Introduction

Mesenchymal stem cells (MSCs) can be isolated from several sources, including the bone marrow (BM) (1), cord blood (CB) (2), umbilical cord (UC), placenta (3), and adipose tissue (4). MSCs are considered to possess multi-potent differentiating ability, and tend to accumulate in the inflamed regions or damaged tissues, where they suppress the inflammatory response or accelerate the repair process. Among them, the UC-derived MSCs (UC-MSCs) have become one of the major MSC sources for autologous and allogeneic use owing to their prominent benefits with respect to painless, safe, and secure collection after delivery. UC is collected and transferred from the obstetrics to cell processing facility, where it is immediately processed by digestion with collagenase (5-7) or explant method (8-11). However, if we could cryopreserve the UC as a living tissue material, it would be advantageous not only for clinical use, but also for research. For example, when we have a problem in the cell processing, we can go back and check the original source of the UC-MSCs. We can check the quality of the UC-MSCs by only using a part of the UC before large-scale culturing, resulting in being cost-effective. We can collect the UC from even in a remote area if it can be frozen. UC-MSCs may be used in treating several disorders in humans such as cerebral palsy (12, 13) and some genetic diseases (14). In addition, the UC cells derived from the donor with genetic or rare and intractable disease can be a great source for research, including induction into induced pluripotent stem (iPS) cells. We may be able to isolate *de novo* stem cells from the UC tissue.

For cell and tissue cryopreservation, there are several types of cryoprotectants, generally containing 5–10% dimethyl sulfoxide (DMSO) with animal or human-derived serum. DMSO and Dextran 40 together with CB-plasma have been used worldwide for CB

cryopreservation (15) in CB banks, but the CB-plasma may not be suitable for freezing UC. Ten percent DMSO and various amounts of fetal bovine serum (FBS) with or without culture medium are the common standard cocktail for cryopreservation of cells in research (16); xenogeneic animal-origin serum should be avoided for clinical use. Cellbanker series (ZENOAQ Resource Co., Ltd), including Cellbanker 1 and STEM-CELLBANKER®, are commercial, ready-to-use cryoprotectants that can be poured onto the cell pellets to be frozen. Of these, Cellbanker 1 plus contains FBS, while STEM-CELLBANKER® does not include FBS, similar to CryoStor® (Stemcell Technologies Inc.). STEM-CELLBANKER® is a novel serum- and animal-free cryoprotectant containing DMSO and anhydrous dextrose, which both permeating and non-permeating cryoprotectants (17). In particular, the STEM-CELLBANKER® was recently reported as a favorable cryoprotectant to freeze adipose and bone marrow derived MSCs (18), human hepatocytes (17, 19), human embryos, and iPS cells (20) as compared to other solutions with DMSO. The goal of this study was to find a suitable cryoprotectant that would yield MSCs isolated from the frozen-thawed UC tissue that are equivalent to those from the fresh UC in growth and functions. Here, we report for the first time, the cryopreservation of the UC tissue in serum- and xeno-free cryoprotectant, STEM-CELLBANKER®, which is qualitatively equivalent to fresh UC.

Materials and Methods

UC cryopreservation

The present study was approved by the Ethics Committee of the Institute of Medical Science, University of Tokyo, and the NTT Medical Center Hospital, Japan. UCs were

collected after informed consent was obtained from pregnant women planning to have cesarean sections. The UC surface was immersed in phosphate-buffered saline (PBS) (Cell Science & Technology Institute, Japan) containing antibiotics and anti-fungal reagent (Antibiotic-Antimycotic, 100X, Life technologies, USA). Next, the UC was cut in cross-sections (1–2 g) and incised longitudinally. Fragments of the UC tissue were immersed in various cryoprotectants in cryotubes for specific durations and frozen in a controlled-rate freezer to −80°C. The cooling temperature was controlled at maximal speed of 2°C/min. The cryoprotectants used in this study were: 1) 10% DMSO/1% Dextran (BloodStor 55-5, BioLife Solutions, WA)/human plasma (derived from the same donor-derived CB plasma, which is the supernatant of CB processed by hydroxyl-ethyl starch and centrifuge method (15); 2) 10% DMSO + 90% fetal bovine serum (FBS; Sigma-Aldrich, Germany); 3) CELLBANKER 1 plus (ZENOAQ Resource Co., Ltd., Japan); and 4) STEM-CELLBANKER® (ZENOAQ Resource Co., Ltd.). The frozen UC tissues were moved into vapor nitrogen tank and stored for at least 2 weeks. To evaluate the quality of the frozen UC-tissue, the UC-tissue fragment was thawed rapidly in a 37°C water bath and rinsed with the α-minimal essential medium (MEM; Wako Pure Chemical Industries, Ltd., Japan) supplemented with 10% FBS, followed by culturing with improved explant method.

Improved Explant method for isolating UC-MSCs

The fresh and frozen-thawed UC tissues were minced into 1–2 mm^3 fragments with Cellamigo® (Tsubakimoto Co., Japan) for improved explant isolation (11). Briefly, minced

fragments (2 g) were used at a density of about 2 g/10-cm dish and covered with Cellamigo, and the α-MEM supplemented with 10% FBS and antibiotics-antimycotics was immediately poured into the dishes (11). The tissue fragments and cells were incubated at 37°C with 5% CO_2 until the fibroblast-like adherent cells migrated from the tissue fragments and reached 80–90% confluency as described previously in detail (11). Within 9–12 days, the adherent cells and tissue fragments were harvested using 10% trypsin solution (rTE; Cell Science & Technology Institute, Japan) and the tissue fragments were removed by filtering with a 100-μm cell strainer (BD Falcon, USA) (10). These cells were defined as passage 1 (P1) cells. The timing of harvesting the cells from the frozen-thawed UC was adjusted to coincide with the days to harvest the cells from the fresh UC. In all comparative experiments, the fresh and frozen-thawed MSCs originated from the same initial donor sample. The harvested cell numbers were counted using Trypan blue staining and adjusted for 1 g of the UC-tissue. The proliferation ratios of MSCs derived from the frozen-thawed UC (frozen-thawed UC-MSCs) were calculated compared to those from the fresh UC-tissue (fresh UC-MSCs). The remaining cells were cryopreserved in STEM-CELLBANKER® freezing medium (ZENOAQ Resource Co., Ltd.) for further analysis. The cells were thawed and washed with α-MEM twice, plated at 4500 cells/cm^2, and were expanded after about 4 days of culture. The passage 3 (P3) cells were used for further analyses of the UC-MSCs.

Flow cytometry analysis and cell sorting

Standard flow cytometry techniques were used to determine the defined MSC markers as described elsewhere (21), for the frozen-thawed UC-MSCs or fresh UC-MSCs. Both the

UC-MSCs were stained using the Human MSC Analysis kit (BD Biosciences, San Jose, CA, USA) containing the following mouse monoclonal antibodies (mAbs): fluorescein isothiocyanate (FITC)-conjugated anti-human CD90; phycoerythrin (PE)-conjugated anti-human CD105, NEGA-mix including anti-human CD45/CD34/CD11b/CD19/ HLA-DR; allophycocyanin (APC)-conjugated anti-human CD73; and FITC-, PE-, and APC-conjugated anti-mouse IgGs (BD Biosciences). Optionally, the UC-MSCs were stained with FITC-conjugated anti-HLA-DR, and anti-human CD44 (BD Biosciences), PE-conjugated anti-HLA-ABC (BD Biosciences), APC-conjugated anti-CD45 (BD Biosciences). Propidium iodide was used to identify and exclude dead cells by flow cytometry.

Mixed lymphocyte reactions (MLRs)

MLR was performed on the frozen-thawed UC-MSCs and fresh control cells derived from a third-party donor to the donors of responder and stimulator cell. Human CBs for responder cells and for allogeneic dendritic cells were partially provided by the RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology of Japan. Carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, USA)-labeled human CB-derived mononuclear cells as the responder cells, were mixed with irradiated (50 Gy) human allogeneic CB-derived dendritic cells (DCs) as stimulator cells, in a 10:1 ratio in the presence of low-dose anti-CD3 antibody. After 4 days of incubation, CFSE-labeled responder cells were stained with APC-conjugated anti human-CD4 or CD8 mAbs on the day of the harvest. The stained cells were acquired with a FACSCanto II flow cytometer (BD Biosystems) and analyzed using FlowJo software (Tomy Digital Biology, Co. Ltd., Japan). In CFSE

histograms, when the responder cell divides, the fluorescence should be apportioned equally to the daughter cells to make half the parent-cell intensity, meaning the proliferation of the responder cells. The responder cells were also treated with phytohemagglutinin-L (PHA-L; Sigma-Aldrich, MO, USA), as positive control.

Adipogenic, chondrogenic, and osteogenic differentiation

UC-MSCs were plated at a density of 2×10^4 cells/well in 12-well plates, induced to differentiate into adipocytes with culture medium supplemented with 100 μM indomethacin (Sigma-Aldrich), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), and 10 μg/ml insulin (Sigma-Aldrich) and the medium was refreshed every 3 days. Then the cells were stained with Oil Red O (Sigma-Aldrich).

To evaluate the chondrogenic differentiation ability of the UC-MSCs, we used a pellet culture system in the chondrogenic differentiation medium. The pellet cells, consisting of 2.5×10^5 UC-MSCs, were cultured in StemMACS™ ChondroDiff Media (Miltenyi Biotec GmbH; Germany) in 15-ml conical tubes. The medium was refreshed every 3 days, and after 24 days, the cells were fixed with 4% formaldehyde and stained with 0.05% toluidine blue (Sigma-Aldrich).

In osteogenic differentiation, UC-MSCs were cultured at a density of 2×10^4 cells/well in 24-well plates and treated with osteogenic medium consisting of αMEM supplemented with 10% FBS, 10 nM dexamethasone (Sigma–Aldrich), 10 mM β-glycerol phosphate (Sigma–Aldrich), 100 μM ascorbic acid (Sigma–Aldrich), and 50 ng/mL human bone morphogenetic protein 2

(rhBMP2; Peprotech, NJ, USA). After 3 weeks, the cells were stained by alkaline phosphatase method (LabAssay™ ALP, WAKO, Japan).

Statistical analysis

Differences between groups were analyzed with JMP 10.0.2 software (SAS Institute, Cary, NC, USA). Statistical analyses were performed with Wilcoxon tests, and a *P*-value of 0.05 was regarded as statistically significant.

Results

Cell Numbers of cells derived from the frozen-thawed UC in various cryoprotectants

First, to evaluate the best cryoprotectant for better yield of the UC-MSCs from the frozen-thawed UC-tissue, we compared the cell numbers and ratio derived from 1 g frozen-thawed-UC in various cryoprotectants with 3 h pre-freezing immersion to those from the fresh-UC (Table 1, n = 4). Without any cryoprotectant, no cells were obtained from the frozen-thawed UC. The highest yield of isolated cells was demonstrated in the frozen-thawed UC stored in STEM-CELLBANKER®, although there were no significant differences among the frozen-thawed UC in different cryoprotectants. No significant difference was observed in the ratio and absolute number between the cells from fresh UC and those from frozen-thawed UC in STEM-CELLBANKER®. The ratio and absolute number of isolated cells from the frozen-thawed UC compared to those from 1 g fresh-UC were 1.5 ± 0.96, and 1.47 ± 0.31 × 10^6 cells/g in STEM-CELLBANKER®, respectively.

Influence of pre-freezing immersion time of UC tissue in cryoprotectant on cell yield

Next, to evaluate the influence of pre-freezing immersion time of the UC-tissue in the cryoprotectant, STEM-CELLBANKER®, on the cell yield from the frozen-thawed UC, we performed a time-course experiment from 0 to 18 h, compared with those without freezing. Unexpectedly, there were no significant differences in the isolated cell numbers between 0 and 18 h of pre-freezing immersion time of the UC-tissue in STEM-CELLBANKER®. The data indicates the ratio of isolated cell number from the frozen-thawed UC compared to those in the fresh-UC (Figure 1). We also could not isolate the cells from the frozen-thawed UC without STEM-CELLBANKER® as demonstrated above. In order for the cryoprotectant to penetrate the UC tissue, we took 3 h to immerse the UC tissue in STEM-CELLBANKER® before freezing in the following experiments.

Frozen-thawed UC-MSCs express MSC biomarkers

The frozen-thawed UC-MSCs isolated from both the fresh UC and frozen-thawed UC in STEM-CELLBANKER® met the minimal criteria of MSCs as defined by the International Society for Cell Therapy (22). UC-MSCs isolated from both the fresh- and frozen-thawed UC showed a spindle-shaped, plastic-adherent cells that were positive for CD73, CD105, CD90, HLA-ABC, and CD44, and negative for CD45, HLA–DR, NEGA-mix containing CD34, CD14, and CD19 (Figure 2). No significant difference was seen between the fluorescent intensity of positive markers of the fresh UC-MSCs and that of the frozen-thawed UC-MSCs.

Frozen-thawed UC MSCs suppress activated T cell proliferation

In the MLR, CFSE-labeled responder T cells proliferated in response to allogeneic DCs stimulation. Both the fresh UC and frozen-thawed UC-MSCs derived from a third-party donor efficiently inhibited the proliferation of responder T cells triggered by allogeneic DCs as the stimulator (Figure 3). In addition, the fresh and frozen-thawed UC-MSCs inhibited the T cell proliferation stimulated with PHA-L. Inhibitions of T cell proliferation by UC-MSCs were observed in $CD4^+$ and $CD8^+$ subsets.

Adipocyte, chondrocyte, and osteoblast differentiation

To confirm the differentiation ability of the frozen-thawed UC-MSCs, we induced frozen-thawed UC-MSCs at P3 into adipocytes and chondrocytes. Using adipocyte differentiation induction medium, we observed the accumulation of Oil Red O-stained lipid drops in both fresh and frozen-thawed UC-MSCs (Figure 4A, B).

The pellet culture system was used to evaluate the differentiation of cells in chondrogenic differentiation medium. Elastic firm pellets with diameters of approximately 1 mm were observed in cells derived from both the fresh and frozen-thawed UC-MSCs (Figure 4C, D). Toluidine blue staining revealed the presence of extracellular matrix in histological sections of cells grown in chondrogenic induction medium (Figure 4E–H).

The fresh and frozen-thawed UC-MSCs were induced into osteoblastic cells, which were positive for alkaline phosphatase. There was no difference in the alkaline phosphatase expression between them (Figure 4I–J).

Discussion

UC is a promising source for MSCs, for both autologous and allogeneic use. The cryopreservation of the UC as the original tissue for the UC-MSCs is reasonable with several advantages including traceability, repeated uses from the same donor sample when needed, the possibility of future discoveries for cells with unknown potential, and feasibility as a tool for gene therapy in autologous use. Among various cryoprotectant solutions as described in the Introduction, serum-free and xeno-free cryoprotectants are ideal for clinical use. Ennis et al. introduced the cryopreservation of the UC vessels in serum-free and xeno-free cryoprotectant solution (such as CryoStor®; Stemcell Technologies Inc. Canada) for isolating human UC perivascular cells (HUPVCs), although they did not show the comparative tests results (23). Roy et al., reported for the first time that cryopreservation of the UC tissue can be done in 10% DMSO and 0.2 M sucrose solution without serum and xenogeneic animal origin reagents, but the cumulative cell yield derived from the frozen-thawed UC-MSCs in their solution was inferior to that of fresh UC-MSCs (24). Our results showed that CELLBANKER series, CELLBANKER 1 plus and STEM-CELLBANKER®, provided relatively high yield of cell recovery compared with the other two cryoprotectants. Cellbanker 1 plus indicated a low ratio but high cell numbers when compared to STEM-CELLBANKER®. The discrepancies of the ratio and absolute numbers may be dependent on the unit deviations. However, we may conclude that the cell recovery from the frozen-thawed UC in STEM-CELLBANKER® is most stable and suitable for clinical use. The reason why the recovered cell numbers were higher in the frozen-thawed UC with STEM-CELLBANKER® than with the fresh UC is unknown. We speculated that this was due to certain factors that stimulated cell growth of MSCs derived from the frozen-thawed UC. Moreover, unexpectedly, there were no significant differences in the number of isolated cells derived from the frozen-thawed UC, when the UC in

STEM-CELLBANKER® was immersed for 0 to 18 h in the refrigerator (4°C) before slow freezing at −80°C. Although DMSO is generally thought to be harmful to the cells, we randomly set the immersion time to be 3 h for the study. We also found it possible to keep the UC tissue at 4°C without any cryoprotectants before processing using explant method (data not shown). For clinical use, the properties of the frozen-thawed UC-MSC in STEM-CELLBANKER® should be similar to the fresh UC-MSCs. The phenotypic characteristics of the frozen-thawed UC-MSCs in STEM-CELLBANKER® were similar to those of the fresh UC-MSCs. As previously reported (25, 26), the immunosuppressive ability was observed in the frozen-thawed UC-MSCs stored in STEM-CELLBANKER® as well as in the fresh UC-MSCs. The differentiation ability into adipocytes, chondrocytes, and osteoblasts also showed no differences between the frozen-thawed UC-MSCs and fresh UC-MSCs, although neither of them reached full osteogenic differentiation, indicating sufficient calcium deposition (data not shown) as described previously (9, 25, 27). Although we evaluated the limited differentiation potential of the frozen-thawed UC-MSCs, the potential of stored UC-MSCs may be unlimited in the future.

For clinical use, we applied the quality control system of CB bank for the UC-tissue and MSCs banking, including health check questionnaire up to more than six months after birth. In addition, UC may be more influenced by the pregnancy and delivery conditions, such as premature, hypoxia, and infections, which may result in a relatively large deviation in the cell recovery from the frozen-thawed UC. In fact, collected UCs differed in diameter and elasticity in our study. Moreover, the epigenetic influence of the cryopreservation on the UC-tissue remains to be evaluated. To overcome some of the limitations on the quality control of the frozen-thawed UC-MSCs, the frozen UC tissue must be evaluated not only by their phenotypic

characteristics, but also by functional assays for immunosuppressive property and differentiation potencies.

To our knowledge, this is the first report on the cryopreservation of the UC tissue in serum- and xeno-free cryoprotectant with equivalent results to those from the fresh UC in growth and functions. The cryopreservation of the UC tissue by the proposed method is safe, simple, and feasible for clinical use and should facilitate the use of UC-MSCs as an alternative MSC source for immunotherapy and regenerative medicine.

Acknowledgments

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Author Disclosure Statement

STEM-CELLBANKER® has been registered as a brand name by Nippon Zenyaku Kogyo Co., Ltd and ZENOAQ Resource Co., Ltd. The patent for STEM-CELLBANKER ® for cryopreservation of UC- tissue, registered by ZENOAQ Resource Co., Ltd. and University of Tokyo, is pending. No other competing financial interests exist.

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Figure legends

Figure 1. Isolation of mesenchymal stem cells (MSCs) derived from frozen-thawed umbilical cord with STEM-CELLBANKER ®. Umbilical cord-derived MSCs (UC-MSCs) were isolated by improved explant method after pre-freezing incubation in STEM-CELLBANKER® for 0, 1, 3, 6, and 18 h, respectively. Without any cryoprotectant, no MSCs were obtained from the frozen-thawed UC. Data are indicated as mean isolated cell number ratio to those from the fresh UC on the y-axis (n = 4).

Figure 2. Characterization of umbilical cord-derived mesenchymal stem cells (UC-MSCs) isolated from frozen-thawed UC.

UC-MSCs isolated from both the fresh and frozen-thawed UC showed positive CD73, CD105, CD90, CD44, and HLA-ABC expression, and negative CD45, HLA–DR expression, and NEGA-mix containing CD34, CD14, CD19, and HLA-DR. Fluorescent intensity (FI) ± standard deviation for positive markers is indicated without significant differences between the fresh and frozen-thawed UC-MSCs (n = 3).

Figure 3. Immunosuppressive effect of Umbilical cord-derived mesenchymal stromal cells (UC-MSCs) derived from frozen-thawed UC.

UC-MSCs derived from the frozen-thawed UC showed inhibition of CD4- and CD8-positive T cell proliferation triggered by the allogeneic dendritic cells and PHA-L. Carboxyfluorescein succinimidyl ester (CFSE)-labeled mononuclear cells (R) were cultured in different conditions, R indicates R only, R + S indicates co-culture with allogeneic dendritic cells (S), R + S + Fresh

UC-MSCs or frozen-thawed UC-MSCs indicates allogeneic stimulation and allogeneic fresh or frozen-thawed UC-MSCs of the third-party donor, respectively. The ratio of the mixture of R:S:UC-MSCs for the MLR was 10:1:1. "R + PHA-L" indicates R with 10 μg of PHA-L. R + PHA-L + Fresh UC-MSCs or frozen-thawed UC-MSCs indicates R + PHA-L with fresh UC-MSCs or frozen-thawed UC-MSCs from third-party donor, respectively. Data are shown as representative of three independent experiments.

Figure 4. Adipocyte, chondrocyte, and osteoblastic differentiation ability of umbilical cord-derived mesenchymal stromal cells (UC-MSCs) derived from frozen-thawed UC.

Frozen-thawed UC-MSCs were differentiated into adipocytes (upper panel) and chondrocytes (lower panel). In adipocyte-differentiated cells (A and B), the accumulation of Oil Red O-stained lipid drops was observed in both the fresh UC-MSCs and frozen-thawed UC-MSCs (red droplets; magnification, 200X). For chondrogenic differentiation, the pellet culture system was used. The elastic firm pellet was observed in the differentiation medium after 3 weeks of culture (C and D) (magnification 40X). Toluidine blue staining revealed the presence of extracellular matrix with metachromasia in histological sections (E to H, magnification of 40X and 100X, respectively). The fresh and frozen-thawed UC-MSCs were induced into osteoblastic cells, which were positive for alkaline phosphatase (I and J) (violet cells; magnification, 200x). There was no difference in the alkaline phosphatase expression between them.

Table 1. Isolated cell number yield from frozen-thawed umbilical cord stored in different cryoprotectants.

Cryoprotectants	Isolated Cell number ratio (Mean ± SD)	Absolute number $\times 10^6$/ g (Mean ± SD)	*P*
Fresh	1	1.0 ± 0.95	
Freezing			
No cryoprotectant	0 ± 0	0 ± 0	*P<0.05
CELLBANKER 1 plus	0.75 ± 0.80	1.38 ± 0.67	
STEM-CELLBANKER®	1.5 ± 0.96	1.47 ± 0.31	
DMSO/DEX.+ plasma	0.36 ± 0.55	0.59 ± 0.23	
90% FBS + 10% DMSO	0.48 ± 0.76	0.86 ± 0.13	

DEX.; dextran 40 solution, FBS; fetal bovine serum, DMSO; dimethyl sulfoxide; (n = 4)

Figure 1

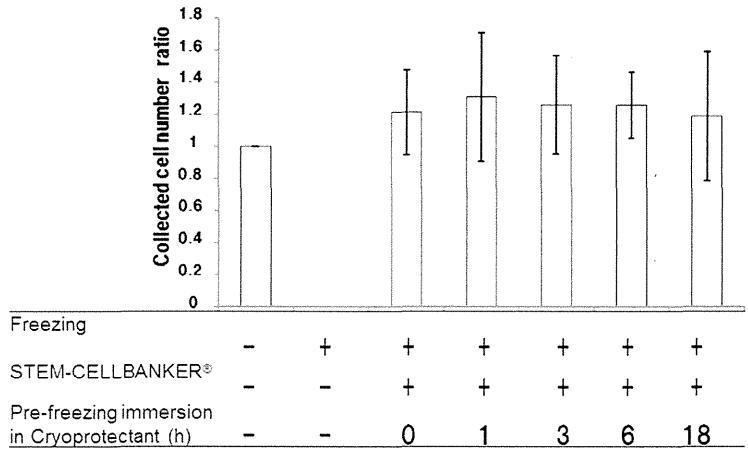

Figure 1. Isolation of mesenchymal stem cells (MSCs) derived from frozen-thawed umbilical cord with STEM-CELLBANKER®. Umbilical cord-derived MSCs (UC-MSCs) were isolated by improved explant method after pre-freezing incubation in STEM-CELLBANKER® for 0, 1, 3, 6, and 18 h, respectively. Without any cryoprotectant, no MSCs were obtained from the frozen-thawed UC. Data are indicated as mean isolated cell number ratio to those from the fresh UC on the y-axis (n = 4).
254x190mm (96 x 96 DPI)