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Original Article

Ascorbic acid enhances the cold preservation period of human adipose tissue–derived mesenchymal stromal cells

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ABSTRACT

Introduction: We previously developed 3% trehalose-added lactated Ringer's solution (LR-3T) and 3% trehalose- and 5% dextran-40–added lactated Ringer's solution (LR-3T-5D), which can be used to preserve adipose-derived mesenchymal stem cells (hADSCs) for 24 h at 5 and 25 °C. However, it is necessary to further extend the storage duration of cells to expand transportation zones and ensure time for quality control testing of final cell products. Therefore, we attempted to prolong the preservation duration of hADSCs by adding supplements to LR-3T-5D. We focused on ascorbic acid as an antioxidant because it is widely clinically as a nutrient.
Methods: We added the antioxidant ascorbic acid to LR-3T-5D and evaluated the viability, colony formation rate, proliferative capacity, and surface markers of hADSCs before and after preservation at 5 °C.
Results: Analysis of the concentration of ascorbic acid added to LR-3T-5D indicated that 1000 mg/L was the optimal concentration for maintaining the viability of hADSCs after 72 h of cold preservation. No changes were observed in the expression of specific cell surface markers or in the potential of hADSCs to differentiate into adipocytes, osteoblasts, or chondrocytes before and after cold preservation.
Discussion: These results suggest that cold preservation of hADSCs in LR-3T-5D supplemented with ascorbic acid helps maintain the quality of cells for use in cell therapy.

1. Introduction

Ensuring that the quality of therapeutic cells remains stable during the post-production process until the cells are administered to patients is a critical issue [1]. In terms of the rationale for setting the preservation period, it is assumed that 48–72 h is the optimal period for quality control from the cell processing center to on-site clinical use of the cells [2–4].

Cryopreservation is used to preserve cells for long periods of time. However, there are cases in which cryopreservation is inappropriate, depending on the cell state to be used, such as when cells must be active immediately after storage [5–7]. Cells can generally be stored at room temperature (20 °C or above) for a few hours to a day, after which the cells can then be considered active. Therefore, room temperature storage requires the provision of nutrients and oxygen similar to cell culture. In contrast, cold preservation at 4–10 °C is intended to be performed for one to several days, and the time required for these cells to become active after preservation can be shorter than that of cryopreserved cells. Generally, when cold-preserving mammalian cells, cold stress–induced cytotoxicity is problematic [8,9]. Therefore, the reported duration of cold preservation in which mesenchymal stem cell (MSC) quality can be maintained is approximately 24 h [10,11].

The maintenance of cells at low temperatures is inextricably linked to a decrease in cellular activity, and long-term low-temperature preservation reportedly results in extensive cellular damage [12]. As cold temperatures are associated with reduced metabolic and enzymatic activity, the notion that cold temperature induces free radical production appears less tenable. Low-

Abbreviations: hADSC, human adipose-derived mesenchymal stromal cell; AsA, ascorbic acid.

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temperature stress results in the accumulation of intracellular reactive oxygen species (ROS) that cause mitochondrial damage, which in turn leads to DNA damage and ultimately cell death [13]. Cold stress–induced cell death is known as ferroptosis, an iron-dependent process characterized by the accumulation of lipid peroxides. Ferroptosis is genetically and biochemically distinct from other forms of regulated cell death, such as apoptosis [14]. Therefore, we hypothesized that the presence of an antioxidant would suppress cell death during cold preservation. We focused on ascorbic acid ([AsA] also known as vitamin C), a typical antioxidant, because it is a component of nutritional infusions and has a long history of clinical use.

We previously developed a cell suspension and preservation solution, 3% trehalose and 5% dextran-40–added lactated Ringer's solution (LR-3T-5D), which maintains the viability and colony formation rate of human adipose-derived MSCs (hADSCs) at 5 °C or 25 °C for 24 h [11]. In this study, we investigated the effect of adding the antioxidant AsA to LR-3T-5D on hADSCs stored at low temperature, with the aim of reducing cold preservation–associated cell damage.

2. Materials and methods

2.1. Study design

The study was approved by the ethics committee of Otsuka Pharmaceutical Factory, Inc. We performed two experiments. First, the effectiveness of AsA (1000 mg/L) as a cold-preservation vehicle was evaluated by monitoring several cell characteristics (viability, colony-forming capacity, cell surface markers, differentiation ability, and proliferation ability) in comparison with no AsA. The second experiment compared the formulation of cold preservation solution consisting of 1000 mg/mL AsA in lactated Ringer's solution containing both 3% trehalose and 5% dextran-40 with solution consisting of 1000 mg/mL AsA in lactated Ringer's solution containing 3% trehalose.

2.2. Components of the solutions

LR solution (Lactec® injection), Cellstor-W (LR-3T; lactate containing 3% trehalose), and Cellstor-S (LR-3T-5D; lactate containing 3% trehalose and 5% dextran-40) were supplied by Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan). AsA (500 mg) was purchased from Sawai Pharmaceutical Co., Ltd (Osaka, Japan).

2.3. Preparation of hADSCs

Human ADSCs (female, 32Y or 44Y, PT5006; Lonza Walkersville, Inc., Walkersville, MD, USA) were seeded in a 75-cm^2 flask in medium prepared from a medium kit (ADSC- BulletKit™, Lonza Walkersville, Inc.) and maintained at 37 °C in a humidified atmosphere with 5% CO_2. The medium was changed every 3 or 4 days. Cells were passaged at approximately 90% confluence, 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, hADSCs were suspended in various solutions at either 7.5 × 10^5 cells/1.5 mL in PROTEOSAVE™ SS 1.5-mL microtubes (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) or 5.0 × 10^5 cells/1.0 mL in STEMFULL™ tubes (Sumitomo Bakelite Co., Ltd.). Suspended cells were stored at 5 °C in a refrigerator for 3 or 7 days. Cell viability was determined manually using a plastic cell counting plate (OneCell Counter, Bio Medical Science, Ltd., Tokyo, Japan) after trypan blue staining.

The percent cell viability and viable cell recovery ratio were calculated according to the following equations:

$$\text{Cell viability } [\%] = \frac{\text{Total cell count} - \text{Dead cell count}}{\text{Total cell count}} \times 100$$

$$\text{Viable cell recovery ratio } [\%] = \frac{\text{number of viable cells at each time point}}{\text{number of viable cells before preservation}} \times 100$$

2.5. Cell surface markers

To examine the surface immunophenotypes, 2 × 10^5 cells in 20 μL of stain 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, CD29, CD31, 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, Indianapolis, IN, USA).

2.6. Colony-forming unit capacity

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.

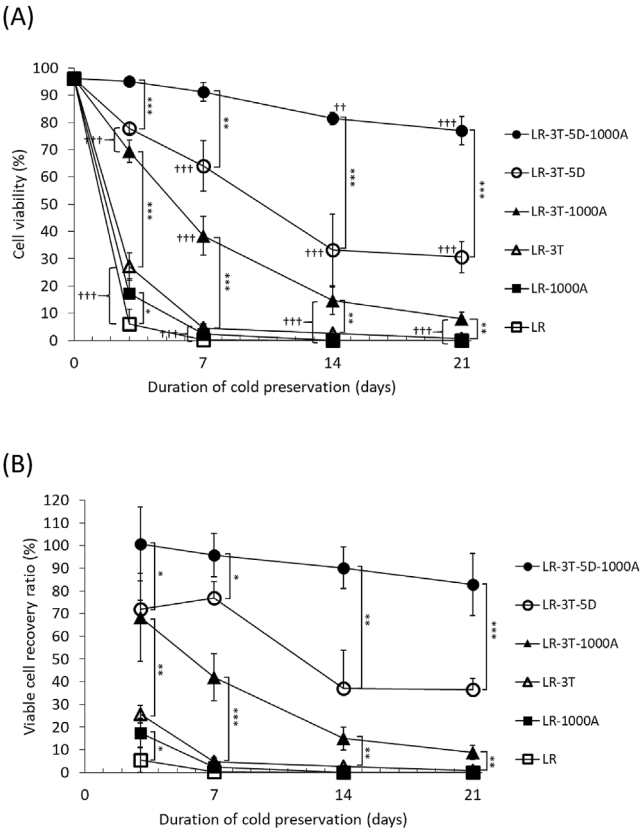

Fig. 1. Cell viability (A) and viable cell recovery ratio (B) of hADSC after preservation at 5 °C for up to 21 days in LR, LR-3T, and LR-3T-5D without and with 1000 mg/L AsA. Each point and vertical bar indicate the mean ± SD (n = 4). *p < 0.05, **p < 0.01, and ***p < 0.001 for comparison of absence and presence of AsA, as determined by Student's *t*-test. ††p < 0.01 and †††p < 0.001 versus before preservation, as determined by Dunnett's test. 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. 1000A, 1000 mg/L AsA.

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, the cells were washed with distilled water. Colonies consisting 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.

2.7. Adipogenic, osteogenic, and chondrogenic 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 manual (Lonza Walkersville, Inc.) and evaluated using a calcified nodule staining kit (AK21, Cosmo Bio Co., Ltd., Tokyo, Japan). Chondrogenic differentiation was induced according to the Poietics™ human ADSCs chondrogenesis protocol (Lonza Walkersville, Inc.) and evaluated using Alcian Blue staining of paraffin-embedded sections.

2.8. Cell growth assay using time-lapse microscopy

The IncuCyte® ZOOM Live Cell Analysis System (Sartorius Lab Holding GmbH, Gottingen, Germany) is an automated cell culture monitoring system (microscope + imaging controller) located within a CO_2 incubator. The system allows for the measurement of changes in cell confluence index in real time, reflecting changes in cell number. hADSCs were plated in 24-well plates (2.5×10^3 cells/well) and placed in the IncuCyte® ZOOM system. The culture medium was changed every 3 or 4 days. Time-lapse imaging of four fields of vision in each well was carried out for 10 or 11 days at a frequency of every 6 h. Curves reflecting the change in monolayer area over time were generated using the IncuCyte® ZOOM image-processing software package (Sartorius Lab Holding GmbH). Cells were re-plated after 156 h, and the degree of confluence was calculated from the ratio of the monolayer area to the entire area.

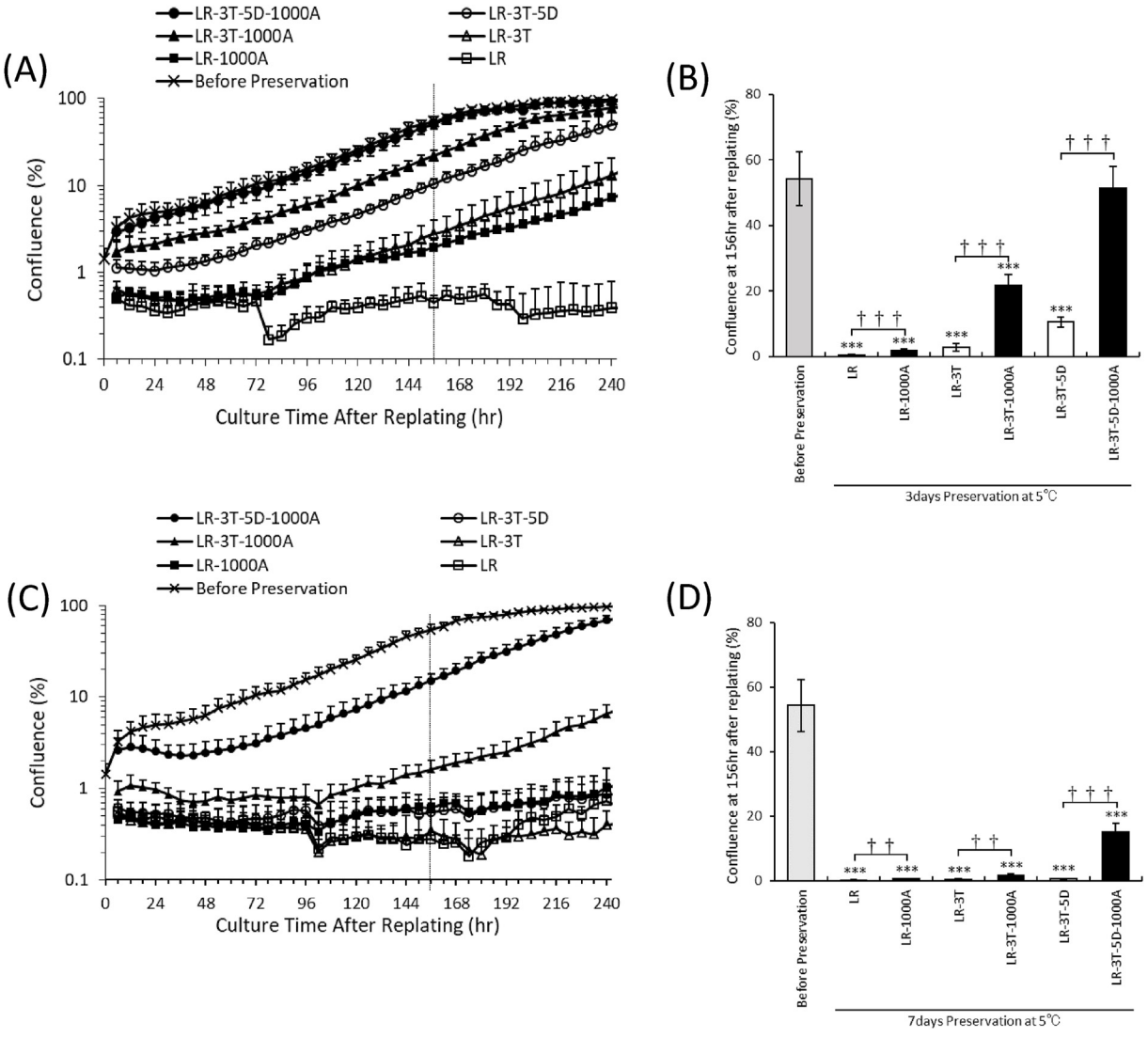

Fig. 2. Time course analysis of proliferation of hADSCs after preservation at 5 °C for either 3 days (A) or 7 days (C) in LR, LR-3T, and LR-3T-5D without and with 1000 mg/L AsA. Comparison of confluence at 156 h (B and D) after re-plating. Each point and vertical bar indicate the mean ± SD (n = 4). ***p < 0.001 versus before preservation, as determined by Dunnett's test. ††p < 0.01 and †††p < 0.001 for comparison between absence and presence of AsA, as determined by Student's *t*-test. 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. 1000A, 1000 mg/L AsA.

2.9. Statistical analyses

Results are presented as the mean ± standard deviation. Dunnett's multiple comparison test and Student's *t*-test were used to evaluate the significance of differences. Data were analyzed using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Effect of AsA on cell preservation performance

We tested the effectiveness of AsA as a cold-preservation additive for cells. hADSCs were preserved for 3–21 days at 5 °C in LR solution, LR solution plus 3% trehalose (LR-3T), and LT-3T solution plus 5% dextran-40 (LR-3T-5D) without or with 1000 mg/L AsA. The percent cell viability and viable cell recovery ratio were significantly increased for almost the entire duration of cold preservation with the addition of AsA to each solution (Fig. 1A and B). In addition, cell viability change compared to before preservation in LR-3T-5D with AsA (LR-3T-5D-1000A) was greater than that of cells in either LR-3T with AsA (LR-3T-1000A) or LR with AsA (LR-1000A) (Fig. 1A). Although viable cell recovery ratio results were similar to the cell viability results, addition of AsA had little positive effect on LR-based preservation (Fig. 1B).

In the next step, we investigated the changes in cell condition after cold preservation with and without AsA. Cold-preserved hADSCs in the same composition as shown in Fig. 2 were re-plated on culture plates and monitored using a time-lapse microscopy system to assess cell proliferation. The results were similar to both the percent cell viability and viable cell recovery ratio analyses shown in Fig. 2. Based on these data, we determined that LR-3T-5D with AsA added is more effective for preserving cells in good condition than either LR or LR-3T.

3.2. Determination of appropriate concentration of AsA with LR-3T-5D

To determine the optimal concentration of AsA in LR-3T-5D for cold preservation of hADSCs, cells were stored in the cold for 3 days in LR-3T-5D preservation solution with AsA added at concentrations ranging from 0 to 4000 mg/mL. A significant decrease in viability was observed for cells stored in LR-3T-5D containing 0–100 mg/L AsA. In addition, cell viability was significantly higher in LR-3T-5D containing ≥100 mg/L AsA compared with 0 mg/L AsA (Fig. 3A), and the highest viable cell recovery rate was observed with 100 mg/L AsA (Fig. 3B).

Preserved hADSCs were then re-plated to evaluate cell proliferation activity and colony-forming capacity. Better preservation results were obtained in solutions containing AsA at a concentration between 100 and 4000 mg/L compared with lower concentrations (Fig. 4). In addition, cold storage in solution containing AsA at a concentration of >2000 mg/L resulted in decreased colony-forming capacity (Fig. 4C, Supplemental Figure 2). Based on these results, we determined that 1000 mg/L is the optimal concentration of AsA in LR-3T-5D for cold preservation of hADSCs for at least 3 days.

3.3. Cell surface markers and differentiation potential

Negative markers for hADSCs, such as CD14, CD31, CD34, CD45, and HLA-DR, exhibited consistently low expression levels before preservation and also at 3 days after preservation at 5 °C in LR-3T-5D with or without 1000 mg/L AsA (Table 1). In contrast, positive markers for hADSCs, such as CD29, CD73, CD90, CD105, and CD44, exhibited consistently high expression levels before preservation and at 3 days after preservation at 5 °C in LR-3T-5D with or without 1000 mg/L AsA (Table 1).

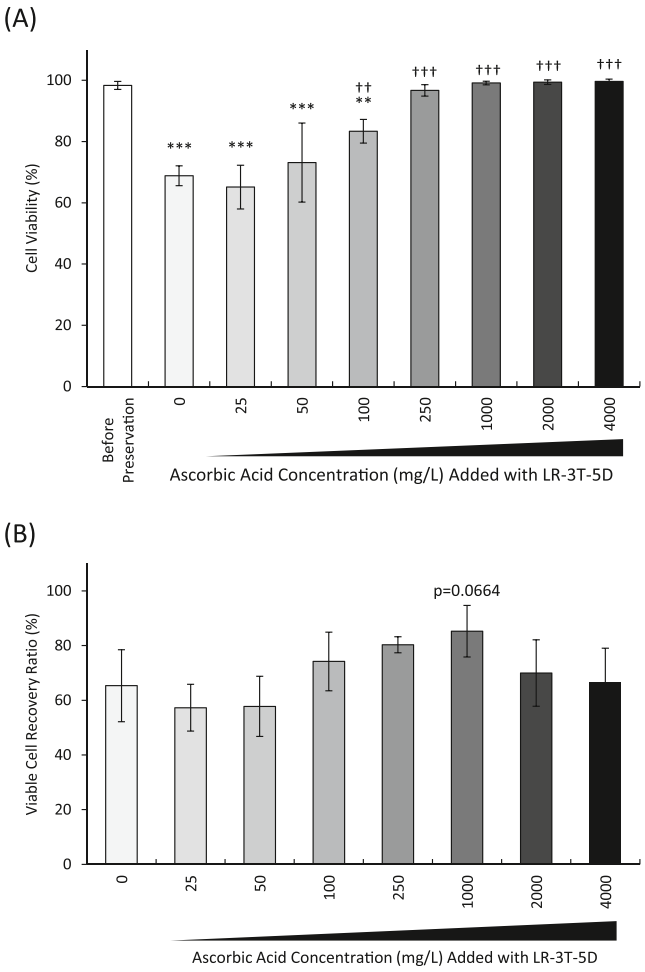

Fig. 3. Viability of hADSCs after 3 days of preservation at 5 °C in LR-3T-5D without and with various concentrations of AsA. (A) Viability of hADSCs before preservation and after preservation for 3 days at 5 °C in solutions containing various concentrations of AsA. Each point and vertical bar indicate the mean ± SD (n = 4). **p < 0.01 and ***p < 0.001 versus before preservation, as determined by Dunnett's test. ††p < 0.01 and †††p < 0.001 versus LR-3T-5D, as determined by Dunnett's test. (B) Viable cell recovery ratio of hADSCs in various concentrations of AsA after 3 days of preservation at 5 °C. Each point and vertical bar indicate the mean ± SD (n = 4). No significant difference versus LR-3T-5D, as determined by Dunnett's test. LR-3T-5D, lactated Ringer's solution containing 3% trehalose and 5% dextran-40.

Adipogenic, osteogenic, and chondrogenic differentiation were induced in hADSCs 3 days after preservation at 5 °C (Fig. 5). Adipocytes containing oil droplets stained with oil red were observed both before and after 3 days of preservation in LR-3T-5D with 1000 mg/L AsA, and there was no difference between before and after preservation. Similarly, there was no significant difference before and after 3 days of preservation with LR-3T-5D-1000A in terms of either osteogenesis or chondrogenesis.

4. Discussion

In this study, LR-3T-5D exhibited better cell-preservation effects under refrigeration than either LR or LR-3T (Fig. 1). Our previous study found enhanced cold-preservation effects as well as improved suspension when cells were stored in solution containing dextran-40 [11]. With regard to the effect of dextran-40 on cold preservation, it has been reported that it inhibits cell membrane peroxidation by removing ROS [15]. The same mechanism might have been involved in the results in the present study.

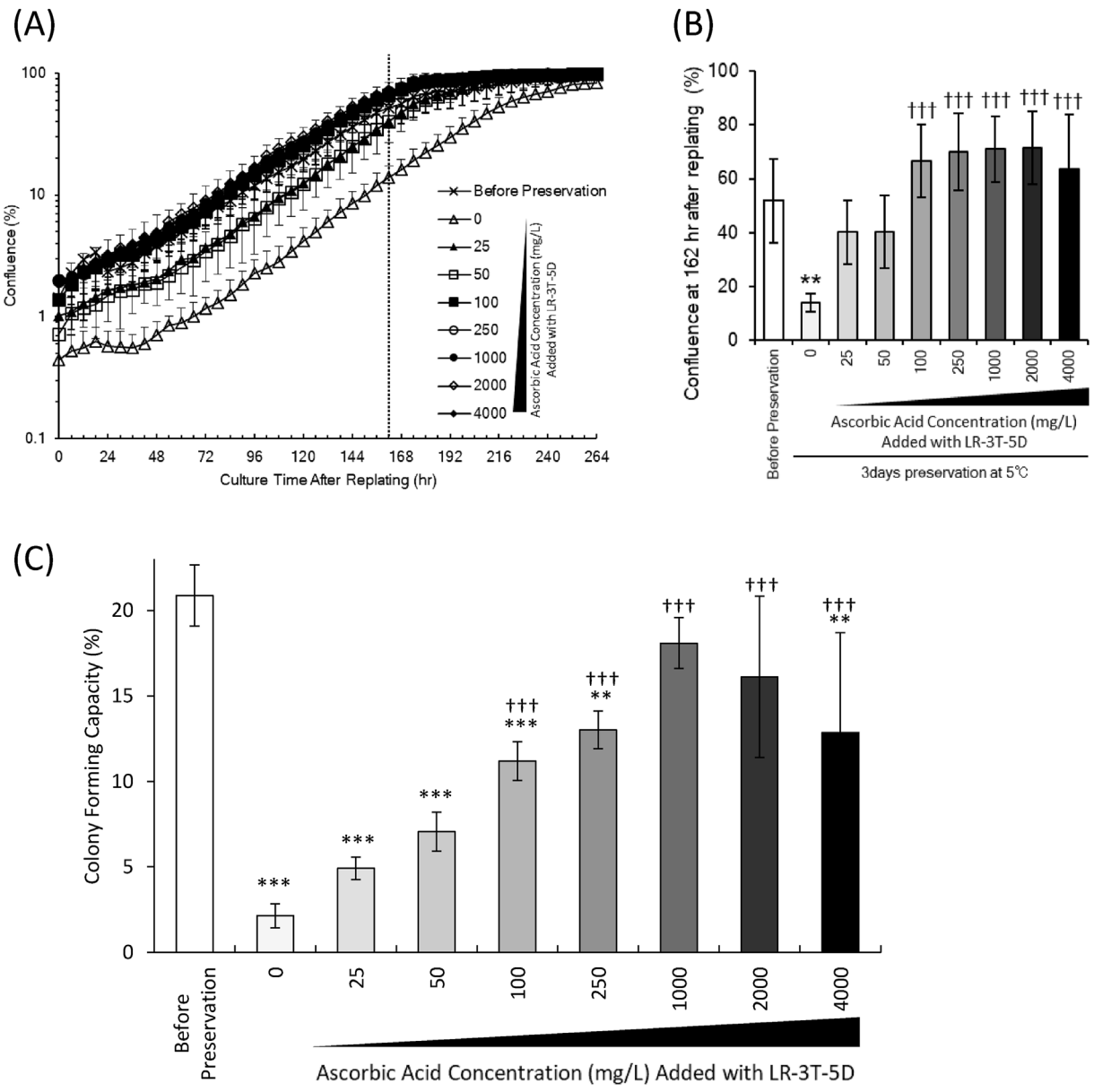

Fig. 4. (A) Time-course analysis of the proliferation of hADSCs after preservation at 5 °C for 3 days in LR-3D-5T without and with various concentrations of AsA. Comparison of confluence at 162 h (B) after re-plating. Each point and vertical bar indicate the mean ± SD (n = 4). *p < 0.05 and **p < 0.01 versus before preservation, as determined by Dunnett's test. ††p < 0.01 and †††p < 0.001 versus LR-3T-5D, as determined by Dunnett's test. (C) Colony-forming capacity of hADSCs after 3 days of preservation at 5 °C in LR-3T-5D without and with various concentrations of AsA. Each point and vertical bar indicate the mean ± SD (n = 4). **p < 0.01 and ***p < 0.001 versus before preservation, as determined by Dunnett's test. †††p < 0.001 versus LR-3T-5D, as determined by Dunnett's test. LR-3T-5D, lactated Ringer's solution containing 3% trehalose and 5% dextran-40.

Table 1
Cell surface marker expression by hADSCs before preservation and 3 days after preservation in LR-3T-5D and LR-3T-5D-1000A at 5 °C. Values indicate the mean ± SD (n = 4). LR-3T-5D, lactated Ringer's solution with 3% trehalose and 5% dextran-40. LR-3T-5D-1000A, LR-3T-5D containing 1000 mg/L AsA.

		Before preservation	After 3 days Preservation at 5 °C	
			LR-3T-5D	LR-3T-5D −1000A
Negative markers for hADSC	CD14	1.7 ± 0.3	0.6 ± 0.1	0.9 ± 0.1
	CD31	1.7 ± 0.2	0.6 ± 0.1	1.0 ± 0.1
	CD34	3.7 ± 0.3	0.9 ± 0.2	1.3 ± 0.2
	CD45	2.0 ± 0.2	0.6 ± 0.1	1.0 ± 0.1
	HLA-DR	1.6 ± 0.1	0.7 ± 0.1	1.0 ± 0.2
Positive markers for hADSC	CD29	100.0 ± 0.0	99.6 ± 0.4	99.0 ± 1.5
	CD73	99.9 ± 0.1	99.5 ± 0.2	99.6 ± 0.4
	CD90	99.9 ± 0.1	100.0 ± 0.0	99.8 ± 0.4
	CD105	99.9 ± 0.1	98.0 ± 1.7	98.9 ± 0.9
	CD44	99.5 ± 0.8	99.3 ± 0.5	96.2 ± 2.7

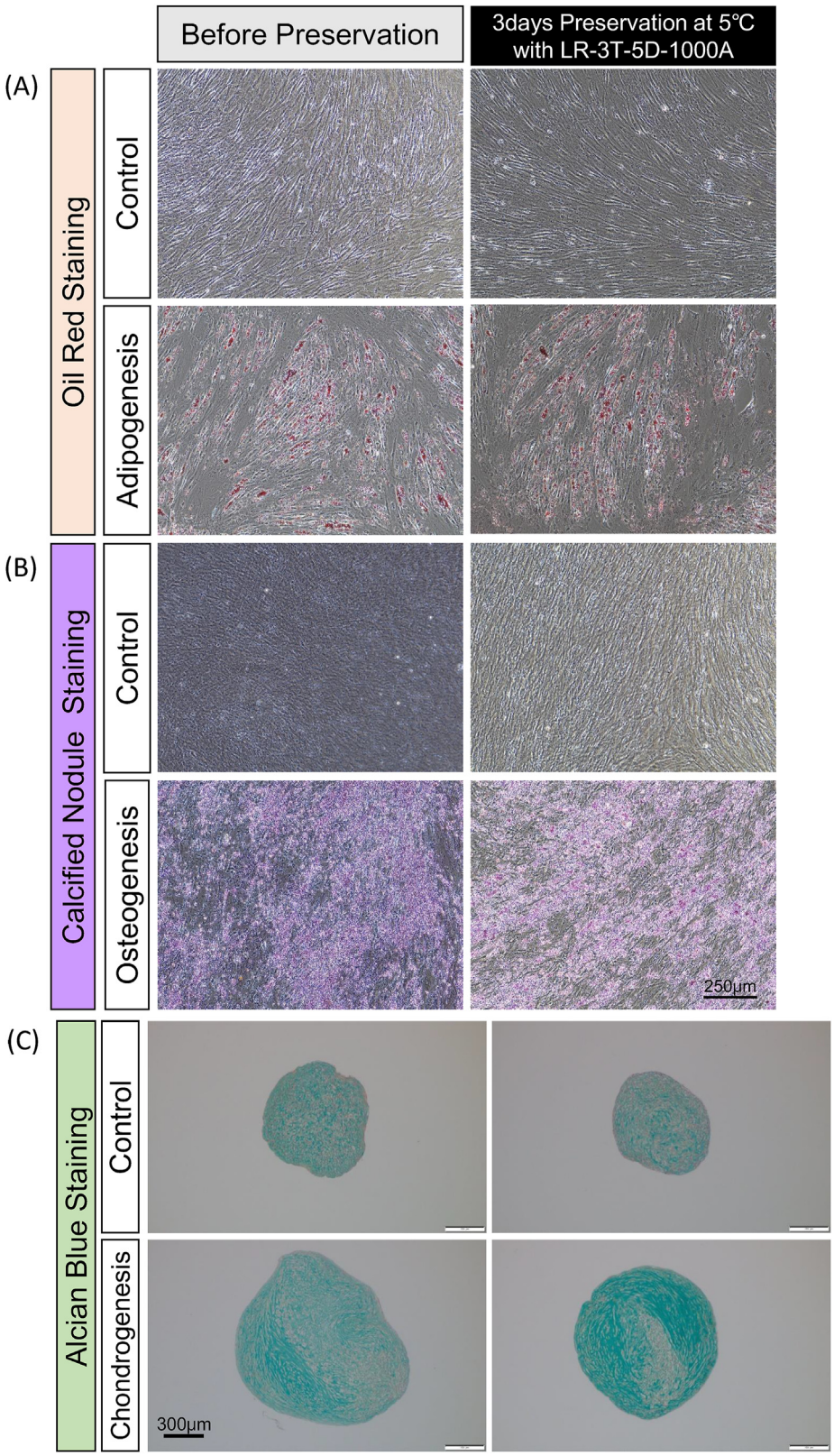

Fig. 5. Representative results of adipogenesis (A), osteogenesis (B), and chondrogenesis differentiation (C) assays of hADSCs after 3 days of preservation at 5 °C in LR-3T-5D with or without 1000A. LR-3T-5D, lactated Ringer's solution containing 3% trehalose and 5% dextran-40.

In the present study, the addition of AsA to all base solutions (LR, LR-3T, and LR-3T-5D) significantly increased cell viability after cold preservation (Fig. 1A). Cold preservation using AsA has been reported for mammalian cells such as red blood cells and hepatocytes [16,17]. AsA is reportedly a major antioxidant [18], and its antioxidant mechanisms include donation of hydrogen atoms to lipid radicals, quenching of singlet oxygen, and removal of molecular oxygen [19–21].

Cell preservation technologies are classified into two categories. One category includes hypothermic preservation methods, in which cells are stored between 1–35 °C. The second category includes cryopreservation methods, in which cells are stored between −196 and −80 °C. Hypothermic preservation, also called shelf-life preservation, is typically used for short preservation periods, generally no longer than 3 days [22]. In comparison with cryopreservation, hypothermic preservation has the advantages of simpler procedures, lower costs, and less damage to cells. Ngo et al. reported the performance of preserving solution containing 0.4% human albumin for the storage of several types of MSCs for up to 72 h under hypothermic conditions [23]. We obtained results similar to theirs using LR-3T-5D containing AsA but lacking human albumin, and we believe this approach is advantageous in terms of production costs, as traceability need not be considered due to the absence of biological raw materials.

Petrenko et al. reported that the viability and functional activity of MSCs should be maintained for 3 days to allow sufficient time for quality control analyses or transport prior to cell administration [2]. Our simple formulation, LR-3T-5D containing 1000 mg/L AsA, appears to satisfy this requirement. However, before clinical use, further in vivo research is needed to determine the efficacy and safety of our solution for different therapeutic cell lines.

5. Conclusions

Our data suggest that LR-3T-5D containing 1000 mg/L AsA is suitable for cold storage of hADSCs for up to 3 days and maintains cell viability, colony-forming capacity, cell surface markers, and proliferation potency after re-plating. These findings indicate our solution is suitable for maintaining the quality of cells used in stem cell therapies during shipping from the manufacturing facility to clinical facilities and during the inspection period of the final product.

Declaration of competing interest

No competing financial interests exist.

Acknowledgment

The experimental work presented in this article was performed while all the authors were employed 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.2023.06.008.

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