Study on preservation solutions for therapy with human adipose tissue-derived mesenchymal stromal cells

May 2023

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Chapter 1. General Introduction

Stem cell transplantation therapy is promising for various refractory diseases [Hoang et al. 2021], such as cardiovascular disorders [Attar et al. 2021, Zhang et al. 2021], neurological disorders [Andrzejewska 2021], autoimmune diseases [Rad 2019], osteoarthritis [Nasiri et al. 2022], liver disorders [Liu et al. 2022], graft-versus-host disease [Zhao et al. 2019, Kelly and Rasko 2021], and coronavirus disease 2019 [Kirkham et al. 2023]. Mesenchymal stromal cells (MSCs) are especially attractive because of their immunosuppressive effect, multi-potency in differentiation, and remodeling effect on extracellular matrices. In addition, MSCs do not express human leucocyte antigen (HLA) Class II, which allows allogeneic administration without donor-recipient matching [Kelly and Rasko 2021]. MSCs are easily accessible from various sources. Bone marrow, umbilical cord, and adipose tissue are major sources of MSCs [Zhou et al. 2021]. Because bone marrow constitutes only a rare MSC population and the painful harvesting procedure requires general anesthesia, the usage of bone marrow-derived MSCs is limited [Mushahary et al. 2018]. On the other hand, adipose tissue is a rich source of MSCs, and a large number of MSCs can be obtained from adipose tissue by a minimally invasive procedure [Mushahary et al. 2018]. Thus, adipose tissue-derived MSCs have become an essential candidate for stem cell-based therapy [Mushahary et al. 2018].

Maintaining cell viability and function during preservation and transportation for cell-based therapy is essential. Preservation of cells allows for their safe portation over distances and improves quality control testing in clinical and research applications [Heydarzadeh et al. 2022]. In general, the storage methods for regenerative medicine are classified into four categories: storage in a low-temperature zone, storage in an ordinary-temperature zone, storage in a culture-temperature zone, and cryopreservation. Of the three regenerative medicine products using mesenchymal stem cells approved in Japan to date, two adopt cryopreservation, and one adopts ordinary temperature storage. TEMCELL® HS Inj. (JCR Pharmaceuticals Co., Ltd.), an allogeneic bone marrow-derived mesenchymal stem cell product used for acute graft-versus-host disease after hematopoietic stem cell transplantation, is cryopreserved, thawed, diluted with saline, and infused intravenously. The administration starts within 3 hours after dilution [JCR Pharmaceuticals Co., Ltd, 2017]. STEMIRAC® Inj. (Nipro Corporation), an autologous bone marrow-derived mesenchymal stem cell product used for neurosis and dysfunction associated with spinal cord injury is cryopreserved, thawed, and intravenously infused while diluting with saline. The administration finishes within 1 hour after thawing

[Nipro Corporation, 2020]. ALOFISEL® Inj. (Takeda Pharmaceutical Co., Ltd.), an allogeneic adipose tissue-derived mesenchymal stem cell product used for complicated anal fistula associated with Crohn's disease is stored at 15°C to 25°C with culture media containing albumin and injected locally within 72 hours after shipping [Takeda Pharmaceutical Co., Ltd., 2021]. Thus, different storage methods are selected according to the product.

For intravascular transplantation, in many cases, stem cells are suspended in an electrolyte solution, such as normal saline or lactated Ringer's solution (Table 1). However, these solutions are not necessarily ideal for maintaining cell viability during storage and preventing the sedimentation of cells during infusion. Therefore, we have designed novel preservation solutions that apply to cold storage, ambient temperature storage, and infusion of MSCs [Chapter 2. Fujita et al. 2020]. Our first study aimed to optimize the compositions of these preservation solutions, which could affect the efficacy and safety of stem cell therapy using human adipose tissue-derived mesenchymal stromal cells (hADSCs). We tested various components of cell preservation solutions to determine the optimal combination of components for maintaining hADSCs.

Table 1 Composition of various intravenous solutions

Components	Saline	R	LR	AR	BR
Na^+	154 mEq/L	147 mEq/L	130 mEq/L	130 mEq/L	130 mEq/L
K^+	-	4 mEq/L	4 mEq/L	4 mEq/L	4 mEq/L
Ca^{2+}	-	4.5 mEq/L	3 mEq/L	3 mEq/L	3 mEq/L
Mg^{2+}	-	-	-	-	2 mEq/L
Cl^-	154 mEq/L	155.5 mEq/L	109 mEq/L	109 mEq/L	109 mEq/L
L-Lactate	-	-	28 mEq/L	-	-
Acetate	-	-	-	28 mEq/L	-
Bicarbonate	-	-	-	-	28 mEq/L
$Citrate^{3-}$					4 mEq/L

R: Ringer's solution, LR : lactated Ringer's solution, AR: acetated Ringer's solution, BR: bicarbonate Ringer's solution

Cellular volume homeostasis and ion homeostasis are essential for maintaining cell viability [Lang et al. 2006, Romero 2004, Dubyak 2004, Lang et al. 2007]. Osmolality and the electrolyte composition of extracellular solutions affect homeostasis [Romero 2004, Strange et al. 2004]. Thus, we examined the cytoprotective effects of Ringer's solutions containing an alkalizing compound compared to normal saline (Table 1). These are generally used as infusion solutions in clinical to compensate for extracellular fluid loss and thus have much safety information.

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside, Figure 1) is a nonreducing

disaccharide known as a protein, membrane stabilizer, and cryoprotective agent for different kinds of cells [Jain and Roy 2009]. In nature, trehalose contributes seeds of many plants, yeasts, mosses, cysts of crustaceans, and certain microscopic animals such as nematodes, rotifers, and tardigrades to survive the loss of their water [Crowe 2015]. The trehalose mechanism of action is not fully understood. However, several hypotheses have been proposed. The water replacement hypothesis, one of the majors is that trehalose forms hydrogen bonds with polar residues in proteins and phospholipids of membranes and gives them a physical state resembling that seen in the presence of water [Crowe 2015]. Trehalose works particularly well than other sugars for the following reasons: (1) the glycosidic bond linking the two glucose monomers is remarkably stable, (2) trehalose forms a glass in the dry state, which is essential to its ability to stabilize biomaterials [Crowe 2015]. Trehalose is an ingredient in food, health, and beauty products, and pharmaceutical products such as monoclonal antibodies administrated via a parenteral route [Falconer 2019, Gervasi et al. 2018, Richards et al. 2002]. We found that trehalose maintains cell viability in cell preservation solutions stored at 5°C and 25°C, and we attempted to optimize its concentration in preservation solutions.

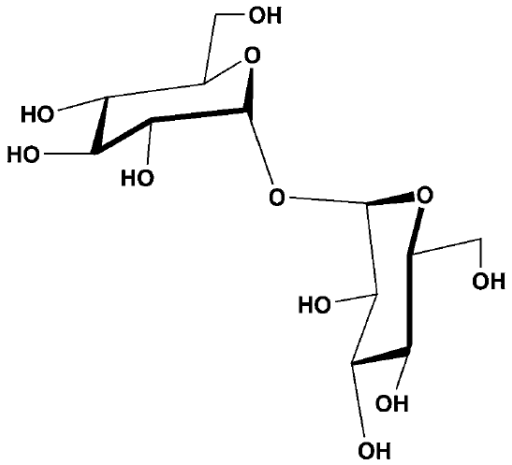


Figure 1 Chemical structure of trehalose
[Skibinskiy et al. 2005]

Cells precipitate onto the bottom of infusion bags during storage and infusion; this may change the cell concentration and cause infusion toxicity, such as a cell embolism in the lung. In this study, we used low molecular weight dextran 40 (Figure 2) to prevent cell sedimentation. Dextran 40 has been clinically used as a plasma expander to increase colloidal osmotic pressure in blood [Boldt 2005]. We hypothesized that the viscosity and specific gravity of dextran 40 would prevent cells from sedimentation in our

proposed solution.

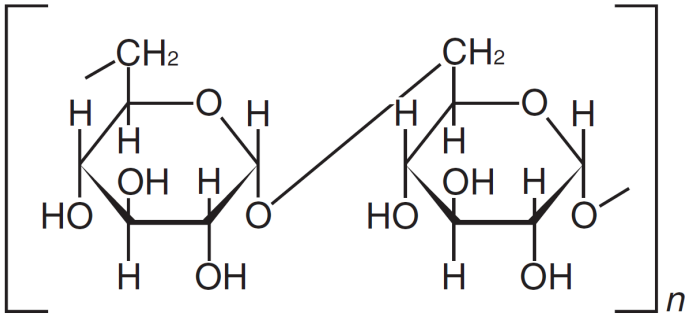


Figure 2 Chemical structure of dextran
[Otsuka Pharmaceutical Factory, Inc. 2023]

As a result, we developed a cell preservation solution consisting of lactated Ringer's solution with 3% trehalose and 5% dextran 40 (LR-3T-5D) for storing hADSCs at 5°C and 25°C for up to 24 h. The International Society for Cellular Therapy defined MSCs as a population of cells that is plastic-adherent when maintained in standard culture conditions expresses CD73, CD90, and CD105 in the absence of CD34, CD45, CD14 or CD11b, CD79α or CD19, and HLA-DR surface molecules, differentiate to osteoblasts, adipocytes, and chondroblasts in vitro [Dominici et al. 2006]. So, we evaluated the colony formation unit, which reflects plastic-adherent, cell surface makers, and differentiation ability of hADSCs before and after storage.

Furthermore, we confirmed the usefulness of LR-3T-5D as a vehicle solution for cryopreservation with dimethyl sulfoxide (Me2SO, Figure 3). If LR-3T-5D is also effective as a vehicle solution for cryopreservation, it can provide a seamless cell preservation method from washing to freezing.

Cell cryopreservation solutions generally contain about 10% Me2SO. This technique was established in the 1950s [Lovelock and Bishop 1959] and is now commonly used in cell banks. However, side effects associated with the transplantation of stem cells with Me2SO have been reported, including nausea, vomiting, cardiac arrhythmias, neurological symptoms, respiratory arrest, renal/hepatic dysfunction, and allergies [Santos et al. 2003; Windrum et al. 2005; Cox et al. 2012; Shu et al. 2014; Awan et al. 2020]. In addition, a portion of the administered Me2SO is reduced to dimethyl sulfide in the body, which is then secreted through the skin and exhaled air, causing a foul, garlic-like odor [Santos et al. 2003; Shu et al. 2014; Awan et al. 2020]. Therefore, there is a need for a cryopreservation solution that can reduce the side effects of Me2SO. In our second study, we confirmed the effects of different Me2SO concentrations and cell-washing procedures after thawing cells using LR-3T-5D to

reduce the amount of Me2SO administered [Chapter 3, Fujita et al. 2021].

Me2SO stimulates differentiation in some types of cells. For example, mouse bone marrow mesenchymal stem cells are differentiated into cardiomyocytes by Me2SO [Young et al. 2004]. Cryopreservation of human embryo-stem (ES) cells in Me2SO-containing cryopreservation solution decreases the expression of Oct-4, an undifferentiated marker [Katkov et al. 2006], and Me2SO affects the epigenetic profile of mouse embryoid bodies [Iwatani et al. 2006]. The human promyelocytic leukemia cell line HL-60 is differentiated into granulocytes by Me2SO [Jiang et al. 2006]. These effects are unfavorable for maintaining cell characteristics during cryopreservation. Therefore, given concerns over maintaining stemness in addition to safety, a Me2SO-free cryopreservation solution is desired. We have compared the effects of the cell membrane penetrating cryoprotectants propylene glycol (PG), ethylene glycol, and glycerol (Figure 3) with those of Me2SO and confirmed that LR-3T-5D with 10% PG results in a similar cell proliferative capacity as LR-3T-5D with 10% Me2SO. In our third study, we compared the performance of LR-3T-5D with 10% PG to that of LR-3T-5D with 10% Me2SO on cell characteristics (cell surface antigen positivity, adipogenic differentiation, osteogenic differentiation, and genetic response to cytokine stimulation) of hADSCs [Chapter 4, Fujita et al. 2022]. Furthermore, we optimized the concentration of PG with LR-3T-5D as a cryopreservation solution for hADSCs.

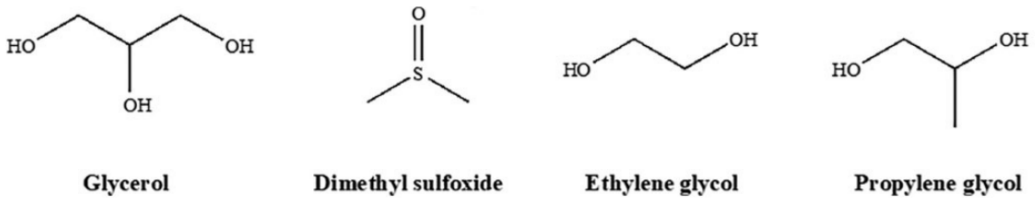


Figure 3 Chemical structure of glycerol, dimethyl sulfoxide, ethylene glycol, and propylene glycol
[Whaley et al. 2021]

Chapter 2. Determination of compositions of room temperature and refrigerated temperature storage for human adipose tissue derived mesenchymal stromal cells

2.1 Introduction

Stem cell transplantation is a promising therapy for various diseases, such as cardiovascular disorders [Hou et al. 2016, Terashvili et al. 2019], autoimmune diseases [Munir et al. 2015], osteoarthritis [Wyles et al. 2015], liver disorders [Kadyk et al. 2015, Forbes et al. 2015, Tsolaki et al. 2015, Kholodenko et al. 2017], and graft-versus-host disease [Chen et al. 2015, Trounson et al. 2015]. Mesenchymal stromal cells (MSCs) are especially attractive because of their immunosuppressive effect, multi-potency in differentiation, and remodeling effect on extracellular matrices. For intravascular transplantation, in many cases, stem cells are suspended in an electrolyte solution, such as normal saline or lactated Ringer's solution. However, these solutions are not necessarily ideal for maintaining cell viability and preventing the sedimentation of cells during storage and infusion.

Cellular volume homeostasis and ion homeostasis are important for maintaining cell viability [Lang et al. 2006, Romero 2004, Dubyak 2004, Lang et al. 2007]. Osmolality and the electrolyte composition of extracellular solutions affect homeostasis [Romero 2004, Strange et al 2004]. Thus, we examined the cytoprotective effects of Ringer's solutions containing an alkalizing compound in comparison to normal saline. These solutions are generally used as infusion solutions and thus have abundant safety information.

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a nonreducing disaccharide that is known to be a protein and membrane stabilizer and cryoprotective agent for different kinds of cells [Jain and Roy 2009]. In this study, we found that trehalose maintains cell viability in cell preservation solutions stored at 5°C and 25°C, and we attempted to optimize its concentration in preservation solutions.

Cells precipitate onto the bottom of infusion bags during storage and infusion; this may change the cell concentration during infusion and possibly cause infusion toxicity, such as a cell embolism in the lung. We used low molecular weight dextran 40 to prevent cell sedimentation in this study. Dextran 40 has been clinically used as a plasma expander to increase colloidal osmotic pressure in blood [Boldt 2005]. We hypothesized that the viscosity and specific gravity of dextran 40 would prevent cells from sedimentation in our proposed solution.

The objective of this study was to optimize the compositions of preservation solutions, which could affect the efficacy and safety of stem cell therapy using human adipose tissue-derived mesenchymal stromal cells (hADSCs). Here, we tested various components of cell preservation solutions to determine the optimal combination of components for maintaining hADSCs.

2.2 Material and methods

2.2.1 Study design

The present study was approved by the ethics committee of Otsuka Pharmaceutical Factory, Inc.

This study consisted of five consecutive steps. First, we determined the effects of various medical electrolyte solutions for dehydration on the viability of hADSCs. Second, we determined the optimal concentration of trehalose for the viability of hADSCs. Third, we determined the concentration of dextran 40 needed to prevent cell sedimentation for 1 h and cell death. Fourth, we evaluated the ability of the final compositions of the cell preservation solutions to preserve cells for up to 96 h when stored at 5°C or 25°C. Finally, we confirmed the characteristics of the cells (cell surface markers, colony-forming capacity, and differentiation ability) as well as the cell concentrations in the infusion line after 24 h and 1 h of preservation, respectively, in the preservation solutions.

2.2.2 Components of the solutions

Normal saline, Ringer's solution, lactated Ringer's solution (Lactec® Injection), bicarbonate Ringer's solution (BICANATE® Injection), 10% dextran 40 in lactated Ringer's solution (Low Molecular Dextran L Injection), lactated Ringer's solution with 3% trehalose (LR-3T; Cellstor®-W), and lactated Ringer's solution with 3% trehalose and 5% dextran 40 (LR-3T-5D; Cellstor®-S) were supplied by Otsuka Pharmaceutical Factory, Inc (Tokushima, Japan). Acetated Ringer's solution (Veen-F Inj.) was purchased from Fuso Pharmaceutical Industries, Ltd (Osaka, Japan). Trehalose dehydrate was purchased from Fujifilm Wako Pure Chemical Co. (Osaka, Japan).

2.2.3 Preparation of hADSCs

Human ADSCs (Female, 51Y or 38Y, PT5006; LonzaWalkersville, Inc., Walkersville, MD, USA) were used in this study; 51Y and 38Y indicates the age of the donors. The results obtained using the 51Y hADSCs are described in 2.3.1, 2.3.2 and 2.3.3 of the Results, while those described in 2.3.4 and 2.3.5 were 38Y. Human ADSCs were seeded in a 75-cm^2 flask with 15 mL of medium prepared from a medium kit (ADSC BulletKit™, Lonza Walkersville, Inc.), and maintained at 37°C in a humidified atmosphere of 5% CO_2. All of the mediumwas changed every 3 or 4 days. Cells were passaged at approximately 90% confluency, and passage 3 or 4 was used for the experiments. Cells were trypsinized with Trypsin/EDTA solution (CC-5012, Lonza Walkersville, Inc.) for 5 min at 37°C.

2.2.4 Cell viability

In this experiment, 5.0×10^5 hADSCs suspended in 1 mL of phosphate-buffered saline or LR-3T were added to low cell adsorption tubes (STEMFULL™, Sumitomo Bakelite Co., Ltd., Tokyo, Japan). After centrifugation, the supernatants were replaced with 1 mL of various preservation solutions, and the cells were resuspended. The samples were stored at 5°C in a refrigerator or 25°C in an incubator. Cell viability was determined manually with a plastic cell counting plate (OneCell Counter, Bio Medical Science, Ltd., Tokyo, Japan) after trypan blue staining.

In the experiments for determining optimal compositions, viability was assayed immediately and 24 h after suspension. For the evaluation of our optimized compositions (LR-3T and LR-3T-5D), viability was assayed immediately and at 6, 24, 48, and 96 h after suspension.

Cell viability was calculated according to the formula below.

Cell viability [%] = (total number of cells − number of dead cells)/ (total number of cells) × 100.

2.2.5 Percentage of cells in the supernatant

In this experiment, 7.5×10^5 hADSCs suspended in 1 mL of phosphate-buffered saline were added to low cell adsorption tubes (STEMFULL™). After centrifugation, the supernatants were replaced with 3 mL of phosphate-buffered saline or various preservation solutions, and the cells were resuspended. The samples were stored at room temperature. Cells were counted in 3 mL of supernatant immediately after resuspension

for cells in phosphate buffered saline to establish the baseline values, or at 1 h after resuspension for cells in the other preservation solutions. A schematic drawing of the methods is shown in Figure 1.

The percentage of cells in the supernatant was calculated according to the formula below.

% cells in the supernatant = [(number of cells in the supernatant of the sample)／(mean baseline value)] × 100

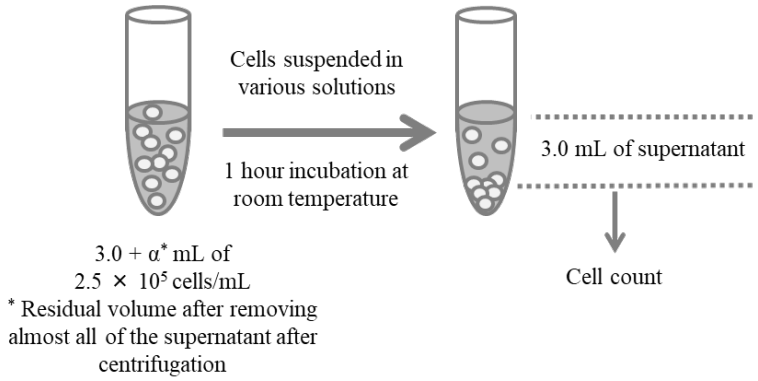


Figure 4 Method used to evaluate the percentage of cells in supernatant.

2.2.6 Annexin V staining

Suspended cells were stained using an Annexin V-FITC Kit (Beckman Coulter, Brea, CA, USA). Measurements were performed using a Gallios flow cytometer (Beckman Coulter).

2.2.7 Cell surface markers

To examine the surface immunophenotypes of the cells, 2×10^5 cells in 20 mL of staining buffer with fetal bovine serum (BD Biosciences, San Jose, CA, USA) were incubated for 60 to 120 min on ice with phycoerythrin-labeled antibodies against human CD14, CD34, CD44, CD45, CD73, CD90, CD105, and HLA-DR (BD Biosciences) or the respective isotype controls (BD Biosciences). After washing, the labeled cells were analyzed using a Gallios flow cytometer (Beckman Coulter).

2.2.8 Colony-forming capacity

Cells were suspended and diluted 100-fold in culture medium. The diluted solution was added to a dish containing 4 mL of the culture medium so that cells were plated at a density of 315 cells in a 21-cm^2 culture dish (15 cells/cm^2). The culture medium was changed every 3 days. After 8 days, the cells were washed with phosphate-buffered saline, fixed with ice-cold methanol for 15 min, and then stained with Giemsa at room temperature. After 30 min of staining, cells were washed with distilled water. Colonies of more than 50 cells were then counted. The colony-forming efficiency of cells was calculated by dividing the number of colonies per dish by the number of cells (315) seeded per dish.

2.2.9 Adipogenic and osteogenic differentiation ability

Adipogenic differentiation was induced according to the Poietics™ human ADSCs adipogenesis protocol (LonzaWalkersville, Inc.) and evaluated by Oil Red staining. Osteogenic differentiation was induced according to the Poietics™ human ADSCs osteogenesis protocol (Lonza Walkersville, Inc.) and evaluated with an alkaline phosphatase staining kit (AK20, Cosmo Bio Co., Ltd., Tokyo, Japan) and a calcified nodule staining kit (AK21, Cosmo Bio Co., Ltd.).

2.2.10 Cell concentrations in the infusion line

Each blood bag (BB-T015CJ, 150 mL, Terumo Co., Tokyo, Japan) was hung on a stand, and the height of the hook of the stand was adjusted so that the liquid level in the blood bag was about 90 cm from the top of the low cell adsorption tubes (STEMFULL™) installed on a tube rack. A blood transfusion set (TB-U200L, Terumo Co.) was used as the infusion line from the bag to the tubes. The blood bags containing hADSCs suspended in LR-3T-5D or normal saline (1.5×10^7 cells/100 mL) were placed on the stand after mixing by inversion, and were allowed to stand for 1 h. A 22G Surflash™ I.V. Catheter (SR-FF2225, Terumo Co.) was connected to the blood transfusion set, and the needle tip was placed in the numbered low cell adsorption tubes (STEMFULL™). The solution was dropped into the tubes at a rate of approximately 3.3 mL/min, and 5 mL of the test solution was collected per tube. At the end ofthe 10th collection fraction, the test solution in the blood bag was mixed approximately five times to stir the precipitated cells. These procedures were performed three times for each solution. A schematic drawing of the methods is shown in Figure 2.

The volume and cell concentration of each fraction were measured. The cell

recovery rate was calculated as the ratio of the total number of collected cells to the total number of cells (1.5×10^7 cells/100 mL) at the time the blood bag was filled.

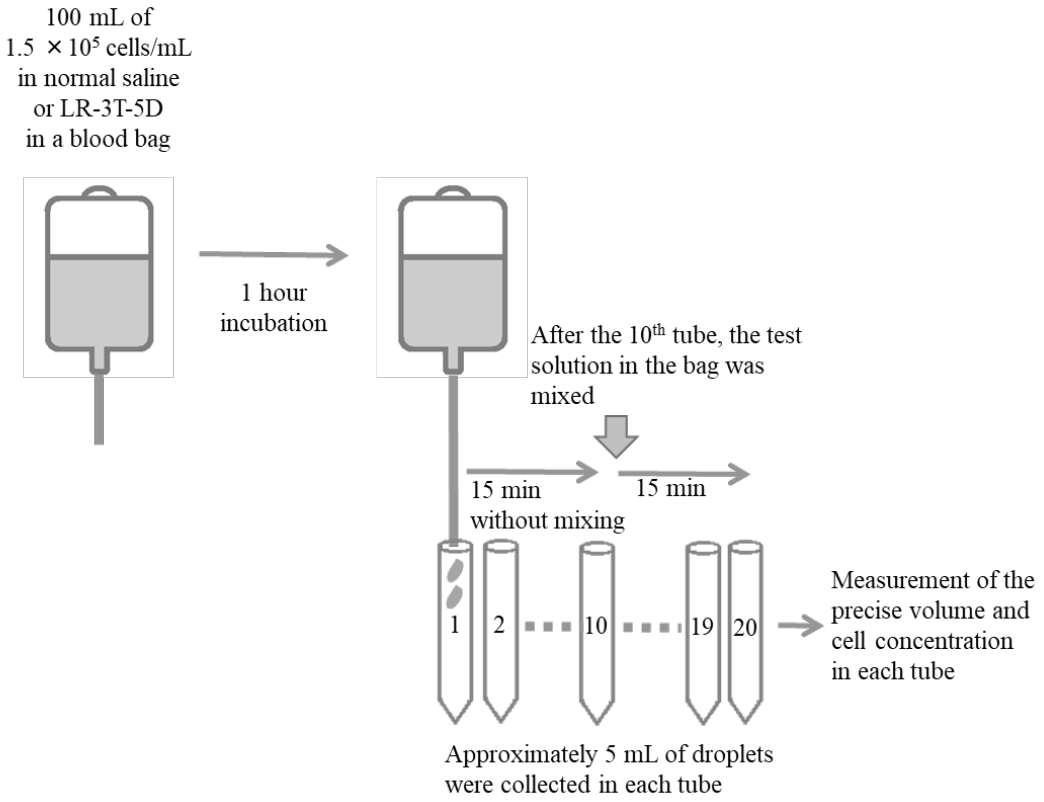


Figure 5 Method used for evaluating the cell concentration in the infusion line.

2.2.11 Statistical methods

Results are presented as the mean ± standard deviation (SD). Dunnett's multiple comparison test and the Student's t-test were used. The doseeresponse relationship of the percentage of cells in the supernatant was evaluated with the maximum contrast method [Yoshimura et al. 1997]. Alpha was set at 0.05, and all tests were two-tailed. Data were analyzed with SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

2.3 Results

2.3.1 Electrolyte composition

Cell viability was significantly higher in the various Ringer's solutions than in normal saline ($p < 0.001$ for all of the Ringer's solutions). On the other hand, we found

no significant difference in cell viability among the Ringer's solutions. Viability after storage for 24 h was highest in lactated Ringer's solution (Table 1).

Table 2 Cell viability of human adipose tissue-derived mesenchymal stromal cells in various solutions after storage at 5°C

Group	Immediately after suspension (%)	24 hours after suspension (%)
Normal saline	98.6 ± 1.2	73.1 ± 8.4
Ringer's solution	99.1 ± 1.1	93.7 ± 3.6***
Lactated Ringer's solution	99.1 ± 1.1	96.3 ± 1.8***
Acetated Ringer's solution	99.5 ± 0.7	95.2 ± 2.8***
Bicarbonate Ringer's solution	99.3 ± 0.7	92.4 ± 5.3***

Values indicate the mean ± SD (n = 6). ***p < 0.001 vs. normal saline with Dunnett's test. No significant differences in cell viability were found among the Ringer's solutions using Tukey's test.

2.3.2 Appropriate concentration of trehalose

Cell viability was significantly higher in the lactated Ringer's solutions containing more than 3% trehalose than in the other lactated Ringer's solutions ($p < 0.001$; Table 2).

Table 3 Cell viability of human adipose tissue-derived mesenchymal stromal cells in various solutions, with and without trehalose, after storage at 5°C.

Group	n	Immediately after suspension (%)	24 hour after suspension (%)
S	6	98.6 ± 0.9	70.6 ± 6.2
LR	6	98.7 ± 1.2	89.0 ± 2.7
LR+1.0%T	6	97.6 ± 0.8	90.2 ± 2.5
LR+3.0%T	5	98.1 ± 1.5	98.0 ± 1.6***
LR+5.0%T	6	98.5 ± 1.6	97.1 ± 2.0***
LR+7.0%T	6	98.6 ± 0.9	97.4 ± 2.9***
LR+10.0%T	6	98.1 ± 1.8	96.4 ± 2.5***

The values indicate mean ± SD (n=5 - 6). *p<0.05, ***p<0.001 vs. lactated Ringer's solution, by Dunnett's test. S; Normal saline, LR; Lactated Ringer's solution, T; trehalose.

2.3.3 Appropriate concentration of dextran 40

The percentage of cells in the supernatant after 1 h of settling in normal saline and

lactated Ringer's solutions were less than 30%, and the percentage in the lactated Ringer's solution containing 3% trehalose was less than 40% (Figure 3). Increasing the concentration of dextran 40 in lactated Ringer's solution containing 3% trehalose increased the percentage of cells in the supernatant (Figure 3). The dose response relationship of the percentage of cells in the supernatant with 13 patterns of hypothesized monotonic increases was evaluated with the maximum contrast method (Figure 4). The maximum sum-of-squared contrast weights was indicated by the 2-start 4-end pattern (Table 3). Therefore, we concluded that the percentage of cells in the supernatant reached a plateau in lactated Ringer's solution containing 3% trehalose and 5% dextran 40. The mean ± SD of the percentage of cells in the supernatant after 1 h of settling in lactated Ringer's solution containing 3% trehalose and 5% dextran 40 was 83.5% ± 7.6% (Figure 3).

Cell viability was significantly lower in the lactated Ringer's solution containing 3% trehalose and 10% dextran 40 than in the lactated Ringer's solution containing only 3% trehalose ($p < 0.001$; Table 4).

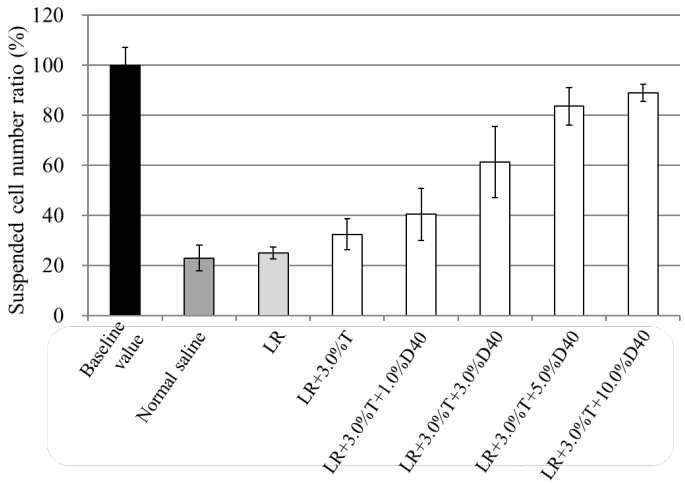


Figure 6 The percentage of cells in the supernatant after 1 h of settling in various cell preservation solutions.

Each point and vertical bar indicates the mean and standard deviation (SD; n = 6). The percentages are the numbers of hADSCs in 3 mL of supernatant of various solutions after 1 h of settling at room temperature relative to the baseline values. LR: lactated Ringer's solution, LR+3.0% T: lactated Ringer's solution containing 3% trehalose, LR+3.0% T+1.0%−10.0% D40: lactated Ringer's solution containing 3% trehalose +1.0%−10.0% dextran 40.

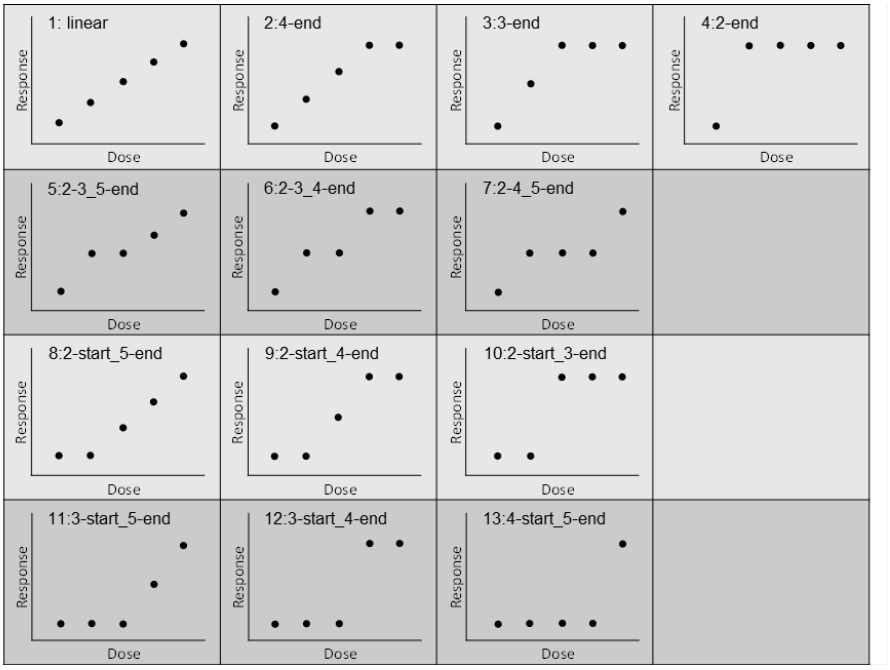


Figure 7 Dose-response curves of the percentage of cells in supernatant with 13 hypothetical patterns of monotonic increases according to the maximum contrast method.

The maximum sum-of-squared contrast weights was indicated by the 2-start 4-end pattern.

Table 4 Sum of squared contrast weights of the percentage of cells in supernatant using human adipose tissue-derived mesenchymal stromal cells after 1 hour in test solutions containing different dextran concentrations at room temperature

Dose-response pattern	Contrast vector*	Sum of squared contrast weight
1 : linear	(-2, -1, 0, 1, 2)	14612.9
2 : 4-end	(-4, 2, 0, 3, 3)	14863.8
3 : 3-end	(-2, -1, 1, 1, 1)	12366.7
4 : 2-end	(-4, 1, 1, 1, 1)	6248.3
5 : 2-3 5-end	(-4, -1, -1, 2, 4)	13397.2
6 : 2-3 4-end	(-4, -1, -1, 3, 3)	13631.2
7 : 2-4 5-end	(-2, 0, 0, 0, 2)	9556.2
8 : 2-start 5-end	(-3, -3, 0, 2, 4)	14608.0
9 : 2-start 4-end	(-3, -3, 0, 3, 3)	14886.6
10 : 2-start 3-end	(-3, -3, 2, 2, 2)	12386.6
11 : 3-start 5-end	(-1, -1, -1, 1, 2)	12148.8
12 : 3-start 4-end	(-2, -2, -2, 3, 3)	12424.4
13 : 4-start 5-end	(-1, -1, -1, -1, 4)	5703.2

*From left to right, values indicate the weights for LR+3.0% T, LR+3.0% T+1.0% D40, LR+3.0% T+3.0% D40, LR+3.0% T+5.0% D40, and LR+3.0% T+10.0% D40, respectively, where LR is lactated Ringer's solution, T is trehalose, and D40 is dextran 40

Table 5 Cell viability of human adipose tissue-derived mesenchymal stromal cells in various solutions, with 3% trehalose, with and without dextran 40, after storage at 5°C.

Group	Immediately after suspension (%)	24 hour after suspension (%)
S	97.3 ±1.7	68.4 ±6.4
LR	97.1 ±1.4	90.2 ±5.4
LR+3.0% T	97.4 ±1.8	95.1 ±3.2
LR+3.0%T+1.0%D40	97.1 ±1.5	95.8 ±1.9
LR+3.0%T+3.0%D40	97.5 ±1.7	96.8 ±2.4
LR+3.0%T+5.0%D40	98.4 ±1.2	96.4 ±3.0
LR+3.0%T+10.0%D40	98.4 ±1.4	83.8 ±5.6***

The values indicate mean ± SD (n=6). ***p<0.001 vs. LR+3.0% T, by Dunnett's test. S; Normal saline, LR; Lactated Ringer's solution, T; trehalose, D40; dextran 40.

2.3.4 Evaluating the optimal cell preservation solutions

We concluded that the optimal composition for the wash and preservation solution was lactated Ringer's solution containing 3% trehalose (LR-3T), and the optimal composition for the suspension and preservation solution was lactated Ringer's solution containing 3% trehalose and 5% dextran 40 (LR-3T-5D; Table 5). We subsequently tested LR-3T and LR-3T-5D against four endpoints: cell viability, cell surface markers, colony-forming capacity, and adipogenic and osteogenic differentiation. Furthermore, we tested the stability of the cell concentration in the infusion line with LR-3T-5D.

Table 6 Composition of the optimal preservation solution for human adipose tissue-derived mesenchymal stromal cells

Components	LR-3T	LR-3T-5D
Na^+	130 mEq/L	130 mEq/L
K^+	4 mEq/L	4 mEq/L
Ca^{2+}	3 mEq/L	3 mEq/L
Cl^-	109 mEq/L	109 mEq/L
L-Lactate$^-$	28 mEq/L	28 mEq/L
Trehalose	3%	3%
Dextran 40	-	5%

LR-3T: lactated Ringer's solution with 3% trehalose.
LR-3T-5D: lactated Ringer's solution with 3% trehalose and 5% dextran 40.

Figure 5A shows the time course of cell viability during 96 h of preservation in

LR-3T, LR-3T-5D, and lactated Ringer's solution at 5°C. The mean values for cell viability were significantly higher in LR-3T and LR-3T-5D than in lactated Ringer's solution at all time points after 6 h of storage. The cell viabilities after 24 h of storage at 5°C in LR-3T and LR-5D were 94.9% ± 2.4% and 97.6% ± 2.4%, respectively. The mean values for cell viability in LR-3T-5D were higher than 90% even at 48 h after storage. Figure 5B shows the time course of cell viability after storage in various solutions at 25°C. The mean values for cell viability were significantly higher in LR-3T-5D than in lactated Ringer's solution at all time points after 6 h of storage. The mean value for cell viability was significantly higher in LR-3T than in lactated Ringer's solution at 6 h. The mean values for cell viability tended to be higher in LR-3T than in lactated Ringer's solution at 48 h ($p = 0.0569$) and 96 h ($p = 0.0979$). The mean values for cell viability were higher than 90% in LR-3T and LR-3T-5D for up to 48 h.

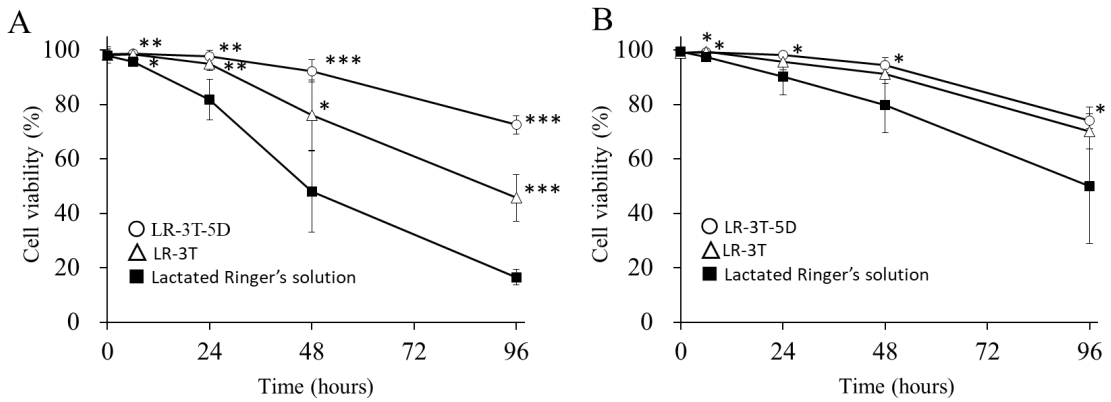


Figure 8 Changes in the cell viability of hADSCs in test solutions after storage at 5°C (A) and 25°C (B).

Each point and vertical bar indicates the mean ± SD (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001 vs. lactated Ringer's solution with Dunnett's test. LR-3T: lactated Ringer's solution with 3% trehalose, LR-3T-5D: lactated Ringer's solution with 3% trehalose and 5% dextran 40.

Table 6 and Figure 6 show the time course of the Annexin V-positive ratio, a marker of the early phase of apoptosis, during 24 h of preservation in LR-3T, LR-3T-5D, and lactated Ringer's solution at 25°C. The Annexin V-positive ratio of hADSC stored in LR-3T and LR-3T-5D remained at the same level before and after the 24 h of storage; in contrast, the Annexin V-positive ratio of cells stored in LR was increased after 24 h of storage.

Table 7 Annexin V-positive ratio of human adipose tissue-derived mesenchymal stromal cells preserved in LR, LR-3T and LR-3T-5D after 0, 6, and 24 hours of storage at 25°C.

Group	Annexin V-positive ratio (%) Time after suspension		
	0 hours	6 hours	24 hours
LR	9.7 ± 3.1	12.0 ± 1.8	62.1 ± 13.0
LR-3T	11.4 ± 4.6	11.0 ± 2.2	13.5 ± 1.3
LR-3T-5D	12.5 ± 4.9	9.0 ± 0.6	12.9 ± 1.5

Values are means ± SD (n = 4). LR: lactated Ringer's solution, LR-3T: lactated Ringer's solution with 3% trehalose, LR-3T-5D: lactated Ringer's solution with 3% trehalose and 5% dextran 40.

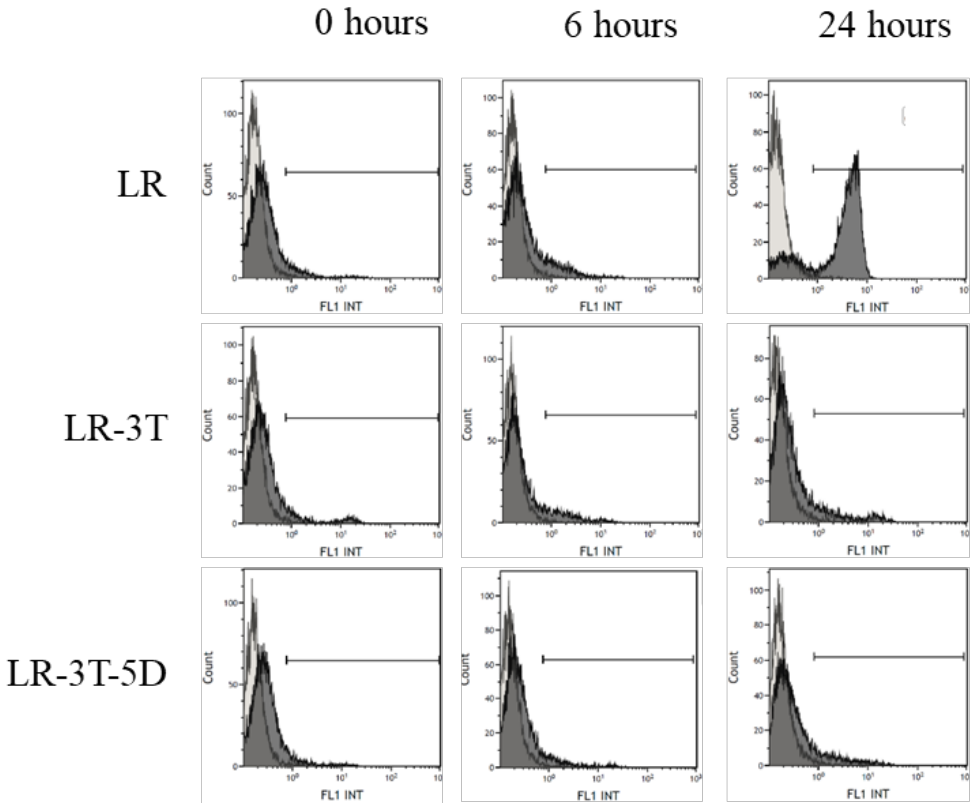


Figure 9 Representative charts of Annexin V analysis of hADSCs preserved in LR, LR-3T and LR-3T-5D after 0, 6, or 24 hours of storage at 25°C.
Antigen stains and isotype controls are represented by solid and shaded curves, respectively.

The cells were negative for CD14, CD34, CD45, and HLA-DR before preservation, and also at 24 h after preservation at 5°C or 25°C in LR-3T and LR-3T-5D. The cells were positive for CD44, CD73, CD90, and CD105 before preservation and at 24 h after storage at 5°C or 25°C in LR-3T and LR-3T-5D (Table 7, Table 8, Figure 7).

Table 8 Cell surface marker analysis of hADSCs before preservation and 24 hours after preservation in LR-3T at 5°C and 25°C.

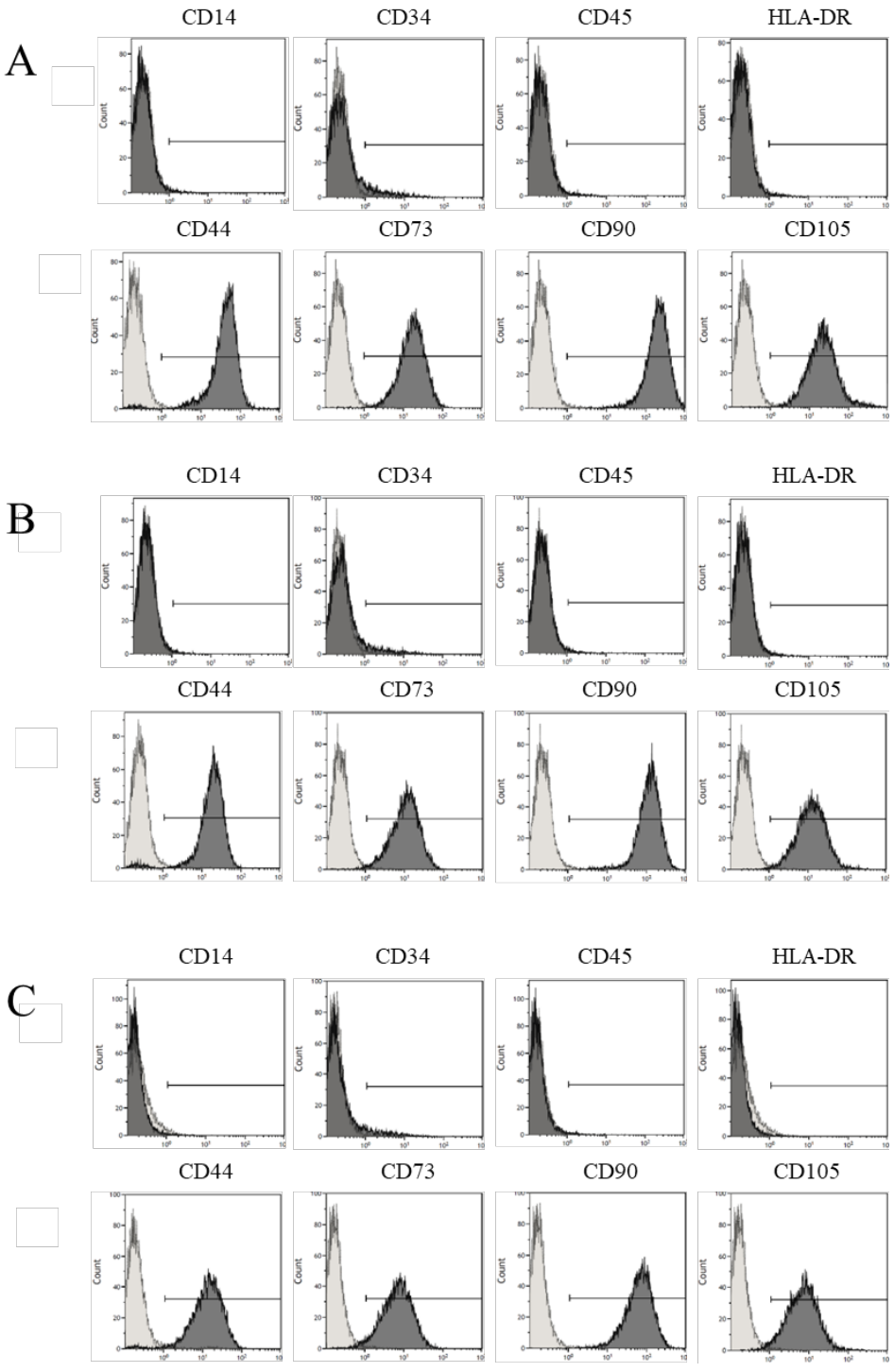
Cell surface marker	Positive ratio (%) Before preservation	24 hours after preservation at 5°C	24 hours after preservation at 25°C
CD14	1.2 ± 0.3	0.9 ± 0.1	0.9 ± 0.2
CD34	9.0 ± 0.4	8.7 ± 2.0	8.0 ± 1.5
CD45	1.3 ± 0.2	1.1 ± 0.1	1.0 ± 0.4
HLA-DR	1.1 ± 0.2	0.9 ± 0.2	0.8 ± 0.3
CD44	97.9 ± 0.8	97.1 ± 12	97.5 ± 0.2
CD73	99.7 ± 0.1	99.4 ± 0.3	97.6 ± 0.8
CD90	99.9 ± 0.1	99.9 ± 0.0	99.9 ± 0.0
CD105	99.9 ± 0.0	99.8 ± 0.1	96.9 ± 3.3

Values are means ± SD (n = 4). LR-3T: lactated Ringer's solution with 3% trehalose.

Table 9 Cell surface marker analysis of hADSCs before preservation and 24 hours after preservation in LR-3T-5D at 5°C or 25°C.

Cell surface marker	Positive ratio (%) Before preservation	24 hours after preservation at 5°C	24 hours after preservation at 25°C
CD14	1.0 ± 0.3	1.0 ± 0.1	1.2 ± 0.2
CD34	9.4 ± 0.4	9.1 ± 0.7	6.5 ± 0.9
CD45	1.1 ± 0.3	1.1 ± 0.3	1.3 ± 0.1
HLA-DR	1.1 ± 0.3	1.0 ± 0.2	1.2 ± 0.2
CD44	95.9 ± 3.1	94.6 ± 2.3	96.8 ± 1.1
CD73	99.3 ± 0.4	99.1 ± 0.5	97.3 ± 1.4
CD90	99.9 ± 0.1	99.9 ± 0.0	99.8 ± 0.1
CD105	99.9 ± 0.1	99.9 ± 0.1	99.2 ± 0.1

Values are means ± SD (n = 4). LR-3T-5D: lactated Ringer's solution with 3% trehalose and 5% dextran 40.


A
CD14
CD34
CD45
HLA-DR
CD44
CD73
CD90
CD105
B
CD14
CD34
CD45
HLA-DR
CD44
CD73
CD90
CD105
C
CD14
CD34
CD45
HLA-DR
CD44
CD73
CD90
CD105

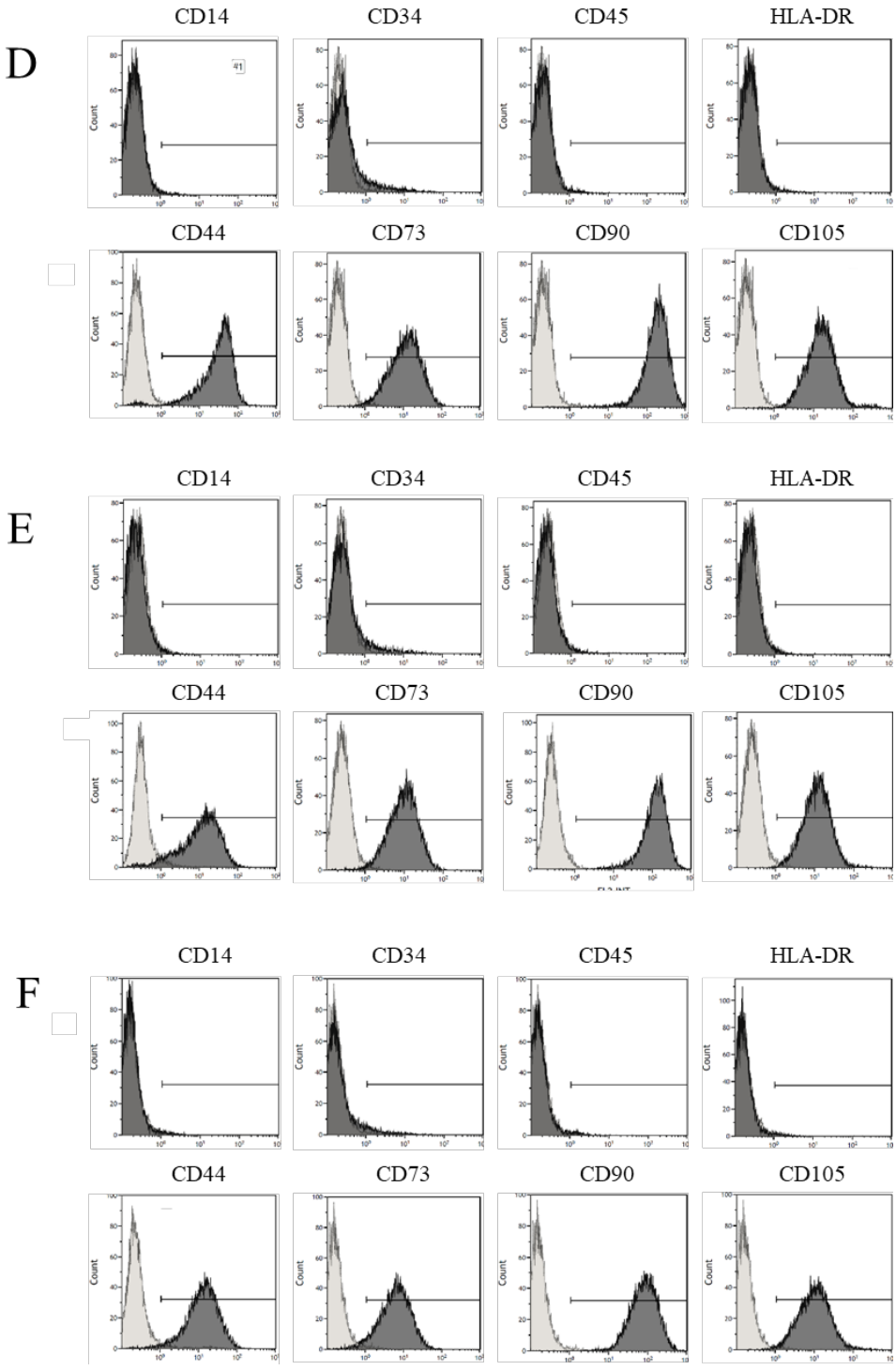


Figure 10 . Representative charts of the cell surface marker analysis of hADSCs preserved in LR-3T (A-C) and LR-3T-5D (D-F). (A) and (D), before preservation; (B) and (E), 24 hours after preservation at 5°C; (C) and (F), 24 hours after preservation at 25°C.

Antigen stains and isotype controls are represented by solid and shaded curves, respectively.

Table 9 and Figure 8 show the results of the colony-forming assay with hADSCs before preservation and at 6 and 24 h after preservation at 5°C and 25°C in LR-3T or LR-3T-5D. In the case with LR-3T at 25°C, the number of colony-forming units remained unchanged at least 24 h after preservation. Although the mean number of colony-forming units at 24 h after preservation at 5°C in LR-3T tended to be lower when compared to that before preservation ($p = 0.0818$), with the other preservation conditions, no significant difference was found in the values before and after preservation.

Table 10 Colony-forming capacity of human adipose tissue-derived mesenchymal stromal cells preserved in LR-3T and LR-3T-5D.

Group		Immediately after suspension (CFU/100 cells)	6 hours after suspension (CFU/100 cells)	24 hours after suspension (CFU/100 cells)
LR-3T	5°C	21.6 ± 2.5	21.0 ± 2.0	16.3 ± 5.4
	25°C		21.5 ± 2.1	21.9 ± 1.7
LR-3T-5D	5°C	21.3 ± 1.9	21.9 ± 3.2	22.0 ± 2.7
	25°C		19.1 ± 1.0	21.6 ± 2.5

Values are means ± SD (n = 4). No significant difference was found when values at 6 or 24 hours were compared to the value immediately after suspension with Dunnett's test.
CFU: colony-forming unit, LR-3T: lactated Ringer's solution with 3% trehalose, LR-3T-5D: lactated Ringer's solution with 3% trehalose and 5% dextran 40.

Adipogenic differentiation and osteogenic differentiation were induced using hADSCs 24 h after storage at 5°C or 25°C (Figure 9, Figure 10). Adipocytes containing oil droplets stained with Oil Red were observed in every preservation condition tested; no obvious difference was seen before and after preservation for any of the preservation conditions. Similarly, osteogenesis was also observed in every preservation condition tested; no obvious difference was seen before and after preservation for any of the preservation conditions.

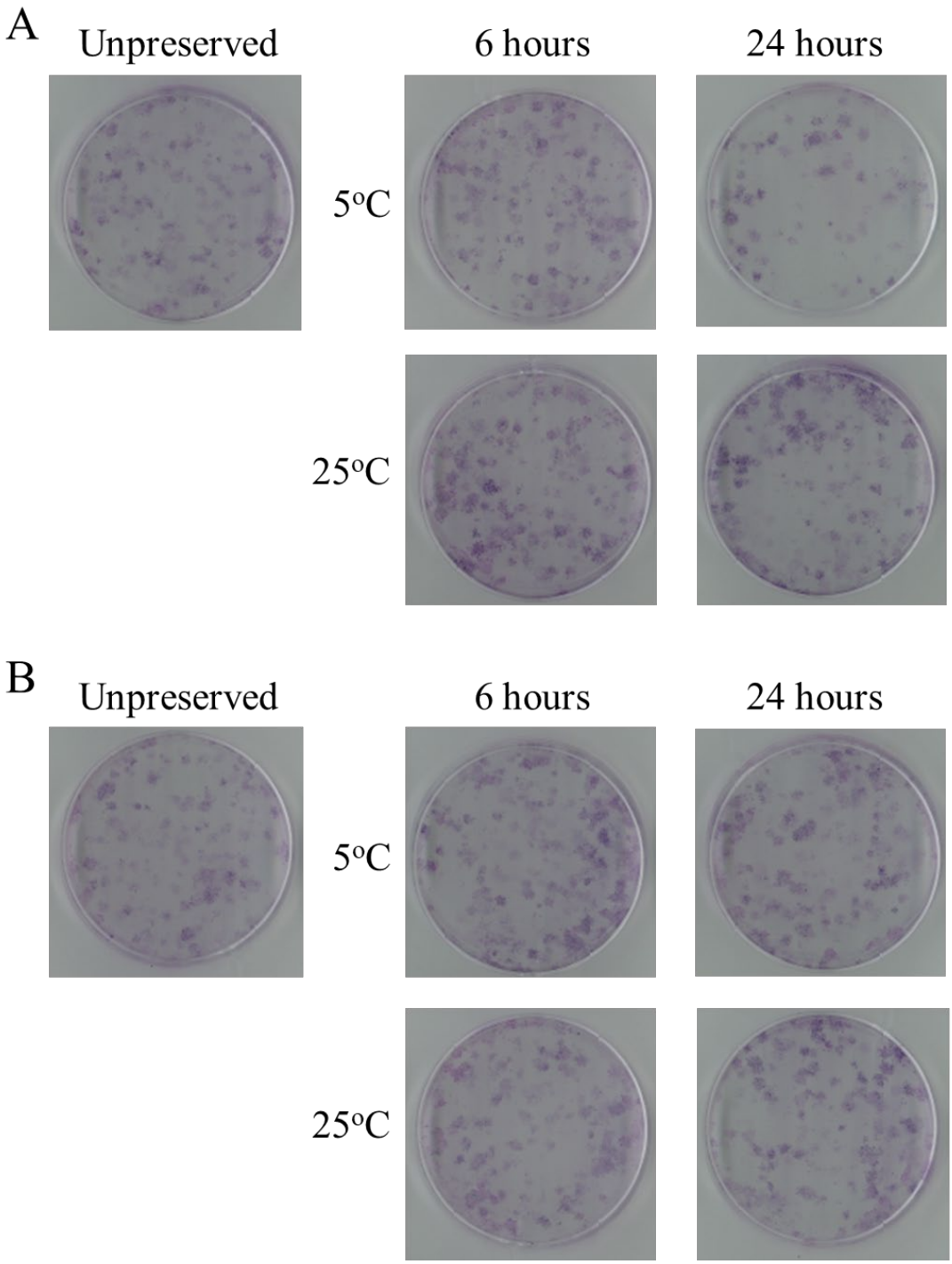


Figure 11 Representative images of the colony-forming assay with hADSCs preserved in LR-3T(A) and LR-3T-5D (B).

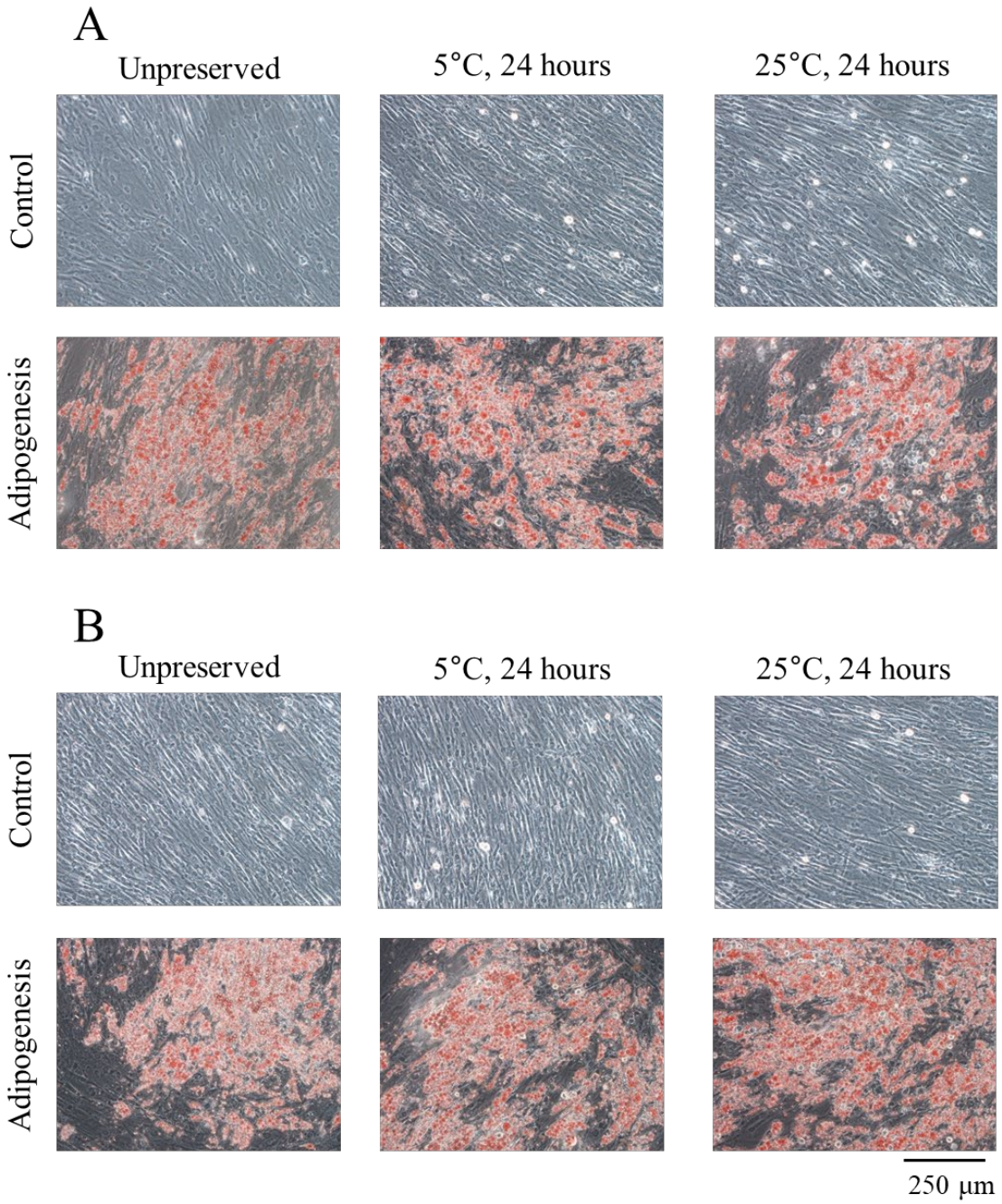


Figure 12 Representative images of the adipogenesis differentiation assay with hADSCs preserved in LR-3T (A) and LR-3T-5D (B) at 5°C or 25°C.

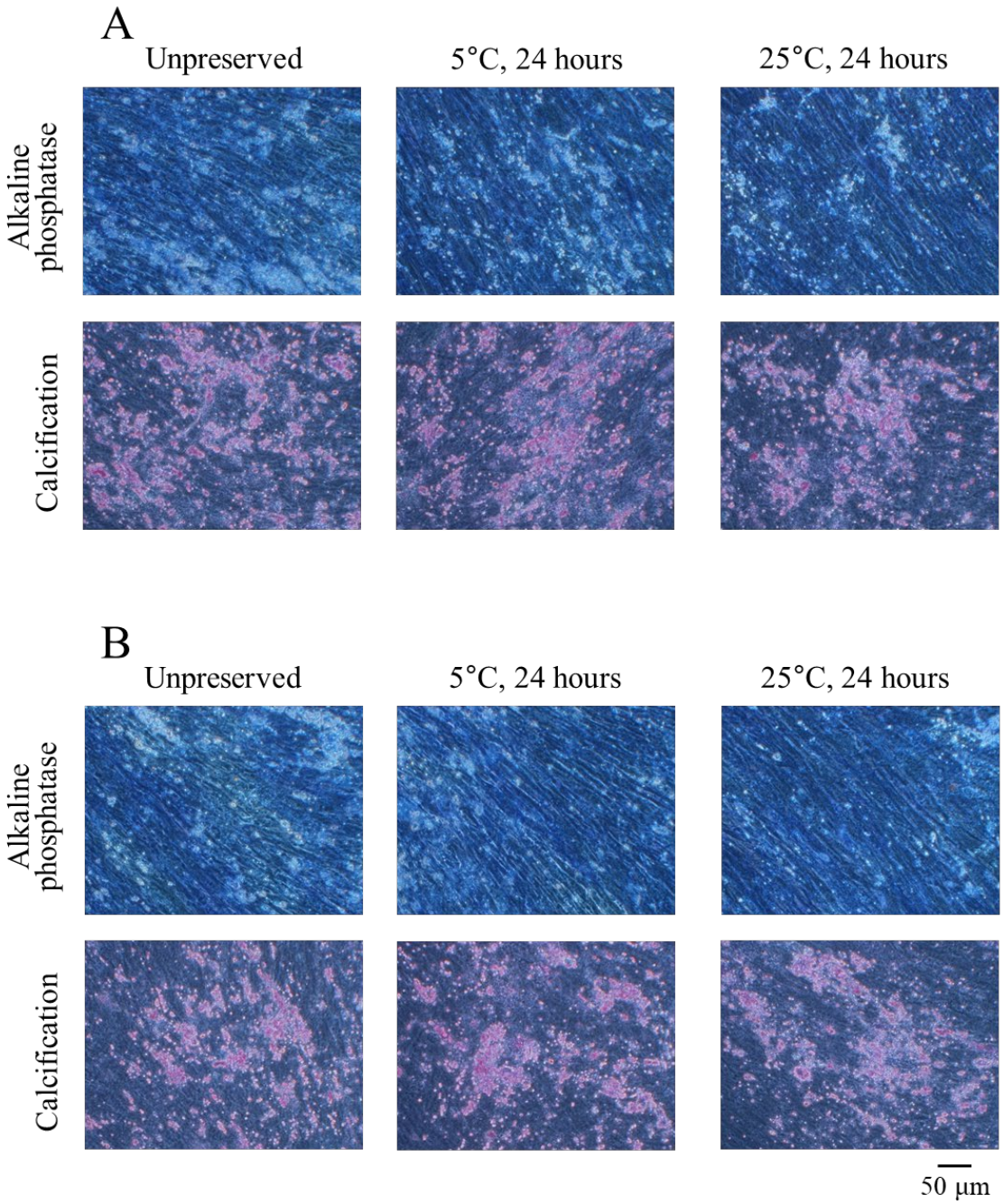


Figure 13 Representative images of the osteogenesis differentiation assay with hADSCs preserved in LR-3T (A) and LR-3T-5D (B) at 5°C or 25°C.

2.3.5 Cell concentration in the infusion line

The cell concentration of hADSCs suspended in LR-3T-5D or normal saline in the infusion line from the blood bag was evaluated in the worst-case scenario model of

infusion, in which the infusion was started without mixing after 1 h of settling, and mixing was performed at the halfway point of infusion (the end of the 10th fraction). The cell concentrations of fractions in normal saline were lower than the ideal concentration until mixing, and they gradually increased after mixing. The cell concentration of the final fraction (the 20th fraction) in normal saline was much higher than the ideal concentration. On the other hand, the cell concentrations of fractions in LR-3T-5D were stable over time throughout the infusion, even in the worst-case scenario (Figure 11A). The cell recovery rate was significantly higher with LR-3T-5D than with normal saline (77.9% ± 5.0% vs. 67.6% ± 3.6%, respectively; Figure 11B).

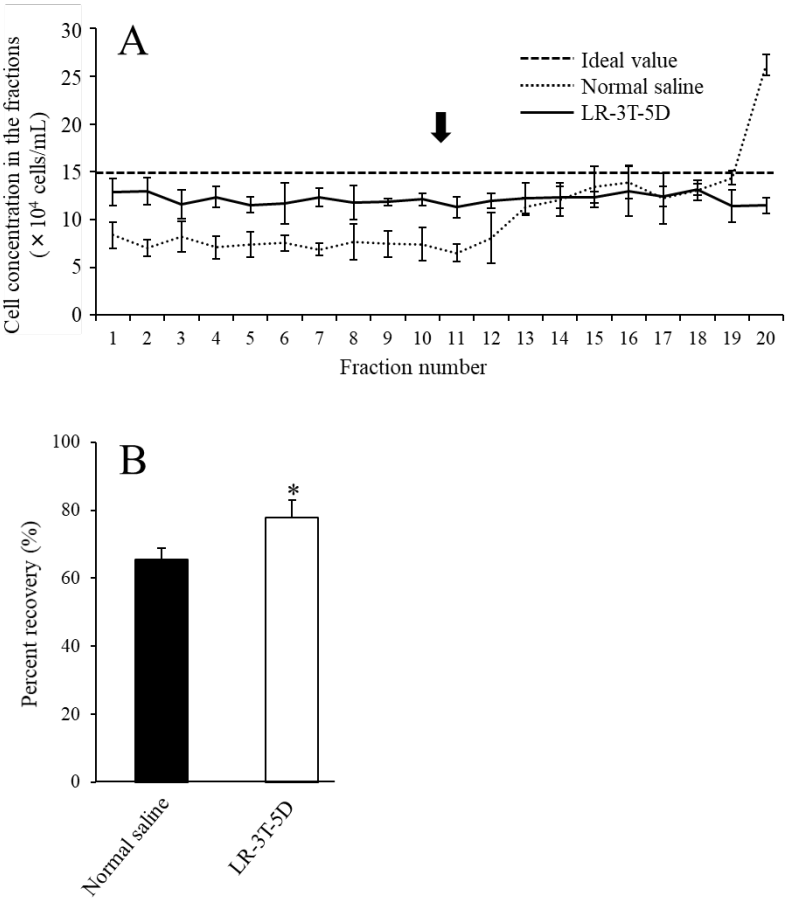


Figure 14 The concentrations of hADSCs suspended in LR-3T-5D or normal saline in the infusion line from blood bags (A).

The values indicate the mean ± SD (n = 3) of the cell concentration in each fraction. The dashed line indicates the ideal value of the cell concentration in each fraction. The arrow indicates the time point at which the bags were mixed. Comparison of percent recovery (B). The values indicate the mean ± SD (n = 3) of the percent recovery. *p < 0.05 with the Student's t-test. LR-3T-5D: lactated Ringer's solution with 3% trehalose and 5% dextran 40.

2.4 Discussion

The osmolality and electrolyte composition of extracellular solutions affect cell viability via cellular volume homeostasis and ion homeostasis [Romero 2004, Dubyak 2004, Lang et al. 2007, Strange 2007]. Normal saline is isotonic, but it contains only sodium ions and chloride ions. On the other hand, various Ringer's solutions contain potassium ions and calcium ions, in addition to sodium ions and chloride ions, and they are more similar to the extracellular fluid in the human body. The ions in Ringer's solutions prevent cell death, and account for the difference between normal saline and Ringer's solutions in maintaining cell viability. We found no major differences among the various Ringer's solutions. However, among the solutions tested, cell viability was the highest in lactated Ringer's solution. Therefore, we chose the composition of electrolytes in lactated Ringer's solution for our cell preservation solution.

Trehalose is a stabilizer of proteins and cell membranes, and a cryoprotective agent for different kinds of cells [Jain and Roy 2009]. Trehalose is also a component of ET-Kyoto solution, which is used for lung and pancreas islet transplantation as an organ-preservation solution [Chen et al. 2004, Matsumoto et al. 2010]. Trehalose is superior to other saccharides, such as glucose, maltose, sucrose, and raffinose, as an organ protectant in an isolated rat model of lung perfusion [Fukuse et al. 1999]. A modified-Euro-Collins solution, in which glucose is replaced by trehalose, had better preservation effects than the original Euro-Collins solution in a 12-h canine model of lung preservation and transplantation [Hirata et al. 1994]. LR-3T and LR-3T-5D containing 3% trehalose were more effective at preserving the viability of hADSCs stored at 5°C and 25°C than lactated Ringer's solutions. The mechanism of the protective effect of trehalose on cell membranes and proteins against desiccation and cryopreservation has been explained at a molecular level in some reports [Jain and Roy 2009, Albertorio et al. 2007]. For example, the clam-shell conformation of trehalose facilitates interactions between the sugar and the polar head region of phospholipids by creating hydrogen-bonding geometry that is appropriate for the adjacent lipids to prevent air from delaminating the lipid membrane [Albertorio et al. 2007]. However, the mechanism by which trehalose prevents cell death in a preservation solution, other than desiccation and freezing, is unclear. Cells precipitate onto the bottom of infusion bags during storage and infusion, and this creates variability in cell concentrations during infusion. This variability affects the efficacy of cell therapy as changes in cell concentrations can induce infusion-related toxicity, such as a pulmonary embolism. In fact, the package insert of TEMCELL® HS Inj. (JCR Pharmaceuticals Co., Ltd., Hyogo, Japan), which

contains human allogeneic bone marrow-derived mesenchymal stem cells used to treat acute graft-versus-host disease after hematopoietic stem cell transplantation, says to gently mix the infusion bag by hand because sedimentation likely makes the cell concentration non-uniform [JCR Pharmaceuticals Co., Ltd, 2017]. In our study, the cell suspension was saturated in lactated Ringer's solution containing 3% trehalose and 5% dextran 40. In addition, lactated Ringer's solution containing 3% trehalose and 5% dextran 40 did not affect cell viability. In fact, LR-3T-5D stabilized the cell concentration in the infusion line even in the worst-case scenario, which was 15 min of infusion without mixing after 1 h of settling, followed by 15 min of infusion after mixing (Figure 11). The cell concentrations of fractions in LR-3T-5D and the percent recovery were lower than the ideal values. This suggested that a portion of the cells attached to the infusion bags when cells were transferred to the bags.

The trends of viability differed between the 5°C (Figure 5A) and 25°C (Figure 5B) conditions in lactated Ringer's solution, LR-3T, and LR-3T-5D. Rauen et al. reported that hypothermic injury starts to occur in rat hepatocytes at temperatures below 16°C, and it reaches a maximum at 4°C-8°C [Rauen et al. 2007]. Our results also suggested that storage at 5°C caused more severe hypothermic injury to hADSCs than storage at 25°C.

CD14, CD34, CD45, and HLA-DR are known as negative markers of MSCs [Dominici et al. 2006]. CD14 is prominently expressed on monocytes and macrophages; CD34 marks primitive hematopoietic progenitors and endothelial cells; CD45 is a pan-leucocyte marker; and HLADR marks HLA class II cell surface receptors. In contrast, CD73, CD90, and CD105 are known as positive markers of MSCs [Dominici et al. 2006, Di Battista et al. 2014]. We found no obvious changes in the ratios of cells positive for CD14, CD34, CD45, HLA-DR, CD44, CD73, CD90, or CD105 when the cells were preserved in LR-3T and LR-3T-5D for 24 h at either 5°C or 25°C when compared to the ratios before preservation. This indicated that storage in LR-3T and LR-3T-5D did not affect the expression of cell surface markers.

There were no obvious differences in colony-forming capacity before and after preservation when cells were preserved in LR-3T and LR-3T-5D for at least 24 h at either 5°C or 25°C. This indicated that storage for at least 24 h in LR-3T and LR-3T-5D did not affect the ratio of cells with proliferation ability.

One of the criteria for defining MSCs is multipotent differentiation potential [Dominici et al. 2006].We confirmed that hADSCs still had adipogenic and osteogenic differentiation ability after 24 h of storage in LR-3T and LR-3T-5D at 5°C or 25°C. This indicated that 24 h of storage in LR-3T and LR-3T-5D did not affect the differentiation

ability of hADSCs.

Taken together, we concluded that the cell characteristics (cell surface markers, colony-forming capacity, and differentiation ability) of hADSCs stored in LR-3T and LR-3T-5D were stable for at least 24 h at 5°C and 25°C.

Another study reported that hADSCs could be stored for 24 h between 2°C and 8°C in acetated Ringer's solution with 5% human serum albumin [Zhang et al. 2017]. In that study, 5% human serum albumin could protect cells; in our study, the human serum albumin was replaced by 3% trehalose and 5% dextran 40. LR-3T and LR-3T-5D are beneficial in that bio-derived materials, which are at risk from contamination with adventitious virus, are not required. Furthermore, our solution prevents cell sedimentation for at least 1 h, a characteristic that we have not found in any reports of other solutions.

2.5 Conclusions

Our data showed that LR-3T, consisting of lactated Ringer's solution with 3% trehalose, and LR-3T-5D, consisting of lactated Ringer's solution with 3% trehalose and 5% dextran 40, could preserve the cell viability and cell characteristics of hADSCs during storage at 5°C and 25°C for at least 24 h. LR-3T-5D stabilized the percentage of cells in the supernatant for at least 1 h. These results suggest that LR-3T and LR-3T-5D can help maintain the quality of stem cells for therapy during preservation and infusion. However, further in vivo research is needed on the efficacy and safety of the solutions in different therapeutic cell lines before clinical use.

2.6 Chapter summary

Stem cells for therapy are often suspended in a preservation solution, such as normal saline or lactated Ringer's solution, for a short time before intravenous infusion. However, these solutions are not necessarily ideal for maintaining cell viability and preventing the sedimentation of cells during storage and infusion. In this study, we attempted to optimize the compositions of preservation solutions, which could affect the efficacy and safety of stem cell therapy.

We determined the characteristics of a preservation solution that would optimize cell viability and the percentage of cells in the supernatant using human adipose-derived mesenchymal stromal cells (hADSCs). We compared solutions that differed by electrolytes (e.g., normal saline and Ringer's solution) and the concentrations of dextran

40 and trehalose. The effects of the solutions on hADSCs were evaluated by assessing cell surface markers, colony-forming capacity, differentiation potential, and cell concentrations in the infusion line.

Optimized preservation solutions consisted of lactated Ringer's solution with 3% trehalose without or with 5% dextran 40 (LR-3T and LR-3T-5D, respectively). The cell viabilities after 24 h of storage at 5°C in LR-3T and LR-3T-5D were 94.9% ± 2.4% and 97.6% ± 2.4%, respectively. The percentage of cells in the supernatant after 1 h of storage at room temperature in LR-3T-5D was 83.5% ± 7.6%. These solutions preserved the percentage of cell surface marker-positive cells, the colony-forming capacity, and the adipogenic and osteogenic differentiation ability in hADSCs for at least 24 h after preservation at 5°C and 25°C. The cell concentrations of fractions in LR-3T-5D were stable over time throughout the infusion, even in the worst-case scenario.

We determined the optimal composition of preservation solutions for hADSCs and confirmed the effects of these solutions on cell viability and the stability of cell characteristics in vitro. Our results suggest that LR-3T and LR-3T-5D can help maintain the quality of stem cells for therapy during preservation and infusion.

Chapter 3. Cryopreservation of human adipose tissue-derived mesenchymal stromal cells with LR-3T-5D containing 10% dimethyl sulfoxide

3.1 Introduction

Mesenchymal stromal cells have been widely studied for their broad ranging clinical potential [Pittenger et al. 2019]. We developed a cell preservation solution consisting of lactated Ringer's solution with 3% trehalose and 5% dextran 40 (LR-3T-5D) for storage of human adipose tissue-derived mesenchymal stromal cells (hADSCs) at 5°C and 25°C for up to 24 h, instead of normal saline and lactated Ringer's solution, which are not necessarily ideal for maintaining cell viability and preventing the sedimentation of cells during storage and infusion [Chapter 2, Fujita et al. 2020]. However, trehalose and dextran are also moderately effective for cryopreservation in combination with a penetrating cryoprotectant [Elliott et al. 2017]. If LR-3T-5D is also effective as a vehicle solution for cryopreservation, it can provide a seamless cell preservation method from the process of washing to freezing. The objective of this study was to test the efficacy of LR-3T-5D as a vehicle solution for cryopreservation using hADSCs.

Lactated Ringer's solution (LR) is mixture of sodium chloride, sodium lactate, potassium chloride, and calcium chloride in water. These electrolyte concentrations resemble extracellular fluid, except for lactate, which substitutes for bicarbonate. LR is generally used as an infusion solution for fluid resuscitation after blood loss in clinical practice. Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a nonreducing disaccharide that is known to be a protein- and membrane-stabilizer and cryoprotective agent [Jain and Roy 2009]. Trehalose is used as an ingredient in food, health and beauty products, and pharmaceutical products such as monoclonal antibodies administrated via a parenteral route [Falconer 2019, Gervasi et al. 2018, Richards et al. 2002]. Dextran is a straight chain glucose polymer with α-1,6 glycosidic linkages and α-1,3 linked branches. For many years, dextran has been used as a plasma volume expander at a concentration 10% and an antithrombotic agent in clinical practice [Abir et al. 2004, Nolan 2001]. Therefore, all of the ingredients of LR-3T-5D can be prepared at clinical grade.

Dimethyl sulfoxide (Me2SO) is one of the most effective and widely used cryoprotectants [Awan et al. 2020, Elliott et al. 2017] but is associated with toxicity in transplant recipients [Awan et al. 2020, Morris et al. 2014]. In addition, a significant and

unpleasant response to injected Me2SO is a garlic-like odor and taste, caused by its metabolite, dimethyl sulfide [Awan et al. 2020, Shu et al. 2014]. Because reducing the concentration of Me2SO is effective for suppressing the frequency of its side effects, it is recommended to lower the concentration to 5% instead of the most commonly used 10%, or to wash cells before infusion [Awan et al. 2020, Morris et al. 2014, Shu et al. 2014]. In this study, we confirmed the effects of different Me2SO concentrations and of cell-washing procedures after thawing cells. Furthermore, we compared the effects of other cell membrane penetrating cryoprotectants, propylene glycol, ethylene glycol, and glycerol, to Me2SO in combination with LR-3T-5D.

3.2 Materials and method

3.2.1 Study design

The present study was approved by the ethics committee of Otsuka Pharmaceutical Factory, Inc.

We performed seventh experiments. First, the effects as a cryoprotective vehicle with 10% Me2SO were compared among LR, LR-3T, and LR-3T-5D. Second, we performed a dose-response experiment for trehalose and dextran 40 to test the cell viability of hADSCs after cryopreservation. Third, we determined cell characteristics (colony-forming capacity, differentiation ability, and cell surface markers) after 6- or 7-month cryopreservation in liquid nitrogen or at − 80°C. Fourth, we tested the effects of the duration of incubation before freezing with LR-3T-5D containing Me2SO on cell viability and annexin V-positive ratio just before freezing and immediately after thawing. Fifth, we investigated the effects of Me2SO concentration on cell viability and proliferation of hADSCs immediately after thawing. Sixth, immediately after thawing, we washed cells with LR-3T and replaced the solution with LR-3T or LR-3T-5D and evaluated the effects. Seventh, we tested other cell membrane penetrating cryoprotectants with LR-3T-5D in regard to hADSC viability and annexin V-positive ratio immediately after thawing.

3.2.2 Components of the solutions

Lactated Ringer's solution (Lactec® Injection), 10% dextran 40 in lactated Ringer's solution (Low Molecular Dextran L Injection), lactated Ringer's solution with 3% trehalose (LR-3T; Cellstor-W), lactated Ringer's solution with 5% dextran 40 (LR-

5D), and lactated Ringer's solution with 3% trehalose and 5% dextran 40 (LR-3T-5D; Cellstor-S) were supplied by Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan). Trehalose dehydrate, Me2SO (CultureSure®DMSO), propylene glycol, ethylene glycol, and glycerol were purchased from Fujifilm Wako Pure Chemical Co. (Osaka, Japan). LR-3T-5D with 10% cryoprotectant was prepared by mixing LR-3T-5D and cryoprotectant at ratios of 9:1 (vol/vol). The compositions of LR-3T, LR-3T-5D, and LR-3T-5D with 10% Me2SO are described in Table 10.

Table 11 Compositions of preservation solutions (LR-3T and LR-3T-5D) and the cryopreservation solution (LR-3T-5D with 10% Me2SO).

Components	LR-3T	LR-3T-5D	LR-3T-5D with 10% Me2SO
Na^+	130 mEq/L	130 mEq/L	117 mEq/L
K^+	4 mEq/L	4 mEq/L	3.6 mEq/L
Ca^{2+}	3 mEq/L	3 mEq/L	2.7 mEq/L
Cl^-	109 mEq/L	109 mEq/L	98 mEq/L
$Lactate^-$	28 mEq/L	28 mEq/L	25 mEq/L
Trehalose	3%	3%	2.7%
Dextran40	–	5%	4.5%
Me2SO	–	–	10%

LR-3T: lactated Ringer's solution with 3% trehalose.
LR-3T-5D: lactated Ringer's solution with 3% trehalose and 5% dextran 40.
LR-3T-5D with 10% Me2SO: LR-3T-5D with 10% dimethyl sulfoxide.

3.2.3 Preparation of hADSCs

Human ADSCs (Female, 38Y or 44Y, PT5006, Lot No. 0000421627 or 0000692059; Lonza Walkersville, Inc., Walkersville, MD, USA) were used in this study; 38Y and 44Y indicate the age of the donors. Human ADSCs were seeded in a 75-cm^2 flask with 15 mL of medium prepared from a medium kit (PT-4505 ADSC BulletKit™, Lonza Walkersville, Inc.) and maintained at 37°C in a humidified atmosphere of 5% CO_2. All of the medium was changed every 3 or 4 days. Cells were passaged at approximately 90% confluency, and passage 2, 3, or 4 (3, 4, or 5 after cell preparation) was used for the experiments. Cells were washed with phosphate-buffered saline (PBS without calcium and magnesium, hereinafter called PBS(−)), and trypsinized with Trypsin/EDTA solution (CC-5012, Lonza Walkersville, Inc.) for 5 min at 37°C. The trypsin reaction was stopped with TNS (CC-5002, Lonza Walkersville, Inc.), and hADSCs were subsequently detached by pipetting. The supernatant

containing the hADSCs was transferred to a 50-mL conical tube, centrifuged (210×g, 5 min, at room temperature), and the supernatant was aspirated. hADSCs were suspended in PBS (−) or LR-3T and used for each experiment.

3.2.4 Cryopreservation of hADSC

The hADSCs suspended in PBS (−) or LR-3T were transferred to a 15- or 50-mL conical tube, centrifuged (210×g, 5 min, at room temperature), and the supernatant was aspirated. hADSCs were resuspended with LR, LR-3T, or LR-3T-5D containing 0.5% Me2SO to 10% Me2SO so that the final cell concentration was 0.96–3.0 × 10^6 cells/mL. One milliliter of suspension was dispensed into each cryovial (Nunc™ CryoTube™ Vials, size 1.8 mL, Thermo Fisher Scientific Inc., Waltham, MA, USA). Immediately after dispensing, the vial was placed in a BICELL (Nihon Freezer Co., Ltd., Tokyo, Japan) or freeze box (capacity: 81 or 100 vials) and stored at − 80 ◦C for about 24 h. Then the vial was transferred from the BICELL or the freeze box to a freeze box preincubated in liquid nitrogen and stored in liquid nitrogen or a − 80 ◦C freezer for up to 7 months.

3.2.5 Thawing, washing, and replacement of cryopreservation solution

The frozen vials in which the hADSCs were stored were quickly thawed in a thermostat bath heated to 37°C. After thawing, the cell suspension was transferred from the vial into a low cell adsorption tube (STEMFULL™, Sumitomo Bakelite Co., Ltd., Tokyo, Japan). One milliliter of LR-3T was used to rinse the frozen vials at room temperature, and cells were transferred dropwise into the low cell adsorption tube over approximately 1 min. In addition, 9 mL of LR-3T at room temperature was added into the low cell adsorption tube over approximately 5 min, centrifuged (210×g, 5 min, at room temperature), and the supernatant was aspirated. hADSCs were suspended in LR-3T or LR-3T-5D. The cell suspension was stored for 24 h after replacement at 25°C.

3.2.6 Cell viability and viable cell recovery ratio

The total numbers of cells and dead cells were counted manually with a plastic cell counting plate (OneCell Counter, Bio Medical Science, Ltd., Tokyo, Japan) after trypan blue staining. Cell viability and viable cell recovery ratio were calculated according to the formulae below.

Cell viability [%] = (total number of cells − number of dead cells) / (total number of cells) × 100

Viable cell recovery ratio [%] = number of viable cells in 1 mL at each time point/number of viable cells in 1 mL before freezing × 100

3.2.7 Annexin V staining

Annexin V staining was evaluated as described in 2.2.6.

3.2.8 Colony-forming capacity

Colony-forming capacity was evaluated as described in 2.2.8.

3.2.9 Cell proliferation curve

hADSCs were seeded at a density of 5.0–6.3 × 10^3 cells/cm^2 (4.8–6.0 × 10^4 cells/9.6 cm^2 /well) on a 6-well plate and cultured using mesenchymal stromal cell (MSC) medium. Medium exchange was carried out within 24 h after seeding and every 3 days thereafter. The 6-well plates in which hADSCs were cultured at 1, 3, 5, and 7 days after seeding were washed with PBS (−), treated with trypsin/EDTA, and the trypsin reaction was stopped with TNS. The total number of cells was counted with a OneCell Counter.

3.2.10 Adipogenic and osteogenic differentiation ability

Adipogenic and osteogenic differentiation ability were evaluated as described in 2.2.9.

3.2.11 Cell surface markers

Cell surface makers were evaluated as described in 2.2.7.

3.2.12 Analysis and statistics

Results are presented as the mean ± standard deviation (SD). Statistical analysis was performed using Dunnett's multiple comparison test, Tukey's multiple comparison test, and Student's t-test with a significance level of $p < 0.05$. Data were analyzed with SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

3.3 Results

3.3.1 Comparison of LR, LR-3T, and LR-3T-5D as cryoprotective vehicle solutions

The effects as cryoprotective vehicle solutions with 10% Me2SO were compared among LR, LR-3T, and LR-3T-5D (Figure 12). hADSCs were suspended in LR with 10% Me2SO, LR-3T with 10% Me2SO, or LR-3T-5D with 10% Me2SO, then cryopreserved for about 3 months. Cells were washed and replaced by LR-3T and incubated at 25°C.

Cell viability of hADSCs before freezing was significantly higher in LR-3T with 10% Me2SO and LR-3T-5D with 10% Me2SO than in LR with 10% Me2SO (Figure 12A). Cell viability immediately after thawing was significantly higher in LR-3T with 10% Me2SO and LR-3T-5D with 10% Me2SO than in LR with 10% Me2SO. Cell viability immediately after replacement tended to be higher in LR-3T with 10% Me2SO ($p = 0.0728$) and was significantly higher in LR-3T-5D with 10% Me2SO than in LR with 10% Me2SO. Cell viability 6 h after replacement was significantly higher in LR-3T with 10% Me2SO and LR-3T-5D with 10% Me2SO than in LR with 10% Me2SO. Cell viability 24 h after replacement was significantly higher in LR-3T-5D with 10% Me2SO than in LR with 10% Me2SO.

The viable cell recovery ratio immediately after thawing tended to be higher in LR-3T with 10% Me2SO ($p = 0.0985$) and was significantly higher in LR-3T-5D with 10% Me2SO than in LR with 10% Me2SO (Figure 12B). The cell recovery ratio immediately after replacement was significantly higher in LR-3T with 10% Me2SO and LR-3T-5D with 10% Me2SO than in LR with 10% Me2SO. The cell recovery ratio 6 h after replacement was significantly higher in LR-3T with 10% Me2SO and LR-3T-5D with 10% Me2SO than in LR with 10% Me2SO and tended to be higher in LR-3T-5D with 10% Me2SO than in LR-3T with 10% Me2SO ($p = 0.0956$). The cell recovery ratio 24 h after replacement was significantly higher in LR-3T with 10% Me2SO and LR-3T-5D with 10% Me2SO than in LR with 10% Me2SO.

The annexin V-positive ratio immediately after thawing tended to be lower in

LR-3T-5D with 10% Me2SO than in LR with 10% Me2SO (Figure 12C, $p = 0.0665$). The annexin V-positive ratio immediately after replacement was significantly lower in LR-3T-5D with 10% Me2SO than in LR with 10% Me2SO.

The colony formation ratio immediately after replacement tended to be higher in LR-3T-5D with 10% Me2SO than in LR with 10% Me2SO (Figure 12D). The colony formation ratio 24 h after replacement was significantly higher in LR-3T with 10% Me2SO and LR-3T-5D with 10% Me2SO than in LR with 10% Me2SO.

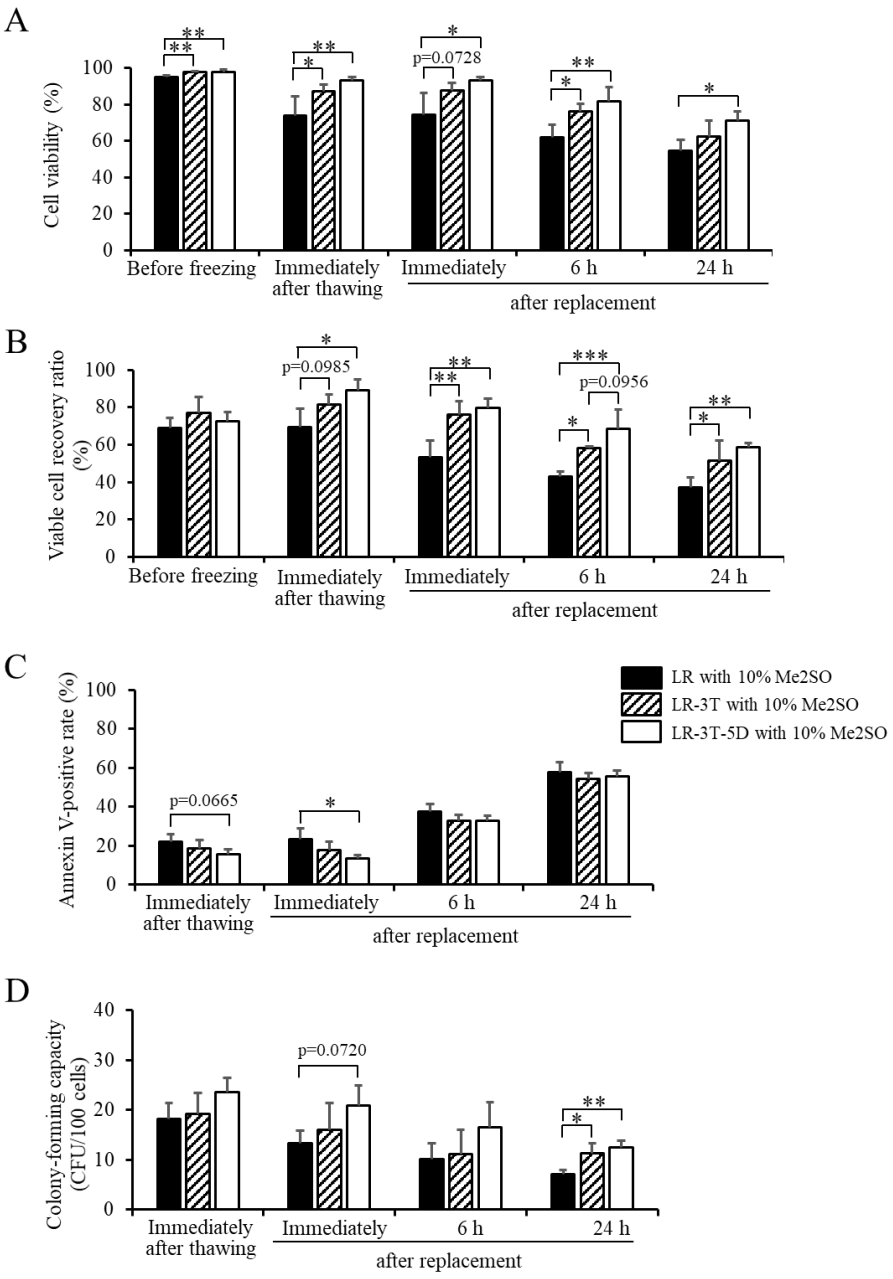


Figure 15 Cell viability (A), viable cell recovery ratio (B), annexin V-positive ratio (C), and colony-forming capacity (D) of hADSCs cryopreserved using LR with 10% Me2SO, LR-3T with 10% Me2SO, or LR-3T-5D with 10% Me2SO.

hADSCs at passage 5 were suspended at a density of 1.0×10^6 cells/mL using LR with 10% Me2SO, LR-3T with 10% Me2SO, or LR-3T-5D with 10% Me2SO. Vials containing 1 mL of the suspension were put into a freeze box (capacity: 81 vials) and frozen in a freezer at −80°C for a day. Then, these vials were transferred from the freeze box to another freeze box preincubated in liquid nitrogen and stored in liquid nitrogen for 85 or 92 days (about 3 months). The data are presented as the mean ± SD (n = 4). Statistical analysis was performed using Tukey's test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.3.2 Dose response of trehalose and dextran 40

The concentrations of trehalose and dextran 40 in LR-3T-5D with 10% Me2SO are 2.7% and 4.5%, respectively. We tested whether these concentrations were appropriate as a cryopreservation solution in dose-response experiments for trehalose and dextran 40.

Cell viability immediately after thawing was more than 90% in LR-5D containing more than 0.9% trehalose, and significantly higher in LR-5D with 2.7% and 4.5% trehalose than in LR-5D without trehalose (Figure 13A). Cell viability immediately after thawing was more than 90% and significantly higher in LR-3T with more than 0.9% dextran 40 than in LR-3T without dextran 40 (Figure 13B).

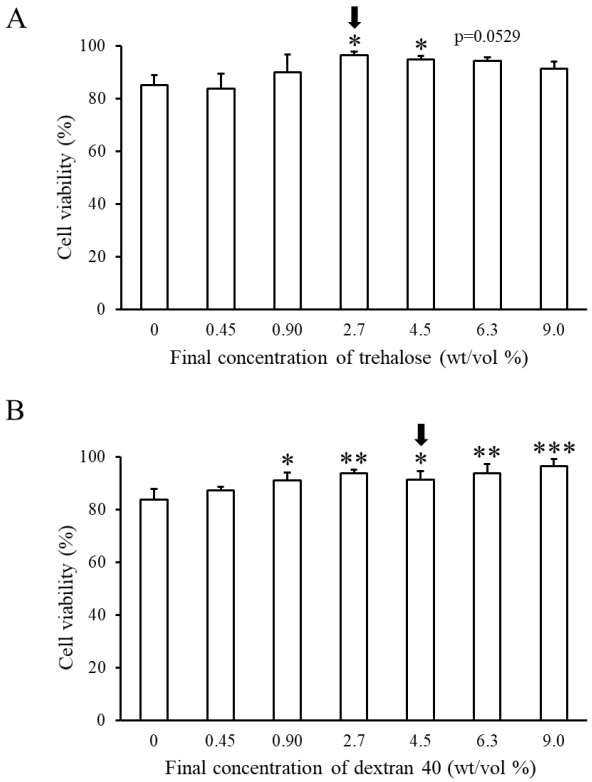


Figure 16 Cell viability of hADSCs immediately after thawing of samples cryopreserved in various concentrations of trehalose (A) in LR with 5% dextran and 10% Me2SO, or various concentrations of dextran 40 (B) in LR with 3% trehalose and 10% Me2SO.

hADSCs at passage 3 were suspended at a density of 9.6×10^5 cells/mL with various preservation solutions with 10% Me2SO. Vials containing 1 mL of the suspension were put into BICELL and frozen in a freezer at −80°C for a day. Then, the vials were transferred from BICELL to a freeze box preincubated in liquid nitrogen and stored in liquid nitrogen. The data are presented as the mean ± SD (n = 3). Statistical analysis was performed using Dunnett's test. *p < 0.05, **p < 0.01, ***p < 0.001 vs. 0%. The result of the same concentration of trehalose and dextran 40 as LR-3T-5D with 10% Me2SO is indicated by arrows.

3.3.3 Cell characteristics after 6- or 7-month cryopreservation in liquid nitrogen or − 80°C freezer

Table 11 shows the cell viability, annexin V-positive ratio, and colony-forming capacity of hADSCs immediately after thawing following cryopreservation for 7 months using LR-3T-5D containing 10% Me2SO. There was no significant difference in cell viability between liquid nitrogen storage and −80°C freezer storage. These viabilities were more than 90% and slightly decreased from the value before freezing (98.2%). There was no significant difference in annexin V-positive ratio and colony formation capacity between liquid nitrogen storage and −80°C freezer storage.

Table 12 Cell viability, annexin V-positive ratio, and colony-forming capacity of hADSCs immediately after thawing.

Group	In liquid nitrogen	In −80 °C freezer
Cell viability (%)	96.4 ± 2.6	95.1 ± 1.9
Annexin V-positive ratio (%)	16.4 ± 1.7	22.1 ± 5.6
Colony-forming capacity (CFU/100 cells)	23.3 ± 1.2	23.4 ± 2.2

hADSCs at passage 4 were suspended at a density of 1.0×10^6 cells/mL in LR-3T-5D with 10% Me2SO. Vials containing 1 mL of the suspension were put into a BICELL and frozen in a freezer at −80°C for a day. Then, these vials were transferred from the BICELL to a freeze box preincubated in liquid nitrogen and stored in liquid nitrogen or a −80°C freezer for 7 months. The data are presented as the mean ± SD (n = 4). Statistical analysis was performed using Student's ttest, and there was no significant difference between storage in liquid nitrogen and in a −80°C freezer. CFU: colony-forming unit.

Figure 14 shows the cell proliferation curve during subcultures seeded immediately after the thawing of hADSCs following cryopreservation for 7 months in liquid nitrogen or a −80°C freezer using LR-3T-5D containing 10% Me2SO. There was no significant difference in cell growth between liquid nitrogen storage and −80°C freezer storage.

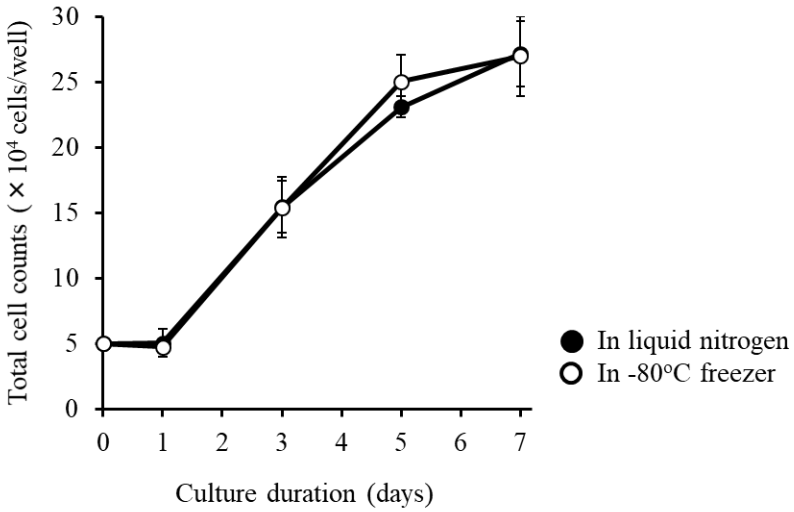


Figure 17 Proliferation curve of hADSCs seeded immediately after thawing following cryopreservation for 7 months.

hADSCs at passage 3 were suspended at a density of 1.0×10^6 cells/mL with LR-3T-5D with 10% Me2SO. Vials containing 1 mL of the suspension were put into BICELL and frozen in a freezer at −80°C for a day. Then, these vials were transferred from BICELL to a freeze box and stored in liquid nitrogen or a −80°C freezer for 7 months. After thawing, the cells were seeded into a 6-well plate at a density of 5.2×10^3 cells/cm^2 (5.2×10^4 cells/well). The data are presented as the mean ± SD (n = 4). Statistical analysis was performed using Student's t-test. There was no significant difference between storage in liquid nitrogen and in a −80°C freezer.

Adipogenic differentiation and osteogenic differentiation were induced using hADSCs immediately after thawing following cryopreservation for 7 months with LR-3T-5D containing 10% Me2SO (Figure 15A and B, respectively). Adipocytes containing oil droplets stained with Oil Red O were observed both in liquid nitrogen and −80°C freezer groups, but not in control cultures without the induction of adipogenic differentiation. Similarly, osteoblasts confirmed by alkaline phosphatase staining and calcification staining were observed both in liquid nitrogen and −80°C freezer groups, but not in control cultures without the induction of osteogenic differentiation.

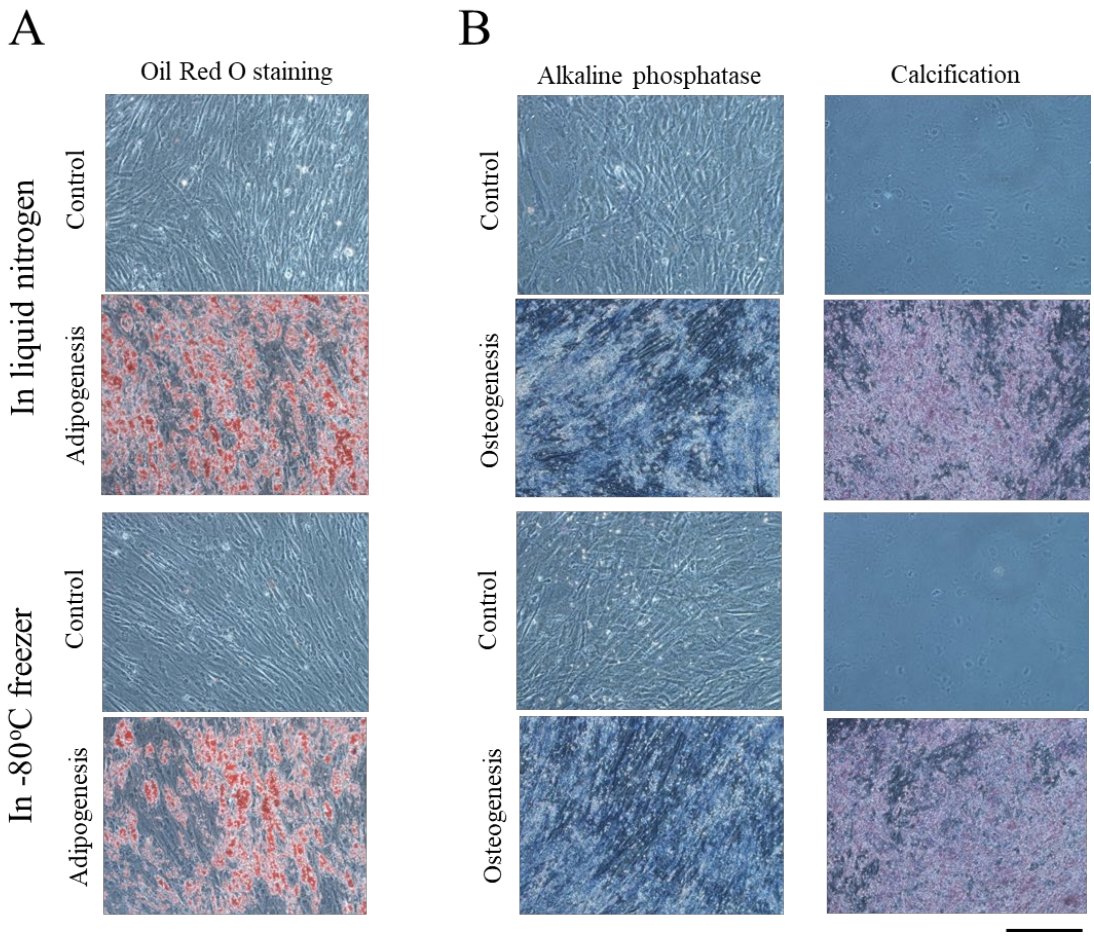


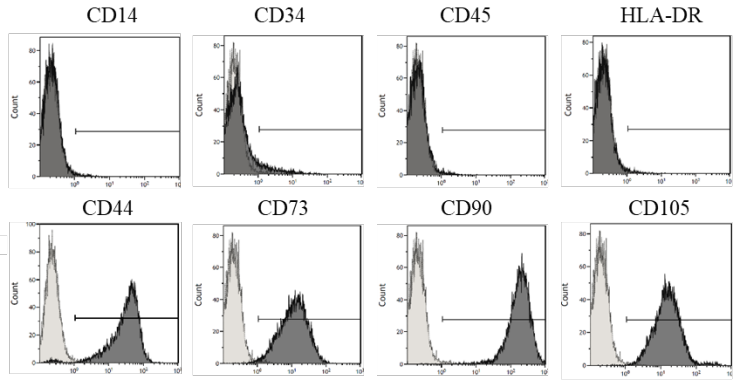
Figure 18 Representative images after adipogenesis differentiation (A) and osteogenesis differentiation (B) of hADSCs seeded immediately after thawing following cryopreservation for 7 months.

hADSCs at passage 4 were suspended at a density of 1.0×10^6 cells/mL with LR-3T-5D containing 10% Me2SO. Vials containing 1 mL of the suspension were put into BICELL and frozen in a freezer at −80°C for a day. Then, these vials were transferred from BICELL to a freeze box and stored in liquid nitrogen or a −80°C freezer for 7 months. The cells immediately after thawing were seeded and cultured in normal culture medium (control), medium for adipogenic differentiation, or medium for osteogenic differentiation for 10 days. Adipogenic differentiation was confirmed by Oil Red O staining. Osteogenic differentiation was confirmed by alkaline phosphatase staining and calcified nodule staining. Scale bar: 250 μm.

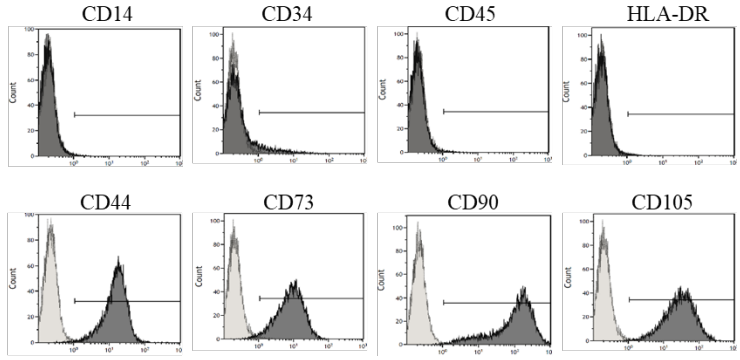
CD14, CD34, CD45, and HLA-DR are known as negative markers of MSCs [Dominici et al. 2006]. CD14 is prominently expressed on monocytes and macrophages, CD34 marks primitive hematopoietic progenitors and endothelial cells, CD45 is a pan-leucocyte marker, and HLA-DR marks HLA class II cell surface receptors. In contrast, CD44, CD73, CD90, and CD105 are known as positive markers of MSCs [Di Battista et al. 2014, Dominici et al. 2006]. The cells were negative for CD14, CD34, CD45, and HLA-DR before preservation and also immediately after thawing following cryopreservation in liquid nitrogen for 6 months. The cells were positive for CD44,

CD73, CD90, and CD105 before preservation and also immediately after thawing following cryopreservation in liquid nitrogen for 6 months (Figure 16).

A



B



C

Cell surface marker	Positive ratio (%)	
	Before freezing	Immediately after thawing
CD14	1.0 ± 0.3	0.3 ± 0.0
CD34	9.4 ± 0.4	9.0 ± 0.4
CD45	1.1 ± 0.3	0.4 ± 0.1
HLA-DR	1.1 ± 0.3	0.4 ± 0.1
CD44	95.9 ± 3.1	99.0 ± 1.0
CD73	99.3 ± 0.4	98.2 ± 1.0
CD90	99.9 ± 0.1	99.9 ± 0.1
CD105	99.9 ± 0.1	99.9 ± 0.0

Figure 19 Representative charts of flow cytometry analysis on cell surface markers of hADSCs before freezing (A) and immediately after thawing (B).

Positive ratios of cell surface markers are presented as the mean ± SD (n = 3, C). The results before freezing are the same results as in Table 8. hADSCs at passage 3 were suspended at a density of 3.0×10^6 cells/mL with LR-3T-5D with 10% Me2SO. Vials containing 1 mL of the suspension were put into BICELL and frozen in a freezer at −80°C for a day. Then, the vials were transferred from BICELL to a freeze box preincubated in liquid nitrogen and stored in liquid nitrogen or a −80°C freezer for 6 months. Isotype controls and antigen stains are represented as the thin line and the thick line, respectively.

3.3.4 Duration of incubation before freezing

Me2SO penetrates into cells through the cell membrane and works as a cryoprotectant. However, long exposure to Me2SO by cells is potentially toxic. We therefore tested the effects of the duration of incubation before freezing with LR-3T-5D, which contains Me2SO, on cell viability and annexin V-positive ratio just before freezing and immediately after thawing (Figure 17). There was no significant difference in cell viability or the annexin V-positive ratio of hADSCs just before freezing and immediately after thawing for either 3 h or 6 h vs. 0 h incubation before freezing. This suggests that Me2SO penetrated into cells immediately after exposure and did not have any obvious toxic effects with up to 6 h of incubation before freezing. There was no significant difference in cell viability between just before freezing and immediately after thawing. The annexin V-positive ratio of hADSCs immediately after thawing increased compared to that just before freezing, showing that ADSCs were potentially damaged by the freezing and thawing process.

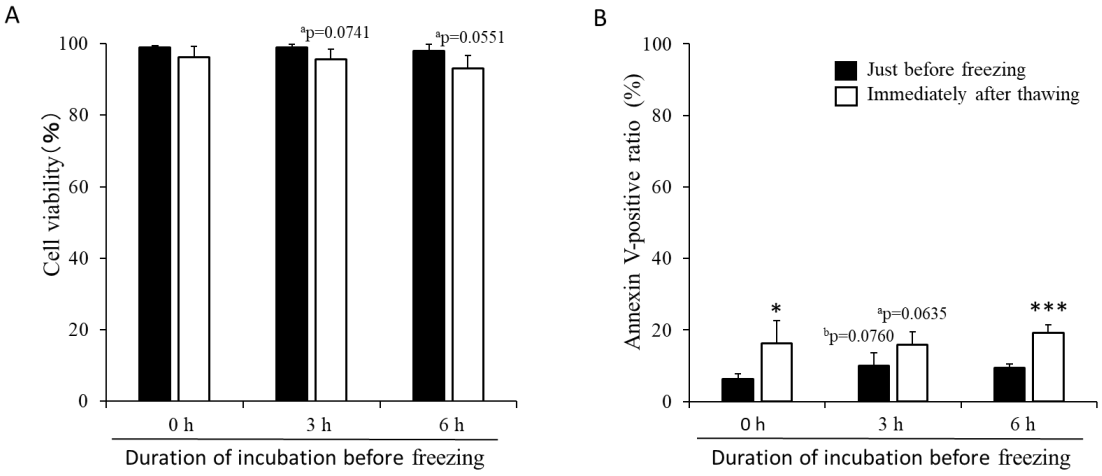


Figure 20 Cell viability (A) and annexin V-positive (B) ratio before freezing and immediately after thawing with various durations of incubation with LR-3T-5D with 10% Me2SO before freezing.

hADSCs of passage 4 were suspended at a density of 1.0×10^6 cells/mL in LR-3T-5D with 10% Me2SO. Vials containing 1 mL of the suspension were incubated at 5°C for 0, 3, or 6 h, then put into BICELL and frozen in a freezer at −80°C for a day. The vials were transferred from BICELL to a freeze box and stored in liquid nitrogen for 47 or 48 days. The cell viability and annexin V-positive ratio of hADSCs were analyzed just before freezing and immediately after thawing. The data are presented as the mean ± SD (n = 4). *p < 0.05, ***p < 0.001. Superscript a, vs. just before freezing (Student's t-test). Superscript b, vs. 0 h (Dunnett's test).

3.3.5 Minimization of Me2SO concentration

Figure 18 shows the cell viability of hADSCs immediately after thawing and the cell proliferation curve of hADSCs during subcultures seeded immediately after thawing following cryopreservation in LR-3T-5D with various concentrations of Me2SO. Cell viability was significantly lower in LR-3T-5D with 0% and 0.5% Me2SO than in LR-3T-5D with 10% Me2SO. The cell proliferation curve was significantly lower in LR-3T-5D with 0%, 0.5%, and 1% Me2SO than in LR-3T-5D with 10% Me2SO. The cell proliferation curve in LR-3T-5D with 5% Me2SO was comparable to that in LR-3T-5D with 10% Me2SO.

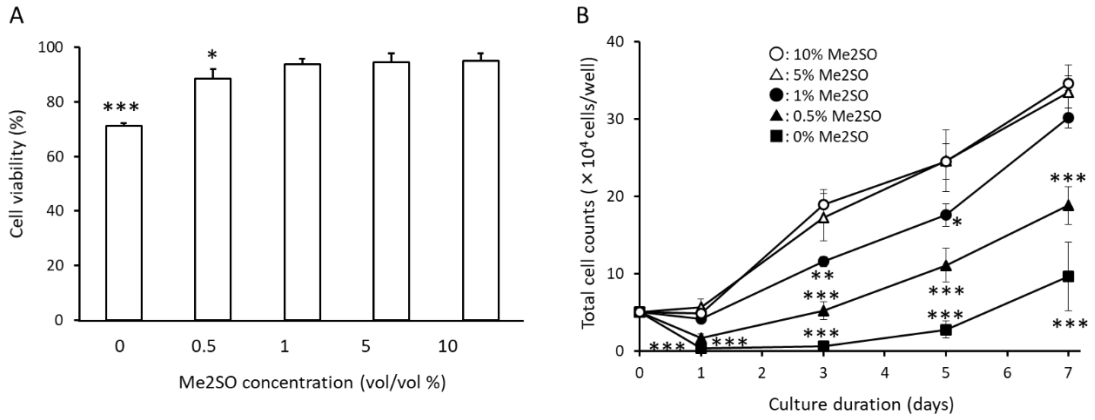


Figure 21 Cell viability (A) of hADSCs immediately after thawing, and proliferation curve (B) of hADSCs seeded immediately after thawing following cryopreservation in LR-3T-5D without Me2SO or with 0.5, 1, 5, and 10% Me2SO.

hADSCs at passage 3 were suspended at a density of 1.0×10^6 cells/mL in LR-3T-5D with or without various concentrations of Me2SO. Vials containing 1 mL of the suspension were put into BICELL and frozen in a freezer at −80°C for a day. Then, the vials were transferred from BICELL to a freeze box preincubated in liquid nitrogen and stored in liquid nitrogen or a −80°C freezer. Immediately after thawing, the cells were seeded at a density of 5.2×10^3 cells/cm^2 (5×10^4 cells/well) in a 6-well plate. The data are presented as the mean ± SD (n = 3). Statistical analysis was performed using Dunnett's test. *p < 0.05, **p < 0.01, ***p < 0.001 vs. 10% Me2SO.

3.3.6 Washing with LR-3T and replacement with LR-3T or LR-3T-5D after thawing

hADSCs cryopreserved in LR-3T-5D with 10% Me2SO were washed immediately after thawing with LR-3T, then suspended with LR-3T or LR-3T-5D and stored at 25°C. There was no significant difference in any parameter between

immediately after thawing without replacing the solution and immediately after replacement by LR-3T or LR-3T-5D (Figure 19).

The cell viability 24 h after replacement by LR-3T was significantly decreased compared to that immediately after replacement. The cell viabilities 6 h and 24 h after replacement by LR-3T-5D were significantly decreased compared to that immediately after replacement.

The viable cell recovery ratio 24 h after replacement by LR-3T was significantly decreased compared to that immediately after replacement. There was no significant difference between LR-3T and LR-3T-5D in viable cell recovery ratio.

The annexin V positivity 24 h after replacement by LR-3T was significantly increased compared to that immediately after replacement. The annexin V positivity 6 h and 24 h after replacement by LR-3T-5D was significantly increased compared to that immediately after replacement. The cell viability and annexin V-positive ratio 6 h and 24 h after replacement were significantly higher in LR-3T-5D than in LR-3T.

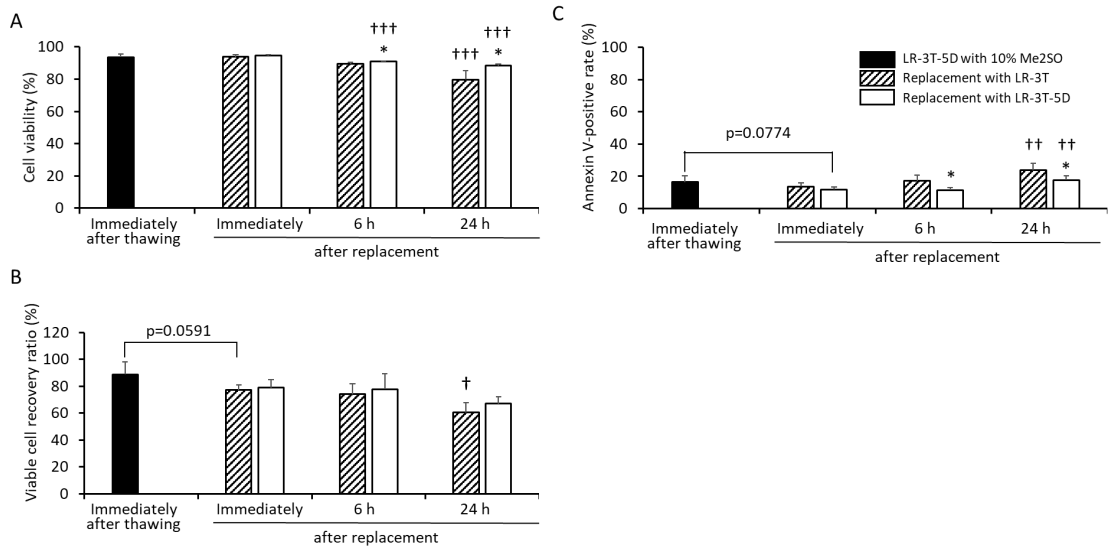


Figure 22 Cell viability (A), viable cell recovery ratio (B), and annexin V-positive ratio (C) of hADSCs immediately after thawing, or immediately, 6 h, or 24 h after replacement by LR-3T or LR-3T-5D following cryopreservation with LR-3T-5D containing 10% Me2SO.

hADSCs at passage 5 were suspended at a density of 1.0×10^6 cells/mL in LR-3T-5D with 10% Me2SO. Vials containing 1 mL of the suspension were put into a freeze box and frozen in a freezer at −80°C for a day. Then, the vials were transferred from the freeze box to another freeze box preincubated in liquid nitrogen and stored in liquid nitrogen for 112 or 113 days (about 4 months). Immediately after thawing, cells were washed with LR-3T and suspended in LR-3T or LR-3T-5D, then incubated at 25°C. The data are presented as the mean ± SD (n = 4). There was no significant difference in value immediately after replacement with LR-3T or LR-3T-5D vs. immediately after thawing (Student's t test). The results at 6 h and 24 h after replacement were compared to those immediately after thawing (Dunnett's test). † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. immediately after thawing (Dunnett's test). * $p < 0.05$ between LR-3T and LR-3T-5D (Student's t-test).

3.3.7 Comparison of cryoprotectants

Figure 20A shows the cell viability of hADSCs immediately after thawing following cryopreservation in LR-3T-5D with various cryoprotectants. Because Me2SO is the most common cryoprotectant, LR-3T-5D with 10% Me2SO was set as a control. The cell viabilities of hADSCs immediately after thawing following cryopreservation in LR-3T-5D with 10% propylene glycol and LR-3T-5D with 10% ethylene glycol were comparable to that in LR-3T-5D with 10% Me2SO, while that in LR-3T-5D with 10% glycerol tended to be lower ($p = 0.0640$) than that in LR-3T-5D with 10% Me2SO.

Figure 20B shows the annexin V-positive ratio of hADSCs immediately after thawing. The annexin V-positive ratio in LR-3T-5D with 10% propylene glycol was 17.4 ± 2.6%, which was comparable to that in LR3T-5D with 10% Me2SO (15.8 ± 3.9%). However, the annexin V-positive ratios in LR-3T-5D with 10% ethylene glycol and LR-3T-5D with 10% glycerol were 24.6 ± 6.9% and 46.1 ± 10.2%, which were relatively higher ($p = 0.1911$) and significantly higher ($p < 0.001$) than that in LR-3T-5D with 10% Me2SO, respectively.

Figure 20C shows the cell proliferation curve of hADSCs during subcultures seeded immediately after thawing. The cell proliferation curve was comparable between LR-3T-5D with 10% propylene glycol and LR3T-5D with 10% Me2SO.

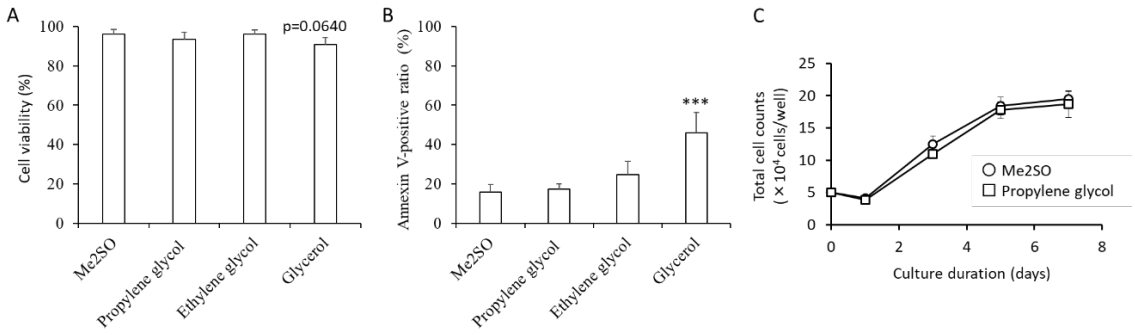


Figure 23 Cell viability (A) and annexin V-positive ratio (B) immediately after thawing of hADSCs and proliferation curve (C) of hADSCs seeded immediately after thawing following cryopreservation in LR-3T-5D containing various cryoprotectants at a concentration of 10%.

hADSCs at passage 3 were suspended at a density of 1.0×10^6 cells/mL with various preservation solutions. Vials containing 1 mL (A and B) or 0.6 mL (C) of the suspension were put into BICELL and frozen in a freezer at −80°C for a day. Then, the vials were transferred from BICELL to a freeze box and stored in liquid nitrogen for 32 days (A and B) or 39 days (C). Immediately after thawing, the cells were seeded at a density of 5.2×10^3 cells/cm^2 (5×10^4 cells/well) in a 6-well plate. The data are presented as the mean ± SD ($n = 4$). ***$p < 0.001$ vs. 10% Me2SO (Dunnett's test, A and B). There was no significant difference between Me2SO and propylene glycol (Student's t-test, C).

3.4 Discussion

We have already developed a cell suspension and preservation solution (LR-3T-5D) for the storage of human adipose tissue-derived mesenchymal stromal cells (hADSCs) at 5°C and 25°C for up to 24 h in Chapter 2. Here, we confirmed that LR-3T-5D was also more effective for cell viability, viable cell recovery ratio, annexin V-positive ratio, and colony-forming capacity as a vehicle solution for cryopreservation with 10% Me2SO than LR. The additive cryoprotectant effects of trehalose and dextran 40 with each other and with Me2SO were confirmed in a dose-dependent manner. It appears that a 2.7% concentration of dextran 40 is sufficient for cryopreservation. This solution can be prepared by mixing LR-3T and LR-3T-5D in a proportion of 3:2, and then the solution is mixed with Me2SO in a proportion of 9:1. But we introduced a simpler protocol, LR-3T-5D with 10% Me2SO, in this study. The cell viability, cell proliferation ability, cell differentiation ability, and cell surface positive/negative markers of hADSCs were well preserved after 6- or 7-month cryopreservations with LR-3T-5D containing 10% Me2SO both in liquid nitrogen storage and a −80°C freezer. In our previous study, we also used LR-3T-5D containing 10% Me2SO for cryopreservation of porcine bone marrow mesenchymal stromal cells [Nishimura et al. 2019].

The cell viability in LR-3T-5D containing 10% Me2SO was 98.6 ± 0.8% before freezing and 93.7 ± 1.7% immediately after thawing (Figure 12). The cell viability slightly decreased with freezing and thawing, but was still at a high level. The viable cell recovery ratio in LR-3T-5D containing 10% Me2SO was 71.8 ± 5.2% before freezing and 88.7 ± 9.3% immediately after thawing (Figure 12). It is unclear why the viable cell recovery ratio was higher immediately after thawing compared to before freezing. We speculate that some cells might be adsorbed to the cryovial before freezing, and these cells might be released after thawing due to changes in the structure of cell surfaces caused by freezing in LR-3T-5D with 10% Me2SO. The annexin V-positive ratio with LR-3T-5D with 10% Me2SO without incubation was 6.2 ± 1.6% before freezing and 16.2 ± 6.4% immediately after thawing (Figure 17). The annexin Vpositive ratio was significantly increased by the freezing and thawing process. We did not measure colony-forming capacity with LR-3T-5D with 10% Me2SO just before freezing (Figure 12, Table 11). However, we measured colony-forming capacity immediately after suspension in LR-3T-5D with cells from the same preparation as those used for the experiments described in Table 11. The results were the same as described in Table 9. The value was 21.3 ± 1.9%, and it was 23.3 ± 1.2% immediately

after thawing following cryopreservation in liquid nitrogen, and 23.4 ± 2.2% immediately after thawing following cryopreservation in a − 80°C freezer. These data showed that the colony-forming capacity of the cryopreserved cells immediately after thawing was comparable to that before freezing.

There are many reports on the cryoprotective effect of trehalose in various kinds of cells, such as platelets [Wolkers et al. 2001], red blood cells [Pellerin-Mendes et al. 1997], peripheral blood stem cells [Martinetti et al. 2017], hematopoietic stem cells of umbilical cord blood [Motta et al. 2014, Rodrigues et al. 2008], mesenchymal stromal cells [Rosa et al. 2009, Petrenko et al. 2014], sperm [Liu et al. 2016], oocytes [Zhang e al. 2017], embryonic stem cells [Wu et al. 2005], hepatocytes [Katenz et al. 2007], pancreatic islets [Beattie et al. 1997], and fetal skin [Erdag et al. 2019]. The mechanism of the trehalose cryoprotective effect is considered to be reduced ice crystal formation, which has a direct impact on cell viability [Solocinski et al. 2017]. The benefits of dextran 40 for cryopreservation have been reported for red blood cells [Pellerin-Mendes et al. 1997], sperm [Gloria et al. 2019, Kundu et al. 2002], mesenchymal stromal cells [Fujii et al. 2017, Pogozhykh et al. 2015], and corneal cells [Halberstadt et al. 2001]. It has been hypothesized that the large number of –OH groups in dextran can interfere with the H-bond formation of water, inhibiting large ice crystal formation during cryopreservation [Gloria e al. 2019, Kundu et al. 2002]. Although the hypothetical mechanisms resemble each other between trehalose and dextran, the cryoprotective effects of trehalose and dextran 40 were additive in our results. They may each cover the limited inhibitory action against ice crystal formation of the other.

The cell specifications after thawing of hADSCs cryopreserved with LR-3T-5D containing 10% Me2SO for 6 months in a − 80°C freezer were comparable to those in liquid nitrogen. This removes the necessity for liquid nitrogen, which is more costly and time consuming to maintain than a − 80°C freezer.

Although the cell viabilities in LR-3T-5D with 1% and 5% Me2SO were comparable to 10% Me2SO, only the proliferation curve for 5% was comparable to that of 10% Me2SO. Therefore, 5% Me2SO is recommended to reduce toxicity. Washing and replacement did not have much influence on cell viability or the viable cell recovery ratio. The cell viability and viable cell recovery ratio were gradually but slightly decreased after replacement, and the annexin V positivity was gradually but slightly increased after replacement. Therefore, washing and replacement might be advisable to avoid the toxicity of Me2SO without large changes in cell specification. From the results of viability and annexin V-positive ratio, LR-3T-5D was superior to LR-3T as a preservation solution after thawing. Although, the cell viabilities at 6 h and 24 h after

replacement by LR-3T-5D were significantly decreased compared to that immediately after replacement, they were still at high levels, 91.0 ± 0.3% and 88.3 ± 1.1%, respectively.

We compared other cell membrane-penetrating cryoprotectants to Me2SO. Glycerol was inferior to Me2SO in cell viability and annexin V-positive ratio. There was no significant difference between ethylene glycol and Me2SO. Ethylene glycol itself has low toxicity, but is in vivo metabolized to cell toxins that can be harmful [Leth et al. 2005]. Therefore, ethylene glycol is not appropriate as a substitute of Me2SO in cell therapy. Propylene glycol was comparable in cell viability, annexin V-positive ratio, and proliferation curve to Me2SO in our results. Propylene glycol is used as a solvent for intravenous, oral, and topical pharmaceutical preparations [Zar et al. 2007]. Although it is considered safe, large intravenous dosages given over a short period can be toxic. Propylene glycol is metabolized by the liver to form lactate, acetate, and pyruvate. Propylene glycol toxicity includes the development of serum hyperosmolality, lactic acidosis, and kidney failure. No maximum dose is recommended in the literature for intravenous therapy with propylene glycol. If one uses the maximum recommended dose of lorazepam, a propylene glycol dose of 2.9 g/h or 69 g/day would be presumably safe in the absence of risk factors. Propylene glycol is thus a candidate for substitution of Me2SO for cell therapy if it is used in safe doses.

There are several commercial Me2SO-free cryoprotectants [Awan et al. 2020]. However, their exact compositions are not disclosed. The advantage of our solutions is that their exact compositions are disclosed so that users can account for their safety. And all components of our solutions can be prepared from raw materials that are used as raw materials of various approved drugs.

3.5 Conclusion

Our data showed that LR-3T-5D, consisting of lactated Ringer's solution with 3% trehalose and 5% dextran 40, is effective as a vehicle solution for cryopreservation. LR-3T-5D containing 10% Me2SO preserved the cell viability and cell characteristics of hADSCs during storage in liquid nitrogen and in a − 80°C freezer for at least 7 months. These results suggest that LR-3T-5D containing 10% Me2SO can help maintain the quality of stem cells for therapy during cryopreservation. To reduce the amount of Me2SO exposure, reducing the concentration of Me2SO to 5%, washing cells and replacing the solution with LR-3T and LR-3T-5D after thawing, and substituting Me2SO with propylene glycol are feasible steps that keep the cryopreservation efficacy

the same as with 10% Me2SO. However, further in vivo research is needed to test the efficacy and safety of these methods combined with each therapeutic cell product before clinical use.

3.6 Chapter summary

We tested the efficacy of lactated Ringer's solution with 3% trehalose and 5% dextran 40 (LR-3T-5D) as a vehicle solution for cryopreservation using human adipose-derived mesenchymal stromal cells (hADSCs) with dimethyl sulfoxide (Me2SO). We also tested the effect of the Me2SO concentration in the cryopreservation solution, and the effect of washing with lactated Ringer's solution with 3% trehalose (LR-3T) and replacement with LR-3T or LR-3T-5D. LR-3T-5D was more effective for cell viability, viable cell recovery ratio, annexin V-positive ratio, and colony-forming capacity as a vehicle solution for cryopreservation with 10% Me2SO than LR. The additive effects as cryoprotectants of trehalose and dextran 40 were confirmed to be dose dependent. The cell viability, cell proliferation ability, cell differentiation ability, and the ratio of cell surface positive/negative markers of hADSCs were well maintained after cryopreservation with LR-3T-5D containing 10% Me2SO in liquid nitrogen or in a −80°C freezer. The cell viability and the proliferation curve in LR-3T-5D with 5% Me2SO were comparable to those with 10% Me2SO. LR-3T-5D was superior to LR-3T as a replacement solution in terms of viability and annexin V positivity. Our data showed that LR-3T-5D is effective as a vehicle solution for cryopreservation. Reducing Me2SO concentration to 5%, and washing and replacement with fresh LR-3T and LR-3T-5D after thawing, are feasible approaches to maintain cryopreservation efficacy.

Chapter 4. Dimethyl sulfoxide-free cryopreservation solution containing trehalose, dextran 40, and propylene glycol for therapy with human adipose tissue-derived mesenchymal stromal cells

4.1 Introduction

Cryopreservation of cells is generally performed using a solution containing about 10% dimethyl sulfoxide (Me2SO). This technique was established in the 1950s [Lovelock and Bishop 1959] and is now commonly used in cell banks. However, side effects associated with the transplantation of stem cells with Me2SO have been reported, including nausea, vomiting, cardiac arrhythmias, neurological symptoms, respiratory arrest, renal/hepatic dysfunction, and allergies [Santos et al. 2003; Windrum et al. 2005; Cox et al. 2012; Shu et al. 2014; Awan et al. 2020]. In addition, a portion of the administered Me2SO is reduced to dimethyl sulfide in the body, which is then secreted through the skin and exhaled air, causing a foul, garlic-like odor [Santos et al. 2003; Shu et al. 2014; Awan et al. 2020]. Therefore, there is a need for a cryopreservation solution that can reduce the side effects of Me2SO and that has the same level of performance as Me2SO-containing products, assuming that it is administered to humans.

Me2SO stimulates cell differentiation in some types of cells. For example, mouse bone marrow mesenchymal stem cells are differentiated into cardiomyocytes by Me2SO [Young et al. 2004]. Cryopreservation of human ES cells in Me2SO-containing cryopreservation solution decreases the expression of Oct-4, an undifferentiated marker [Katkov et al. 2006], and Me2SO affects the epigenetic profile of mouse embryoid bodies [Iwatani et al. 2006]. The human promyelocytic leukemia cell line HL-60 is differentiated into granulocytes by Me2SO [Jiang et al. 2006]. However, these effects are unfavorable for maintaining cell characteristics during cell cryopreservation. Therefore, given concerns over maintaining stemness in addition to safety, a Me2SO-free cryopreservation solution is desired.

We have shown the usefulness of lactated Ringer's solution supplemented with 3% trehalose and 5% dextran (LR-3 T-5D) with 10% Me2SO as a cryopreservation solution in Chapter 3. We have also evaluated the procedure of cell washing to remove the effect of Me2SO after thawing the cells. However, there are concerns over the effects of Me2SO when cells are thawed and used directly in clinical applications. Therefore, we have compared the effects of the cell membrane penetrating cryoprotectants propylene glycol (PG), ethylene glycol, and glycerol with those of Me2SO, and confirmed that

LR-3T-5D with 10% PG results in a similar cell proliferative capacity as LR-3T-5D with 10% Me2SO. In the present study, we compared the performance of LR-3T-5D with 10% PG to that of LR-3T-5D with 10% Me2SO on cell specifications (cell surface antigen positivity, adipogenic differentiation, osteogenic differentiation, and genetic response to cytokine stimulation) of hADSCs.

4.2 Materials and method

4.2.1 Study design

The present study was approved by the ethics committee of Otsuka Pharmaceutical Factory, Inc.

We performed three experiments. First, the effects as a cryoprotective vehicle with 10% PG were compared among LR, LR-3T, and LR-3T-5D. Second, the cell characteristics (viability, annexin V-positive ratio, colony-forming capacity, differentiation ability, cell surface markers, and mRNA expression level) after cryopreservation in liquid nitrogen were compared between LR-3T-5D with Me2SO and LT-3T-5D with PG. Third, the concentration of PG was optimized.

4.2.2 Components of the solutions

Lactated Ringer's solution (LR; Lactec® Injection), lactated Ringer's solution with 3% trehalose (LR-3T; Cellstor-W), and lactated Ringer's solution with 3% trehalose and 5% dextran 40 (LR-3T-5D; Cellstor-S) were supplied by Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan). Me2SO (CultureSure®DMSO) was purchased from Fujifilm Wako Pure Chemical Co. (Osaka, Japan). PG was purchased from Maruishi Pharmaceutical. Co., Ltd. (Osaka, Japan) and Fujifilm Wako Pure Chemical Co. (Osaka, Japan). The base solution (LR, LR-3T, or LR-3T-5D) with 4 or 10% cryoprotectant was prepared by mixing base solution and cryoprotectant at ratios of 24:1 or 9:1 (vol/vol), respectively. The compositions of LR-3T, LR-3T-5D, LR-3T-5D with 4 or 10% Me2SO, and LR-3T-5D with 4 or 10% PG are described in Table 1.

Table 13 Compositions of preservation solutions (LR-3T and LR-3T-5D) and the cryopreservation solution (LR-3T-5D with 10% Me2SO and LR-3T-5D with 10% PG)

Components	LR-3 T	LR-3 T-5D	LR-3 T-5D with 4% Me2SO	LR-3 T-5D with 10% Me2SO	LR-3 T-5D with 4% PG	LR-3 T-5D with 10% PG
Na^+	130 mEq/L	130 mEq/L	1.25 mEq/L	117 mEq/L	1.25 mEq/L	117 mEq/L
K^+	4 mEq/L	4 mEq/L	3.8 mEq/L	3.6 mEq/L	3.8 mEq/L	3.6 mEq/L
Ca^{2+}	3 mEq/L	3 mEq/L	2.9 mEq/L	2.7 mEq/L	2.9 mEq/L	2.7 mEq/L
Cl^-	109 mEq/L	109 mEq/L	105 mEq/L	98 mEq/L	105 mEq/L	98 mEq/L
$Lactate^-$	28 mEq/L	28 mEq/L	27 mEq/L	25 mEq/L	27 mEq/L	25 mEq/L
Trehalose	3%	3%	2.88%	2.7%	2.88%	2.7%
Dextran40	–	5%	4.8%	4.5%	4.8%	4.5%
DMSO	–	–	4%	10%	–	–
PG	–	–	–	–	4%	10%

LR-3T: lactated Ringer's solution with 3% trehalose., LR-3T-5D: lactated Ringer's solution with 3% trehalose and 5% dextran 40, LR-3T-5D with 4% Me2SO: LR-3T-5D with 4% dimethyl sulfoxide. LR-3T-5D with 10% Me2SO: LR-3T-5D with 10% dimethyl sulfoxide, LR-3T-5D with 4% PG: LR-3T-5D with 4% propylene glycol, LR-3T-5D with 10% PG: LR-3T-5D with 10% propylene glycol

4.2.3 Preparation of hADSCs

Human ADSCs (Female, 44Y or 32Y, PT5006, Lot No. 0000692059 or 19TL200176; Lonza Walkersville, Inc., Walkersville, MD, USA) were used in this study; 38Y, 44Y, and 32Y indicate the ages of the donors. Human ADSCs were seeded in a 75-cm^2 flask with 15 mL of medium prepared from a medium kit (PT-4505 ADSC BulletKit™, Lonza Walkersville, Inc.) and maintained at 37°C in a humidified atmosphere of 5% CO_2. All of the medium was changed every 3 or 4 days. Cells were passaged at approximately 90% confluency, and passages 2, 3, or 5 (3, 4, or 6 after cell preparation) were used for the experiments. Cells were collected as described in 3.2.3.

4.2.4 Cryopreservation of hADSCs

The hADSCs suspended in LR-3T were transferred to a 15- or 50-mL conical tube, centrifuged (210 × g, 5 min, at room temperature), and the supernatant was aspirated. hADSCs were resuspended with LR, LR-3T, or LR-3T-5D containing 10% Me2SO or 10% PG so that the final cell concentration was 1.0 to 3.0 × 10^6 cells/mL. One milliliter of suspension was dispensed into each cryovial (Nunc™ CryoTube™ Vials, size 1.8 mL, Thermo Fisher Scientific Inc., Waltham, MA, USA). Immediately after dispensing, the vial was placed in a BICELL (Nihon Freezer Co., Ltd., Tokyo, Japan) and stored at −80°C for approximately 24 h. Then the vial was transferred from

the BICELL or the freeze box to a freeze box preincubated in liquid nitrogen and stored in liquid nitrogen for 46 days at the longest. The length of the storage period in each experiments was different among experiments and described in figure legends.

4.2.5 Thawing of cryopreservation solution

The frozen vials in which the hADSCs were stored were quickly thawed in a thermostat bath heated to 37°C. After thawing, they were used for the following studies.

4.2.6 Cell viability and viable cell recovery ratio

Cell viability and viable cell recovery ratio were evaluated as described in 3.2.6.

4.2.7 Annexin V staining

Annexin V staining was evaluated as described in 2.2.6.

4.2.8 Colony-forming capacity

Colony-forming capacity was evaluated as described in 2.2.8.

4.2.9 Cell proliferation curve

Cell proliferation curve was evaluated as described in 3.2.9.

4.2.10 Adipogenic and osteogenic differentiation ability

Adipogenic and osteogenic differentiation ability were evaluated as described in 2.2.9.

4.2.11 Cell surface markers

Cell surface makers were evaluated as described in 2.2.7.

4.2.12 mRNA expression level after cytokine stimulation

The changes in mRNA expression of the following human genes (Table 13) were examined in hADSCs under the following conditions with subcultures both before and after frozen storage. Either freshly prepared or thawed hADSC suspensions (1×10^6 cells) were diluted with culture medium (1:9) and were centrifuged at 800 × g for 5 min. Each cell pellet was diluted in medium and divided in half (5×10^5 cells each). hADSCs were cultured in medium with or without both 5 ng/mL recombinant human interferon gamma (IFN-γ; R&D Systems, Inc., Minneapolis, MN, USA) and 5 ng/mL recombinant human tumor necrosis factor alpha (TNF-α; R&D Systems, Inc.) using 25-cm^2 culture flasks for 24 h. Then, hADSCs were collected with trypsinization and were lysed in RLT buffer (Qiagen Inc., Germantown, MD, USA) with 2-mercaptoethanol. Total RNA was isolated using RNeasy columns (Qiagen Inc.) following the manufacturer's instructions, and the RNA amount was measured by a NanoDrop 200c (Thermo Fisher Scientific Inc.). cDNA was synthesized from 1 µg of RNA using a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific Inc.) with the following conditions: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min, and was stored at −30°C. 25 ng of total RNA equivalent cDNA per reaction was mixed with TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific Inc.) (1:1) into a TaqMan Array Plate 32 Plus (Fast, 0.1-mL) (Thermo Fisher Scientific Inc.). PCR assay was performed using the Applied Biosystems 7500 Fast Real-Time PCR System with the following profile: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 20 s, and 40 cycles at 95°C for 3 s and 60°C for 30 s. The threshold cycle (Ct) was calculated by the instrument's software (7500 Fast System ver. 2.3). The relative expression of each mRNA was calculated using the ΔCt method (where ΔCt is the value obtained by subtracting the Ct value of hypoxanthine phosphoribosyltransferase 1 (HPRT1) mRNA from the Ct value of the target mRNA). Specifically, $2^{-(\Delta Ct)}$ is expressed as the amount of target mRNA relative to HPRT1 mRNA. Target genes were selected from immunomodulatory genes, indoleamine 2,3-dioxygenase 1 (IDO1), hepatocyte growth factor (HGF), prostaglandin E synthase (PTGES), prostaglandin-endoperoxide synthase 2 (PTGS2, also known as cyclooxygenase 2, COX2), programmed death 1 ligand-1 (PD-L1), and chemokine (C-C motif) ligand 5 (CCL5, also known as regulated on activation, normal T cell expressed and secreted, RANTES).

Table 14 Real-time PCR primers

Gene Symbols	Assay ID
HPRT1	Hs99999909_m1
CCL5 (RANTES)	Hs99999048_m1
PD-L1 (CD274)	Hs00204257_m1
HGF	Hs00300159_m1
IDO1	Hs00984148_m1
PTGES	Hs00610420_m1
PTGS2 (COX2)	Hs00153133_m1

Assay IDs of the primers used for TaqMan-based PCR (Thermo Fisher Scientific Inc.) are indicated with gene symbols.
HPRT1, hypoxanthine phosphoribosyltransferase 1 (housekeeping gene); CCL5, chemokine (C-C motif) ligand 5 (RANTES, regulated on activation, normal T cell expressed and secreted); PD-L1, programmed death 1 ligand-1; HGF, hepatocyte growth factor; IDO1, indoleamine 2,3-dioxygenase 1; PTGES, prostaglandin E synthase; PTGS2, prostaglandin-endoperoxide synthase 2 (COX2, cyclooxygenase 2).

4.2.13 Analysis and statistics

Results are presented as the mean ± standard deviation (SD). Statistical analysis was performed using Dunnett's multiple comparison test, Tukey's multiple comparison test, and Student's t test with a significance level of $p < 0.05$. Data were analyzed with SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

4.3 Results

4.3.1 Comparison of LR, LR-3T, and LR-3T-5D as cryoprotective vehicle solutions with 10% PG

The effects as cryoprotective vehicle solutions with 10% PG were compared among LR, LR-3T, and LR-3T-5D (Figure 21). hADSCs were suspended in LR with 10% PG, LR-3T with 10% PG, or LR-3T-5D with 10% PG, then cryopreserved for more than 1 month. There was no significant difference in cell viability, viable cell recovery ratio, or annexin V-positive ratio of hADSCs before freezing among LR with 10% PG, LR-3T with 10% PG, and LR-3T-5D with 10% PG. Cell viability immediately after thawing was significantly higher ($p < 0.05$) in LR-3T with 10% PG and LR-3T-5D with 10% PG than in LR with 10% PG (Figure 21A). The viable cell recovery ratio immediately after thawing was significantly higher ($p < 0.05$) in LR-3T-5D with 10% PG than in LR with 10% PG (Figure 21B). The annexin V-positive ratio immediately after thawing was significantly lower ($p < 0.05$) in LR-3T with 10% PG and LR-3T-5D

with 10% PG than in LR with 10% PG (Figure 21C).

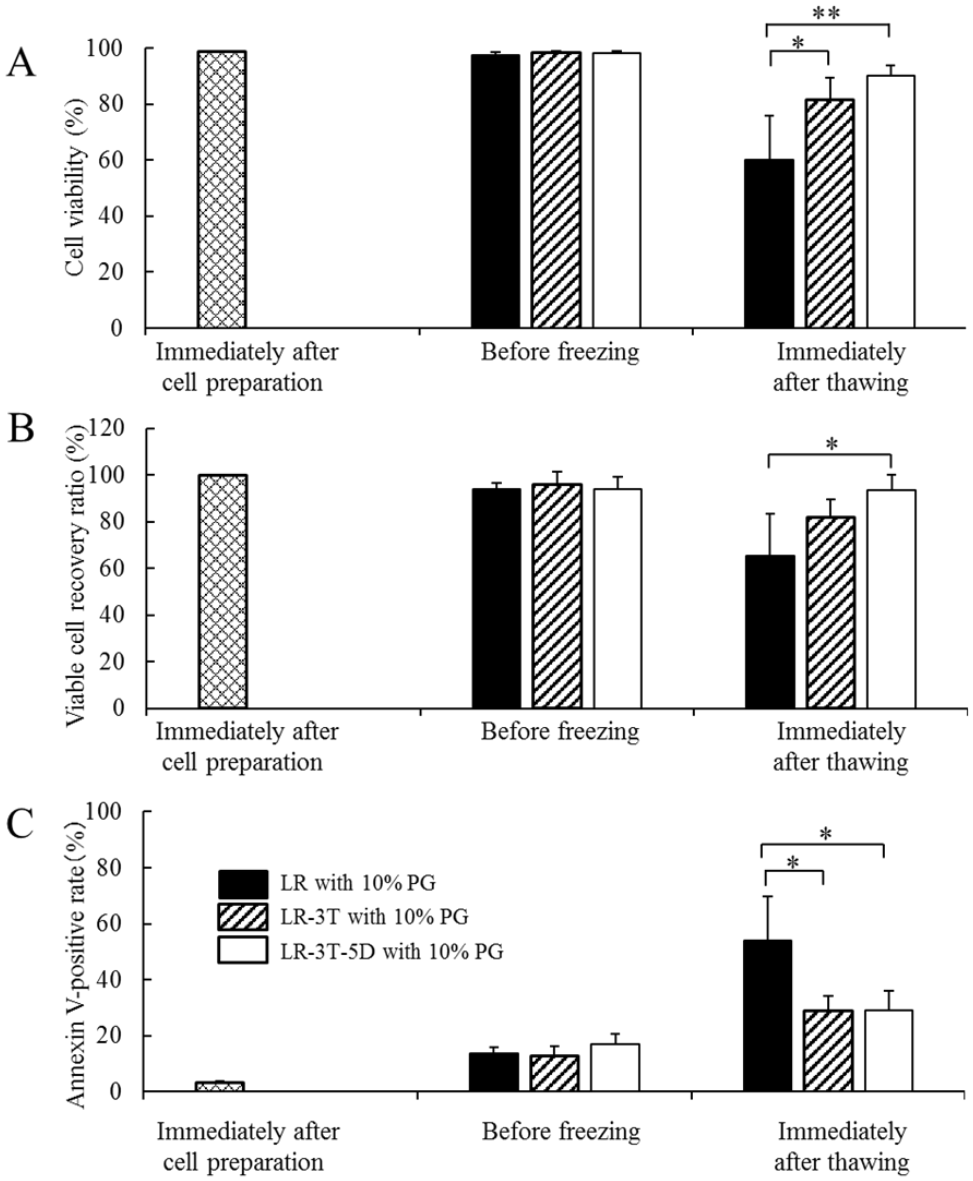


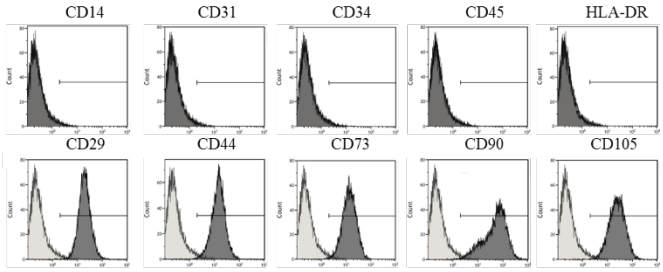
Figure 24 Cell viability (A), viable cell recovery ratio (B), and annexin V-positive ratio (C) of hADSCs cryopreserved using LR with 10% PG, LR-3T with 10% PG, or LR-3T-5D with 10% PG.

hADSCs at passage 3 were suspended at a density of 1.0×10^6 cells/mL using LR with 10% PG, LR-3T with 10% PG, or LR-3T-5D with 10% PG. Vials containing 1 mL of the suspension were put into a BICELL and frozen in a freezer at −80°C for a day. Then, the vials were transferred from the BICELL to a freeze box preincubated in liquid nitrogen then stored in liquid nitrogen for 36 or 37 days. The data are presented as the mean ± SD (n=4). Statistical analysis was performed using Tukey's test. *p<0.05, **p<0.01.

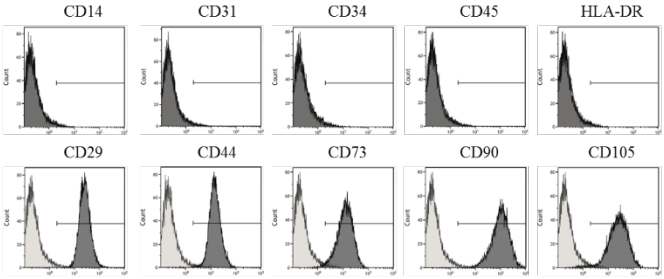
4.3.2 Cell characteristics

CD14, CD31, CD34, CD45, and HLA-DR in cells with LR-3T-5D containing 10% PG were negative, as were those in cells with LR-3T-5D with 10% Me2SO. CD29, CD44, CD73, CD90, and CD105 in cells with LR-3T-5D containing 10% PG were positive, as were those in cells with LR-3T-5D with 10% Me2SO (Figure 22).

A 10% Me2SO



B 10% PG



C

Cell surface marker	Positive ratio (%)	
	10% Me2SO	10% PG
CD14	1.6 ± 0.5	1.0 ± 0.4
CD31	1.5 ± 0.4	1.1 ± 0.4
CD34	2.9 ± 1.1	2.3 ± 0.5
CD45	1.8 ± 0.7	1.2 ± 0.4
HLA-DR	1.4 ± 0.4	1.0 ± 0.3
CD29	99.9 ± 0.0	99.9 ± 0.1
CD44	99.5 ± 1.3	99.7 ± 0.2
CD73	98.5 ± 1.4	97.9 ± 0.7
CD90	98.9 ± 1.2	99.1 ± 0.9
CD105	99.8 ± 0.1	98.6 ± 1.0

Figure 25 Representative charts of flow cytometry analyses of cell surface markers of hADSCs cryopreserved using LR-3T-5D with 10% Me2SO (A) and LR-3T-5D with 10% PG (B).

Positive ratios of cell surface markers are presented as the mean ± SD (n=3, C). hADSCs at passage 6 were suspended at a density of 3.0×10^6 cells/mL using LR-3T-5D with 10% Me2SO or LR-3T-5D with 10% PG. Vials containing 1 mL of the suspension were put into a BICELL and frozen in a freezer at −80°C for a day. Then, the vials were transferred from the BICELL to a freeze box preincubated in liquid nitrogen and stored in liquid nitrogen for 18, 44, or 46 days. Isotype controls and antigen stains are represented as the light and dark shaded areas, respectively.

Adipogenic differentiation and osteogenic differentiation were induced using hADSCs immediately after thawing following cryopreservation with LR-3T-5D containing 10% Me2SO or 10% PG (Figure 23A and B, respectively). Adipocytes containing oil droplets stained with Oil Red O were observed both in 10% Me2SO and 10% PG groups, but not in control cultures without the induction of adipogenic differentiation. Similarly, osteoblasts confirmed by alkaline phosphatase staining and calcification staining were observed both in 10% Me2SO and 10% PG groups, but not in control cultures without the induction of osteogenic differentiation.

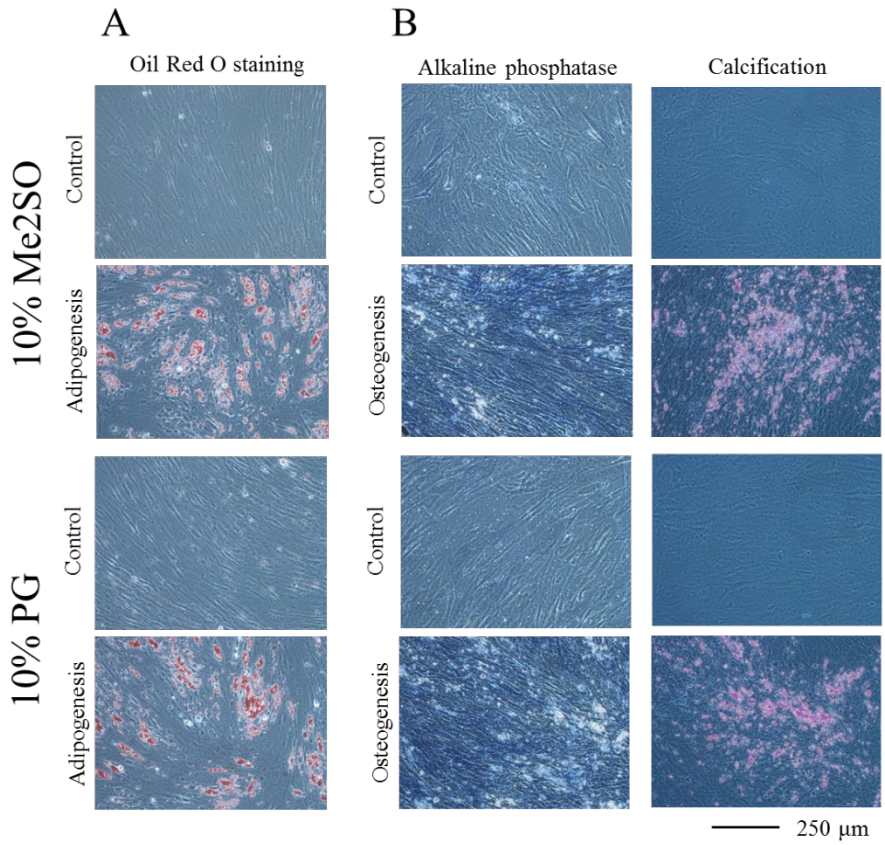


Figure 26 Representative images after adipogenesis differentiation (A) and osteogenesis differentiation (B) of hADSCs seeded immediately after thawing following cryopreservation for 46 days.

hADSCs at passage 4 were suspended at a density of 1.0×10^6 cells/mL using LR-3T-5D with 10% Me2SO or LR-3T-5D with 10% PG. Vials containing 1 mL of the suspension were put into a BICELL and frozen in a freezer at −80°C for a day. Then, the vials were transferred from the BICELL to a freeze box preincubated in liquid nitrogen and stored in liquid nitrogen for 46 days. The cells immediately after thawing were seeded and cultured in normal culture medium (control), medium for adipogenic differentiation, or medium for osteogenic differentiation. Adipogenic and osteogenic differentiated cells were cultured for 9 and 17 days, respectively. Adipogenic differentiation was confirmed by Oil Red O staining. Osteogenic differentiation was confirmed by alkaline phosphatase staining and calcified nodule staining.

Figure 24 shows the colony-forming capacity of hADSCs immediately after thawing following cryopreservation using LR-3T-5D containing 10% Me2SO or 10% PG. There was no significant difference in colony formation capacity between LR-3T-5D with 10% Me2SO and LR-3T-5D with 10% PG.

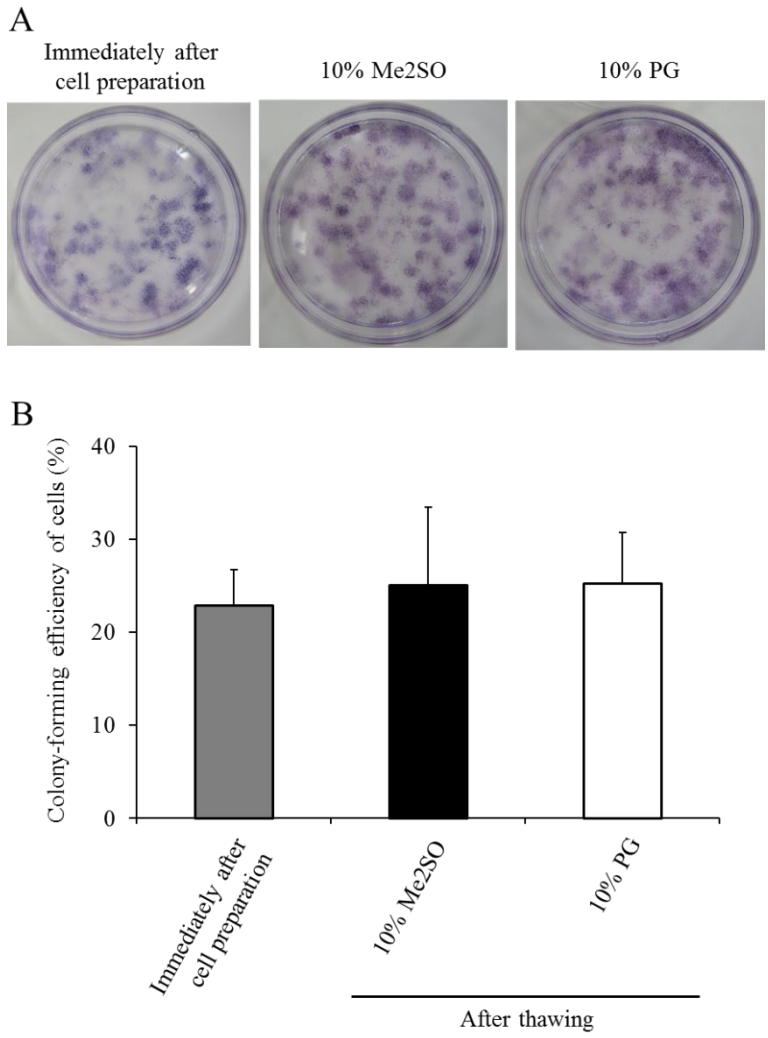


Figure 27 Colony-forming capacity of hADSCs immediately after thawing.

hADSCs at passage 4 were suspended at a density of 1.0×10^6 cells/mL in LR-3T-5D with 10% Me2SO or 10% PG. Vials containing 1 mL of the suspension were put into a BICELL and frozen in a freezer at −80°C for a day. Then, these vials were transferred from the BICELL to a freeze box preincubated in liquid nitrogen and stored in liquid nitrogen for 31 days. The data are presented as the mean ± SD (n=4). The values 'after cell preparation' were those at the time of suspension in LR-3T during cell preparation. Statistical analysis was performed using Student's t test, and there was no significant difference between storage in 10% Me2SO and in 10% PG.

4.3.3 mRNA expression level

Mesenchymal stromal cells respond to inflammatory cytokines and show broad anti-inflammatory and immunomodulatory properties via the secretion of various factors [Pittenger et al. 2019]. Thus, we focused on the responses of representative genes such as immunomodulatory genes (IDO-1, PTGES, PTGES2, and CCL5) or a pro-angiogenesis and anti-apoptotic gene (HGF) to cytokine stimulation following cryopreservation of hADSCs. To confirm the cytokine response of hADSCs, fresh cells (immediately after cell preparation) or cells after thawing following cryopreservation were cultured with or without 5 ng/mL recombinant human IFN-γ and 5 ng/mL recombinant human TNF-α for 24 h. All gene expressions were markedly and significantly higher ($p < 0.05$) in subcultures with cytokines in hADSCs immediately after cell preparation or after thawing following cryopreservation both in LR-3T-5D with 10% Me2SO and LR-3T-5D with 10% PG groups than in subcultures without cytokines (Figure 25).

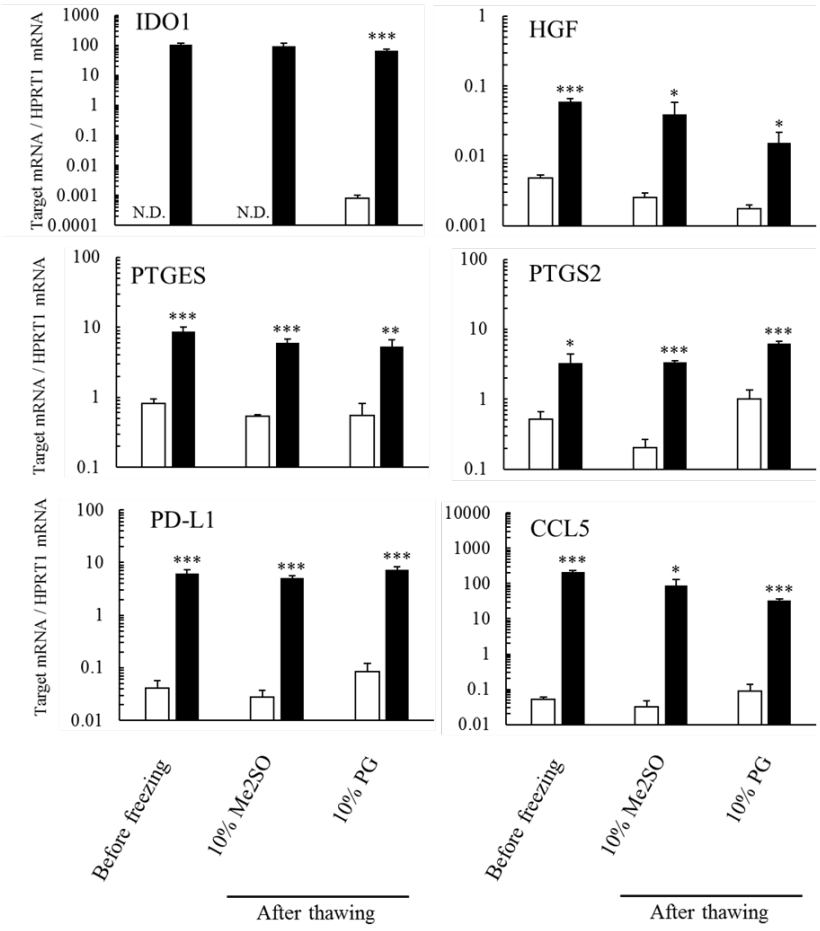


Figure 28 mRNA expression of hADSCs subcultured with cytokine stimulation after thawing.

hADSCs at passage 4 were suspended at a density of 1.0 × 10^6 cells/mL in LR-3T-5D with 10% Me2SO or 10% PG. Vials containing 1 mL of the suspension were put into a BICELL and frozen in a freezer at −80°C for a day. Then, these vials were transferred from the BICELL to a freeze box preincubated in liquid nitrogen and stored in liquid nitrogen for 5 days. ADSCs before freezing or after thawing were cultured in medium with or without both 5 ng/mL recombinant human interferon gamma and 5 ng/mL recombinant human tumor necrosis factor alpha for 24 h. The open and closed columns are without and with cytokine stimulation, respectively. HPRT1, hypoxanthine phosphoribosyltransferase 1 (housekeeping gene); CCL5, chemokine (C-C motif) ligand 5 (RANTES, regulated on activation, normal T cell expressed and secreted); PD-L1, programmed death 1 ligand-1; HGF, hepatocyte growth factor; IDO1, indoleamine 2,3-dioxygenase 1; PTGES, prostaglandin E synthase; PTGS2, prostaglandin-endoperoxide synthase 2 (COX2, cyclooxygenase 2). Data are expressed as the ratio of the target mRNA to HPRT1 mRNA. The data are presented as the mean ± SD (n=3). Statistical analysis was performed using Student's t test between subcultures with and without cytokine stimulation. *p<0.05, **p<0.01, ***p<0.001

4.3.4 Optimization of the concentration of PG

Figure 26 shows the cell proliferation curve of hADSCs during subcultures seeded immediately after thawing of hADSCs cryopreserved using LR-3T-5D containing various concentrations of Me2SO or PG. The proliferation of the LR-3T-5D containing 1.25% Me2SO group was slower than that of the LR-3T-5D containing 10% Me2SO group up to 3 days after seeding, while the LR-3T-5D containing 2.5% or 5% Me2SO groups showed the same levels of proliferation as that of the LR-3T-5D containing 10% Me2SO group (Figure 26A). However, the total cell counts at 5 and 7 days after seeding of the LR-3T-5D containing 2.5% or 5% PG groups were significantly higher ($p < 0.05$) than that of the LR-3T-5D containing 10% PG group (Figure 26B).

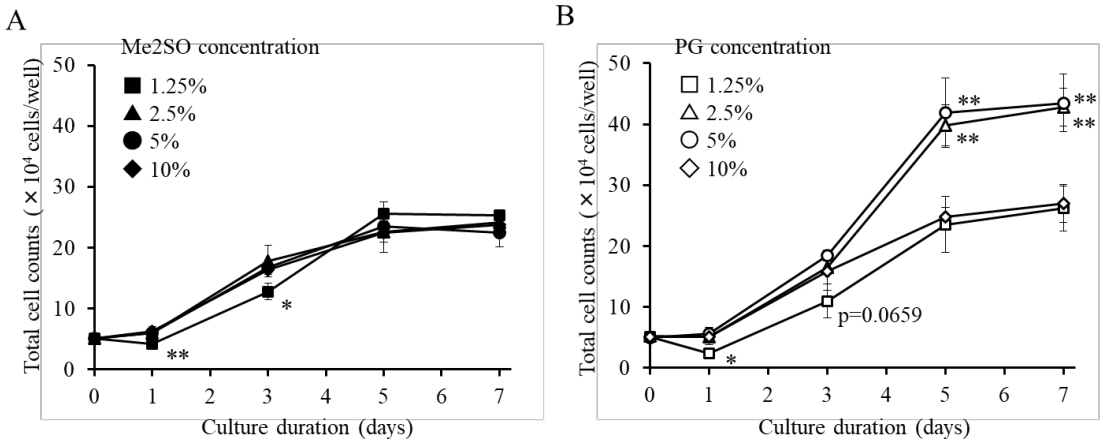


Figure 29 Proliferation curve of hADSCs seeded immediately after thawing that were cryopreserved using LR-3T-5D containing various concentrations of Me2SO (A) or PG (B).

hADSCs at passage 4 were suspended at a density of 1.0 × 10^6 cells/mL using LR-3T-5D with 1.25 to 10%

Me2SO or LR-3T-5D with 1.25 to 10% PG. Vials containing 1 mL of the suspension were put into a BICELL and frozen in a freezer at −80°C for a day. Then, the vials were transferred from the BICELL to a freeze box preincubated in liquid nitrogen and stored in liquid nitrogen for 31 days. Immediately after thawing, the cells were seeded at a density of 5.6×10^3 cells/cm^2 (5×10^4 cells/well) in a 6-well plate. The data are presented as the mean ± SD (n=3). Statistical analysis was performed using Dunnett's test. *p<0.05, **p<0.01 vs. closed or open diamonds.

We compared cell viability, viable cell recovery ratio, and the cell proliferation curve between before freezing and immediately after thawing in hADSCs cryopreserved using LR-3T-5D containing 4% (the representative concentration for 2.5% to 5%) Me2SO or PG. Cell viability immediately after thawing was approximately 95%, but significantly lower ($p < 0.05$) in hADSCs cryopreserved using LR-3T-5D containing 4% Me2SO or 4% PG than before freezing (98.8%) (Figure 27A). The viable cell recovery ratio in hADSCs cryopreserved using LR-3T-5D containing 4% Me2SO or PG was also about 95% (Figure 27B).

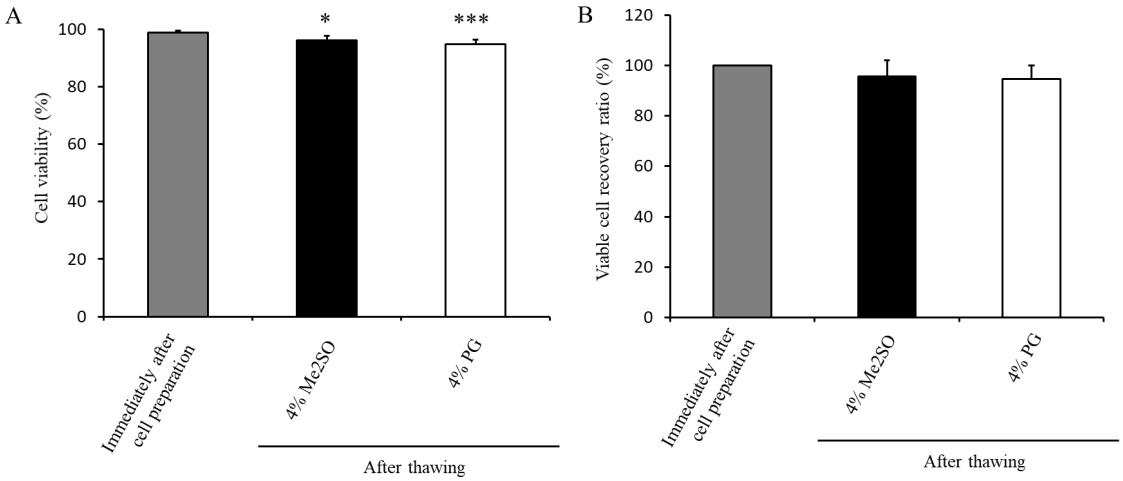


Figure 30 Cell viability (A) and viable cell recovery ratio (B) of hADSCs immediately after thawing of samples cryopreserved in 4% Me2SO or 4% PG.

Test solutions containing Me2SO or PG were prepared by adding Me2SO or PG to LR-3T-5D. hADSCs at passage 5 were suspended at a density of 1.0×10^6 cells/mL using test solutions containing Me2SO or PG. For the data before freezing, hADSCs suspended in LR-3T-5D were used. Vials containing 1 mL of the suspension were put into a BICELL and frozen in a freezer at −80°C for 3~4.5 h. Then, the vials were transferred from the BICELL to a freeze box preincubated in liquid nitrogen and stored in liquid nitrogen for 23 days. The data are presented as the mean ± SD (n=6). Statistical analysis was performed using Dunnett's test. *p<0.05, ***p<0.001 vs. before freezing.

As shown in the photographs (Figure 28A), the hADSCs at 5 days after seeding before freezing and those frozen in LR-3T-5D containing 4% Me2SO or 4% PG reached 100% confluence. The total cell count of LR-3T-5D containing 4% PG was comparable to that before freezing, while that of LR-3T-5D containing 4% Me2SO was significantly

lower ($p < 0.05$) than that before freezing at 3 to 7 days after seeding (Figure 28B).

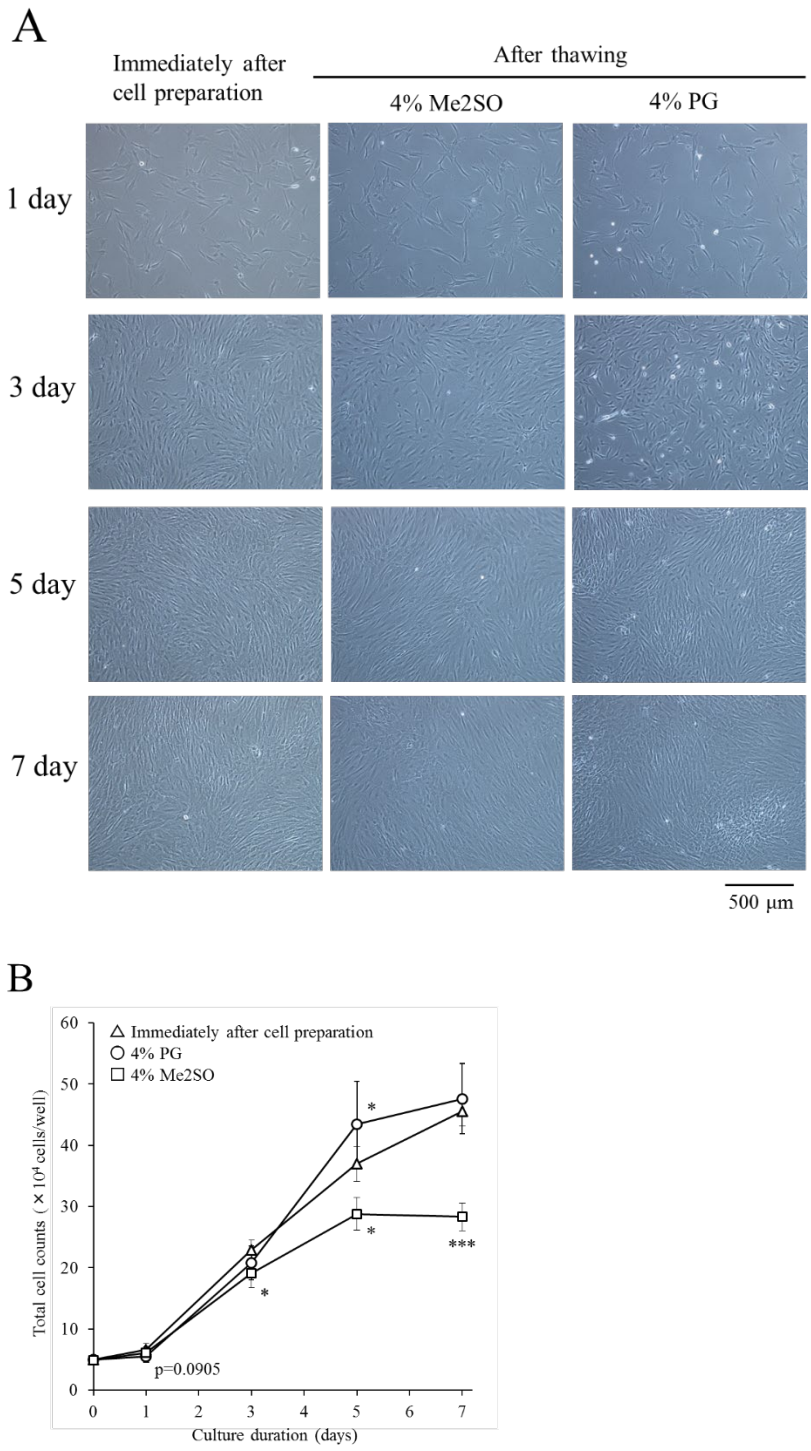


Figure 31 Photographs over time (A) and proliferation curve (B) of hADSCs seeded immediately after thawing of samples cryopreserved in 4% Me2SO or 4% PG.

Test solutions containing Me2SO or PG were prepared by adding Me2SO or PG to LR-3T-5D. hADSCs at passage 5 were suspended at a density of 1.0×10^6 cells/mL using test solutions containing Me2SO or PG. For the data before freezing, hADSCs suspended in LR-3T-5D were used. Vials containing 1 mL of the suspension were put into a BICELL and frozen in a freezer at −80°C for 3~4.5 h. Then, the vials were transferred from the BICELL to a freeze box preincubated in liquid nitrogen and stored in liquid nitrogen for 23 days. Immediately after thawing, the cells were seeded at a density of 5.6×10^3 cells/cm^2 (5×10^4 cells/well) in a 6-well plate. The data are presented as the mean ± SD (n=6). Statistical analysis was performed using Dunnett's test. *p<0.05, ***p<0.001 vs. before freezing.

4.4 Discussion

There is concern about side effects of Me2SO administration. Therefore, it is desirable to wash the cells and replace the solution after thawing to avoid Me2SO exposure. However, taking this approach prior to clinical use is cumbersome. In contrast, a Me2SO-free cryopreservation solution with 10% PG has the advantage of avoiding Me2SO-related side effects without such procedures.

We have already confirmed the additive effects of trehalose and dextran 40 added to LR for refrigerated storage [Chapter 2], room temperature storage [Chapter 2], and cryopreservation with 10% Me2SO [Chapter 3]. In this study, similarly, we found that cell viability immediately after thawing was improved by LR-3T with 10% PG and LR-3T-5D with 10% PG compared with LR with 10% PG. The viable cell recovery ratio immediately after thawing also increased in LR-3T-5D with 10% PG than in LR with 10% PG. Moreover, the annexin V-positive ratio immediately after thawing decreased in LR-3T with 10% PG and LR-3T-5D with 10% PG than in LR with 10% PG. These data indicated the additive effects of trehalose and dextran 40 for cryopreservation with 10% PG. Therefore, we concluded that the presence of both trehalose and dextran 40 is essential to keep the higher quality of hADSCs during the freeze-thaw process with propylene glycol.

CD14, CD31, CD45, and HLA-DR are negative markers of hADSCs [Bourin et al. 2013, Di Battista et al. 2014]. CD14 is prominently expressed on monocytes and macrophages [Dominici et al. 2006, Di Battista et al. 2014], and CD-31 is a platelet endothelial cell adhesion molecule (PECAM-1) that is found on the surface of platelets, leukocytes, and endothelial cells [Bourin et al. 2013]. CD45 is a common leukocyte antigen [Dominici et al. 2006, Bourin et al. 2013, Di Battista et al. 2014], and HLA-DR is a HLA class II cell surface receptor [Dominici et al. 2006, Di Battista et al. 2014]. CD34 has been both reported as a negative marker [Di Battista et al. 2014] and a positive marker [Bouri et al. 2013] of hADSCs. The percentage of CD34-positive hADSCs depends on the class of CD34 antibody, the method of adipose tissue harvest, the degree of vascular hemorrhage, the subsequent digestion and isolation techniques,

the culture conditions, and passage number [Bouri et al. 2013]. In this study, CD34 was used as a negative marker. CD34 marks primitive hematopoietic progenitors and endothelial cells [Dominici et al. 2006, Bourin et al. 2013, Di Battista et al. 2014]. CD29, CD44, CD73, CD90, and CD105 are positive markers of hADSCs [Bouri et al. 2013, Di Battista et al. 2014]. These negative and positive cell surface markers remained negative and positive respectively after thawing following cryopreservation in LR-3T-5D with 10% PG, indicating that the cell characteristics of hADSCs were preserved during the freeze-thaw process.

One criterion for defining MSCs is multipotent differentiation potential [Bouri et al. 2013, Di Battista et al. 2014, Dominici et al. 2006]. We confirmed that hADSCs still had adipogenic and osteogenic differentiation abilities after thawing following cryopreservation in LR-3T-5D with 10% PG. This result indicated that the freeze-thaw process did not affect the differentiation ability of hADSCs. There were no obvious differences in colony-forming capacity before and after cryopreservation using LR-3T-5D with 10% PG. This result also indicated that the cryopreservation did not affect the ratio of cells with proliferation ability.

MSCs respond to inflammatory cytokines and show broad anti-inflammatory and immunomodulatory properties via the secretion of various factors [Krampera et al. 2013, Pittenger et al. 2019]. The International Society for Cellular Therapy (ISCT) recommends that a standard immune plasticity assay should use IFN-γ ± TNF-α as a model in vitro priming agent [Krampera et al. 2013]. Thus, we used culture media containing IFN-γ and TNF-α as an immune-stimulation model for hADSCs and evaluated the expression of representative genes IDO1, HGF, PTGES, PTGS2, PD-L1, and CCL5, which are upregulated in cytokine-exposed MSCs [Krampera et al. 2013, Chinnadurai et al. 2018, Guan et al. 2018, Galipeau et al. 2016]. These genes have various function related to efficacy of MSCs. For example, IDO1 catalyzes the rate-limiting step of tryptophan catabolism along the degradation pathway, leading to tryptophan starvation and production of tryptophan metabolites kynurenines, resulting in inhibiting effector T-cell responses and promoting Treg development [Guan et al. 2018]. HGF is antiapoptotic and angiogenic factor [Merimi et al. 2021]. Prostaglandin E2 is synthesized by PTGES and PTGS2 (also known as COX2, cyclooxygenase 2). Prostaglandin E2 secreted from bone marrow stromal cells reprograms the macrophages to increase their interleukin-10 production [Németh et al. 2009]. Via the interaction of PD-L1 with its receptor PD-1 expressed on T lymphocytes, MSCs suppress the activation of CD4+ T cells, down-regulate interleukin-2 secretion and induce irreversible hyporesponsiveness and cell death [Davies et al. 2017]. CCL5 (also known

as RANTES, regulated on activation, normal T cell expressed and secreted) recruits T cells to the vicinity of MSCs [Can and Coskun 2020]. We confirmed that hADSCs still had a gene expression response to cytokines after thawing following cryopreservation in LR-3T-5D with 10% PG, the same as that before cryopreservation. This observation suggested that the freeze-thaw processes did not affect the putative effects of hADSCs. In summary, the cell surface markers, adipogenesis and osteogenesis differentiation potency, cell proliferation ability, and gene expression response to cytokine stimulation of hADSCs after thawing following cryopreservation in LR-3T-5D with 10% PG were comparable to those before cryopreservation and in LR-3T-5D with 10% Me2SO.

Furthermore, we optimized the concentration of PG for cryopreservation of hADSCs. Interestingly, the total cell counts at 5 days and 7 days after subculture of the 2.5% and 5% PG groups were significantly higher than those of the 10% PG group. However, such a response to concentration was not observed for Me2SO. When we chose 4% as a representative concentration for 2.5 to 5%, the total cell counts at 5 days and 7 days after subculture of the 4% PG group were comparable to those before freezing and significantly higher than those of the 4% Me2SO group. This suggests that the morphology of hADSCs was changed and cell proliferation was mechanically suppressed by 4% Me2SO, but not by 4% PG. Therefore, our data suggest that the optimal concentration of PG to maintain the cell characteristics of hADSCs frozen with LR-3T-5D is 2.5% to 5%.

There are several commercial Me2SO-free cryoprotectants [Awan et al. 2020], but their exact compositions have not been disclosed. The advantage of our solutions is that their exact compositions are disclosed so that users may account for their safety. Moreover, all components of our solutions can be prepared from raw materials that are used in various approved drugs. We also confirmed that the optimal concentration of PG to keep the viability of freeze-thawed CD4 positive T cells and CD8 positive T cells was 3-5 percent [unpublished data]. It suggested that LR-3T-5D containing 3% to 5% PG is possibly effective for other cells.

4.5 Conclusion

The hADSCs cryopreserved in LR-3T-5D containing 2.5% to 5% PG were found to maintain their same performance from before freezing. Our results suggest that cryopreservation with PG-containing LR-3T-5D may maintain the quality of cells for cell therapy. For clinical applications, further in vivo studies on efficacy and safety in combination with each therapeutic cell type are necessary.

4.6 Chapter summary

We evaluated a dimethyl sulfoxide (Me2SO)-free cryopreservation solution to freeze human adipose-derived mesenchymal stromal cells (hADSCs). In the first experiment, we compared the combined effects of 3% trehalose (3T) and 5% dextran (5D) in lactated Ringer's solution (LR) as a cryopreservation base solution containing 10% propylene glycol (PG). The cell viability of hADSCs immediately after thawing was significantly higher ($p < 0.05$) in LR supplemented with 3T (LR-3T) and with 3T and 5D (LR-3T-5D) than in LR. In the second experiment, we compared the cell characteristics of hADSCs freeze-thawed in LR-3T-5D containing either 10% Me2SO or 10% PG. The cell viability, annexin V-positive ratio, colony-forming capacity, cell proliferation, cell surface antigen positivity, adipogenic differentiation, osteogenic differentiation, and genetic response to cytokine stimulation of hADSCs immediately after thawing were similar between the LR-3T-5D containing 10% Me2SO and 10% PG. In the third experiment, we examined various concentrations of PG on the cell proliferative capacity of freeze-thawed hADSCs. The cell proliferative capacity of hADSCs frozen with LR-3T-5D containing 2.5% to 5% PG was significantly higher ($p < 0.05$) than LR-3T-5D containing 10% PG. Furthermore, the cell proliferative capacity of hADSCs frozen with LR-3T-5D containing 4% PG was similar to that of fresh hADSCs. These results indicate that the combination of 3T-5D in an LR solution as a basic solution is effective for post-thaw cell viability, and that the optimal concentration of PG to maintain the cell characteristics of hADSCs frozen with LR-3T-5D is 2.5% to 5%, which is promising for cell therapy applications.

Chapter 5. Overall conclusion

Stem cell transplantation therapy is promising for various refractory diseases [Hoang et al. 2021]. Mesenchymal stromal cells (MSCs) are attractive because of their immunosuppressive effect, multi-potency in differentiation, and remodeling effect on extracellular matrices. In addition, MSCs do not express human leucocyte antigen (HLA) Class II, which allows allogeneic administration without donor-recipient matching [Kelly and Rasko 2021]. MSCs are easily accessible from various sources. Therefore, many non-clinical and clinical studies of MSCs have been conducted to research and develop cellular medicines.

Maintaining cell viability and function during preservation and transportation for cell-based therapy is essential. Preserving cells allows for safe transportation over distances and improves quality control testing in clinical and research applications [Heydarzadeh et al. 2022]. Storage methods for the product should be selected and optimized according to the mechanism of action and the route of administration of the product.

For intravascular transplantation, in many cases, stem cells are suspended in an electrolyte solution, such as normal saline or lactated Ringer's solution. However, these solutions are not necessarily ideal for maintaining cell viability during storage and preventing the sedimentation of cells during infusion. Therefore we have designed novel preservation solutions that apply to cold storage, ambient temperature storage, and infusion of MSCs. Our first study aimed to optimize the compositions of these preservation solutions, which could affect the efficacy and safety of stem cell therapy using human adipose tissue-derived mesenchymal stromal cells (hADSCs). We tested various components of cell preservation solutions to determine the optimal combination of components for maintaining hADSCs.

Cellular volume homeostasis and ion homeostasis are essential for maintaining cell viability [Lang et al. 2006, Romero 2004, Dubyak 2004, Lang et al. 2007]. Osmolality and the electrolyte composition of extracellular solutions affect homeostasis [Romero 2004, Strange et al. 2004]. Thus, we examined the cytoprotective effects of Ringer's solutions and Ringer's solutions containing an alkalizing compound compared to normal saline (Table 2). These are generally used as infusion solutions in clinical to compensate for extracellular fluid loss and thus have much safety information. Our data showed that cell viability was significantly higher in the various Ringer's solutions than in normal saline ($p < 0.001$ for all of the Ringer's solutions). Normal saline is isotonic but contains only sodium and chloride ions. However, various Ringer's solutions

contain potassium, calcium, sodium, and chloride ions. They are more similar to the extracellular fluid in the human body. The ions in Ringer's solutions prevent cell death and account for the difference between normal saline and Ringer's solutions in maintaining cell viability. We found no significant differences among the various Ringer's solutions. However, cell viability was the highest in lactated Ringer's solution among the solutions tested. Therefore, we chose the composition of electrolytes in lactated Ringer's solution for our cell preservation solution.

We focused on trehalose and optimized the concentration to improve viability after storage because of its protein and membrane stabilizer effect and low toxicity. Cell viability after 24-hour storage at 5°C was significantly higher in the lactated Ringer's solutions containing more than 3% trehalose than in the other lactated Ringer's solutions ($p < 0.001$; Table 3). So we choose 3% as the concentration of trehalose.

Cells precipitate onto the bottom of infusion bags during storage and infusion; this may change the cell concentration and cause infusion toxicity, such as a cell embolism in the lung. In this study, we used low molecular weight dextran 40 to prevent cell sedimentation. Dextran 40 has been clinically used as a plasma expander to increase colloidal osmotic pressure in blood [Boldt 2005]. We hypothesized that the viscosity and specific gravity of dextran 40 would prevent cells from sedimentation in our proposed solution. We evaluated hADSCs in the supernatant after 1 h of settling to optimize the concentration of dextran 40 in the lactated Ringer's solution containing 3% trehalose. The percentage of cells in the supernatant reached a plateau at 5% dextran 40 (Figure 6). So we choose 5% as the concentration of dextran 40.

We concluded that the optimal composition for the wash and preservation solution was lactated Ringer's solution containing 3% trehalose (LR-3T), and the optimal composition for the suspension and preservation solution was lactated Ringer's solution containing 3% trehalose and 5% dextran 40 (LR-3T-5D; Table 6). We evaluated the time course (0, 6, 24, and 48 h) of cell viability during 96 h of preservation in LR-3T, LR-3T-5D, and lactated Ringer's solution at 5°C and 25°C (Figure 8). The mean values for cell viability were significantly higher in LR-3T and LR-3T-5D than in lactated Ringer's solution at all time points after 6 h of storage at 5°C. The mean values for cell viability in LR-3T-5D were higher than 90%, even at 48 h after storage. The mean values for cell viability were significantly higher in LR-3T-5D than in lactated Ringer's solution at all time points after 6 h of storage at 25°C. The mean value for cell viability was significantly higher in LR-3T than in lactated Ringer's solution at 6 h. The mean values for cell viability tended to be higher in LR-3T than in lactated Ringer's solution at 48 h ($p = 0.0569$) and 96 h ($p = 0.0979$). The mean values for cell viability were higher than

90% in LR-3T and LR-3T-5D for up to 48 h.

The International Society for Cellular Therapy defined MSCs as a population of cells that is plastic-adherent when maintained in standard culture conditions expresses CD73, CD90, and CD105 in the absence of CD34, CD45, CD14 or CD11b, CD79α or CD19, and HLA-DR surface molecules, differentiate to osteoblasts, adipocytes, and chondroblasts in vitro [Dominici et al. 2006]. So, we evaluated the colony formation unit (Table 10), which reflects plastic-adherent, cell surface makers (Table 8, Table 9), adipogenesis (Figure 12), and osteogenesis (Figure 13) differentiation ability of hADSCs before and after storage. The cells were negative for CD14, CD34, CD45, and HLA-DR before preservation and 24 h after preservation at 5°C or 25°C in LR-3T and LR-3T-5D. The cells were positive for CD44, CD73, CD90, and CD105 before preservation and 24 h after storage at 5°C or 25°C in LR-3T and LR-3T-5D (Table 8, Table 9). In the case of LR-3T at 25°C, colony-forming units remained unchanged for at least 24 h after preservation. Although the mean number of colony-forming units at 24 h after preservation at 5°C in LR-3T tended to be lower when compared to that before preservation ($p = 0.0818$), with the other preservation conditions, no significant difference was found in the values before and after preservation. Adipogenic differentiation and osteogenic differentiation were induced using hADSCs 24 h after storage at 5°C or 25°C. There was no noticeable difference in the differentiation status before and after preservation for any preservation conditions.

In summary, LR-3T and LR-3T-5D preserved the cell viability and characteristics of hADSCs during storage at 5°C and 25°C for at least 24 h.

As mentioned above, LR-3T-5D stabilized the percentage of cells in the supernatant for at least 1 h. We confirmed the concept of preventing cell sedimentation during infusion with LR-3T-5D using the worst-case scenario model of infusion, in which the infusion was started without mixing after 1 h of settling, and mixing was performed at the halfway point of infusion. The cell concentrations of fractions in LR-3T-5D were stable throughout the infusion, even in the worst-case scenario. These results suggest that LR-3T and LR-3T-5D can help maintain the quality of stem cells for therapy during preservation and infusion.

Furthermore, we confirmed the usefulness of LR-3T-5D as a vehicle solution for cryopreservation with dimethyl sulfoxide (Me2SO). If LR-3T-5D is also effective as a vehicle solution for cryopreservation, it can provide a seamless cell preservation method from washing to freezing. LR-3T-5D containing 10% dimethyl sulfoxide (Me2SO) preserved the cell viability (Table 12), colony-forming capacity (Table 12), adipogenic and osteogenic differentiation (Figure 18), and cell surface markers (Figure 19) of

hADSCs during storage in liquid nitrogen and a − 80°C freezer for at least 7 months. These results suggest that LR-3T-5D containing 10% Me2SO can help maintain the quality of stem cells for therapy during cryopreservation.

Cell cryopreservation solutions generally contain about 10% Me2SO. However, side effects associated with the transplantation of stem cells with Me2SO have been reported, including nausea, vomiting, cardiac arrhythmias, neurological symptoms, respiratory arrest, renal/hepatic dysfunction, and allergies [Santos et al. 2003; Windrum et al. 2005; Cox et al. 2012; Shu et al. 2014; Awan et al. 2020]. In addition, a portion of the administered Me2SO is reduced to dimethyl sulfide in the body, which is then secreted through the skin and exhaled air, causing a foul, garlic-like odor [Santos et al. 2003; Shu et al. 2014; Awan et al. 2020]. Therefore, there is a need for a cryopreservation solution that can reduce the side effects of Me2SO. We confirmed the effects of different Me2SO concentrations and cell-washing procedures after thawing cells using LR-3T-5D to reduce the amount of Me2SO administered.

Cell viability was significantly lower in LR-3T-5D with 0% and 0.5% Me2SO than in LR-3T-5D with 10% Me2SO (Figure 21). The cell proliferation curve was significantly lower in LR-3T-5D with 0%, 0.5%, and 1% Me2SO than in LR-3T-5D with 10% Me2SO. The cell proliferation curve in LR-3T-5D with 5% Me2SO was comparable to that in LR-3T-5D with 10% Me2SO.

There was no significant difference in the cell viability and the viable cell recovery ratio between immediately after thawing without replacing the solution and immediately after replacement by LR-3T or LR-3T-5D (Figure 24). Therefore, washing and replacement had little influence on hADSCs. The cell viability and viable cell recovery ratio were gradually but slightly decreased after replacement, and the annexin V positivity was gradually but slightly increased after replacement. Therefore, washing and replacement might be advisable to avoid the toxicity of Me2SO without significant changes in cell characteristics. LR-3T-5D was superior to LR-3T as a preservation solution after thawing from the viability and annexin V-positive ratio. Although the cell viabilities at 6 h and 24 h after replacement with LR-3T-5D were significantly decreased compared to that immediately after replacement, they were still at high levels, 91.0 ± 0.3% and 88.3 ± 1.1%, respectively.

In summary, to reduce the amount of Me2SO exposure, reducing the concentration of Me2SO to 5%, washing cells, and replacing the solution with LR-3T and LR-3T-5D after thawing are feasible steps that keep the cryopreservation efficacy the same as with 10% Me2SO.

Me2SO stimulates differentiation in some types of cells. For example, mouse bone

marrow mesenchymal stem cells are differentiated into cardiomyocytes by Me2SO [Young et al. 2004]. Cryopreservation of human embryo-stem (ES) cells in Me2SO-containing cryopreservation solution decreases the expression of Oct-4, an undifferentiated marker [Katkov et al. 2006], and Me2SO affects the epigenetic profile of mouse embryoid bodies [Iwatani et al. 2006]. The human promyelocytic leukemia cell line HL-60 is differentiated into granulocytes by Me2SO [Jiang et al. 2006]. These effects are unfavorable for maintaining cell characteristics during cryopreservation. Therefore, given concerns over maintaining stemness in addition to safety, a Me2SO-free cryopreservation solution is desired.

We have compared the effects of the cell membrane penetrating cryoprotectants propylene glycol (PG), ethylene glycol, and glycerol with those of Me2SO (Figure 23). 10% glycerol was inferior to 10% Me2SO in cell viability and annexin V-positive ratio. There was no significant difference between 10% ethylene glycol and 10% Me2SO. Ethylene glycol itself has low toxicity but is in vivo metabolized to cell toxins that can be harmful [Leth et al. 2005]. Therefore, ethylene glycol is not appropriate as a substitute for Me2SO in cell therapy. 10% propylene glycol was comparable in cell viability, annexin V-positive ratio, and proliferation curve to 10% Me2SO in our results.

Furthermore, we optimized the concentration of PG with LR-3T-5D as a cryopreservation solution for hADSCs (Figure 29). The total cell counts at 5 and 7 days after seeding the LR-3T-5D containing 2.5% or 5% PG groups were significantly higher ($p < 0.05$) than that of the LR-3T-5D containing 10% PG group. The total cell count of LR-3T-5D containing 4% PG was comparable to that before freezing, while that of LR-3T-5D containing 4% Me2SO was significantly lower ($p < 0.05$) than before freezing at 3 to 7 days after seeding. So we conclude that the hADSCs cryopreserved in LR-3T-5D containing 2.5–5% PG maintained the same performance as before freezing.

We proposed solution compositions for cold storage, ambient temperature storage, and cryopreservation with/without Me2SO of hADSCs. Cell preservation methods should be selected according to the expected effects, route of administration, and other considerations in cell therapy. Further in vivo studies on efficacy and safety in combination with each therapeutic cell type are necessary for clinical applications.

Acknowledgements

I would like to thank Dr. Takeshige Otoi, Director and Professor of Bio-innovation Research Center, Tokushima University, who gave me appropriate advice and careful guidance throughout the preparation of this dissertation. I would like to express my gratitude to Professor Yoshihiro Uto of the Graduate School of Technology, Industrial and Social Sciences, Tokushima University and Professor Taro Mito of the Graduate School for their guidance as vice-inspectors.

As the special advisor to the Otsuka Pharmaceutical Factory, Inc., we received guidance from Professor Eiji Kobayashi at the Jikei University School of Medicine. I would like to express my sincere gratitude for this.

I have been motivated and advised by Dr. Kinji Hashimoto, Mr. Shigeo Tanei, and Dr. Masako Doi in this project at Otsuka Pharmaceutical Factory, Inc. I am grateful for these contributions.

I would like to express my sincere gratitude to all colleagues involved in this research, including Dr. Masahiro Nishimura, Dr. Tamaki Wada, Dr. Natsuki Komori, Ms. Chikage Shirakawa, and Mr. Taichi Takenawa at the Regenerative Medicine Research Laboratory, Otsuka Pharmaceutical Factory, Inc. I would also like to thank Mr. Shinya Kaneda and Dr. Osamu Sawamoto as a supervisor for supporting and approving submitting the primary papers.

I would like to express my deepest gratitude to Dr. Shinichi Matsumoto, Special Advisor to the Otsuka Pharmaceutical Factory, Inc., for providing appropriate guidance, generous support, and encouragement from my external assignment to the Baylor Research Institute to the present.

Finally, the preparation of this paper could only be done with the support of my family, and I appreciate it from my heart.

April, 2023
Yasutaka Fujita

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