FULL-LENGTH ARTICLE

Manufacturing

Evaluation of the effectiveness of a new cryopreservation system based on a two-compartment vial for the cryopreservation of cell therapy products

Cristina Rosell-Valle^{1,*}, Cristina Antúnez², Fernando Campos¹, Natalia Gallot⁵, Mariano García-Arranz³, Damian García-Olmo³, Rosario Gutierrez⁴, Roberto Hernán⁵, Concha Herrera⁶, Rosario Jiménez⁴, Laura Leyva-Fernández⁷, Rafael Maldonado-Sánchez⁷, Raquel Muñoz-Fernández⁷, Sonia Nogueras⁴, Lourdes Ortiz⁴, Inmaculada Piudo¹, Isidora Ranchal¹, Antonio Rodríguez-Acosta⁷, Cristina Segovia², Beatriz Fernández-Muñoz^{1,*}

¹ Unidad de Producción y Reprogramación Celular, Red Andaluza Para el Diseño y Traslación de Terapias Avanzadas, Sevilla, Spain

² Unidad de Expansión y Terapia Celular, Centro de Transfusión, Tejidos y Células, Málaga, Spain

³ Fundación Jiménez Díaz, Madrid, Spain

⁴ Unidad de Terapia Celular, Hospital Universitario Reina Sofía, Córdoba, Spain

⁵ Cellulis S.L., Santoña, Spain

⁶ Unidad de Terapia Celular, Instituto Maimónides de Investigación Biomédica de Córdoba, Hospital Universitario Reina Sofía, Universidad de Córdoba, Córdoba, Spain

⁷ Unidad de Producción Celular, Hospital Regional Universitario de Málaga, Málaga, Spain

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ABSTRACT

Background aims: Successful cell cryopreservation and banking remain a major challenge for the manufacture of cell therapy products, particularly in relation to providing a hermetic, sterile cryovial that ensures optimal viability and stability post-thaw while minimizing exposure to toxic cryoprotective agents, typically dimethyl sulfoxide (Me₂SO).

Methods: In the present study, the authors evaluated the effectiveness and functionality of Limbo technology (Cellulis S.L., Santoña, Spain). This system provides a hermetic vial with two compartments (one for adding cells with the cryoprotective agent solution and the other for the diluent solution) and an automated defrosting device. Limbo technology (Cellulis S.L.) allows reduction of the final amount of Me₂SO, sidestepping washing and dilution steps and favoring standardization. The study was performed in several Good Manufacturing Practice laboratories manufacturing diverse cell therapy products (human mesenchymal stromal cells, hematopoietic progenitor cells, leukapheresis products, fibroblasts and induced pluripotent stem cells). Laboratories compared Limbo technology (Cellulis S.L.) with their standard cryopreservation procedure, analyzing cell recovery, viability, phenotype and functionality.

Results: Limbo technology (Cellulis S.L.) maintained the viability and functionality of most of the cell products and preserved sterility while reducing the final concentration of Me₂SO.

Conclusions: Results showed that use of Limbo technology (Cellulis S.L.) offers an overall safe alternative for cell banking and direct infusion of cryopreserved cell products into patients.

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Introduction

The potential of cell therapy as treatment for diseases for which there is currently no cure is increasingly recognized, and the development of new cell-based products has been a particularly active field in recent years. Cell-based therapies are expected to not only bring

* Correspondence: Cristina Rosell-Valle, PhD and Beatriz Fernández-Muñoz, PhD, Unidad de Producción y Reprogramación Celular, Red Andaluza Para el Diseño y Traslación de Terapias Avanzadas, C/Max Planck, 3. Edificio Iris 1. 41092, Sevilla, Spain.

E-mail addresses: cristina.rosell@juntadeandalucia.es (C. Rosell-Valle), beatriz.fernandez.munoz@juntadeandalucia.es (B. Fernández-Muñoz).

important health benefits but also substantially impact the pharmaceutical industry [1,2]. Cell-based medications are, by their nature, biological samples that have a limited shelf life. The continuous expansion of cells in culture over long periods after their isolation implies high economic costs, risk of contamination and acquisition of chromosomal aberrations and phenotypic changes [3,4]. Cell cryopreservation circumvents these issues while also saving time and money. Accordingly, the generation of cryopreserved master and working cell banks is a common intermediate step in the manufacture of most cell-based products. However, most cell products are transplanted as non-frozen fresh products, and the pharmaceutical industry is betting on the direct administration of cryopreserved cells to lower the associated costs [3–5]. An important advantage of cryopreservation is that it allows sufficient time for quality control tests while at the same time extending the period of usefulness of cells and facilitating large-scale manufacturing, ultimately lowering costs. Cryopreservation also ensures the immediate availability of the medicinal product, as it is possible to bank cell products in hospitals and application centers until their use, allowing better timing of therapy [5].

Nevertheless, successful cell cryopreservation is challenging because cell-based medicinal products must have functionality similar to that of “fresh” samples when used clinically. Ineffective cryopreservation processes can induce cell damage and apoptosis, which compromises cell survival and functionality [6,7]. For example, it has been shown that cryopreserved human mesenchymal stromal cells (hMSCs) display impaired immunomodulatory properties and are subjected to faster clearance after blood exposure [8–10]. Indeed, some clinical trials have reported negative outcomes when employing cryopreserved hMSCs for direct infusion [reviewed in 11]. A major limitation in cellular cryopreservation is the use of cryoprotective agents required for good cell recovery. Dimethyl sulfoxide (Me₂SO) is the most widely used cryoprotectant for cell therapy [12]; however, it is known to induce toxicity and epigenetic changes in cells [13,14]. Also, numerous studies have reported Me₂SO-related adverse reactions following infusion of Me₂SO-preserved cell products, ranging from mild events such as nausea, vomiting, headache, hypotension, hypertension and diarrhea to severe reactions such as cardiac arrhythmias, cardiac arrest, respiratory stress and epileptic seizures [5,11,13,15,16]. Thus, it would be desirable to reduce the levels of Me₂SO (e.g., from 10% to 5% [17,18]) in cellular products for clinical use both in final and intermediate products.

Another potential weakness in the storage, shipment and delivery of cell therapy products is the material of which the storage vials are made. Cryotubes made of polypropylene have long been used to store biological samples [19,20], but there is evidence that the leaching of chemical additives from the resin can interfere with cell components [20,21]. These vials also typically use screw caps that are open to the environment and are not scalable for use in later phases of development. Given these limitations, attention has now turned to the manufacture of vials made of cyclic olefin polymers (COPs) and copolymers, as these materials have more desirable properties, including excellent thermal characteristics, lower gas and moisture permeability, glass-like transparency and break resistance, and can be sterilized by conventional autoclaving [20,22–24].

The process of cell thawing after cryopreservation is usually performed at 37°C in water baths, but the risk of contamination makes this option less appealing for pharmaceutical manufacturing under Good Manufacturing Practice (GMP) [25–28]. Accordingly, heating plates and thawing by hand are sometimes used for GMP manufacturing, but these manipulations are difficult to standardize and thus hinder comparisons between studies in different centers and hospitals.

Developing new optimized protocols, vials and devices that improve cell cryopreservation/thawing and allow standardization could increase the cellular recovery and functionality of cryopreserved cells, ensuring their effective clinical application, ultimately

benefiting the patient [29]. Although considerable progress has been made in optimizing freezing protocols, freezing media composition, cooling devices and cryovials to ensure that cell products are safe and retain their therapeutic characteristics following cryopreservation, more research focused on improving cell recovery and Me₂SO-associated injury is still needed [5,11].

Here the authors evaluated the effectiveness and functionality of Limbo technology (Cellulis S.L., Santoña, Spain), a system that provides a prototype cryovial with two advantages over traditional cryovials: (i) it is a sealed and sterile vial made of COPs, and (ii) its two-compartment system and controlled defrosting device allow slow dilution of Me₂SO to avoid osmotic shock, without the need for additional manipulation, reducing the final amount of Me₂SO and its associated toxicity and favoring thawing standardization between different centers and hospitals. To this aim, the authors enlisted the cooperation of several GMP facilities in Spain producing different cell therapy products (hMSCs, hematopoietic progenitor cells [HPCs], leukapheresis products, fibroblasts and induced pluripotent stem cells [iPSCs]) to compare Limbo technology (Cellulis S.L.) with standard cryopreservation procedures and validate the use of this new system for GMP manufacturing. Since hMSCs can be isolated from different tissues [30], the authors tested the system with hMSCs derived from two sources: bone marrow (bone marrow-derived hMSCs [BM-hMSCs]) and adipose tissue (human adipose-derived mesenchymal stem cells [hADMSCs]). The authors' results showed that Limbo technology (Cellulis S.L.) maintains the viability and functionality of most of the cell products tested as well as preserves sterility and may offer a safe alternative for cell banking and the direct application of cryopreserved cell products into patients.

Methods

Cell freezing with Limbo technology

The Limbo cell therapy cryopreservation system is designed and produced by Cellulis S.L., Santoña, Cantabria, Spain (<https://www.cellulis.com>). Limbo cryovial prototypes (Cellulis S.L.) are made of COPs and have two compartments separated at the base and open at the top. The larger compartment, called the diluent compartment, has a large capacity for diluent medium (4 mL), whereas the other, smaller (1 mL) compartment is designed for the cells to be deposited (Figure 1A; also see supplementary Video 1). The cell compartment is a thin semi-ring cavity located in the outer part of the vial, as it has been shown that cells in the center of cryovials freeze more slowly than those nearer the edge, which affects their viability and function [31].

Medium was first added to the diluent compartment. The cells were then resuspended with a cryopreservation solution with 10% Me₂SO and transferred to the cell compartment of the Limbo cryovials (Cellulis S.L.). A rubber stopper with an aluminum cap was used to encapsulate and hermetically seal the cryovials. All manipulations were performed in a grade A environment in accordance with GMP standards. Limbo vials (Cellulis S.L.) were then transferred to a purpose-made ethylene-vinyl acetate container (Cellulis S.L.), being cautious not to mix the contents of the compartments. Limbo vials (Cellulis S.L.) were placed in the ethylene-vinyl acetate container in a freezer at –80°C or without a container in a biological programmable controlled-rate freezer. Cryovials were then transferred to liquid nitrogen (LN₂). The standard cryopreservation method of each laboratory was performed in parallel for comparison. The conditions used in each laboratory and the freezing programs are detailed in Table 1 and supplementary Table 1, respectively.

Cell thawing with Limbo technology

An automated cell thawing device (Imatec Innovation S.L., Santoña, Spain) was used to thaw cells in Limbo cryovials (Cellulis S.L.)

Figure 1. Limbo technology. (A) Schematic representation of the Limbo freezing protocol. A prototype cryovial with two compartments separated by a base was used. The compartment with the largest capacity was designed for diluent medium (frontal view). The cell compartment, with a smaller volume, is a thin semi-ring hole open at the top of the cryovial (frontal and axial view). Stoppers and aluminum caps were used to close the vials with the use of an encapsulator. An EVA container or controlled-rate freezer was used for the initial cryopreservation of Limbo vials that were finally transferred to LN₂ tanks. (B) Schematic representation of the Limbo thawing protocol. An automated cell thawing device was used to thaw cells, which functions by inserting Limbo vials upside down into the thermoblock inlet. Diluent falls drop by drop, slowly diluting Me₂SO. The time evolution of the thawing process, which takes ~7 min, is monitored by an LED. EVA, ethylene-vinyl acetate; LED, light-emitting diode. (Color version of figure is available online.)

(Figure 1B; also see supplementary Video 1). The device consists of an electronic appliance that incorporates software to control the temperature and time of thawing. Cell thawing is performed by inserting the vial upside down into the thermoblock inlet, which triggers an audible alarm to indicate that the position is correct. The cell thawing device automatically detects the Limbo cryovial (Cellulis S.L.) and starts the thawing process, which takes ~7 min. Cells descend into the stopper space by gravity

after passing from the ice phase to the liquid phase, such that their exposure to heat ends in their transition phase. The design of the vial allows for gradual thawing, as the smaller compartment (where the cells are contained) is thawed first, and then the diluent falls drop by drop, slowly diluting Me₂SO and avoiding cellular osmotic shock. Once the two compartments are mixed, the final concentration of Me₂SO is 2% (5-fold dilution). The device shows the time evolution of the process using a

Table 1
Description of the cryopreservation protocols and reagents used in each laboratory.

Cell type	Standard protocol			Limbo technology				Number of cells cryopreserved ($\times 10^6$ cells/cryotube)	GMP laboratories
	Cryoprotective agent solution	Freezing method	Thawing method	Cryoprotective agent solution in cell compartment	Diluent compartment	Freezing method	Thawing method		
hADMSCs	DMEM 20% FBS 10% Me ₂ SO	Nunc cryovials and Mr. Frosty	Water bath	DMEM 20% FBS 10% Me ₂ SO	DMEM 20% FBS	Limbo cryovials and EVA container	Limbo cell thawing device	10	Fundación Jiménez Díaz Madrid
hADMSCs	DMEM 20% FBS 10% Me ₂ SO	Nunc cryovials and Mr. Frosty	Water bath	DMEM 20% FBS 10% Me ₂ SO	MEM α 5% hPL	Limbo cryovials and EVA container	Limbo cell thawing device	1	Hospital Regional Universitario de Malaga
BM-hMSCs	20% human albumin + 10% Me ₂ SO	Nunc cryovials and programmable controlled-rate freezer	Water bath	20% human albumin + 10% Me ₂ SO	5% of human albumin	Limbo cryovials and programmable controlled-rate freezer	Limbo cell thawing device	20	Hospital Reina Sofia Cordoba
hFBs	CryoStor CS10	Nunc cryovials and Mr. Frosty	Water bath	CryoStor CS10	Supplemented DMEM FBS	Limbo cryovials and EVA container	Limbo cell thawing device	1	UPRC Seville
HPCs	Dextran + 10% Me ₂ SO	CryoStore freezing bags and programmable controlled-rate freezer	Water bath	Dextran + 10% Me ₂ SO	5% albumin + 0.9% saline + Voluven	Limbo cryovials and programmable controlled-rate freezer	Cell thawing device	45–50	Centro de Transfusión, Tejidos y Células Malaga
Leukapheresis	Autologous plasma + 10% Me ₂ SO	Nunc cryovials and programmable controlled-rate freezer	ThawSTAR automated cell thawing system	Autologous plasma + 10% Me ₂ SO	5% albumin + 0.9% saline + Voluven	Limbo cryovials and programmable controlled-rate freezer	Limbo cell thawing device	100–150	Centro de Transfusión, Tejidos y Células Malaga
hiPSCs	CryoStor CS10	Nunc cryovials and Mr. Frosty	Water bath	CryoStor CS10	DMEM/F12 + 20% KSR	Limbo cryovials and EVA container	Limbo cell thawing device	20 (colonies)	UPRC Seville

Cell type	Standard protocol						Limbo technology						GMP laboratories
	Freezing			Thawing			Freezing			Thawing			
	rate (°C min ^{−1})	CPA additional rate (mL sec ^{−1})/T (°C) ^b	Total time (min) ^a	rate (°C min ^{−1})	Dilution rate (mL sec ^{−1})/T (°C) ^c	Total time (min)	rate (°C min ^{−1})	CPA additional rate (mL sec ^{−1})/T (°C) ^b	Total time (min) ^a	rate (°C min ^{−1})	Dilution rate (mL sec ^{−1})/T (°C) ^c	Total time (min)	
hADMSCs	−1	Pellet directly resuspended in CPA/RT	1.2 ± 0.4	46 ± 0.8	0.4 ± 0.2/RT	5 ± 0.5	−1	Pellet directly resuspended in CPA/RT	2.5 ± 0.03	31.7 ± 0.7	0.009 ± 0.0002/37°C	7.3 ± 0.2	Fundación Jiménez Díaz Madrid
hADMSCs	−1	Pellet directly resuspended in CPA/RT	1.5 ± 0.5	44.1 ± 2	0.5 ± 0.2/RT	4 ± 0.5	−1	Pellet directly resuspended in CPA/4°C	3.2 ± 0.04	31.7 ± 0.7	0.009 ± 0.0002/37°C	7.3 ± 0.2	Hospital Regional Universitario de Malaga
BM-hMSCs	Program 1 ^d	0.053 ± 0.005/4°C	14 ± 0.6	48.3 ± 2.9	0.3 ± 0.2/RT	4.8 ± 0.3	Program 1 ^d	0.053 ± 0.005/4°C	15.4 ± 0.05	31.7 ± 0.7	0.009 ± 0.0002/37°C	7.3 ± 0.2	Hospital Reina Sofia Cordoba
hFBs	−1	Pellet directly resuspended in CPA/RT	3.1 ± 0.2	49.5 ± 5.3	0.1 ± 0.5/RT	4.8 ± 0.6	−1	Pellet directly resuspended in CPA/RT	4.4 ± 0.02	31.7 ± 0.7	0.009 ± 0.0002/37°C	7.3 ± 0.2	UPRC Seville
HPCs	Program 2 ^d	0.018 ± 0.005/4°C	10 ± 0.5	23 ± 0.5	0.3 ± 0.1/RT	10 ± 0.5	Program 2 ^d	0.018 ± 0.005/4°C	11.3 ± 0.03	31.7 ± 0.7	0.009 ± 0.0002/37°C	7.3 ± 0.2	Centro de Transfusión, Tejidos y Células Malaga
Leukapheresis	Program 2 ^d	0.028 ± 0.005/4°C	1.1 ± 0.6	46 ± 0.8	0.3 ± 0.1/RT	5 ± 0.5	Program 2 ^d	0.028 ± 0.005/4°C	2.4 ± 0.03	31.7 ± 0.7	0.009 ± 0.0002/37°C	7.3 ± 0.2	Centro de Transfusión, Tejidos y Células Malaga
hiPSCs	−1	Pellet directly resuspended in CPA/RT	3.1 ± 0.5	49.5 ± 5.3	0.1 ± 0.5/RT	4.8 ± 0.6	−1	Pellet directly resuspended in CPA/RT	4.3 ± 0.02	31.7 ± 0.7	0.009 ± 0.0002/37°C	7.3 ± 0.2	UPRC Seville

Mean $\pm$ SEM is shown.

CPA, cryoprotective agent; DMEM, Dulbecco's Modified Eagle's Medium; EVA, ethylene-vinyl acetate; hPL, human platelet lysate; KSR, Knockout serum replacement; MEM α , Minimum Essential Medium α ; RT, room temperature; SEM, standard error of the mean; T, temperature; UPRC, Unidad de Producción y Reprogramación Celular.

^aTime needed to prepare cryovial before introducing it into the –80°C freezer or controlled-rate freezer.

^bSpeed at which CPA was added.

^cSpeed at which thawed cells were added to culture medium to dilute Me₂SO.

^dSee supplementary Table 1.

light-emitting diode. An audible alarm indicates when thawing is complete. The Limbo cryovial (Cellulis S.L.) is removed from the device and maintained upside down for 30 seconds under gentle agitation to prevent cells from overheating. The cryovial is then turned the right way up and placed into a laminar flow cabinet for opening.

Cells were removed from Limbo vials (Cellulis S.L.) with syringes through the rubber stopper or with pipettes and then processed for further analysis.

Cryopreserved cells in CryoStore freezing bags (OriGen Biomedical, Austin, TX, USA) and Nunc cryotubes (control procedures) were thawed at 37°C in a water bath with gentle shaking. Cells were then diluted with supplemented medium and centrifuged. With regard to leukapheresis products, the authors used the ThawSTAR automated thawing system (MedCision, San Rafael, CA, USA), which is the standardized method in our laboratory for thawing this cell type.

Cell lines

To evaluate the potential effectiveness of Limbo technology (Cellulis S.L.) in clinical applications, the authors used different human cell types. Each research group involved in the study isolated and grew cells according to their routine protocols. All samples were obtained after written informed consent according to Spanish law (RD 1301/2006) and has been performed in accordance with the 1964 Declaration of Helsinki.

The hADMSCs were cultured and cryopreserved in parallel in two facilities: Fundación Jimenez Díaz (FJD), Madrid, Spain, and the Unidad de Terapia Celular of the Hospital Regional Universitario (HRU) de Málaga, Málaga, Spain. Procurement of hADMSCs was approved by the ethical committee of the FJD University Hospital (PIC 056-18_FJD) and by the Provincial Research Ethics Committee of Málaga (PI 11-05/17). At FJD, hADMSCs were grown in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (SAFC Biosciences, Lenexa, KS, USA), 0.1 mM nonessential amino acids (Sigma-Aldrich), 2 mM GlutaMAX (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 100 $\mu\text{g mL}^{-1}$ penicillin/streptomycin (Sigma-Aldrich). At HRU de Málaga, hADMSCs were grown in Minimum Essential Medium α (Corning Life Science, Glendale, AZ, USA) supplemented with 2 mM of L-alanine/L-glutamine (Sigma-Aldrich) and 5% human platelet lysate (Cook Medical LLC, Bloomington, IN, USA).

The hMSCs derived from bone marrow (BM-hMSCs) were cultured and cryopreserved at the Unidad de Terapia Celular of the Hospital Universitario Reina Sofia, Córdoba, Spain. Cell procurement was approved by the local ethical committee of clinical research. BM-hMSCs were grown in Minimum Essential Medium α (Lonza) supplemented with 13% (v/v) FBS (Gibco), 1 ng mL^{-1} human fibroblast growth factor (Miltenyi Biotec, Bergisch Gladbach, DE, Europe), 2 mM UltraGlutamine (Lonza, Basel, CH, Europe), 5 $\mu\text{g mL}^{-1}$ gentamicin (B. Braun Medical, Melsungen, DE, Europe) and 0.1 mg mL^{-1} streptomycin (Laboratorio Reig Jofré, Barcelona, ES, Europe).

Human fibroblasts (hFBs) and human iPSCs (hiPSCs) were cultured and cryopreserved at the Unidad de Producción y Reprogramación Celular of Seville, Spain. Procurement of hFBs was approved by the Andalusian Biomedical Research Coordinator Ethical Committee through the Andalusian Public Health System Biobank. The hFBs were grown in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) supplemented with 10% (v/v) FBS (Sigma-Aldrich), 0.1 mM nonessential amino acids (Sigma-Aldrich), 2 mM GlutaMAX (Gibco) and 100 $\mu\text{g mL}^{-1}$ penicillin/streptomycin (Sigma-Aldrich).

HPCs and leukapheresis products were isolated and cryopreserved at the Centro de Transfusión, Tejidos y Células de Málaga, Málaga, Spain. Cell procurement was approved by the Andalusian Public Health System Ethical Committee. HPCs were isolated from three umbilical cord blood units using the Sepax automated cell processing system (Biosafe, Murcia, ES, Europe), which concentrates HPCs by reducing plasma and red blood cells [32]. Leukapheresis products

were obtained using the Spectra Optia apheresis system (Terumo BCT, Lakewood, CO, USA).

The authors also used hiPSCs derived from CD133⁺ umbilical cord cells (cord blood-derived hiPSCs [CB-hiPSCs]) to assess the suitability of Limbo technology (Cellulis S.L.). These cells are available from the Spanish National Repository, and data regarding cell characterization can be downloaded at <http://www.eng.isci.es>. The use of CB-hiPSCs was approved by the Andalusian Ethical Committee of Research with Biological Samples of an Embryonic Origin and Similar Cells (PR-02-2017). CB-hiPSCs were maintained on a feeder layer of mouse embryonic fibroblasts (MEFs), which were inactivated by γ -irradiation. The medium for CB-hiPSCs consisted of Dulbecco's Modified Eagle's Medium/F12 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20% Knockout serum replacement (Thermo Fisher Scientific), 0.1 mM nonessential amino acids (Sigma-Aldrich), 1 mM GlutaMAX (Gibco), 4 nM fibroblast growth factor (Miltenyi Biotec) and 0.1 mM β -mercaptoethanol (Gibco). The medium was changed every other day and colonies were mechanically passaged.

Assessment of cell viability, recovery and stability post-thaw

Cell concentration and viability after thawing were analyzed using 7-aminoactinomycin D dye for HPCs and leukapheresis products and Trypan Blue (Sigma-Aldrich) for the remaining cell types. Cell recovery was calculated according to the equation percentage of cell recovery post-thawing = (alive cells $\times$ 100)/cryopreserved cells. In the case of hFBs, the authors performed an additional analysis of the stability of cells cryopreserved with Limbo (Cellulis S.L.) to evaluate cell viability by Trypan Blue exclusion after specific times at room temperature, which allowed us to assess the time window during which the cells could be used.

To analyze hiPSC recovery, thawed cells were seeded onto MEF-coated Corning six-well plates (Thermo Fisher Scientific), and colonies were counted during the following 15 days after thawing. For morphology analyses, phase-contrast microphotographs were taken on different days after thawing using a Nikon Eclipse Ti microscope.

Cell recovery 24 h post-thaw and proliferation analysis

To better evaluate the effect of Limbo cryopreservation (Cellulis S.L.) on the recovery of hADMSCs, BM-hMSCs and hFBs, thawed cells were seeded and grown at 37°C and 5% CO₂ for 24 h. Cells were then detached and counted again. The percentage of cell recovery 24 h post-thaw was determined with the equation (alive cells at 24 h $\times$ 100)/seeded cells.

For proliferation analysis, hADMSCs and BM-hMSCs were seeded and expanded for 1 week (one passage after thawing). Cells were then counted, and the population doublings were calculated according to the equation $\text{PD} = (\log y - \log x) / \log 2$, where "y" is the number of cells at the end of the cultivation period and "x" the number of cells at the beginning.

Statistical analysis

Data were expressed as mean $\pm$ standard error of the mean. Analyses were carried out using a two-sided Student's unpaired *t*-test and two-way analysis of variance (ANOVA) (cryopreservation method $\times$ time), followed by Sidak's multiple comparisons test, when appropriate. A one-sample *t*-test was used to compare cell viability against the 70% viability Food and Drug Administration (FDA) limit [33]. $P \leq 0.05$ was considered significant. All statistical analyses were performed with Prism 5.0. (GraphPad Software Inc, San Diego, CA, USA). Additional materials and methods information can be found in the supplementary material.

Figure 2. Cryopreservation of hADMSCs at FJD, Madrid, Spain. (A) Schematic representation of cell expansion, cryopreservation and post-thaw analysis. (B) Cell viability measured immediately after thawing. (C) Flow cytometry analysis of surface marker expression. (D) Differentiation capacity in adipogenic medium, with representative images of differentiated cells (top). Scale bar: 50 μm. Absorbance of isopropanol-extracted Oil Red O (bottom). (E) Differentiation capacity in osteogenic medium, with representative images of differentiated cells (top). Scale bar: 50 μm. Absorbance of cetylpyridinium chloride-extracted Alizarin Red S (bottom). (F) Karyotype analysis. Data are shown as mean ± SEM. Standard group n = 2. Limbo group n = 5. Student's unpaired *t*-test. Dashed line indicates FDA limit of viability. **P* ≤ 0.05. SEM, standard error of the mean. (Color version of figure is available online.)

Results

Cryopreservation of hADMSCs

The authors first examined whether the Limbo cryopreservation (Cellulis S.L.) approach affected the phenotype and functionality of hADMSCs that were independently cultured and cryopreserved in two different laboratories (Figures 2, 3). Analyses of the results from the FJD laboratory (Figure 2A) revealed no significant differences between Limbo (Cellulis S.L.) and standard cryopreservation with

regard to cell viability (unpaired *t*-test, *P* > 0.05), which was ~75% in both systems after cryopreservation for 3 months (Figure 2B). Regarding the post-thaw phenotype, flow cytometry analysis revealed a similar and high surface expression of positive ADMS markers, such as CD29, CD73 and CD90, in cells cryopreserved by both methods and a low surface expression of negative markers (Figure 2C). Of note, cells cryopreserved with Limbo (Cellulis S.L.) had a greater capacity to differentiate into adipogenic lineages (unpaired *t*-test, *P* < 0.05) (Figure 2D) than cells cryopreserved by the standard method. By contrast, the osteogenic differentiation capacity was

Figure 3. Cryopreservation of hADMSCs at HRU de Málaga, Spain. (A) Experimental design of cryopreservation and post-thaw analysis. (B,C) Cell viability and recovery analysis at different time points. (D) Recovery after 24 h of cell culture. (E) Cell proliferation (population doublings) after 7 days of culture. (F) Flow cytometry analysis of surface marker expression. (G) Karyotype analysis at 1, 6 and 12 months. (H) Adipogenic and osteogenic differentiation of thawed cells after 12 months of cryopreservation, with representative images of differentiated cells. Scale bar: 50 μm. Data are shown as mean ± SEM (n = 3 per group). Dashed line indicates FDA limit of viability. SEM, standard error of the mean. (Color version of figure is available online.)

similar (Figure 2E). Also, no changes were observed in karyotype between cryopreservation methods (Figure 2F).

The authors next analyzed the cell secretome at 24, 48 and 72 h after thawing (see supplementary Figures 1–4). Compared with

standard cryopreservation, cells frozen with the Limbo system (Cellulis S.L.) secreted higher levels of some cytokines, including IL-1ra (unpaired *t*-test, 24 h, *t* = 3.568, degrees of freedom [df] = 3, *P* < 0.05, 48 h, *t* = 4.541, df = 2, *P* < 0.05) (see supplementary Figure 1A);

Table 2
Summary of microbiological analysis after thawing.

Cell type	Cryopreservation method	Cryopreservation time	Sterility	Gram	Calcofluor	Mycoplasma	Endotoxin levels, IU/mL
hADMSCs	Standard	1 month	Negative	N.D.	N.D.	Negative	<0.75
		6 months	Negative	N.D.	N.D.	Negative	<0.75
		12 months	Negative	N.D.	N.D.	Negative	<0.5
	Limbo technology	1 month	Negative	N.D.	N.D.	Negative	<0.75
		6 months	Negative	N.D.	N.D.	Negative	<0.75
		12 months	Negative	N.D.	N.D.	Negative	<0.5
BM-hMSCs	Standard	1 week	Negative	Negative	Negative	Negative	<0.75
	Limbo technology	1 week	Negative	Negative	Negative	Negative	<0.75

Acceptance criteria: sterility (absence of turbidity), Gram stain (absence of bacteria), Calcofluor (absence of fungal parasites), mycoplasma (absence of mycoplasma-specific DNA amplification), endotoxin levels (<0.75 IU/mL).
N.D., not determined.

chemokines, such as granulocyte colony-stimulating factor (unpaired *t*-test, 48 h, $t = 4.874$, $df = 2$, $P < 0.05$, 72 h, $t = 8.824$, $df = 2$, $P < 0.05$) (see supplementary Figure 3); and growth factors, such as platelet-derived growth factor BB (unpaired *t*-test, 24 h, $t = 5.042$, $df = 2$, $P < 0.05$) and vascular endothelial growth factor (unpaired *t*-test, 24 h, $t = 15.12$, $df = 2$, $P < 0.05$) (see supplementary Figure 4). The reason for this might be because cells cryopreserved with the Limbo system (Cellulis S.L.) do not need to be centrifuged or washed before seeding.

Similar results were obtained at HRU de Málaga. No significant differences were observed in viability and recovery post-thaw between cryopreservation methods at different cryopreservation times (Figure 3A–C) (two-way ANOVA, viability, cryopreservation method, $F_{(1,4)} = 0.461$, $P > 0.05$, and time, $F_{(2,8)} = 3.491$, $P > 0.05$) (Figure 3B) (recovery, cryopreservation method, $F_{(1,4)} = 0.064$, $P > 0.05$, and time, $F_{(2,8)} = 3.491$, $P > 0.05$) (Figure 3C). Both procedures resulted in similar cell viability the first and sixth months of freezing, although after 12 months of cryopreservation, cell viability decreased close to or below the 70% limit established by the FDA for final product release (Figure 3B) [33]. The recovery of thawed cells with both Limbo technology (Cellulis S.L.) and the standard method was lower at 12 months, although this was not significantly different compared with the other cryopreservation times (Sidak's multiple comparisons, $P > 0.05$) (Figure 3C). These results are in line with a study carried out by De Rosa *et al.* [34], who demonstrated that hADMSC viability was ~70% after 12 months of cryopreservation in LN₂ [34].

The ability of cells to adhere to the plastic of cell culture flasks and cell recovery after 24 h were similar between Limbo (Cellulis S.L.) and conventional cryopreservation (two-way ANOVA, viability, cryopreservation method, $F_{(1,4)} = 0.291$, $P > 0.05$, and time, $F_{(2,8)} = 0.146$, $P > 0.05$) (Figure 3D). Similarly, no differences were seen in cell proliferation (two-way ANOVA, viability, cryopreservation method, $F_{(1,4)} = 0.011$, $P > 0.05$, and time, $F_{(2,8)} = 2.030$, $P > 0.05$) (Figure 3E). Flow cytometry analysis revealed that irrespective of the cryopreservation method and storage time, hADMSCs presented high expression of common mesenchymal markers, indicating that freezing/thawing with Limbo technology (Cellulis S.L.) did not change the phenotype of cells (unpaired *t*-test, $P > 0.05$) (Figure 3F). Regarding microbiology, all samples were negative for sterility and mycoplasma tests and showed acceptable endotoxin levels (<0.75 IU/mL) (Table 2). Finally, the cryopreservation method did not have an impact on the karyotype of hADMSCs or the differentiation potential of adipogenic and osteogenic lineages over 12 months of cryopreservation (Figure 3G,H).

Cryopreservation of BM-hMSCs

The authors next compared standard cryopreservation with Limbo technology (Cellulis S.L.) in BM-hMSCs (Figure 4A). Both methods supported high cell viability (~95%) immediately after thawing (unpaired *t*-test, $P > 0.05$), significantly above the FDA limit (one-sample *t*-test, standard, $t = 20.25$, $df = 2$, $P \leq 0.01$, and Limbo, $t = 24$, $df = 2$, $P \leq 0.01$) (Figure 4B). Likewise, the post-thaw recovery of cells

cryopreserved with Limbo technology (Cellulis S.L.) was similar to that of conventionally frozen cells (unpaired *t*-test, $P > 0.05$) (Figure 4C), although after culture for 24 h there was a tendency for a loss in the recovery of frozen cells from the Limbo system (Cellulis S.L.) (unpaired *t*-test, $t = 2.246$, $df = 4$, $P = 0.08$) (Figure 4C).

The phenotype of BM-hMSCs was analyzed by flow cytometry immediately after thawing and after one cell passage. No significant differences were noted between the two methods in the expression of hMSC markers (unpaired *t*-test, $P > 0.05$) (Figure 4D, E). Likewise, all cells showed similar multipotentiality to differentiate into adipogenic and osteogenic lineages (Figure 4F), proliferation capacity (Figure 4G) and karyotype (Figure 4H). Finally, all samples were negative for sterility, Gram stain, Calcofluor and mycoplasma tests and showed acceptable endotoxin levels (<0.75 IU/mL) (Table 2).

Cryopreservation of hFBs

In the next series of experiments, the authors analyzed cell functionality, apoptosis, stability and phenotype of dermal hFBs cryopreserved with either Limbo (Cellulis S.L.) or standard cryopreservation (Figure 5A). No significant differences were seen in viability between the two methods (unpaired *t*-test, $P > 0.05$) (Figure 5B), and all thawed cells had values significantly higher than the 70% limit of the FDA (one-sample *t*-test, standard, $t = 83.41$, $df = 2$, $P \leq 0.001$, and Limbo, $t = 5.556$, $df = 2$, $P \leq 0.05$) (Figure 5B). Also, the recovery post-thaw and after 24 h of culture was similar in both conditions (*t*-test, $P > 0.05$) (Figure 5B).

Analysis of cell apoptosis by flow cytometry showed that the cell percentage of early (Annexin V fluorescein isothiocyanate [FITC]-positive/propidium iodide [PI]-negative staining) and late (Annexin V FITC⁺/PI⁺ staining) apoptosis was not significantly different between the two methods (unpaired *t*-test, $P > 0.05$). The percentage of dead cells by flow cytometry (Annexin V FITC⁺/PI⁺ staining) was similar to that obtained using the Trypan Blue exclusion method (Figure 5C,D), and no significant differences were observed between the two methods (unpaired *t*-test, $P > 0.05$) (Figure 5D). To assess cell stability after thawing with Limbo technology (Cellulis S.L.), the vials were allowed to sit at room temperature for 24 h, and cell viability and recovery were assessed at different time points. Cell viability was significantly above the 70% FDA limit at all time points measured (one-sample *t*-test, $P \leq 0.05$) (Figure 5E), indicating that there was a broad time window in which cells could be used after thawing in Limbo cryovials (Cellulis S.L.). Likewise, the total cell count was stable for 24 h after thawing (Figure 5F). Cells from both cryopreservation methods presented high expression of characteristic fibroblast markers, such as vimentin, fibronectin, collagen type I and CD13 (Figure 5G), indicating that hFB phenotype was not affected by the cryopreservation method. Similarly, karyotype analysis showed no changes in cells cryopreserved with the two methods (Figure 5H).

Figure 4. Cryopreservation of BM-hMSCs. (A) Experimental design of cryopreservation and post-thaw analysis. (B) Cell viability after 1 week of cryopreservation. (C) Cell recovery post-thaw and after 24 h of culture. Flow cytometry analysis of surface marker expression post-thaw (D) and after one cell passage (E), with adipogenic and osteogenic differentiation of thawed cells and representative images of differentiated cells. Scale bar: 50 μ m. (G) Cell proliferation (population doublings) at one passage after thawing. (H) Karyotype at one passage after thawing. Data are shown as mean $\pm$ SEM (n = 3 per group). One-sample *t*-test versus 70% limit of FDA. Dashed line indicates FDA limit of viability. **P* $\leq$ 0.01. SEM, standard error of the mean. (Color version of figure is available online.)

Figure 5. Cryopreservation of hFBs. (A) Experimental design of cryopreservation and post-thaw analysis. (B) Cell viability. (C) Cell recovery post-thaw and after 24 h of culture. (D) Apoptosis analysis. Cell viability (E) and total cell count (F) over 24 h after thawing. (G) Immunocytochemistry analysis of vimentin, fibronectin, collagen type I and CD13. Scale bar: 10 μ m. Data are shown as mean $\pm$ SEM ($n = 3$ per group). One-sample t -test versus 70% limit of FDA. Dashed line indicates FDA limit of viability. * $P \leq 0.05$, ** $P \leq 0.01$. SEM, standard error of the mean. (Color version of figure is available online.)

Cryopreservation of HPCs and leukapheresis products

The authors next evaluated the effect of cryopreservation with Limbo technology (Cellulis S.L.) using HPCs isolated from umbilical cord blood (Figure 6A). As observed in the other cell types, no

differences in cell recovery were noted between the two cryopreservation methods (unpaired t -test, $P > 0.05$) (Figure 6B). However, the authors noted that the number of viable CD34⁺ and CD45⁺ cells was significantly lower after Limbo cryopreservation (Cellulis S.L.) compared with after cryopreservation in CryoStore freezing bags

Figure 6. Cryopreservation of human HPCs and leukapheresis products. (A) Experimental design of cryopreservation and post-thaw analysis. (B) Cell recovery post-thaw. (C) Cell viability of CD34⁺ and CD45⁺ cells. (D) No significant difference was found in the percentage of CD34⁺/CD45⁺ cells. (E) CFU assay. (F) Representative colonies from cryopreserved cells (×100 magnification). (G) Schematic representation of leukapheresis studies. (H) CD3⁺ cell recovery. (I) CD45⁺ viability. (J) Percentage of CD3⁺/CD45⁺ cells. Data are shown as mean ± SEM (n = 3 per group). Student's unpaired t-test Standard versus Limbo. *P ≤ 0.05. One-sample t-test versus 70% limit of FDA. **P ≤ 0.01. Dashed line indicates FDA limit of viability. BFU-E, burst-forming unit erythroid; CFU, colony-forming unit; CFU-E, colony-forming unit erythroid; CFU-GM, colony-forming unit granulocyte-macrophage; SEM, standard error of the mean. (Color version of figure is available online.)

(unpaired *t*-test, CD34⁺, *t* = 13.47, *df* = 4, *P* ≤ 0.0001, and CD45⁺, *t* = 5.501, *df* = 4, *P* ≤ 0.01) (Figure 6C). No significant difference was found in the percentage of CD34⁺/CD45⁺ cells, although the

percentage was slightly lower after thawing with the Limbo system (Cellulis S.L.) (unpaired *t*-test, *P* > 0.05) (Figure 6D). No significant differences were observed in differentiation capacity between the two

methods, as assessed by colony-forming unit assays (unpaired *t*-test, $P > 0.05$) (Figure 6E). Examples of the different granulocyte-macrophage and erythroid progenitor cells are shown in Figure 6F. In the case of leukapheresis products (Figure 6G), no significant differences were found between Limbo (Cellulis S.L.) and the standard method with regard to the recovery of CD3⁺ cells, viability of CD45⁺ cells or percentage of CD3⁺/CD45⁺ cells (unpaired *t*-test, $P > 0.05$) (Figure 6H–J).

Cryopreservation of hiPSCs

The cryopreservation of hiPSCs is particularly challenging, as low recovery rates are common [35,36] and the use of Me₂SO is especially toxic [37]. Thus, the authors studied whether the two-compartment Limbo system (Cellulis S.L.) performed better than the standard cryopreservation method (see supplementary Figure 5A). After thawing, hiPSCs were seeded onto MEFs, and the emerging colonies were counted in both cases. No significant differences were observed between Limbo (Cellulis S.L.) and the standard cryopreservation method for the cumulative colony count (unpaired *t*-test, $P > 0.05$) (see supplementary Figure 5B), although the number of colonies was consistently higher using the standard method. As shown in supplementary Figure 5C,D, neither hiPSC colony morphology nor pluripotency marker expression differed between the two cryopreservation methods.

Discussion

Successful cell cryopreservation and banking remain a major limitation in the manufacture of cell therapy products, specifically in achieving a hermetic and sterile cryovial that maintains optimal cell viability and stability post-thawing [36]. Another challenge is the use of Me₂SO as a cryoprotectant, as it is known to cause toxicity and adverse effects in both cells and patients, and therefore the amount of Me₂SO in the final and intermediate products should be minimized [5,11,16]. In the present study, the authors assessed the usefulness of a new cryopreservation approach based on a prototype cryovial made from COPs that offers certain advantages over traditional vials for cell-based therapies. Limbo cryovials (Cellulis S.L.) are sealed vials with two compartments that function to reduce the final amount of Me₂SO. The system also has a controlled defrosting device where thawing and dilution steps are made automatically, allowing standardization of the thawing process. The authors worked with several GMP laboratories producing different cell-based products to compare the Limbo system (Cellulis S.L.) with established cryopreservation protocols using polypropylene cryotubes or cryobags. The Limbo system (Cellulis S.L.) allowed the elimination of differences in the dilution and thawing rates and total processing times between laboratories (Table 1). The results showed that the Limbo cryopreservation system (Cellulis S.L.) preserved the viability, functionality and phenotype of most of the cell products tested, offering an alternative cryopreservation system for cell therapy products.

The authors did not find significant differences in any of the parameters tested between the Limbo system (Cellulis S.L.) and standard cryopreservation methods for hADMSCs (in two independent laboratories) or BM-hMSCs, suggesting that the Limbo cryopreservation (Cellulis S.L.) approach can be used safely for hMSCs. However, since the immunomodulatory capacity of hMSCs can be altered after freezing/thawing [8–10], future studies should analyze this specific issue.

Similar to hMSCs, the authors did not find significant differences in any of the parameters tested between the Limbo system (Cellulis S.L.) and standard cryopreservation methods for leukapheresis products, hFBs or hiPSCs. However, the authors did note that the viability of HPCs after thawing was significantly lower with the Limbo system (Cellulis S.L.) (Figure 6C). Furthermore, although not statistically significant, slightly lower cell recovery rates and higher levels of

apoptosis were found in some cases with Limbo technology (Cellulis S.L.) (Figures 4C, 5D; also see supplementary Figure 5B). This indicates that further improvements are needed for this technology to be widely used for the cryopreservation of these cell types.

Limbo cryovials (Cellulis S.L.) are sealed vials with a rubber stopper and cap, and a syringe needle system should be used for the collection of thawed cells. Several studies indicate that needle shear forces may cause damage to cells, reducing viability [38,39]. In this sense, one potential limitation of Limbo technology (Cellulis S.L.) is that cells, when collected by syringe needle, have to be passed twice (once for collecting and once for delivering the cells) through the needle, and this could be the cause of the observed lower viability and recovery rates of some cells. It is important to note that the size of the needle, as well as the rate of cell delivery for clinical transplantation, can impact cell disruption, with wider gauges and slow delivery rates being preferable [38–42]. The authors did not account for these factors in the present study, and further studies will be needed to enhance cell recovery from Limbo cryovials (Cellulis S.L.) after using a syringe needle or vial adaptor. Furthermore, the authors used the same freezing program and protocol for Limbo vials (Cellulis S.L.) and reference containers (Nunc vials or CryoStore freezing bags); however, the different plastic material (COPs) and thickness of Limbo vials (Cellulis S.L.) might require the development of an optimized and specific controlled-rate freezer program. For instance, to cryopreserve HPCs, the authors used a freezing program optimized for cryobags made out of ethylene-vinyl acetate, a material thinner than COPs and with different conductivity properties [28]. This may be the cause of the reduced HPC viability observed with the Limbo system (Cellulis S.L.). Future studies should optimize the freezing program, which will probably increase the post-thaw viability and recovery rates.

Regarding other commercially available products, a widely used system for cell therapy products is the Daikyo Crystal Zenith cryovial (West Pharmaceutical Services, Inc, Exton, PA, USA), which consists of a sterile pharmaceutical vial made from COPs, as in the Limbo vials (Cellulis S.L.). Results using this system have demonstrated good cryopreservation of hMSCs for 6 months in LN₂, with a post-thaw viability of ~95% [20,43]. However, an automated cell thawing system is not available for these vials, and manual dilution steps are needed to decrease the final concentration of Me₂SO.

Another commercially available system is the AT-Closed Vial (Aseptic Technology, LLC, Yorba Linda, CA, USA), which consists of a sealed cryogenic vial and an automated cell thawing device. The vial is made from copolymers and is pre-closed, sterile and encapsulated and employs a septum with a thermoplastic elastomer capable of being frozen and stored in LN₂ [20,36]. Unlike Limbo (Cellulis S.L.) or Daikyo Crystal Zenith (West Pharmaceutical Services, Inc), a needle is not necessary to collect the cells because of the disposable AT-Adapt device, which permits access to the AT-Closed Vial (Aseptic Technology, LLC) and might reduce cell damage. A similar system could be designed for Limbo cryovials (Cellulis S.L.) to reduce cell damage. Also, the AT-Closed Vial system (Aseptic Technology, LLC) uses an automated cell loading device that allows standardization and decreases contamination risk during the filling process [44]. However, it is a robotic device that requires a significant economic investment. In this sense, no contamination risk was found with Limbo technology (Cellulis S.L.) during the freezing/thawing process, as shown by the authors' microbiological analysis results (Table 2). Both Limbo technology (Cellulis S.L.) and the AT-Closed Vial system (Aseptic Technology, LLC) provide an automated thawing system, allowing the standardization of thawing procedures between laboratories and hospitals and favoring the regularization of clinical applications. This is likely a safer alternative to thawing cells in a 37°C water bath and might reduce the contamination risk caused by this method [25–28], being a good option for GMP manufacture or clinical application.

In contrast to commercially available cryopreservation systems, Limbo cryovials (Cellulis S.L.) have two compartments that reduce the final amount of Me₂SO without the need for washing steps, thus avoiding possible contamination and reducing time and costs. Despite the advantages of Me₂SO as a cryoprotective agent [45], many studies have highlighted adverse reactions in patients after direct transplantation of cells cryopreserved with Me₂SO [5,11,16]. Therefore, the Limbo cryopreservation system (Cellulis S.L.) could help to dilute the cryoprotective agent and mitigate the negative impacts of Me₂SO by lowering the final dose [17,18]. This technology facilitates the production of ready-to-use cryopreserved cell therapy intermediate and final products, avoiding the need for washing the cells after thawing. Although the authors' objective was to investigate the usefulness of the Limbo system (Cellulis S.L.) for the cryopreservation of cell banks, future studies should analyze whether different infusion media, such as HypoThermosol FRS (BioLife Solutions, Bothell, WA, USA) [46–50] or lactated Ringer's solution [50,51], added to the diluent compartment could maintain cell function and reduce the Me₂SO-related toxicity of directly infused frozen cell products. In this sense, it would be appropriate to compare the results with an equivalent manual manipulation in a conventional bag system to confirm the advantages of the Limbo system (Cellulis S.L.) over traditional methods for direct infusion of cryopreserved cell products.

Conclusions

Overall, this study supports the use of Limbo technology (Cellulis S.L.) as a cryopreservation method for cell therapy products. In contrast to other commercially available cryopreservation systems, Limbo vials (Cellulis S.L.) have two compartments that allow reduction of the final amount of cryoprotectant and its associated toxicity, sidestepping washing and dilution steps and favoring ease and standardization of the thawing process. Although more studies are needed to enable large-scale use of the technology, the Limbo system (Cellulis S.L.) might be an attractive alternative for the preparation of cryopreserved cell banks and cell therapy products intended for direct infusion into patients.

Declaration of Competing Interest

RH and NG are employees of Cellulis S.L. DG is a member of the advisory board of TiGenix. DG and MG have applied for two patents related to the studies titled "Identification and isolation of multipotent cells from nonosteochondral mesenchymal tissue" (WO 2006/057649) and "Use of adipose tissue-derived stromal stem cells in treating fistula" (WO 2006/136244). DG and MG are shareholders of Biosurgery, an educational company providing services to Takeda.

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Author Contributions

Conception and design of the study: CR and BF. Acquisition of data: CR, CA, FC, MG, DG, RG, CH, RJ, LL, RM, RAM, SN, LO, IP, IR, AR and BF. Analysis and interpretation of data: CR, CA, FC, NG, MG, DG, RG, RH, CH, RJ, LL, RM, RAM, SN, LO, IP, IR, AR, CS and BF. Drafting or revising the manuscript: CR and BF. All authors have approved the final article.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2020.12.004.

References

- [1] Mount NM, Ward SJ, Kefalas P, Hyllner J. Cell-based therapy technology classifications and translational challenges. *Philos. Trans. R. Soc. B Biol. Sci.* Oct. 2015;370 (1680):20150017.
- [2] ten Ham RMT, et al. What does cell therapy manufacturing cost? A framework and methodology to facilitate academic and other small-scale cell therapy manufacturing costings. *Cytotherapy* Jul. 2020;22(7):388–97.
- [3] Shaw JM, Oranratnachai A, Trounson AO. Fundamental cryobiology of mammalian, oocytes and ovarian tissue. *Theriogenology* 2000;53(1):59–72.
- [4] Ibars EP, et al. Hepatocyte transplantation program: Lessons learned and future strategies. *World Journal of Gastroenterology* 2016;22(2):874–86. 14-Jan-.
- [5] Marquez-Curtis LA, Janowska-Wieczorek A, McGann LE, Elliott JAW. Mesenchymal stromal cells derived from various tissues: Biological, clinical and cryopreservation aspects. *Cryobiology* 2015;71(2):181–97.
- [6] François M, Copland IB, Yuan S, Romieu-Mourez R, Waller EK, Galipeau J. Cryopreserved mesenchymal stromal cells display impaired immunosuppressive properties as a result of heat-shock response and impaired interferon- γ licensing. *Cytotherapy* 2012;14(2):147–52.
- [7] Li Y, Ma T. Bioprocessing of cryopreservation for large-scale banking of human pluripotent stem cells. *Biores. Open Access* 2012;1(5):205–14.
- [8] Moll G, et al. Do cryopreserved mesenchymal stromal cells display impaired immunomodulatory and therapeutic properties? *Stem Cells* 2014;32(9):2430–42.
- [9] Moll G, et al. Cryopreserved or fresh mesenchymal stromal cells: Only a matter of taste or key to unleash the full clinical potential of MSC therapy? *Advances in Experimental Medicine and Biology*, 951. Springer New York LLC; 2016. p. 77–98.
- [10] Moll G, Hoogduijn MJ, Ankrum JA. Editorial: Safety, Efficacy and Mechanisms of Action of Mesenchymal Stem Cell Therapies. *Front. Immunol.* 2020;11(February):1–4.
- [11] Hornberger K, Yu G, McKenna D, Hubel A. Cryopreservation of Hematopoietic Stem Cells: Emerging Assays, Cryoprotectant Agents, and Technology to Improve Outcomes. *Transfus. Med. Hemotherapy* 2019;46(3):188–96.
- [12] Matsumura K, Bae JY, Hyon SH. Polymorpholites as cryoprotective agents for mammalian cell cryopreservation. *Cell Transplant* Jun. 2010;19(6–7):691–9.
- [13] Yong KW, et al. Phenotypic and Functional Characterization of Long-Term Cryopreserved Human Adipose-Derived Stem Cells. *Sci. Rep.* 2015;5:1–10.
- [14] Miyagi-Shiohira C, et al. Cryopreservation of Adipose-Derived Mesenchymal Stem Cells. *Cell Med Aug.* 2015;8(1–2):3–7.
- [15] Madsen BK, Hilscher M, Zetner D, Rosenberg J. Adverse reactions of dimethyl sulfoxide in humans: a systematic review. *F1000Research* 2019;7:1746.
- [16] Weng L, Beauchesne PR. Dimethyl sulfoxide-free cryopreservation for cell therapy: a review. *Cryobiology* 2020;94:9–17.
- [17] Bakken AM, Bruserud O, Abrahamsen JF. No Differences in Colony Formation of Peripheral Blood Stem Cells Frozen with 5% or 10% Dimethyl Sulfoxide. *J. Hematother. Stem Cell Res.* Jun. 2003;12(3):351–8.
- [18] Mitrus I, et al. Reduction of DMSO concentration in cryopreservation mixture from 10% to 7.5% and 5% has no impact on engraftment after autologous peripheral blood stem cell transplantation: results of a prospective, randomized study. *Bone Marrow Transplant.* Mar. 2018;53(3):274–80.
- [19] Waxman L, Erwin RL, Vilivalam VD. Characterization of a cyclic olefin polymer microcentrifuge tube. *Biotechniques* 2017;62(5):223–8.
- [20] Waxman L, Degrazio FL, Vilivalam V. Chapter 23: "Plastic Packaging for Parenteral Drug Delivery". Book. L. J. Nema S., Parenteral Medications, Fourth Edition. CRC Press; 2019. p. 497–510.
- [21] Lewis LK, Robson MH, Vecherkina Y, Ji C, Beall GW. Interference with spectrophotometric analysis of nucleic acids and proteins by leaching of chemicals from plastic tubes. *Biotechniques* Apr. 2010;48(4):297–302.
- [22] Qadry SS, Roshdy TH, Char H, Del Terzo S, Tarantino R, Moschera J. Evaluation of CZ-resin vials for packaging protein-based parenteral formulations. *Int. J. Pharm.* Feb. 2003;252(1–2):207–12.
- [23] Woods EJ, Bagchi A, Nase R, Vilivalam V. "A novel container system for cell therapy products," West Pharmaceutical Site Repository. Dai-kyo Resin CZ Technical Report; 2008. <https://www.westpharma.com/Site-Repository/Knowledge-Articles/>.
- [24] Nunes PS, Ohlsson PD, Ordeig O, Kutter JP. Cyclic olefin polymers: Emerging materials for lab-on-a-chip applications. *Nanofluidics* 2010;9(2–3):145–61.
- [25] Casewell MW, Slater NGP, Cooper JE. Operating theatre water-baths as a cause of *Pseudomonas* septicemia. *J. Hosp. Infect.* Jan. 1981;2(C):237–40.
- [26] Bray BM. Risk of infection from water bath blood warmers. *Anaesthesia* Jul. 1987;42(7):778–9.
- [27] Walther-Wenke G, et al. Monitoring bacterial contamination of blood components in Germany: effect of contamination reduction measures. *Vox Sang.* May 2011;100(4):359–66.
- [28] Baboo J, et al. The Impact of Varying Cooling and Thawing Rates on the Quality of Cryopreserved Human Peripheral Blood T Cells. *Sci. Rep.* 2019;9(1):1–13.
- [29] Onofre J, Baert Y, Faes K, Goossens E. Cryopreservation of testicular tissue or testicular cell suspensions: a pivotal step in fertility preservation. *Hum. Reprod.* Update 2016;22(6):744–61.

- [30] Moll G, et al. Intravascular Mesenchymal Stromal/Stem Cell Therapy Product Diversification: Time for New Clinical Guidelines. *Trends Mol. Med.* 2019;25(2):149–63.
- [31] Kilbride P, et al. Spatial considerations during cryopreservation of a large volume sample. *Cryobiology* Aug. 2016;73(1):47–54.
- [32] Selves P, Planelles D, Mirabet V, Blanquer A, Carbonell-Uberos F. Qualitative and quantitative cell recovery in umbilical cord blood processed by two automated devices in routine cord blood banking: a comparative study. *Blood Transfus.* 2013;11(3):405–11.
- [33] FDA and Cber, "Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs)," <https://www.gmp-compliance.org/files/guidemgr/cmcsomcell.pdf>. March 2020
- [34] De Rosa A, et al. A new method for cryopreserving adipose-derived stem cells: an attractive and suitable large-scale and long-term cell banking technology. *Tissue Eng. - Part C Methods* 2009;15(4):659–67.
- [35] Hunt CJ. Cryopreservation of human stem cells for clinical application: a review. *Transfus. Med. Hemotherapy* 2011;38(2):107–23.
- [36] Hunt CJ. Technical Considerations in the Freezing, Low-Temperature Storage and Thawing of Stem Cells for Cellular Therapies. *Transfus. Med. Hemotherapy* 2019;46(3):134–49.
- [37] Katkov II, Kan NG, Cimadamore F, Nelson B, Snyder EY, Tersikh AV. DMSO-free programmed cryopreservation of fully dissociated and adherent human induced pluripotent stem cells. *Stem Cells Int.* 2011;2011:981606.
- [38] Mamidi MK, et al. Impact of passing mesenchymal stem cells through smaller bore size needles for subsequent use in patients for clinical or cosmetic indications. *J. Transl. Med.* 2012;10(1):1–10.
- [39] Aguado BA, Mulyasmita W, Su J, Lampe KJ, Heilshorn SC. Improving viability of stem cells during syringe needle flow through the design of hydrogel cell carriers. *Tissue Eng. - Part A* 2012;18(7–8):806–15.
- [40] Agashi K, Chau DYS, Shakesheff KM. The effect of delivery via narrow-bore needles on mesenchymal cells. *Regen. Med.* Dec. 2009;4(1):49–64.
- [41] Amer MH, White LJ, Shakesheff KM. The effect of injection using narrow-bore needles on mammalian cells: Administration and formulation considerations for cell therapies. *J. Pharm. Pharmacol.* 2015;67(5):640–50.
- [42] Wahlberg B, Ghuman H, Liu JR, Modo M. *Ex vivo* biomechanical characterization of syringe-needle ejections for intracerebral cell delivery. *Sci. Rep.* 2018;8(1):9194.
- [43] Woods EJ, Bagchi A, Goebel WS, Vilivalam VD. Container system for enabling commercial production of cryopreserved cell therapy products. *Regen. Med.* Jul. 2010;5(4):659–67.
- [44] Verjans B, Reed CH. "Assessing Filling Technologies For Contamination Risk". *BioPharm International*. 2012;25(3).
- [45] Lovelock JE, Bishop MWH. Prevention of freezing damage to living cells by dimethyl sulphoxide. *Nature* 1959;183(4672):1394–5.
- [46] Mathew AJ, Baust JM, Van Buskirk RG, Baust JG. Cell Preservation in Reparative and Regenerative Medicine: Evolution of Individualized Solution Composition. *Tissue Eng. Nov.* 2004;10(11–12):1662–71.
- [47] Ostrowska A, Gu K, Bode DC, Van Buskirk RG. Hypothermic storage of isolated human hepatocytes: a comparison between University of Wisconsin solution and a hypothermosol platform. *Arch. Toxicol.* May 2009;83(5):493–502.
- [48] Ginis I, Grinblat B, Shirvan MH. Evaluation of bone marrow-derived mesenchymal stem cells after cryopreservation and hypothermic storage in clinically safe medium. *Tissue Eng. - Part C Methods* 2012;18(6):453–63.
- [49] Rossetti T, Nicholls F, Modo M. Intracerebral cell implantation: preparation and characterization of cell suspensions. *Cell Transplant.* 2016;25(4):645–64.
- [50] Petrenko Y, et al. Clinically relevant solution for the hypothermic storage and transportation of human multipotent mesenchymal stromal cells. *Stem Cells Int.* 2019;2019:5909524.
- [51] Didwania A, Miller J, Kassel D, Jackson EV, Chernow B. Effect of intravenous lactated Ringer's solution infusion on the circulating lactate concentration: Part 3. Results of a prospective, randomized, double-blind, placebo-controlled trial. *Crit. Care Med.* Nov. 1997;25(11):1851–4.