPAs during cryopreservation. In -80°C and LN_2, the FDs-group could sustain cell viability of 81% and 80%, respectively. The FDs group's cell viability was marginally lower than the CB group. In -80°C and LN_2, the CB-group showed cell viability of 90.5% and 88.5%, respectively. The cell viability of DDs-group and FD-group was less both in -80°C and LN_2. The cell viability DDs-group was 70.5% and 68% in -80°C and LN_2, respectively. The cell viability of FD-group was 51% and 49.5% in -80°C and LN_2, respectively (Table I).

Proliferation of ncGMSCs and cGMSCs

During incubation from 0 to 144 hours, the proliferation rate of ncGMSCs and cGMSCs were identical (Fig. 3). There were essentially no lag time in the culture of both ncGMSCs and cGMSCs. The time for the cell population to double was represented by the doubling time. For ncGMSCs, the doubling time was 13.79±0.03 hours to 26.13±0.04 hours, while 13.78±0.01 hours to 26.08±0.02 hours for cGMSCs. There was no significant difference between the two groups (p>0.05) (Figure 3). These results suggested that GMSCs are able to maintain their proliferative potential after cryopreservation.

Table I : The viability of cGMSCs at -80°C and LN_2.

Cryoprotectant Agents	Cell Viability (%)	
	Gingival Mesenchymal Stem Cells	
	-80°C	LN_2
CB-group	90.5	88.5
FD-group	51	49.5
DDs-group	70.5	68
FDs-group	81	80

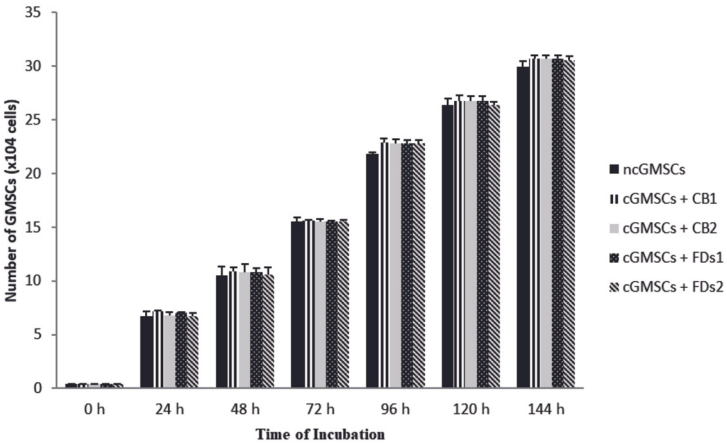

Figure 3 : Proliferation of ncGMSCs and cGMSCs. Proliferation rate of ncGMSCs and cGMSCs were identical throughout 0 to 144 hours incubation. There was no significant difference between the two groups (p>0.05).

Multipotencies of ncGMSCs and cGMSCs

The development of lipid droplets throughout 28 days of incubation indicated the adipogenic differentiation of ncGMSCs and cGMSCs (Figure 4). During 28 days of incubation, ncGMSCs had better osteogenic differentiation ability and more calcium deposition after osteogenic induction than cGMSCs (Figure 5). Subsequently, ncGMSC and cGMSC showed identical chondrogenic development with large and spherical pellets of proteoglycan (Figure 6).

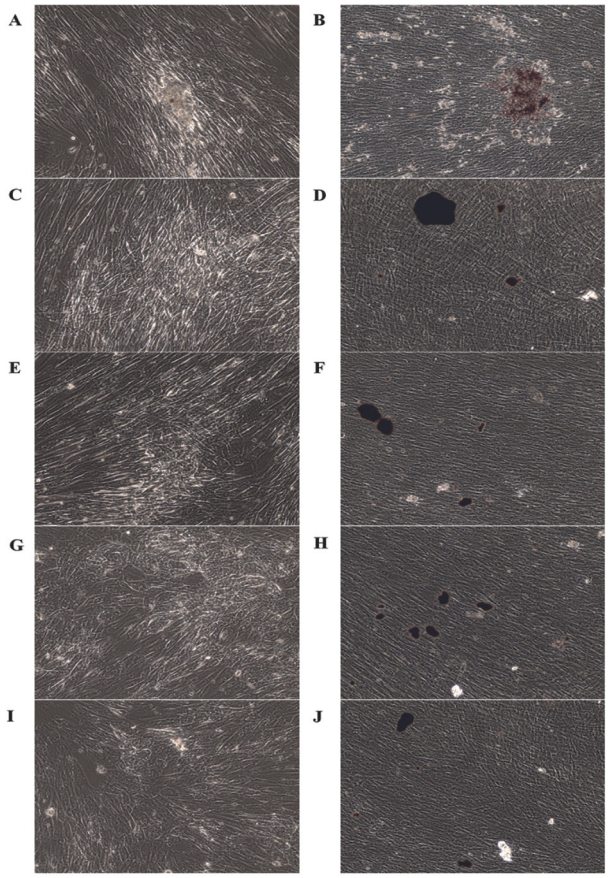

Figure 4 : Adipogenic differentiation of ncGMSCs and cGMSCs. The ncGMSCs and cGMSCs showed development of lipid droplets after 28 days incubation. The ncGMSCs were incubated in culture medium (A), and adipogenic induction medium (B). The cGMSCs were incubated in culture medium (C, E, G, I), and adipogenic induction medium (D, F, H, J). The cGMSCs were cryopreserved in Cell Banker (C-F), 90% FBS + 10% DMSO (G-J). The cGMSCs were cryopreserved at -80°C (C, D, G, H), and LN_2 (E, F, I, J). Magnification: x100; scale bar: 200 µm.

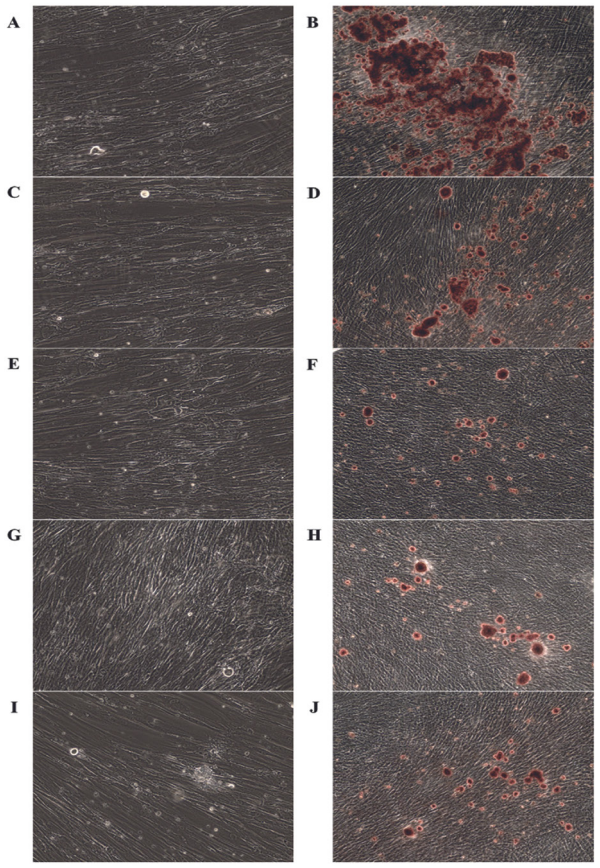

Figure 5 : Osteogenic differentiation of ncGMSCs and cGMSCs. During 28 days of incubation, ncGMSCs had better osteogenic differentiation ability and more calcium deposition after osteogenic induction than cGMSCs. The ncGMSCs were incubated in culture medium (A), and adipogenic induction medium (B). The cGMSCs were incubated in culture medium (C, E, G, I), and adipogenic induction medium (D, F, H, J). The cGMSCs were cryopreserved in Cell Banker (C-F), 90% FBS + 10% DMSO (G-J). The cGMSCs were cryopreserved at -80°C (C, D, G, H), and LN_2 (E, F, I, J). Magnification: x100; scale bar: 200 µm.

DISCUSSION

Cryopreservation is the most effective method of cell preservation with chemical, biological and physical processes terminated at cryogenic temperatures (12). Ideally, CPAs should be non-toxic, non-antigen, chemically inert, and maintain high cell viability after thawing. The post-thawing release criteria should be similar to the post-culture release criteria, with parameters including viability, recovery, doubling time, phenotype, and differentiation capacity (13).

Our study isolated stem cells from normal human gingival tissue characterized by spindle cell morphology, positive expression of MSC surface markers such as CD105, CD73, CD44, and CD90, but negative for the hematopoietic cell marker CD19/CD45/HLA-DR/CD34/CD11b, and multipotent differentiation into osteocytes, chondrocytes, and adipocytes. Our data also show that GMSCs have a high proliferative capacity or a low population doubling time (Figure 3). Some evidence has reported that human GMSCs have a mean population doubling time of 39.6 ± 3.2 hours, which is significantly less than BMSCs (80.4 ± 1.2 hours). In addition, GMSCs also have stable morphology and characteristics of MSCs, and retain telomerase activity after long-term culture (4).

The slow freezing method is preferred for the cryopreservation of MSCs in clinics and research laboratories, because of the low risk of contamination and easier processing (9). This

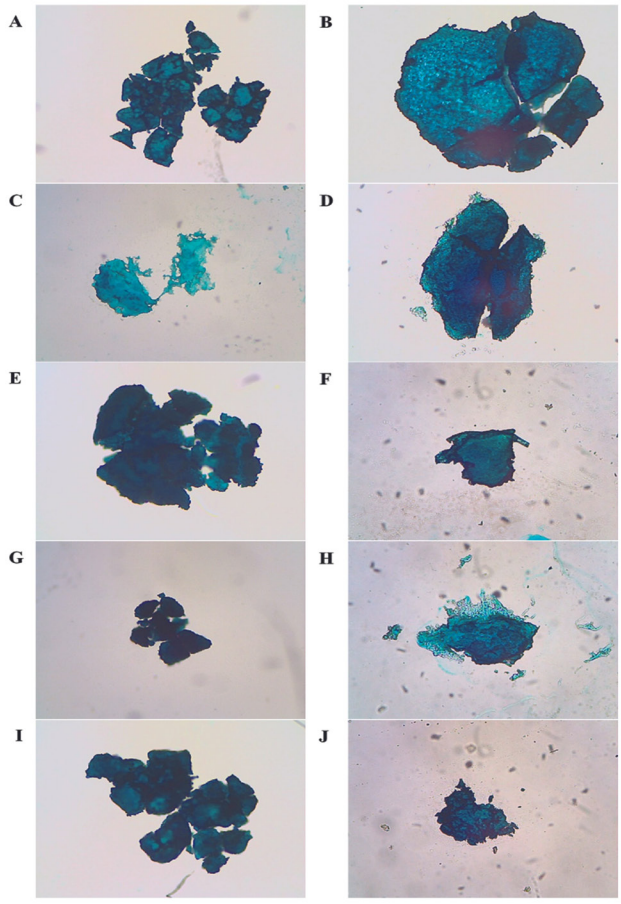

Figure 6 : Chondrogenic differentiation of ncGMSCs and cGMSCs. The ncGMSC and cGMSC showed identical chondrogenic development with large and spherical pellets of proteoglycan. The ncGMSCs were incubated in culture medium (A), and adipogenic induction medium (B). The cGMSCs were incubated in culture medium (C, E, G, I), and adipogenic induction medium (D, F, H, J). The cGMSCs were cryopreserved in Cell Banker (C-F), 90% FBS + 10% DMSO (G-J). The cGMSCs were cryopreserved at -80°C (C, D, G, H), and LN_2 (E, F, I, J). Magnification: x100; scale bar: 200 µm.

research has used a controlled freezing apparatus which produces a freezing rate of 1°C/min, so that there is no direct contact between cells and non-sterile liquid nitrogen during the freezing process. This will reduce the potential risk of GMSCs contamination. Our data showed that the FDs group could maintain GMSC viability of 81% and 80% in -80°C and LN_2, respectively (Table I). Assessment of post-thawing viability is very important in clinical transplantation applications, because it evaluates the quality and quantity of post-thawing cells. In addition, the freeze-thawing process will induce an extensive early apoptotic stress activation pathway that may lead to a time-dependent decrease in viability and function at culture temperature. Viability assessment at 24 hours post-thawing revealed a cascade of cell stress, including peaks of apoptotic (8-12 hours) and necrotic (4-8 hours) activity (14,15).

On the other hand, the slow freezing method is associated with a higher risk of freeze injury due to intra and extracellular ice formation during the freezing process (16), hence optimization of CPA concentration is required to avoid ice crystal formation. Our study resulted a slow freezing method in combination with 90% FBS + 10%. DMSO for cryopreservation of GMSCs.The most interesting aspect is that GMSCs can be propagated in culture for a long time while maintaining their viability and differentiation capacity. Currently, DMSO is a commonly used CPA for the cryopreservation of MSCs. The CPA added to the cryomedia is one of the main factors regulating the survival of cryopreserved cells. The cryopreservation of adipose derived stem cells (ASCs) using 10% DMSO as CPA had no effect on phenotype, viability, proliferation or osteogenic differentiation (17). In addition, cryopreservation protocols for dental pulp derived MSCs (DPSCs) added cryomedia containing 10% DMSO in fetal calf serum (FCS) (18). DMSO is a good choice of freezing media because DMSO can prevent intracellular ice formation and cell rupture by penetrating cells and removing water from them (19).

Our results imply that post-cryopreservation GMSCs (cGMSCs) can maintain their viability and proliferation, and are able to differentiate after slow-freezing cryopreservation as in control (ncGMSCs). The proliferation and doubling time between ncGMSCs and cGMSCs were statistically no significant difference ($p>0.05$) at -80°C and LN_2. In this case, we recommend that the GMSCs be stored at -80°C, as storage in liquid nitrogen will cause the vial to shrink so that the seal does not close tightly allowing the liquid nitrogen to seep into the vial. On rewarming, the vial expands, and then explodes when the nitrogen vaporizes in the vial (20). In addition, cross-contamination of the infected product will occur in other cells stored in the same liquid nitrogen tank (21).

Multilineage differentiation is an important potential for MSCs. Cryopreservation of ASCs (22) and BMSCs (23) has been achieved without loss or alteration of their multipotency, as was our study of cGMSCs (Figure 4-6). Adipogenic induction was associated with the accumulation of lipid droplets stained with Oil Red O solution. The lipid droplet pattern observed in the FDs-group was smaller and less dense than that of the CB-group. Osteocyte induction treatment resulted in mineralized nodules stained by Alizarin Red S as observed in FDs-group and CB-group. Next,

chondrogenic induction on FDs-group and CB-group resulted in similar round and big pellets. Nonetheless, none of the cryomedia compositions negatively affected the differential potential of GMSCs into chondrogenic, osteogenic, and adipogenic cell lineages.

From this discussion, it is clear that cryopreservation procedure should be fully optimized because banking GMSCs can be used to set up repeatable cell availability in the future. Next, technological advances in cell lyophilization will provide additional benefits, such as better viability, simpler long-term storage, and cheaper shipping (24). Finally, the preliminary data of this study are very encouraging and support the development of banking GMSCs for cell-based therapies in the future.

CONCLUSION

The present study showed that post-thawing GMSCs (cGMSCs) retain their viability, proliferation and ability to differentiate after slow-freezing cry opreservation in combination with 90% FBS+10% DMSO at -80°C and LN_2.

ACKNOWLEDGMENT

This research was supported by Research Institute in University of Jember, Indonesia (Grant No. 3240/ UN25.3.1/LT/2019).

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