



Original Article

A pair of cell preservation solutions for therapy with human adipose tissue-derived mesenchymal stromal cells

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ABSTRACT

Introduction: 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.

Methods: 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.

Results: 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.

Discussion: 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. However, further *in vivo* research is needed on the efficacy and safety of the solutions in different therapeutic cell lines before clinical use.

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1. Introduction

Stem cell transplantation is a promising therapy for various diseases, such as cardiovascular disorders [1,2], autoimmune diseases [3], osteoarthritis [4], liver disorders [5–8], and graft-versus-host disease [9,10]. Among stem cells, mesenchymal stromal cells (MSCs) are attractive because of their multi-potency in differentiation, immunosuppressive effect, 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

Abbreviations: hADSCs, human adipose-derived mesenchymal stromal cells; LR-3T, lactated Ringer's solution with 3% trehalose; LR-3T-5D, lactated Ringer's solution with 3% trehalose and 5% dextran 40; MSCs, mesenchymal stromal cells.

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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 [11–14]. Osmolality and the electrolyte composition of extracellular solutions affect homeostasis [12,15]. 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 non-reducing disaccharide that is known to be a protein and membrane stabilizer and cryoprotective agent for different kinds of cells [16]. 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 [17]. 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. Materials and methods

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. 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).

2.3. Preparation of hADSCs

Human ADSCs (Female, 51Y or 38Y, PT5006; Lonza Walkersville, 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 subsection 3.1, subsection 3.2 and subsection 3.3 of the Results, while those described in subsection 3.4-subsection 3.5 were 38Y. Human ADSCs were seeded in a 75-cm² 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% CO₂. All of the medium was 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.

2.4. 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.

$$\text{Cell viability [\%]} = \frac{(\text{total number of cells} - \text{number of dead cells})}{(\text{total number of cells})} \times 100$$

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 Supplement 1.

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

$$\% \text{ cells in the supernatant} = \frac{(\text{number of cells in the supernatant of the sample})}{(\text{mean baseline value})} \times 100$$

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).

2.7. Cell surface markers

To examine the surface immunophenotypes of the cells, 2×10^5 cells in 20 μ L of staining buffer with fetal bovine serum (BD Biosciences, San Jose, CA, USA) were incubated for 60–120 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).

2.8. Colony-forming capacity

After 6 and 24 h of storage at 5 °C and 25 °C in LR-3T or LR-3T-5D, 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-cm² culture dish (15 cells/cm²). 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.

2.9. Adipogenic and osteogenic differentiation ability

Adipogenic differentiation was induced according to the Poietics™ human ADSCs adipogenesis protocol (Lonza Walkersville, 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.).

2.10. 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 SurfFlash™ 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 of the 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 Supplement 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.

2.11. Statistical methods

Results are presented as the mean \pm standard deviation (SD). Dunnett's multiple comparison test and the Student's *t*-test were used. The dose–response relationship of the percentage of cells in the supernatant was evaluated with the maximum contrast method [18]. 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).

3. Results

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 (Supplement 3).

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 1).

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 was less than 30%, and the percentage in the lactated Ringer's solution containing 3% trehalose was less than 40% (Fig. 1). Increasing the concentration of dextran 40 in lactated Ringer's solution containing 3% trehalose increased the percentage of cells in the supernatant (Fig. 1). 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 (Supplement 4). The maximum sum-of-squared contrast weights was indicated by the 2-start 4-end pattern (Supplement 5). 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 \pm 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 $83.5\% \pm 7.6\%$ (Fig. 1).

Cell viability was significantly lower in the lactated Ringer's solution containing 3% trehalose and 10% dextran 40 than in the

Table 1

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

Values are means \pm SD (n = 5–6). ***p < 0.001 vs. lactated Ringer's solution with Dunnett's test. S: normal saline, LR: lactated Ringer's solution, T: trehalose.

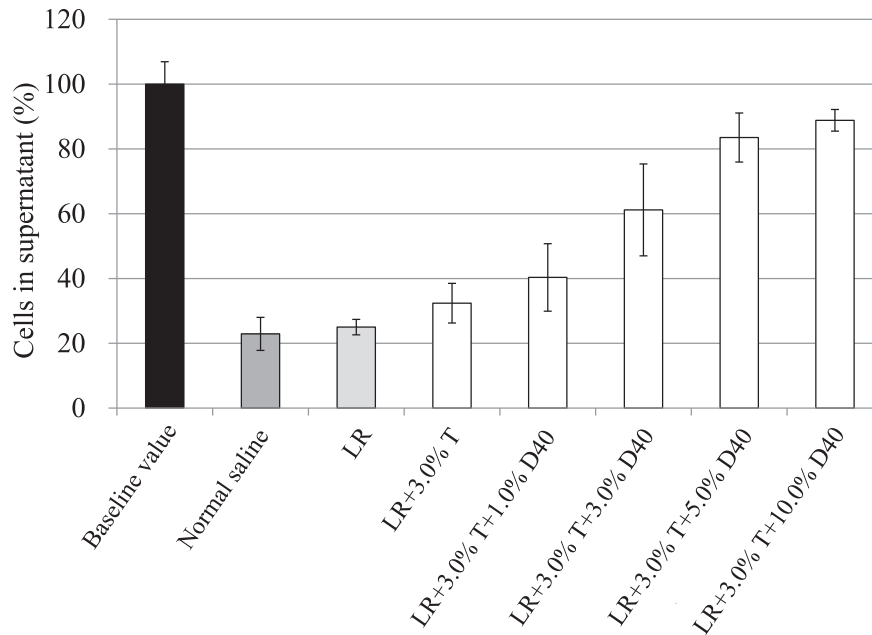


Fig. 1. The percentage of cells in the supernatant after 1 h of settling in various cell preservation solutions. Each point and vertical bar indicates the mean and standard deviation (SD; n = 6). The percentages are the numbers of hADSCs in 3 mL of supernatant of various solutions after 1 h of settling 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.

lactated Ringer's solution containing only 3% trehalose ($p < 0.001$; Table 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; Supplement 6). 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.

Fig. 2A 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 $94.9\% \pm 2.4\%$ and $97.6\% \pm 2.4\%$, respectively. The mean values for cell viability in LR-3T-5D were higher than 90% even at 48 h after storage. Fig. 2B 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.

Table 3 and Supplement 7 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.

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 (Tables 4 and 5, and Supplement 8).

Table 6 and Supplement 9 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

Table 2

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 hours 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***

Values are means ± SD (n = 6). *** $p < 0.001$ vs. LR+3.0% T with Dunnett's test. S: normal saline, LR: lactated Ringer's solution, T: trehalose, D40: dextran 40.

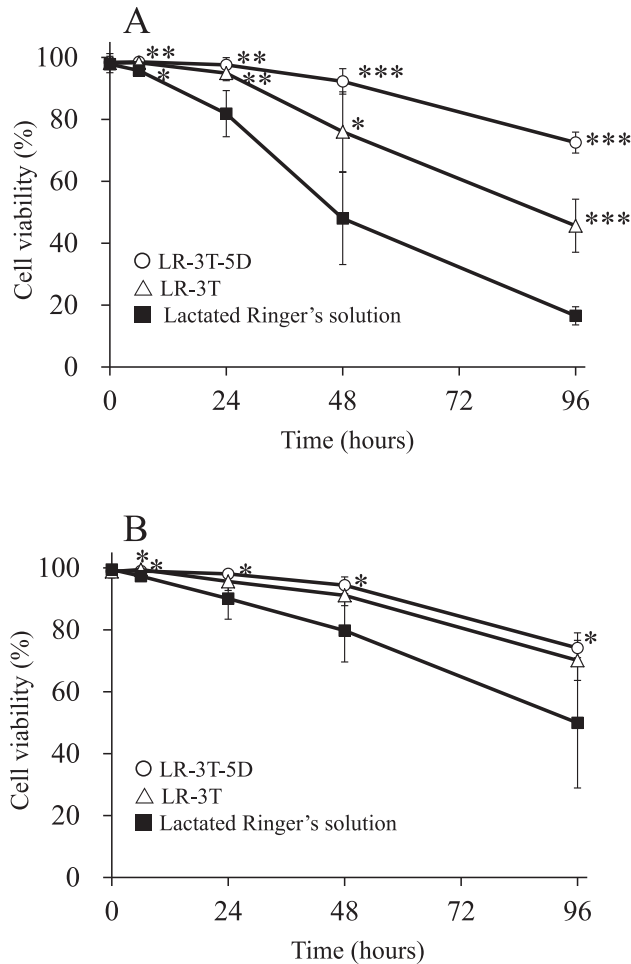


Fig. 2. Changes in the cell viability of hADSCs in test solutions after storage at 5 °C (A) and 25 °C (B). Each point and vertical bar indicates the mean \pm SD ($n = 4$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. lactated Ringer's solution with Dunnett's test. LR-3T: lactated Ringer's solution with 3% trehalose, LR-3T-5D: lactated Ringer's solution with 3% trehalose and 5% dextran 40.

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.

Adipogenic differentiation and osteogenic differentiation were induced using hADSCs 24 h after storage at 5 °C or 25 °C (Supplements 10 and 11). 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.

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 (Fig. 3A). The cell recovery rate was significantly higher with LR-3T-5D than with normal saline ($77.9\% \pm 5.0\%$ vs. $67.6\% \pm 3.6\%$, respectively; Fig. 3B).

4. Discussion

The osmolality and electrolyte composition of extracellular solutions affect cell viability via cellular volume homeostasis and ion homeostasis [12–15]. 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 [16]. Trehalose is also a component of ET-Kyoto solution, which is used for lung and pancreas islet transplantation as an organ-preservation solution [19,20]. 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 [21]. 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 [22]. 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

Table 3

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 \pm 3.1	12.0 \pm 1.8	62.1 \pm 13.0
LR-3T	11.4 \pm 4.6	11.0 \pm 2.2	13.5 \pm 1.3
LR-3T-5D	12.5 \pm 4.9	9.0 \pm 0.6	12.9 \pm 1.5

Values are means \pm 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.

Table 4
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 5
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.

Table 6
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
	25 °C		21.5 ± 2.1
LR-3T-5D	5 °C	21.3 ± 1.9	21.9 ± 3.2
	25 °C		19.1 ± 1.0

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.

some reports [16,23]. 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 [23]. 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 [24]. 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 (Supplement 2). 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 (Figs. 3A) and 25 °C (Fig. 3B) 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 [25]. 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 [26]. CD14 is prominently expressed on monocytes and macrophages; CD34 marks primitive hematopoietic progenitors and endothelial cells; CD-45 is a pan-leucocyte marker; and HLA-DR marks HLA class II cell surface receptors. In contrast, CD73, CD90, and CD105 are known as positive markers of MSCs [26,27]. 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.

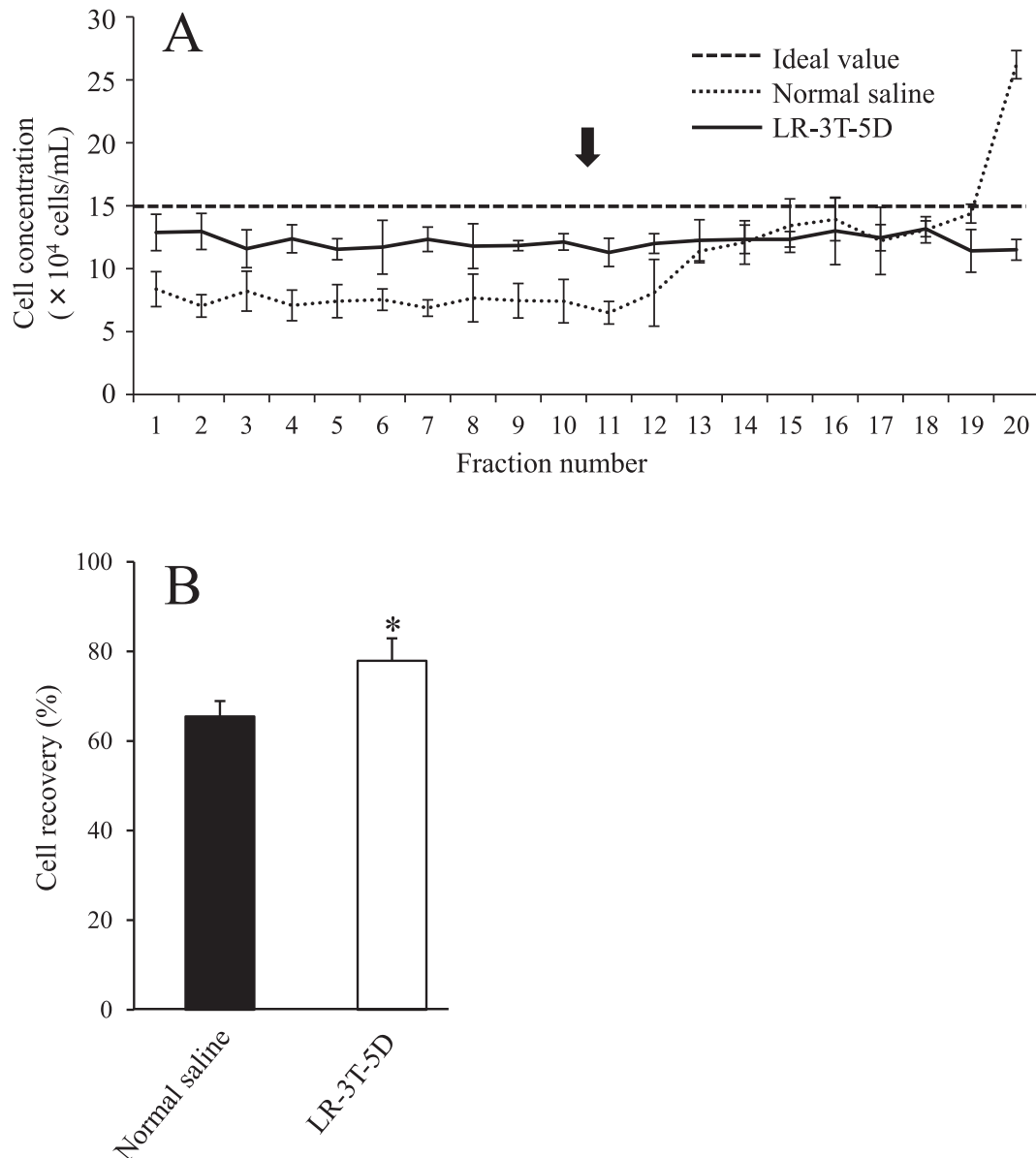


Fig. 3. The concentrations of hADSCs suspended in LR-3T-5D or normal saline in the infusion line from blood bags (A). The values indicate the mean \pm SD ($n = 3$) of the cell concentration in each fraction. The dashed line indicates the ideal value of the cell concentration in each fraction. The arrow indicates the time point at which the bags were mixed. Comparison of percent recovery (B). The values indicate the mean \pm 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.

One of the criteria for defining MSCs is multipotent differentiation potential [26]. 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 [28]. 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.

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.

Disclosure of interests

The experimental work presented in this article was performed while all authors were working at Otsuka Pharmaceutical Factory, Inc.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.reth.2019.10.004>.

References

- [1] Hou L, Kim JJ, Woo YJ, Huang NF. Stem cell-based therapies to promote angiogenesis in ischemic cardiovascular disease. *Am J Physiol Heart Circ Physiol* 2016 Feb 15;310(4):H455–65.
- [2] Terashvili M, Bosnjak ZJ. Stem cell therapies in cardiovascular disease. *J Cardiothorac Vasc Anesth* 2019 Jan;33(1):209–22.
- [3] Munir H, McGettrick HM. Mesenchymal stem cell therapy for autoimmune disease: risks and rewards. *Stem Cells Dev* 2015 Sep 15;24(18):2091–100.
- [4] Wyles CC, Houdek MT, Behfar A, Sierra RJ. Mesenchymal stem cell therapy for osteoarthritis: current perspectives. *Stem Cells Cloning* 2015 Aug 28;8:117–24.
- [5] Kadyk LC, Collins LR, Littman NJ, Millan MT. Proceedings: moving toward cell-based therapies for liver disease. *Stem Cells Transl Med* 2015 Mar;4(3):207–10.
- [6] Forbes SJ, Gupta S, Dhawan A. Cell therapy for liver disease: from liver transplantation to cell factory. *J Hepatol* 2015 Apr;62(1 Suppl):S157–69.
- [7] Tsolaki E, Yannaki E. Stem cell-based regenerative opportunities for the liver: state of the art and beyond. *World J Gastroenterol* 2015 Nov 21;21(43):12334–50.
- [8] Kholodenko IV, Yarygin KN. Cellular mechanisms of liver regeneration and cell-based therapies of liver diseases. *BioMed Res Int* 2017;2017. <https://doi.org/10.1155/2017/8910821>. 8910821.
- [9] Chen X, Wang C, Yin J, Xu J, Wei J, Zhang Y. Efficacy of mesenchymal stem cell therapy for steroid-refractory acute graft-versus-host disease following allogeneic hematopoietic stem cell transplantation: a systematic review and meta-analysis. *PLoS One* 2015 Aug 31;10(8). <https://doi.org/10.1371/journal.pone.0136991>. e0136991.
- [10] Trounson A, McDonald C. Stem cell therapies in clinical trials: progress and challenges. *Cell Stem Cell* 2015 Jul 2;17(1):11–22.
- [11] Lang F, Shumilina E, Ritter M, Gulbins E, Vereninov A, Huber SM. Ion channels and cell volume in regulation of cell proliferation and apoptotic cell death. *Contrib Nephrol* 2006;152:142–60.
- [12] Romero MF. In the beginning, there was the cell: cellular homeostasis. *Adv Physiol Educ* 2004 Dec;28(1–4):135–8. Review.
- [13] Dubyak GR. Ion homeostasis, channels, and transporters: an update on cellular mechanisms. *Adv Physiol Educ* 2004 Dec;28(1–4):143–54.
- [14] Lang F, Föller M, Lang K, Lang P, Ritter M, Vereninov A, et al. Cell volume regulatory ion channels in cell proliferation and cell death. *Methods Enzymol* 2007;428:209–25.
- [15] Strange K. Cellular volume homeostasis. *Adv Physiol Educ* 2004 Dec;28(1–4):155–9.
- [16] Jain NK, Roy I. Effect of trehalose on protein structure. *Protein Sci* 2009 Jan;18(1):24–36.
- [17] Boldt J, Suttner S. Plasma substitutes. *Minerva Anesthesiol* 2005 Dec;71(12):741–58.
- [18] Yoshimura I, Wakana A, Hamada C. A performance comparison of maximum contrast methods to detect dose dependency. *Drug Inf J* 1997;31:423–32.
- [19] Chen F, Nakamura T, Wada H. Development of new organ preservation solutions in Kyoto University. *Yonsei Med J* 2004 Dec 31;45(6):1107–14.
- [20] Matsumoto S, Noguichi H, Shimoda M, Ikemoto T, Naziruddin B, Jackson A, et al. Seven consecutive successful clinical islet isolations with pancreatic ductal injection. *Cell Transplant* 2010;19(3):291–7.
- [21] Fukuse T, Hirata T, Nakamura T, Ueda M, Kawashima M, Hitomi S, et al. Role of saccharides on lung preservation. *Transplantation* 1999 Jul 15;68(1):110–7.
- [22] Hirata T, Fukuse T, Liu CJ, Muro K, Yokomise H, Yagi K, et al. Effects of trehalose in canine lung preservation. *Surgery* 1994 Jan;115(1):102–7.
- [23] Albertorio F, Chapa VA, Chen X, Diaz AJ, Cremer PS. The α,α -(1→1) linkage of trehalose is key to anhydrobiotic preservation. *J Am Chem Soc* 2007;129(34):10567–74.
- [24] JCR Pharmaceuticals Co., Ltd TEMCELL® HS Inj. Pharmaceuticals and medical device agency website [package insert]. 2017. <https://www.pmda.go.jp/files/000215849.pdf> Revised January. [Accessed 15 August 2019].
- [25] Rauen U, Kerkweg U, de Groot H. Iron-dependent vs. iron-independent cold-induced injury to cultured rat hepatocytes: a comparative study in physiological media and organ preservation solutions. *Cryobiology* 2007;54(1):77–86.
- [26] Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;8(4):315–7.
- [27] Di Battista JA, Shebaby W, Kizilay O, Hamade E, Abou Merhi R, Mebarek S, et al. Proliferation and differentiation of human adipose-derived mesenchymal stem cells (ASCs) into osteoblastic lineage are passage dependent. *Inflamm Res* 2014;63(11):907–17.
- [28] Zhang F, Ren H, Shao X, Zhuang C, Chen Y, Qi N. Preservation media, durations and cell concentrations of short-term storage affect key features of human adipose-derived mesenchymal stem cells for therapeutic application. *PeerJ* 2017 May 17;5. <https://doi.org/10.7717/peerj.3301>. e3301.