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Biopreservation Considerations for Regenerative Medicine GMP Manufacturing

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BioLife Solutions has been pioneering Biopreservation Best Practices for Cell Therapy, Regenerative Medicine, Drug Discovery, and Biobanking for over 20 years. A key component of our customer's success has been our unique and collaborative relationship with industry and the research community; especially supporting the optimization of methods and integration with Good Manufacturing Practice (GMP).

Aby J. Mathew, Ph.D. and our team of Ph.D. scientists have enabled biopreservation standardization and optimization for a diverse spectrum of customer applications, and are available to answer any questions you may have.

For example:

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- Can HypoThermosol® be infused/injected into humans?
- What post-thaw assays are best for cell recovery?

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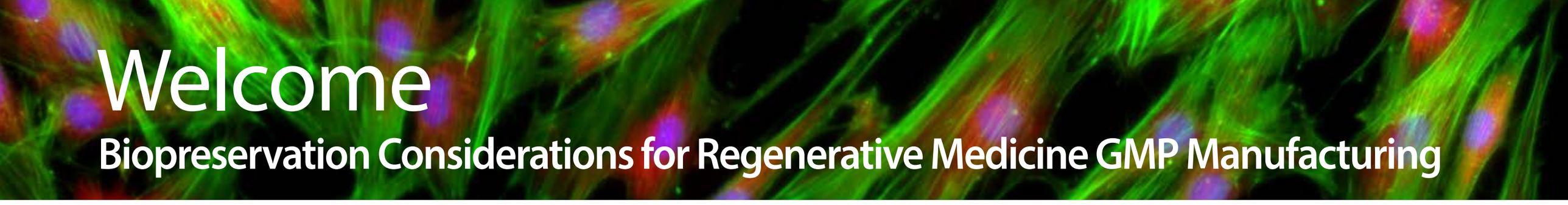
BIOPRESERVATION TOOLS FOR REGENERATIVE MEDICINE



Welcome

Biopreservation Considerations for Regenerative Medicine GMP Manufacturing

We'd also like to welcome you to the next phase of human health—the age of Regenerative Medicine.

At first, it was pharmaceuticals, such as antibiotics and drugs, manufactured relatively simply and cheaply for many. Next was the era of biologics (roughly thirty plus years ago), with antibodies and other medicines taking center stage. Then followed the medical device phase—sensors, silicon chips, software, and technology all powering medical devices that maintained and extended people's health. All of these, however, occur external to the patient, and we are now seeing a monumental shift in where health care is moving. This next wave, called "Regenerative Medicine" —involves leveraging the body's innate ability to "heal itself." Cells can now be taken directly from a patient's body, be transported to a cGMP manufacturing facility, get engineered or edited, scaled up, in some cases purified, and then reinserted into the same patient with a newfound ability to cure cancer, fix broken genes, or regenerate tissues. Although we see just a few of these finally reaching the commercial phase, there is no doubt there will be many more soon, as we are at the forefront of what we will see from the industry over the next twenty years. One thing is certain—it will astound us all.

That's not to say there won't be challenges along the way; since "living drugs" (cell therapies) bring with them new levels of complexity. Cures that work well in the lab face a myriad of challenges on the road to scale up and commercialization—including maintaining these living cells when they leave the donor to the place of engineering, followed by all the commercialization steps necessary before they return. These include apheresis collection, cGMP manufacturing, process development, scale-up, and administration. Finally, the more critical (and often overlooked) components along the path include biopreservation optimization of cells and tissues from the patient and back; and the ever-important shipping (cold chain and tracking visibility) component.

At BioLife Solutions, Inc., we strive to be the leading provider of biopreservation tools for regenerative medicine, to facilitate basic and applied research and commercialization of new therapies by maintaining the health and function of biologic source material and finished products during manufacturing, distribution, and clinical administration. We've made it our mission for over two decades to support Biopreservation Best Practices—applying the best products, expertise, and technical support to help organizations be successful with all their biopreservation challenges. This approach is working, with our products being used in over 300 regenerative medicine applications and with 375+ papers, posters, and abstracts with cryopreservation or hypothermic preservation citing our products. A few of our favorites are included within.

Since the biopreservation component of Regenerative Medicine is often overlooked during the critical transition to GMP manufacturing, we've created this eBook to help guide you along your path.

BioLife Solutions®

BIOPRESERVATION TOOLS FOR REGENERATIVE MEDICINE

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Mary Ann Liebert, Inc. publishers

WHITE PAPER

Biopreservation Considerations for Regenerative Medicine GMP Manufacturing

Aby J. Mathew, Ph.D.

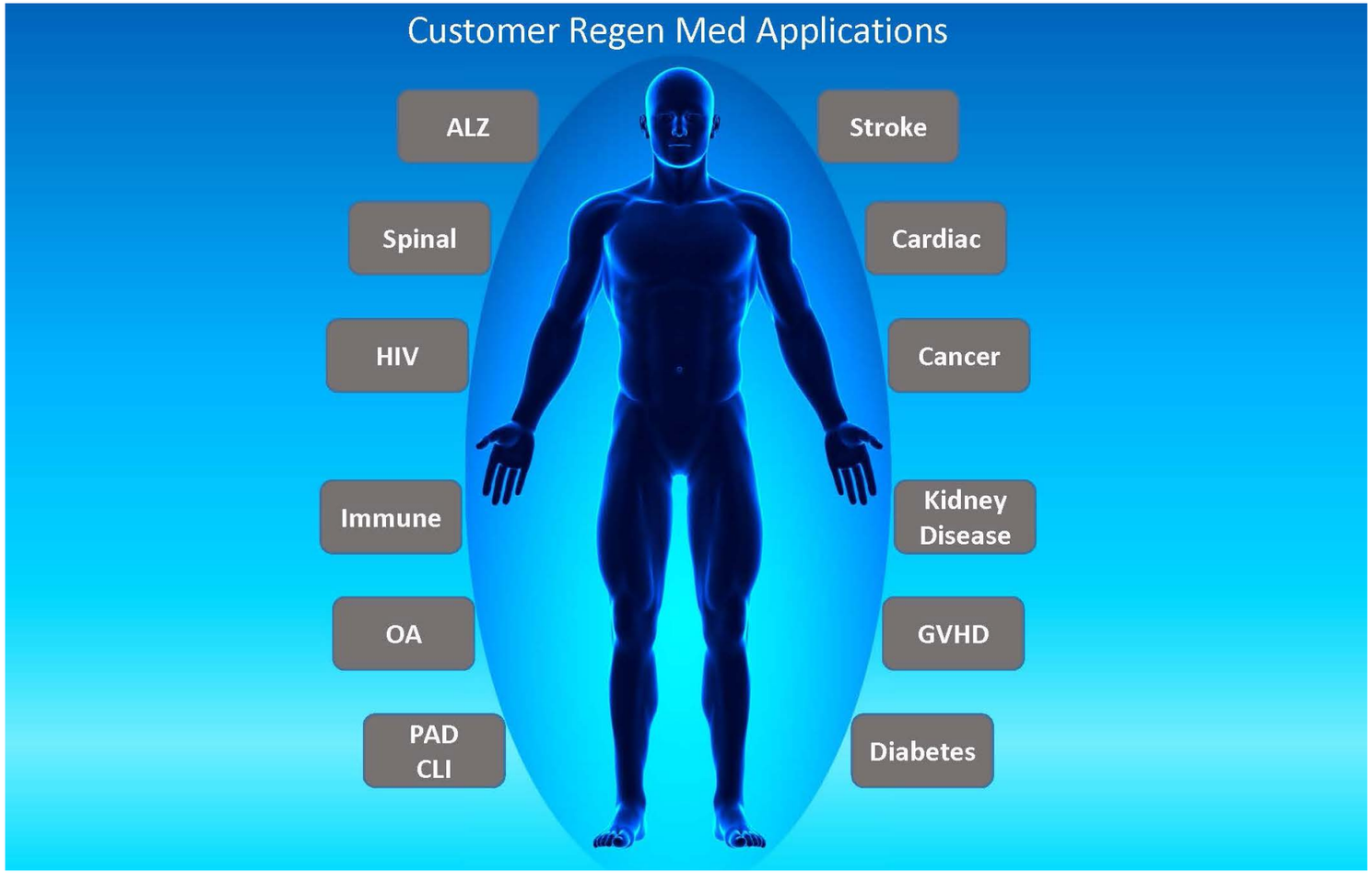
Appropriate clinical biopreservation of cells and tissues is a critical factor in cellular therapies, tissue engineering, and regenerative medicine. These applications utilize cell and tissue material sourced from blood, bone marrow, and various tissues. The clinical and commercial utility of these products is potentially impacted by steps that may reduce stability, including transport of the source material and biopreservation of the final cell or tissue product (either frozen or non-frozen).

Often in cell and tissue processing, a gap exists between biopreservation method optimization from a cryobiology perspective and the process development that results in the cryopreserved or non-frozen cell/tissue product. Traditional home-brew reagent cocktails (including serum) utilized for biopreservation are a risk concern within Good Manufacturing Practices (GMP) clinical manufacturing processes, and they may be a suboptimal option compared to pre-formulated GMP intracellular-like formulations. Furthermore, manufacturing

development often focuses on stability of the final cell/tissue product (even then, without optimization), rather than biopreservation of the cell-based materials throughout the entire lifecycle workflow of the manufacturing system (i.e., from source material procurement through intermediate manufacturing to final cell/tissue product, including transport/delivery steps).

Preserve and Protect

Biopreservation encompasses methods that suppress the degradation of biological material for the post-preservation recovery of structure, viability, and function.[1] Living biological samples—cells and tissues—are far more complex than simple sugars, proteins, antibodies, or nucleic acids. During normothermic isotonic conditions (normal body homeostasis, cell culture, etc.), the environment of human cells and tissues is maintained by active cellular mechanisms to establish an osmotic balance inside and outside the cell. Cells are bathed in an extracellular milieu of ions and molecules that differs from the intracellular environment. From the moment a biospecimen is removed from its native environment, a degradation process begins, and stresses accumulate. The subsequent level of cell damage and cell death impacts the



BioLife's biopreservation solutions are embedded in manufacturing processes for a number of customer regenerative medicine cellular therapies targeting a wide range of clinical applications.

ultimate utility of the cells or tissues within the manufacturing process or clinical application. Cell degradation can be slowed or stopped using appropriate biopreservation methods, most often in current GMP/clinical methodologies via low-temperature preservation that suppresses oxygen demand, metabolic processes, and enzymatic/chemical reactions. Hypothermic storage (2–8°C) and cryopreservation (slow-freezing) are the most common methods used for manufacture of cellular therapies.[1-6]

The stresses that can occur and accumulate as a result of stages outside of normothermic conditions can manifest themselves at many cellular levels—membrane, mitochondria, ion dysregulation, enzymatic, and various "omics" (genomic, proteomic, metabolomic, etc.). Many traditional preservation methods focus on providing an isotonic environment for the cells and tissues, similar to the extracellular milieu under normothermic conditions. However, under low-temperature conditions, the cellular state is not the same as under normothermic conditions, caused in part by inactivation of ATP-driven ion pumps that would normally maintain osmotic balance, membrane phase changes, organelle instability, free radical generation, and water flux. Ultimately, the accumulation of stresses may cause cell death via apoptosis, necrosis, and/or secondary necrosis. Even if the cell survives, the accumulation of cell damage and stress may cause cellular resources to be devoted to cell repair and recovery, and potentially delay the cellular capacity to return to functional performance (i.e., functional lag). Traditional methods of cell assessment post-preservation—such as membrane integrity dyes immediately post-preservation—also may not reflect the time-related and multi-faceted cellular mechanisms associated with Delayed Onset Cell Death resulting from hypothermic storage and/or cryopreservation.[1-6]

More recent methods of biopreservation take an intracellular-like approach to balancing the cells and tissues at low temperatures[1,5]. An intracellular-like design—in contrast to an isotonic/extracellular-like solution such as culture media or saline—seeks to balance the altered cellular ion concentrations that results from low temperatures and nutrient-deprived conditions that exist when cells and tissues are without normal blood supply and normothermic conditions.[1] The evolution of intracellular-like preservation media has significantly advanced the field of organ preservation.[7-8] Further development and use of optimized methods that utilize intracellular-like biopreservation solutions has been demonstrated for the collection, storage, and transport of cells and tissues.[1,5,9,10] Examples of such media include the intracellular-like formulations HypoThermosol® FRS and CryoStor®.

Beyond Basic Science

Considerations toward best practices and GMP manufacturing are recommended to assess aspects beyond the basic science mechanisms for cell culturing. Biopreservation media for cell and tissue therapies are ancillary materials in a cell manufacturing process—they are not drugs or medical devices. In this regard, biopreservation media for manufacturing are qualified as a material within the manufacturing process. The manufacturer of the cell/tissue product may also choose to qualify the biopreservation media for

excipient application (not washed post-preservation; part of final cell/tissue product and patient application). The Quality/Regulatory footprint of materials utilized within GMP manufacturing is assessed in consideration to a risk-based approach[11-12]. Characteristics of biopreservation media that can mitigate Quality/Regulatory risk include being serum-free, protein-free, animal-origin-free, fully-defined, manufactured per GMP with minimal batch variability, and supported by Quality/Regulatory documentation.

Best practices in methods of post-preservation assessment also serve to align assessment methods with cell product viable recovery and functional performance following hypothermic preservation and/or cryopreservation. Although the clarification of Delayed Onset Cell Death was introduced 20 years ago, in relation to the mechanisms of cell death resulting from hypothermic storage and cryopreservation[3,4,6,13-14], there is often still a lack of integration of those principles within manufacturing, cell product release testing, and expectations for product performance. Ongoing efforts to further understand mechanisms of cell damage, cell death, post-preservation assessment, and assay timing, have suggested improved methods for aligning and integrating post-preservation assessment with the status of cell quality.[15]

Evolving Approaches

The mission of developing and optimizing Biopreservation Best Practices is an ongoing, multi-faceted endeavor, aligning scientific principles (cryobiology, cell/molecular biology) with discovery tools (patient samples, disease models, drug discovery) and cell manufacturing methods (GMP, scale-up, closed systems), and then ultimately with applications for use (research, clinical, cold chain delivery). Historical methods incorporating isotonic-based home-brew cocktails and individually-targeted cryoprotectants have been in standard research and clinical practice for decades. Paradigm shifts in scientific technology and clinically-related methods are often slow to evolve or to be adopted. However, more recent reports of research studies related to intracellular-like biopreservation methods optimization, and clinical studies utilizing cell and tissue products that have been cryopreserved or stored non-frozen in intracellular-like biopreservation media, reflect a growing shift away from isotonic-based home-brew cocktails which may contain serum or protein, are formulated at point-of-use without stringent testing, and may not align with the Quality/Regulatory footprint in support of qualification for excipient application.

Models that have previously shown limitations in preservation have demonstrated improved post-preservation viable recovery and functionality utilizing intracellular-like biopreservation methods. Examples include extended hypothermic storage of cardiomyocytes with maintenance of contractile function[16], cryopreservation of cells adhered to a substrate[17], and cryopreservation of neurospheres.[18]

Studies with a biobanking focus reveal the enhanced stability potential of intracellular-like biopreservation for shipment of cells, including

stability at lower cost dry ice conditions (~–80°C) in comparison to liquid nitrogen dry shipper temperatures (–150°C or colder).[19] Some biobanking method validations have also incorporated intracellular-like cryopreservation methods for automated isolation of cells.[20]

Discovery research models for aligning upstream source material (tissues, blood and marrow, biopsies) with downstream testing and alignment with patient disease treatment efficacy, have long recognized the risk in achieving accurate research conclusions or patient treatment if cell and tissue samples do not accurately reflect the patient condition. The stability intervals between patient sample procurement and cell/tissue processing may result in various stresses, as described above, that result in non-alignment with the original patient sample. In fact, this disconnect might lead to reduced drug efficacy or increased variability in study data, when drug development characterization drifts away from disease states. Until recently, the development of Patient-Derived Xenografts (PDX) for testing cancer tumor models relied on fresh tumor samples with just-in-time experimental analysis. Utilizing intracellular-like biopreservation methods, there have been enhanced models related to tissue storage[21], PDX models[22-23], and blood-derived source material[24].

Subsequent integration of improved intracellular-like biopreservation methods have shown utility in regenerative medicine pre-clinical cell models[25-28], pre-clinical animal models[29-34] and veterinary applications[35]. Validation of the utility of the intracellular-like Biopreservation Best Practices approach, as well as the alignment with stringent quality and regulatory risk mitigation, has been shown in numerous human clinical applications[36-48], including hematopoietic stem cell transplantation[49], and novel therapies that have received regulatory approval and marketing authorization[50-51], using HypoThermosol® FRS and CryoStor®.

Conclusion

The living components of cells and tissues required for the next generation of research and therapies are vulnerable to stability limitations of traditional methods that may not be optimized for the evolving therapeutic portfolio. Appropriate clinical biopreservation of cells and tissues is a critical factor in cellular therapies, tissue engineering, and regenerative medicine. Biopreservation methods should be optimized and validated in consideration to scientific, Quality/Regulatory, and GMP manufacturing, needs, as well as the needs for maximum clinical and commercial efficacy. ■

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Evaluation of Bone Marrow–Derived Mesenchymal Stem Cells After Cryopreservation and Hypothermic Storage in Clinically Safe Medium

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Abstract

Achievements in tissue engineering using mesenchymal stem cells (MSC) demand a clinically acceptable "off-the-shelf" cell-therapy product. Efficacy of cryopreservation of human bone marrow–derived MSC in clinically safe, animal product–free medium containing 2%, 5%, and 10% dimethyl sulfoxide (DMSO) was evaluated by measuring cell recovery, viability, apoptosis, proliferation rate, expression of a broad panel of MSC markers, and osteogenic differentiation. Rate-controlled freezing in CryoStor media was performed in a programmable cell freezer. About 95% of frozen cells were recovered as live cells after freezing in CryoStor solutions with 5% and 10% DMSO followed by storage in liquid nitrogen for 1 month. Cell recovery after 5 months storage was 72% and 80% for 5% and 10% DMSO, respectively. Measurements of caspase 3 activity demonstrated that 15.5% and 12.8% of cells after 1 month and 18.3% and 12.9% of cells after 5 months storage

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in 5% and 10% DMSO, respectively, were apoptotic. Proliferation of MSC recovered after cryopreservation was measured during 2 weeks post-plating. Proliferation rate was not compromised and was even enhanced. Cryopreservation did not alter expression of MSC markers. Quantitative analysis of alkaline phosphatase (ALP) activity, ALP surface expression and Ca^{++} deposition in previously cryopreserved MSC and then differentiated for 3 weeks in osteogenic medium demonstrated the same degree of osteogenic differentiation as in unfrozen parallel cultures. Cell viability and functional parameters were analyzed in MSC after short-term storage at 4°C in HypoThermosol-FRS solution, also free of animal products. Hypothermic storage for 2 and 4 days resulted in about 100% and 85% cell recovery, respectively, less than 10% of apoptotic cells, and normal proliferation, marker expression, and osteogenic potential. Overall, our results demonstrate that human MSC could be successfully cryopreserved for banking and clinical applications and delivered to the bedside in clinically safe protective reagents.

Introduction

Adult mesenchymal stem cells (MSC) are adherent stromal cells that are able to self-renew and differentiate into a variety of cells and tissues. A possibility for autologous transplantation and non-tumorogenic character of MSC makes them an attractive tool for tissue engineering. The most progress achieved thus far in animal studies has been in bone and cartilage repair by MSC derived from bone marrow (BM) and adipose tissue.[1–5] Another important value of MSC is their ability to modulate immune responses of T lymphocytes, natural killer cells, and antigen presenting cells.[6,7]

Despite the progress in experimental regenerative studies and accumulating data on possible applications of MSC as immunomodulators and sources of trophic factors in various clinical conditions,[8] clinically accepted methods of cryopreservation and storage of MSC are poorly developed. Standardized procedures for clinical grade cryopreservation of MSC are needed for accumulation of large numbers of autologous cells for one time or repetitive transplantations during a long process of tissue repair, especially for bone regeneration, and also for creation of banks of allogeneic MSC.

A defined medium for a short storage of MSC without freezing is also of importance, as it will improve transportability of stem cells and will allow the necessary time for the completion of safety and quality control tests.[9,10]

Many researchers have previously reported that cryopreserved MSC maintain their potential for proliferation and osteogenic differentiation *in vitro*[11–17] and *in vivo*.[18,19] However, the non-approved reagents were used in most cases without procedural optimization and validations.[20] In a recent study sponsored by EU consortium "Crystal" (CRYo-banking of Stem cells for human Therapeutic AppLications), a cryopreservation procedure in an animal product–free and chemically defined cryopreservation media has been described for umbilical cord blood–derived erythroid and endothelial progenitors as well as adipose tissue mesenchymal stromal cells. Recovery and differentiation potential after cryopreservation were analyzed with promising results.[21]

In the current study, cryopreservation of human BM–derived MSC in the commercial xeno-free media containing 2%, 5% and 10% dimethyl sulfoxide (DMSO) has been optimized and tested with emphasis on cell recovery, apoptosis, long-term proliferation kinetics, marker expression, and quantitative analysis of osteogenic differentiation. Rate-controlled freezing of MSC was performed using different regimens. The results of the study demonstrate good and reproducible physical and functional recovery of cryopreserved MSC after 5 months storage in liquid nitrogen, followed by optimized thawing procedure.

MSC were also subjected to a short-term storage at 4°C in a xeno-free HypoThermosol medium. Quantitative viability and functional tests were performed before and after storage in the same MSC cultures. We demonstrate that hypothermic storage of MSC in this animal product–free medium for 4 days does not affect MSC viability, proliferation, and osteogenic potential.

MATERIALS AND METHODS

Isolation of MSC

BM aspirates were purchased from Lonza, USA, and delivered within 36 h to Israel at 4°C. MSC were isolated according to adhesion selection method as described elsewhere.[22] As previously shown, these cells expressed the main markers of MSC, were negative for CD45, and were capable of differentiation into osteoblasts, adipocytes, and chondrocytes.[22] Cells were passaged in MSC growth medium (GM) purchased from Lonza by plating 5000 cells/cm^2. The average yield of MSC at confluency was 50–60,000 cells/cm^2, suggesting 3–4 cell doublings between passages. Cells of passages 2–4 that had not been previously frozen were used in all the experiments.

Cryopreservation protocol

MSCs were re-suspended in cold animal product-free cryopresrvation media: CryoStor-2 (CS-2), CryoStor-5 (CS-5), and CryoStor-10 (CS-10) containing 2%, 5%, and 10% DMSO, respectively (BioLife Solutions Inc.), or in conventional freezing medium (FM; 90% complete GM from Lonza containing 10% fetal calf serum [FCS]/30% by weight bovine serum albumin [BSA]/10% DMSO) at 10^6 cells/mL and aliquoted into cryo-vials (NUNC/Thermo Fisher Scientific) at 0.5 mL/vial (500,000 cells). The vials were pre-cooled on ice for 10 min and then placed in the programmable cell freezer Kryo-560-16-230 (Planer PLC). Samples were slowly cooled to −5°C (freezing point for CryoStor reagents) and then given a blast of chilling to −25°C and quick return to −5°C to prevent super-cooling and to ensure extracellular ice nucleation. From that point, samples were slowly cooled to −60°C at 1°C/min, and then, fast frozen at −25°C/min down to −196°C. A temperature sensor was inserted in a cryo-vial containing an appropriate cryo-medium. Frozen cells were stored in liquid nitrogen (liquid phase) for about 1 month or for 5 months.

Thawing procedure

Cells were thawed fast in a 37°C water bath with gentle agitation without allowing the sample to warm above chilled temperatures (0°C −10°C;

until ice crystals still remained visible) and then gradually (drop wise) resuspended in cold GM in order to avoid drastic changes in osmolarity and prevent toxicity of cryo-protective reagents. Cell suspension in GM was centrifuged at 250 g and resuspended in 0.5 mL phosphate buffer saline (PBS) containing calcium and magnesium. The cell suspension in PBS was aliquoted as follows: a 100 microL suspension was taken for a Live/Dead assay (see below), and 300 microL were taken for apoptosis assay. The rest of the cells were used for plating for proliferation and differentiation assays at 3000 cells/cm^2 based on live cell counts with Trypan blue.

Live/dead assay

CImmediately after thawing, 100 microL of cell suspension in PBS was mixed in a 24-well plate with 0.5 mL PBS (with Ca^{++} and Mg^{++}) containing 5 microM of Calcein-AM (Invitrogen), an indicator of live cells, and 10 microM of Ethidium homodimer-1 (Invitrogen), an indicator of dead cells. The plate was incubated in a CO_2 incubator at 37°C for 35 min, then centrifuged at 250xg for 5 min to pellet the cells to the bottom in order to get more precise fluorescence readings. Cell fluorescence was measured on a Synergy-BioTek plate reader with a fluorescence detector at the bottom of the plates at an excitation/emission of 485/530 nm and 530/645 nm for Calcein-AM and Ethidium respectively. Background fluorescence of a PBS solution containing 5 microM Calcein-AM and 10 microM Ethidium was subtracted from each value. Live cell number detected by Calcein AM was calculated based on a standard curve (see Proliferation Assay next). The maximal Ethidium fluorescence signal was obtained by permeabilization of 100,000 MSC with 0.1% saponin. Percentage of dead cells was calculated as follows:

$$\%\ deadcells = \frac{F\,x - Fbckgr}{F\,max - Fbckgr},$$

where Fx was fluorescence of a cell sample, Fbckgr was fluorescence of the background wells without cells, and Fmax was a fluorescence of the same number of cells (100,000) treated with 0.1% saponin.

Measuring cell proliferation with Calcein-AM

Thawed MSC were plated in duplicates into 24-well plates at 3000 cells/cm^2 based on the Trypan blue counts of live cells. Cells were allowed to proliferate for 14 days in GM with medium changed twice a week. Cell number was measured the next day after plating (day 1), and on days 4, 7, and 14. At each time point, a number of viable cells was measured by Calcein-AM staining. For the standard curve, serial dilutions of unfrozen live MSC pre-stained with Calcein-AM were plated onto a 24-well plate. The cells were spun down to the bottom of the wells, and Calcein-AM fluorescence was measured.

Measurement of apoptotic cells: flow cytometry analysis of Annexin-V binding

The AnnexinV-FITC kit (IQProducts) was used. Thawed MSC suspension were immediately labeled with Annexin V according to manufacturer's instructions and analyzed on FACSAria (Becton Dickinson Biosciences) using FITC channel.

Measurement of apoptotic cells: flow cytometry analysis of Hoechst staining

Thawed MSC were resuspended in fluorescence-activated cell sorter (FACS) buffer containing 10 microg/mL Hoechst 33342 (Sigma) at 200,000 cells/200 microL. Cell suspension was incubated for 10 min at 37°C in the dark and then analyzed on FACSAria.

Measurement of apoptotic cells: flow cytometry analysis of caspase 3 activity

The EnzChek Caspase 3 Assay Kit (Invitrogen) for adherent cells was modified for flow cytometry. Cryopreserved 300,000 MSC were thawed and immediately resuspended in 300 microL Pipes/EDTA/Chaps reaction buffer supplemented with 3 microL dithiothreitol (no previous lysis of cells was performed); then, 1.5 microL Z-DEVD-R110 rhodamine substrate was added to cell suspensions. The samples were incubated for 20 min at 37°C in the dark, and transferred on ice to stop the reaction. Two controls were performed: (1) cells plus substrate reaction mixture were kept on ice for 20 min and (2) DMSO was added instead of a substrate, and samples were incubated for 20 min at 37°C. In some experiments, cells were preincubated with 1.5 microL of the 1 mM stock solution of the caspase 3 specific inhibitor Ac-DEVD-CHO for 10 min at 37°C and then incubated with caspase 3 rhodamine substrate. At the end of the incubation, 7-AAD (eBioscience) was added to the reaction mixture (15 microL of the 50 microg/mL stock solution per sample) for detection of dead cells. The samples were analyzed on FACSAria using the FITC channel for measuring fluorescence of the caspase 3 product and PerCP channel for 7-AAD.

Hypothermic storage protocol

MSC cultures were grown to 70%–80% confluency, and GM was replaced with pre-cooled HypoThermosol-FRS medium (HTS-FRS, BioLife Solutions). Cell cultures were stored at 4°C for 2 and 4 days in the laboratory refrigerator with a temperature control and alarm system. At the end of the storage period, HTS-FRS medium was replaced with warm GM, and the cultures were moved to a CO_2 incubator and allowed to recover at 37°C for 3 h. At the end of the recovery period, the cells were detached by trypsinization and used for flow cytometry analysis of dead cells (7-AAD staining), apoptotic cells (caspase 3 activity), and expression of MSC markers. For proliferation and differentiation assays, MSC were subjected to hypothermic storage and recovery and then continued growing in GM and osteogenic medium (OM), respectively, without replating.

Measuring live cells with Alamar Blue

In order to measure the number of live cells before and after hypothermic storage in the same cultures, an oxidation-reduction indicator Alamar Blue (AbD Serotec) that chemically interacts with metabolic products released by living cells into the culture medium was used. MSC were plated in duplicates into 24-well plates at 3000 cells/cm^2 and allowed to grow to subconfluency. Right before hypothermic storage, a 0.5 mL/well of fresh GM containing 10% Alamar Blue was added to the cells, and plates were placed for 2 h in a cell incubator. At the end of the incubation, the medium containing Alamar Blue was transferred into a

new 24-well plate, and its fluorescence was measured on a Synergy-BioTek plate reader at an excitation/emission of 530/590 nm. HTS-FRS was added to the cells, and the cells were stored for indicated times at 4°C. At the end of the storage and recovery, the number of live cells was measured again with Alamar Blue. A standard curve was produced by plating MSC in serial dilutions into 24-well plates. Cells were allowed to adhere overnight in the cell incubator. The next day, a fresh medium with 10% Alamar Blue was added to the cells, and Alamar Blue fluorescence was measured. In order to account for possible cell proliferation during the overnight adhesion period, the live cell number was measured with Calcein-AM and plotted against Alamar Blue fluorescence. The standard curve was highly linear (R^2=0.9952).

Staining cells with antibodies against MSC markers

Immediately after thawing, MSC were resuspended at 10^6/mL in FACS buffer [2% BSA (Sigma), 2% human blocking serum (Chemicon) in DMEM without phenol red (Sigma)], and aliquoted at 100 microL per well (100,000 cells) onto a polypropylene U-shaped 96-well

Table 1. Antibodies Against Mesenchymal Stem Cells Markers Used for Flow Cytometry

Product name	*Fluorochrome*	*Clone*	*Producer*	*Cat#*
CD105	**APC**	**SN6**	**eBioscience**	**17-1057-73**
CD44	**APC-Cy7**	**IM7**	**eBioscience**	**10-0441-81**
CD9	**PerCp-Cy5.5**	**ML13**	**Pharmigen**	**341649**
CD166	**FITC**	**3A6**	**Serotech**	**MCA1926F**
CD90	**PE**	**5-E10**	**Pharmigen**	**555596**
ALP	**APC**	**B4-78**	**R&D**	**1448A**

APC, antigen presenting cells.

plate. Each cell aliquot was incubated with saturating concentrations of antibodies against MSC markers or isotype-matched controls on ice for 30 min; then, the plate was centrifuged at 200xg for 3 min and inverted onto a paper towel to drain the supernatant. The cells were washed twice in 200 microL FACS buffer and transferred into a polypropylene FACS test tube containing 500 microL FACS buffer. Analysis of the results was performed using DIVA software (Becton Dickinson). The list of antibodies used in the experiments is shown in *Table 1*.

Osteogenic differentiation of MSC

Thawed MSC were plated in GM overnight, and then the GM was replaced either by OM (alpha-MEM [Sigma], 10% FCS heat inactivated [Biological Industries], 0.2 mM L-ascorbic acid-2-phosphate [Mg salt n-hydrated; Fluka], 10 mM Glycerol 2-phosphate disodium salt hydrate [Sigma], 100 nM dexamethasone [Sigma], 2 mM GlutaMAX™-I Supplement [Invitrogen], 100 units/mL penicillin, 0.1 mg/mL streptomycin, 0.25 mg/mL amphotericin B) or by fresh GM for comparison. The cells were grown for various periods of time while the OM was changed twice a week.

Alkaline phosphatase activity assay

MSC cells growing in 24-well plates in OM and control cultures growing in GM were lysed on days 7 and 14 with 500 microL cold lysis buffer (1 mM $MgCl_2$/0.5% Triton×100 in Alkaline Buffer Solution [Sigma]) and incubated on ice for 1 hour. The lysates were centrifuged at 13,000 rpm for 2 min, and 100 microL cell lysates were combined with 400 microL Phosphatase Substrate Solution (20 mg/mL of p-nitrophenol phosphate [p-NP; Sigma] in 5 mL Alkaline Buffer Solution diluted 1:3 in ddH_2O) and incubated at 37°C for 10 min. The reaction was stopped with 500 microL EDTA-NaOH stop solution (20 g NaOH plus 37.22 g Na_2EDTA in 500 mL ddH_2O). 200 microL of each sample was transferred to a 96-well plate, and absorbance was read at 404 nm using a Synergy plate reader. The results were expressed as nmol p-NP/mL/min and normalized to the number of living cells in the corresponding wells determined by Calcein-AM assay as just described.

Alkaline phosphatase surface expression

MSC cells growing in 25cm^2 flasks in OM and control cultures growing in GM were trypsinized on day 14 and stained with antibodies against human alkaline phosphatase (ALP; liver/bone/kidney isozyme; see antibody information in *Table 1*). Stained cells were analyzed by flow cytometry.

Calcium deposition assay

MSC cells growing in 24-well plates in OM for 21 days and parallel cultures growing in GM were washed with PBS without Ca^{++}, Mg^{++} and then lysed with 250 microL/well 0.5N HCl. The lysates were shaken at 4°C overnight to extract calcium and then centrifuged at 13,000 rpm for 2 min at 4°C. The assay was set up in 96-well plates using Calcium Liquicolor kit (Stanbio Labs) according to the manufacturer's instructions. The amount of Ca^{++} was normalized to the number of living cells in the corresponding wells determined by Calcein-AM assay as just described.

Statistical analysis

A comparison between control and experimental groups in parallel cultures was performed with Student's *t-test*. For multiple measurements, the Anova test was used.

RESULTS

MSC injury induced by the slow freezing phase of the cryopreservation program

The graded freezing technique[23] was used to calibrate Ethidium assay and to differentiate between slow and fast freezing injury. MSC resuspended in various freezing solutions were frozen at –1°C/min to –60°C. At that temperature, the cells were immediately thawed and stained with Ethidium homodimer as described in Methods. In order to exclude possible toxic effects of cryo-solutions on cells during pre-cooling, parallel cultures were kept at 4°C. Maximal Ethidium fluorescence of the same number of cells permeabilized with 0.1% saponin was around 1200 relative fluorescence units (RFU; see *Methods* for details). As shown in *Figure 1A*, Ethidium fluorescence of cells frozen in GM without cryo-protectants was about 400 RFU,

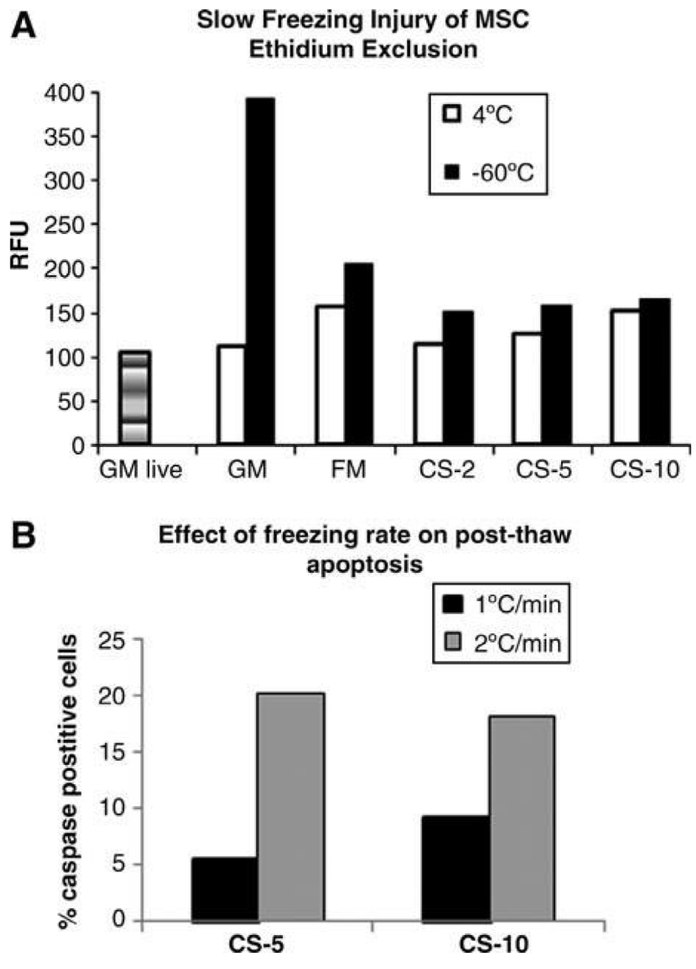


Figure 1. The effect of slow freezing rate on MSC viability. (A) Effect of slow freezing on Ethidium fluorescence. MSC resuspended either in regular GM containing 10% FCS and no cryoprotectants or in the conventional FM containing 10% FCS, 30% bovine serum albumin and 10% dimethyl sulfoxide (DMSO) or in animal product–free CryoStor reagents were frozen at –1°C/min to –60°C. At that temperature, cells were immediately thawed and stained with Ethidium homodimer; parallel cultures re-suspended in all the reagents just mentioned were kept at 4°C during the duration of the slow freezing. A representative experiment is shown. (B) Effect of freezing rate on MSC apoptosis. MSC were frozen in CryoStor-5 (CS-5) and CryoStor-10 (CS-10) at 1°C/min and 2°C/min down to –60°C, and the percentage of apoptotic cells was measured by caspase 3 assay. A representative experiment is shown. MSC, mesenchymal stem cells; GM, growth medium; FM, freezing medium; FCS, fetal calf serum; RFU, relative fluorescence unit.

suggesting that about 30% of cells frozen without cryo-protectants had already died during the slow freezing phase. Fluorescence of MSC slowly frozen in conventional FM and in CryoStor reagents was not different from that of live cells kept in suspension in GM at 37°C during the duration of the slow freezing phase (see *Fig. 1A*). Keeping cells in suspension allowed for accounting for a possible cell death that occurs after MSC are kept in a non-adherent state for a prolonged time (our unpublished observations).

Manufacturer instructions for CryoStor solutions suggested that after fast thawing, the cells could be diluted with GM in a single-step procedure. We have tested this protocol for MSC and compared it with an alternative procedure where thawed cells were diluted with GM gradually to minimize the osmotic injury. MSC were frozen to –60°C in the conventional FM and in CS-10, then quickly thawed at 37°C, and resuspended in 10 mL GM by adding it in either one step or stepwise. Ethidium fluorescence of the cells resuspended in GM in one step was about 800 RFU, while fluorescence of cells resuspended stepwise was similar to the live cell fluorescence (around 100 RFU), suggesting that stepwise addition of medium during MSC thawing is critical for minimizing osmotic injury.

Viability of MSC after cryopreservation for 1 and 5 months

Recovery of MSC after cryopreservation for 1 month in a CS-2 containing 2% DMSO was about 91.7%, while recovery of MSC cryopreserved in CryoStor solutions with 5% and 10% DMSO was above 95% (*Table 2*). Since CS-2 had inferior cryoprotective ability, only CS-5 and CryoStor-10 were tested during a 5 month cryopreservation. Percentage of viable cells after 5 month storage was lower than that of cells frozen for 1 month: 72% and 80%, respectively.

Percentage of dead cells in samples cryopreserved in CryoStor reagents for 1 month ranged between 13% and 15% and was comparable to that of MSC frozen in conventional FM (17%), while samples frozen without cryo-protectants had significantly more dead cells (72%) (*Table 2*). Similar results were obtained for MSC cryopreserved for 5 months (*Table 2*).

MSC apoptosis after cryopreservation for 1 and 5 months

Calibration of caspase 3 assay for flow cytometry was performed using a sample of apoptotic MSC treated in a suspension with staurosporine, a PK-C inhibitor, and a pro-apoptotic agonist.[24] Representative histograms obtained for staurosporine-treated and control untreated cells are shown in *Figure 2A*. A top histogram was produced with freshly trypsinized MSC. The main peak gated on this histogram was defined as a background caspase 3 activity peak. All the events to the right of this gate were defined as cells with high caspase activity or apoptotic cells. All the events to the left of the background peak are mostly debris lacking caspase activity. The same gate was copied onto histogram produced with MSC incubated with 1 microM staurosporine for 4 h (*Fig. 2A* middle histogram). A sharp increase in the number of the events to the right of the gate (apoptotic cells with high caspase activity)

was registered. When cells incubated with staurosporine were pretreated with caspase 3–specific inhibitor, the number of events with high caspase activity significantly decreased (*Fig. 2A* bottom histogram). A statistical analysis of the histograms is summarized in *Figure 2B*.

The results of caspase 3 assay and also of Annexin V and Hoechst 33342 staining of apoptotic cells in MSC suspensions recovered after 1 month of storage in liquid nitrogen are summarized in *Table 3*. When apoptosis was evaluated by Annexin V or Hoechst 33342 staining, lower percentages of apoptotic cells were observed for all tested media, compared with the results of caspase 3 assay, and no significant differences between FM and CryoStor regents were found. However, when apoptosis was measured by caspase 3 assay, there was on an average 13%–17% apoptotic cells in samples previously cryopreserved in CryoStor reagents, while cryopreservation in conventional FM resulted in only 3% of apoptotic cells (*Table 3*). The differences between samples stored in CS-2, CS-5 and CS-10 were not statistically significant, while the differences between CryoStor reagents and conventional FM were statistically significant.

Table 2. Mesenchymal Stem Cells Recovery[a] and Viability[a] After Cryopreservation for 1 and 5 Months

	1 month		*5 month*	
Conditions	***Live cells[b] (calcein)%***	***Dead cells[b] (ethidium)%***	***Live cells[c] (calcein)%***	***Dead cells[c] (ethidium)%***
Before freezing	**99.5±0.5**	**14.1±1.9**	**99.5±0.5**	**13.5±4.2**
GM	**ND**	**72.2±6.4**	**ND**	**69.8±1.6**
FM	**102.8±3.6**	**17.1±4.4**	**ND**	**ND**
CS-2	**91.7±8.0**	**14.7±1.5**	**ND**	**ND**
CS-5	**95.6±8.8**	**12.9±1.0**	**71.9±8.5**	**14.4±2.1**
CS-10	**95.4±3.6**	**13.0±1.1**	**80.2±12.0**	**15.6±3.2**

[a]Mesenchymal stem cells (MSC) were frozen in growth medium (GM) without cryoprtectants, in a conventional freezing medium (FM), or in CryoStor-2 (CS-2), CryoStor-5 (CS-5), and CryoStor-10 (CS-10) reagents for 1 and 5 months. Percentages of live and dead cells were determined immediately after thawing before cell plating. Parallel cultures were analyzed before freezing.

[b]Mean±SD of 4 experiments performed with 4 MSC batches each derived from a different donor. SD, standard deviation.

[c]Mean±SD of 3 experiments performed with 3 MSC batches each derived from a different donor.

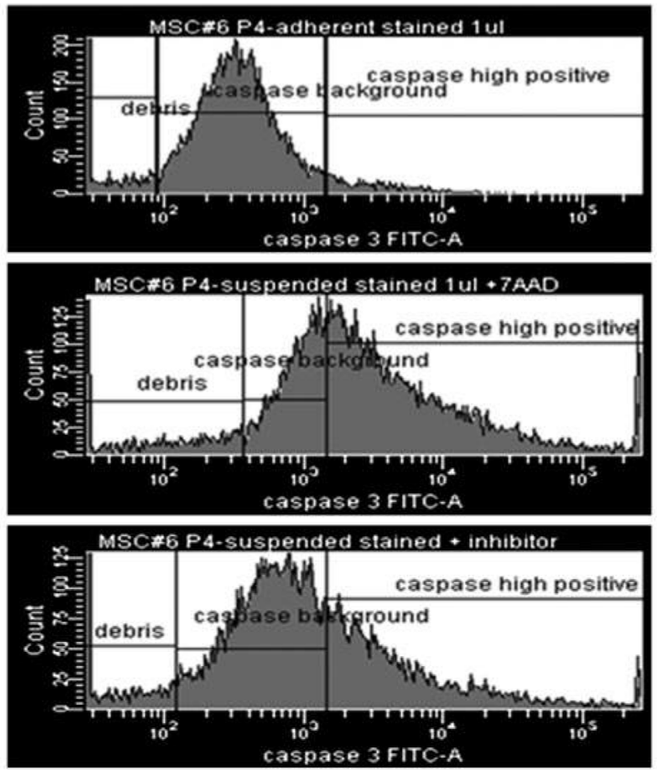
ND, not done.

When MSC were thawed after being cryopreserved in CS-5 and CS-10 for 5 months, similar levels of apoptotic cells compared with one-month storage were observed (*Table 3*).

Proliferation rate of MSC after cryopreservation for 1 and 5 months

Based on viability data showing a slightly lower viability of MSC cryopreserved with 2% DMSO, only MSC cryopreserved in CS-5 and CS-10 were used for assessment of cell proliferation. As shown in *Figure 3*, cryopreservation of MSC for both 1 and 5 months did not compromise and even enhanced cell proliferation. After expansion for 14 days in GM, the number of control unfrozen cells increased 13-fold, while the number of cells cryopreserved with CS-5 and CS-10 for 1 month increased 22 and 26.5-fold, respectively (*Fig. 3A*). Increased proliferation of MSC after cryopreservation was statistically significant and most probably occurred because of selection of "stronger" cells at plating. It is worth noticing that next day after plating (day 1) less cryopreserved than unfrozen MSC adhered to the plates: 5528±647 and 6417±2692 for MSC cryopreserved in CS-5 and CS-10, respectively,

A



B

gated subpopulations	% ALL cells (10,000 events acquired)		
	Live cells	SS 1 μM	SS+ caspase inhibitor
Caspase background	79	28.6	55.9
caspase positive	6.3	62	35.1
debris	14.5	9.4	9.0

Figure 2. Calibration of the flow cytometry analysis of caspase 3 activity, a representative experiment. (A) Flow cytometry histograms: top—freshly trypsinized MSC—background caspase 3 activity is gated; middle—staurosporine-treated MSC—events to the right of the background gate represent cells with high caspase activity; bottom—staurosporine-treated cells pre-incubated with caspase 3-specific inhibitor. (B) Statistical analysis of the histograms.

Table 3. Mesenchymal Stem Cells Apoptosis[a] After Cryopreservation for 1 and 5 Months

	1 month[b]			*5 months*[b]
Freezing medium	***Annexin V% positive***	***Hoechst 33342% positive***	***Caspase 3% positive***	***Caspase 3% positive***
	n=*3*	**n=*3***	**n=*6***	**n=*3***
FM	**9.0±4.1**	**2.6±1.6**	**3.2±2.7**	**ND**
CS-2	**10.7±2.7**	**4.9±0.9**	**16.7±9.7**	**ND**
CS-5	**8.0±1.7**	**3.7±2.1**	**15.6±9.7**	**18.3±12.5**
CS-10	**5.5±1.9**	**2.7±1.0**	**12.8±12.3**	**12.9±9.7**

[a]MSC were frozen in a conventional FM, or in CS-2, CS-5, and CS-10 reagents for 1 and 5 months. Percentage of apoptotic cells was determined immediately after thawing before cell plating.

[b]For cells cryopreserved for 1 months, apoptosis was measured by 3 different methods; for cells stored for 5 months, apoptosis was measured only by caspase 3 assay, which had previously demonstrated the highest percentage of apoptotic cells.

compared with 8789±2336 unfrozen cells (mean±SD; n=5). While the difference in seeded cell number between unfrozen cells and cryopreserved in CS-10 cells was not statistically significant, the difference between unfrozen cryopreserved in CS-5 cells was statistically significant ($p \leq 0.02$).

Similar results were obtained after cryopreservation for 5 months. Again, proliferation of cryopreserved cells was higher than that of unfrozen cells (*Fig. 3B*). Similarly, less adherent cells were found the next day after plating of MSC cryopreserved in CS-5 (3573±1622) and CryoStor10 (4277±1387) compared with unfrozen cells (6398±21; mean±SD; n=3). These differences in seeded cell numbers were compensated already by day 7 of culturing due to the higher proliferation rates of previously cryopreserved cells.

Osteogenic differentiation of MSC after cryopreservation for 1 and 5 months

MSC cryopreserved either for 1 month or for 5 months retained their osteogenic potential. As summarized in *Table 4*, all tested samples of MSC upregulated ALP activity 5–10-fold over undifferentiated cells cultured

in parallel in GM. No significant difference between ALP activity of MSC cryopreserved in CryoStor reagents and unfrozen MSC was observed after both 1 and 5 months of storage in liquid nitrogen. CryoStor regents were equal to conventional FM, containing FCS in terms of osteogenic upregulation of ALP activity (see *Table 4*, 1 month cryopreservation).

Flow cytometry analysis of ALP surface expression measured on day 14 after addition of OM demonstrated that there was no difference in the percentage of ALP-positive cells between unfrozen control cells and those stored in CS-5 and CS-10 for one month (89.6%, 87.8%, and 97.8% respectively). Similarly, storage for 5 months did not affect upregulation of ALP expression: 78.1% of unfrozen MSC and 65.2% of MSC cryopreserved in CS-10 became ALP-positive after osteogenic stimulation for 14 days.

Ca^{++} deposition into extracellular space was measured after 21 days of osteogenic differentiation. No statistically significant differences in the extracted Ca^{++} concentrations (microM/10000 cells) between unfrozen cells (1.9±0.4) and cells cryopreserved for 1 month in CS-5 (2.0±0.7) or CS-10 (2.1±0.7; Mean±SD;

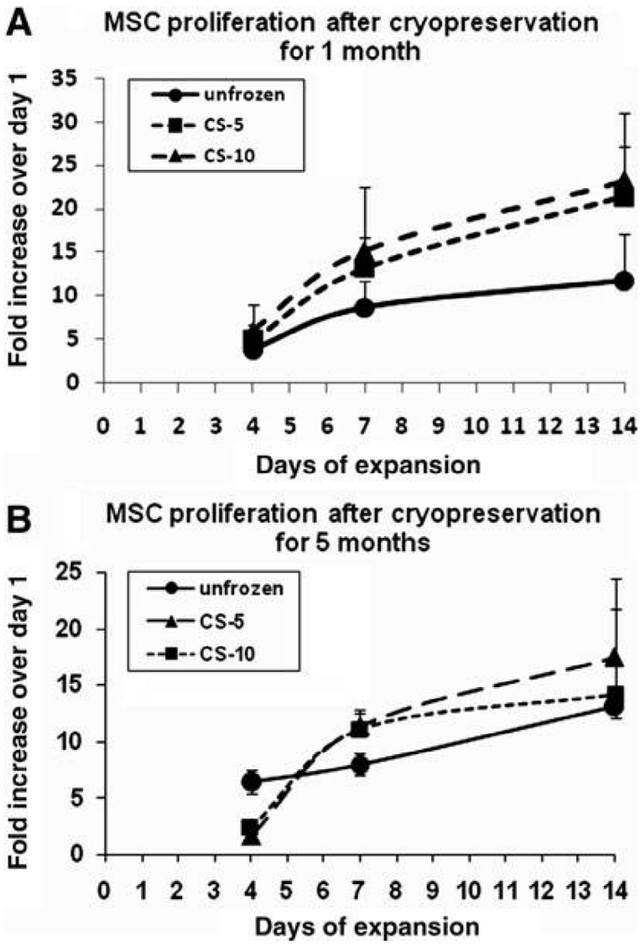


Figure 3. MSC proliferation of MSC cryopreserved for 1 and 5 months. MSC were cryopreserved in CryoStor solutions (CS-5 and CS-10) and stored for 1 month (A) and 5 months (B) in liquid nitrogen. Parallel cultures were analyzed before freezing. The thawed cells were plated into 24-well plates at 3000 cells/cm^2 and allowed to proliferate in GM. Cell number was measured with Calcein-AM on days 1 (next day after plating), 4, 7, and 14. All the cell number values were normalized to the values of day 1. The data represent Mean±SD of 5 and 3 experiments for 1 and 5 months cryopreservation, respectively; each experiment was performed with MSC derived from a different donor.

n=3) were observed. Similarly, cryopreservation in CryoStor reagents for 5 months did not significantly affect Ca^{++} deposition, which was 1.0±0.2, 0.7±0.3 and 0.8±0.2 microM/10000 cells for unfrozen cells, and cells cryopreserved in CS-5 or CS-10, respectively (Mean±SD; n=3).

Analysis of expression of MCS markers after cryopreservation for 5 months

Flow cytometry analysis of MCS markers was performed after a 5 month storage in CS-10 and compared with marker expression in the same cells before freezing. Unfrozen MSC expressed high levels of all markers tested, except CD9 (the latter was expressed on 34% of MSC). This pattern of expression did not change after cryopreservation, except the percentage of CD9-positive cells dropped to 18% (*Table 5*).

Hypothermic storage

The same battery of tests (viability, proliferation, osteogenic differentiation, and marker analysis) was performed for evaluation of efficacy of HypoThermosol®-FRS (HTS-FRS) medium for a short-term storage of MSC at 4°C.

Table 4. ALP Activity[a] in Post-Thaw Mesenchymal Stem Cells

	1 month[b]		*5 months*[c]	
Freezing medium	***7 days***	***14 days***	***7 days***	***14 days***
Before freezing	**1.2±1.1**	**2.9±1.3**	**1.9±1.1**	**7.7±4.5**
FM	**1.3±0.8**	**2.8±0.7**	**ND**	**ND**
CS-2	**1.4±0.8**	**2.7±0.8**	**ND**	**ND**
CS-5	**1.4±0.7**	**2.9±0.8**	**1.8±0.9**	**7.2±3.7**
CS-10	**1.8±0.8**	**2.7±0.7**	**1.9±0.9**	**6.4±3.4**

[a]MSC were frozen in a conventional FM, and in CS-2, CS-5, and CS-10 reagents for 1 and 5 months. After thawing, MSC were seeded in 24-well plates and incubated in osteogenic medium for 7 and 14 days. The data is ALP activity in nmol pNP/min/10,000 cells.

[b]Mean±SD of 6 experiments performed with 6 MSC batches, each derived from a different donor. ALP activity in parallel MSC cultures kept in GM was 0.22±0.19 and 0.52±0.27 for 7 and 14 days, respectively.

[c]Mean±SD of 3 experiments performed with 3 MSC batches, each derived from a different donor. ALP activity in parallel MSC cultures kept in GM was 0.32±0.24 and 1.88±0.91 for 7 and 14 days respectively.

The number of viable cells was measured with Alamar Blue test in the same cultures before hypothermic storage (40004±8380) and after incubation at 4°C in HTS medium for 2 days (47638±9899) and 4 days (34092±7883). In order to evaluate proliferation ability of MSC after hypothermic storage, control cells and those stored in HTS medium for 2 and 4 days were further expanded in GM for 14 days without re-plating. The number of cells in parallel cultures that remained in a cell incubator all the time increased during 14 days about 1.8-fold (from 43482±6840 to 78106±4500); the number of cells that were first stored at 4°C in HTS medium for 2 days also increased 1.8-fold (from 47638±9899 to 85009±1658) and the number of cells that were first stored at 4°C in HTS medium for 4 days increased 1.9-fold (from 34092±7883 to 66402±4953; n=4).

Dead cells and apoptotic cells were measured at the end of the recovery period using 7-AAD and caspase 3 staining, respectively. An increase in the percentage of dead cells from 2.3% in control cultures to 16.8% in stored cultures (p=0.043) and apoptotic cells from 2.9% in control cultures to 6–8% in stored cultures (p=0.008) was observed (*Fig. 4B)*.

Table 5. Expression of Mesenchymal Stem Cells Markers[a] After Cryopreservation for 5 Months and Hypothermic Storage for 4 Days

	Cryopreservation 5 months[b]		*Hypothermic storage 4 days[c]*	
Markers	***Before freezing***	***CS-10***	***37°C***	***4°C***
CD166	**99.5±0.3**	**98.0±3.0**	**98.4±1.2**	**96.7±1.5**
CD9	**33.9±4.0**	**17.9±3.2**	**8.5±2.2**	**4.5±1.5**
CD90	**99.6±0.3**	**99.8±0.1**	**99.7±0.2**	**99.3±0.4**
CD44	**93.2±2.2**	**94.7±4.0**	**96.7±2.6**	**95.6±0.5**
CD105	**99.7±0.3**	**99.4±0.8**	**99.5±0.6**	**98.9±0.6**

[a]All data are a percentage of cells positive for the corresponding marker; Mean±SD of 3 experiments performed with 3 MSC batches, each derived from a different donor.

[b]MSC were frozen in CS-10 for 5 months. After thawing, MSC were seeded into cell culture flasks at 3000 cells/cm^2, allowed to proliferate in GM for 1 week, and then stained with antibodies against MSC markers. Parallel cultures were stained before freezing.

[c]Subconfluent cultures of MSC were stored in HypoThermosol-FRS medium (HTS-FRS) at 4°C for 4 days, then, the HTS-FRS medium was replaced with warm GM, the cultures were allowed to recover in a CO_2 incubator for 3h, trypsinized, and stained with antibodies against MSC markers. Parallel cultures kept in GM at 37°C all the time were analyzed in the same way.

In order to evaluate the osteogenic differentiation of MSC after hypothermic storage, control cells and those stored in HTS medium for 2 and 4 days were allowed to differentiate in OM for 14 days without re-plating. The results are summarized in *Figure 4C*.

No significant differences in ALP activity between control cells (5.6±1.7 nmol pNP/min/10,000 cells) and those stored in HTS for 2 and 4 days (6.2±1.6 and 6.5±1.4, respectively) were found. ALP activity in parallel cultures of both control and stored cells that were not differentiated but kept for 2 weeks in GM was very low (*Fig. 4C*).

Similarly, ALP surface expression in control and stored cultures was not different. In control cultures, 70.2%±18.3% of differentiated cells were ALP positive; in cultures stored in HTS for 4 days and then differentiated, 61.6%±25.9% cells were ALP positive (mean±SD; n=3).

Finally, the expression of MSC markers was analyzed in MSC at the end of the recovery period. No differences between control and stored cells were found except the expression of CD9 decreased (see *Table 5*).

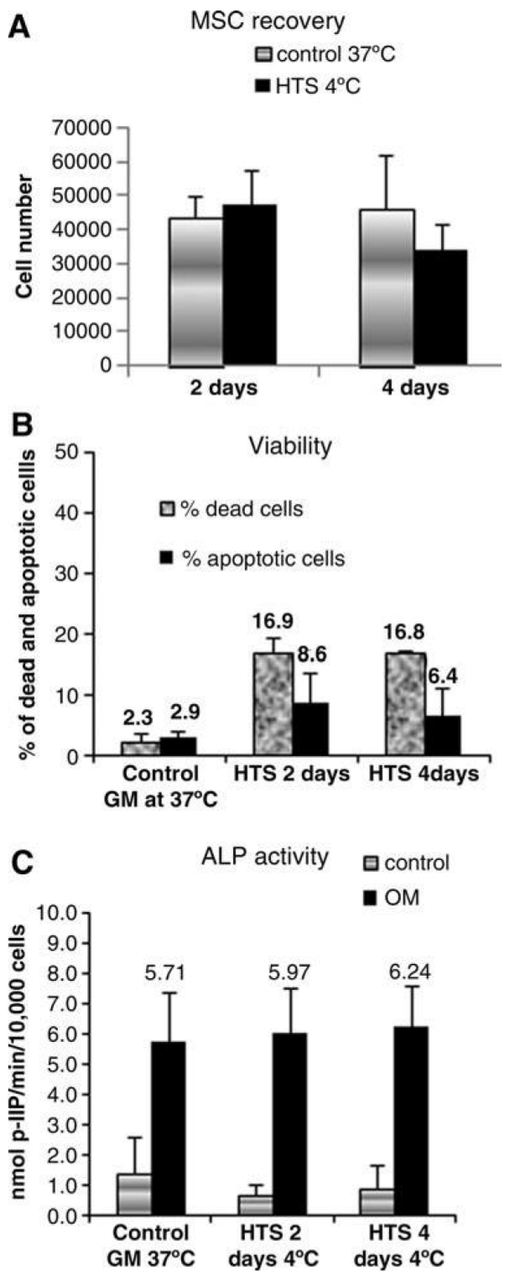


Figure 4. Hypothermic storage of MSC. Subconfluent cultures of MSC were stored in HTS-FRS at 4°C for 2 and 4 days. At the end of the storage period, HTS-FRS medium was replaced with GM, and the cultures were moved to a CO2 incubator and allowed to recover at 37°C for 3 h. Control parallel cultures that were not subjected to hypothermic storage but were kept in GM in the cell incubator were analyzed in the same way. (A) Cell recovery of MSC after storage. Live cell number was measured with Alamar Blue before and after storage in the same cultures of MSC. Each bar is Mean±SD of 2 experiments with 2 different batches of MSC (in triplicate for each MSC batch). (B) Viability of MSC after storage. MSC, control and stored in HTS medium, were trypsinized at the end of the recovery period, stained with 7-AAD for dead cells and with caspase 3 substrate for apoptotic cells, and analyzed by flow cytometry. Each bar is Mean±SD of 3 experiments with 3 different batches of MSC. (C) Osteogenic differentiation of MSC after storage. MSC cultures, control or subjected to hypothermic storage in HTS medium, were differentiated in OM for 14 days without replating, and ALP activity was measured. Parallel cultures were not differentiated but kept in GM for 14 days. Each bar is Mean±SD of 3 experiments with 3 different batches of MSC. ALP, alkaline phosphatase; HTS-FRS, HypoThermosol-FRS medium; OM, osteogenic medium.

Discussion

To our knowledge, this is the first study examining in depth the efficacy of long-term cryopreservation of human BM–derived MSC in an animal product–free clinically accepted medium. Commercial CryoStor™ cryo-medium developed by BioLife Solutions that was chosen for this study has been in the market for some time and is distributed by several vendors, including key companies specializing in stem cell products. However, the efficacy of this medium for cryopreservation of human MSC has been poorly investigated, and no long-term cryopreservation studies were performed. In a recent study under the aegis of European consortium "Crystal" (CRYo-banking of Stem cells for human Therapeutic AppLications), xeno-free chemically defined media containing Pluronic F-68 and variable percentages of DMSO (0%, 5% and 10%) were tested for cryopreservation of various progenitor cells, including adipose tissue–derived mesenchymal stromal cells. A CryoStor solution containing 10% DMSO was also considered for this study; however, it was ruled out after pilot experiments.[21]

The goal of the study was three-fold: (1) to develop and standardize a xeno-free cryopreservation protocol customized for MSC; (2) to optimize a battery of quantitative tests for evaluation of MSC viability, proliferation, and differentiation in order to evaluate the cryo-protocol efficacy; and (3) to assess the efficacy of MSC xeno-free cryopreservation after prolonged (5 months) storage in liquid nitrogen using these tests.

In the preliminary experiments conducted to optimize the freezing/thawing protocol and evaluation parameters, MSC were stored in liquid nitrogen for one month. In most of these experiments, the efficacy of CryoStor reagents was compared with the cryoprotective effect of the commonly used MSC FM formulated by Lonza on the basis of their MSC GM with addition of 10% FCS, 30% BSA, and 10% DMSO. Since this medium has been successfully tested in multiple labs including ours and in many research applications, it was chosen as a gold standard, although it cannot be used in clinical studies because of the presence of animal proteins. CryoStor reagents formulated without FCS, and other animal products were practically equal to FM, except that the number of apoptotic cells measured by caspase 3 activity was significantly lower in post-thaw cells cryopreserved in FM than in MSC cryopreserved in CryoStor reagents (see *Table 3*). However, one cannot exclude that this difference was due to the faster death of the apoptotic cells during cryo-storage in FM. When apoptosis was evaluated by Annexin V binding or by staining with Hoechst 33342, lower percentages of apoptotic cells were observed for all the media, and no significant differences between FM and CryoStor reagents were found. One possible explanation for these results is that translocation of phosphoserine to the outer layer of the cell membrane detected by AnnexinV and slowing of Hoechst efflux resulting in its accumulation in apoptotic cells occurs at early stages of the apoptotic program[25] and then reverse or become shielded by further cell deterioration, while high Caspase 3 activity is detected at the later stage of apoptosis.

Evaluation of dead cells in the MSC samples thawed immediately after being slowly frozen to –60°C allowed isolation of cell injury, which

occurs during slow cooling phase of the cryoprotocol. These experiments demonstrated the importance of MSC protection during slow cooling, and confirmed the high sensitivity of MSC to the rate of freezing: the increase of the freezing rate only from 1°C/min to 2°C/min caused a drastic increase in cell apoptosis (see *Fig. 1B*). It also suggests that the apoptotic program in MSC already starts during the slow cooling phase of cryopreservation and supports the findings of Carvalho et al., who demonstrated the progressive loss of MSC viability at cooling rates of −3°C/min, −5°C/min, and −10°C/min.[26]

Another sensitive step of cryopreservation protocol is the thawing procedure. Contrary to the manufacturer's instructions, slow cooling injury of MSC was further exacerbated by osmotic injury resulting from fast addition of the dilution medium (GM) to the thawed cells, suggesting high sensitivity of MSC to changes in osmotic pressure.

A successful cryopreservation program commonly focuses on cell recovery scores. However, a significant number of recovered cells are often not able to adhere to the cell culture surface and can be found floating the next day after plating. Considering a well-established contribution of apoptosis to cryoinjury,[27] one cannot exclude that at least a part of the so-called "floating cells" are those cells in which cryopreservation triggered an apoptotic program. Revealed by caspase 3 assay, a relatively low percent (13%–17%) of apoptotic cells in post-thaw suspensions of MSC is consistent with the presence of anti-apoptotic agents in CryoStor solutions.[28] However, these apoptotic cells will most probably die after plating and, thus, should be taken into account when estimating the numbers of cryopreserved MSC for transplantation.

Evaluation of cell recovery, dead cell, and apoptotic cell number performed after complete cryopreservation program (slow and then fast cooling, followed by storage of the cells in liquid nitrogen and then gentle thawing) demonstrated a practically full recovery and high viability of MSC stored in liquid nitrogen for one month and about 70%–80% recovery after 5 month storage (see *Table 2*). One could notice that percentages of dead cells shown in *Table 2* were higher than one would expect based on percentages of live cells, which was most probably due to the loss of a portion of cells in the sample permeabilized with 0.1% saponin, which was considered a 100% dead cell sample and which produced maximal fluorescence signal used for calculation of percentage of dead cells (see Methods). This assumption is supported by a comparable percentage of dead cells in control unfrozen samples (14%).

Besides testing viability, proliferation of MSC cells post cryopreservation was analyzed. The goal was to estimate proliferation rate over an extended time after plating rather than recovery of the cell division function within a few days after thawing as was done in the study just cited.[21] We show here for the first time that proliferation rate of post-cryopreserved MSC (after both 1 month and 5 month storage) is significantly higher than that of unfrozen control cells (*Fig. 3A, B*), suggesting possible cell selection. The higher proliferation rate was observed despite the fact that the next day after plating, less cryopreserved cells compared

with unfrozen cells adhered to the culture plates, further supporting the hypothesis of selection of "stronger" cells after cryopreservation. This means that in the future clinical studies, when MSC transplantation is planned immediately after thawing, QC tests of cell viability and apoptosis should be performed before cell plating.

In addition, it is worth noting that comparison of expression of MSC markers in unfrozen cells and cells after cryopreservation (and after hypothermic storage) demonstrated no changes except in expression of CD9, which was downregulated post-freezing (see *Table 5*). We have previously shown that expression of CD9 in MSC and in fibroblasts has inverse relationship where MSC express low levels of CD9 and fibroblasts express high levels.[22] Furthermore, it was shown that in the higher passages of MSC (p6), CD9 expression goes up (from 39.4% to 73.5% CD9 positive cells, ref. 21). In the current study, we used MSC of passage 2–3 where CD9 expression was low. A decrease in the percentage of CD9-positive cells observed after cryopreservation or storage further supports selection of the "younger" phenotype. However, the exact changes occurring in more proliferative post-cryopreserved MSC remain obscure and should be addressed in the future most probably at the gene level. Tumorigenicity of post-cryopreserved MSC should also be challenged.

Since this lab has been focusing on osteogenic differentiation of MSC, we had at hand a number of quantitative assays for a thorough analysis of osteogenic differentiation, developed, optimized, and standardized over the years. These methods allowed us to reveal even subtle changes in cryopreserved cells, which would not have been possible to demonstrate by simple cytochemistry staining commonly used for demonstration of three lineage differentiation of MSC, especially considering variability of MSC batches. All quantitative tests (ALP activity, ALP surface expression, and mineralization of extracellular space) demonstrated no differences in osteogenic potential of cryopreserved MSC (whether stored in liquid nitrogen for 1 or for 5 months) and their paired unfrozen controls. Our findings are consistent with those of Woods and colleagues, who used CryoStor solution with 10% DMSO to preserve dental pulp and then demonstrated good three lineage differentiations of stem cells derived from previously cryopreserved dental pulp.[13] Regeneration of non-union bone fractures is a long process taking months and in cases of substantial bone loss, even years. It is likely that repetitive MSC transplantations will be necessary to accomplish full healing. Therefore, successful clinical grade cryopreservation of MSC for bone repair is the most urgent need. We hope that our multifactorial analysis of CryoStor cryo-medium for bone engineering will encourage a similar evaluation of this medium for application of MSC in various other fields of tissue engineering.

Post-cryopreservation viability, apoptosis, proliferation, marker expression, and differentiation of human stem cells from apical papilla have been recently studied by Ding et al., who demonstrated successful cryopreservation of these MSC using DMSO, ethylene glycol, and glycerol as cryoprotectants; however, FM in these studies contained 90% FCS.[29] Our findings clearly demonstrate that all these parameters are relatively

well preserved in MSC cryopreserved in a clinical-grade medium without serum and other animal products.

In a separate set of experiments, hypothermic storage of MSC in HypoThermosol-FRS™ storage/shipment medium also developed by BioLife Solutions was investigated. HypoThermosol medium was tested for hypothermic preservation of multiple cell types such as cardiac myocytes,[30] coronary vessels,[31] human renal cells,[32] and canine kidney cells[33]; however, storage of MSC in HTS has not been previously studied. We have tested MSC storage in HTS for 2 and 4 days at 4°C. The same battery of tests was applied.

In order to test cell recovery in the same culture, live cell number was measured before and after storage with Alamar Blue indicator. The number of viable cells did not change significantly after 2 days of hypothermic storage and slightly decreased after 4 days of storage. Hypothermic storage of MSC in HTS for 6 days resulted in a significant decrease of cell viability (data not shown). It is worth noticing that MSC stored at 4°C in a regular GM died very quickly (data not shown). HTS was shown to preserve MSC marker expression, proliferation and osteogenic differentiation after storage for at least 4 days.

ALP activity of MSC after storage in continuous cultures in GM was quite low (see *Fig. 3C*), suggesting that HTS storage did not promote spontaneous differentiation of MSC.

An attempt was previously made to store porcine hepatocytes at 4°C in various preservation solutions, but none of the tested reagents was capable of adequately preventing cell death after 1 day of hypothermic storage and subsequent 1 day culturing in normal conditions.[34] In another report, human tubular epithelial cells stored for 2 days in the University of Wisconsin solution (UWS) followed by 1 day re-warming in GM showed increase of apoptotic signaling that was suppressed by addition of antioxidants to storage solution.[35] When UWS storage solution was compared with HTS-FRS, cell viability, energy status, and xenobiotic metabolism were consistently higher and, in some cases, markedly higher after storage in HTS-FRS.[36] In this study in addition to good recovery of MSC after hypothermic storage in HTS, percentage of apoptosis in stored cultures was also low. This could be explained by the presence of anti-oxidants and anti-apoptotic additives in HTS-FRS.[37] In summary, our results indicate that MSCs could be stored at 4°C for at least 4 days in a solution free of animal products.

In conclusion, we have critically evaluated clinical-grade media for cryopreservation and short-term storage of human MSC. We have used a programmable cell freezer and an optimized freezing and thawing program. Quantitative tests of cell viability and apoptosis were adapted for MSC still remaining in a post-thaw suspension. Enhanced proliferation rates of post-cryopreserved MSC were observed and should alarm a scientific community. We believe that this study will contribute to acceleration of MSC application for treatment of non-union bone fractures and other types of tissue injury. Successful cryopreservation and storage of MSC should promote the creation of MSC banks. A standardized protocol for MSC cryopreservation will also resolve the variability

of the data between different laboratories and enhance collaborations within the research community. ■

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Disclosure Statement

No competing financial interests exist.

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Viable Mononuclear Cell Stability Study for Implementation in a Proficiency Testing Program: Impact of Shipment Conditions

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Abstract

The impact of shipping temperatures and preservation media used during transport of either peripheral blood mononuclear cells (PBMCs) or Jurkat cells was assessed, in view of implementing of a proficiency testing scheme on mononuclear cell viability. Samples were analyzed before and after shipment at different temperatures (ambient temperature, dry ice, and liquid nitrogen) and in different preservation media (serum with cryoprotectant, commercial cryopreservation solution, and room temperature transport medium). Sample quality was assessed by viability assays (Trypan Blue dye exclusion, flow cytometry, Cell Analysis System cell counting [CASY]), and by ELISpot functional assay. The liquid nitrogen storage and shipment were found to be the most stable conditions to preserve cell viability and functionality. However, we show that alterna-

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tive high-quality shipment conditions for viable cells are dry ice shipment and commercial cryopreservation solution. These were also cost-efficient shipment conditions, satisfying the requirements of a proficiency testing scheme for viable mononuclear cells. Room-temperature transport medium dramatically and adversely affected the integrity of mononuclear cells.

Introduction

Shipment of viable cells between research laboratories and biobanks is necessary for many collaborative projects. Storage and specimen transport conditions need to be considered since they can influence biological specimen viability and functionality and lead to pre-analytical bias.[1–3] The International Society for Biological and Environmental Repositories (ISBER) Best Practices include detailed guidelines on sample transport.[4] The ISBER Biospecimen Science Working Group (BSWG) has published a standard biospecimen research experimental protocol for the study and reduction of pre-analytical variability related to sample processing.[5] This Working Group has also published specific recommendations on logistics and sample transport.[6] ISBER and the Integrated Biobank of Luxembourg (IBBL) have developed a Proficiency Testing (PT) program for biorepositories to enable external quality assessment of the methods used by biobanks as biospecimen Quality Control (QC) methods.[7,8]

Viable mononuclear cells are the object of one of the biorepository PT schemes. Viable mononuclear cells are important biospecimens because they allow researchers to identify circulating disease biomarkers. Examples include lymphocyte subset-specific gene expression signatures in cancer[9] or autoimmune diseases,[10] lymphocyte subset-specific miRNA signatures in multiple sclerosis,[11] or T cell subset-specific flow cytometric signatures in Parkinson's disease.[12] Frozen viable PBMCs are fit-for-purpose, not only for immunomagnetic sorting of purified monocyte and lymphocyte populations, following cryopreservation,[13] but also for functional studies,[14] immunophenotyping,[15] establishment of lymphoblastoid cell lines (LCL) by Epstein Barr virus (EBV) transformation,[16] and purification of CD34+ cells.[17]

The surrogate QC assay for either EBV transformation success[18] or immunophenotyping and proliferation assays[14] has shown cell viability, with a qualification cut-off at around 70% viability. Therefore, implementation of a PT scheme on cell viability for repositories, which process and cryopreserve mononuclear cells for all the above-mentioned end-uses, is of critical importance. Implementation of such a PT scheme includes shipment of viable mononuclear cells (as "PT test items") to different participants, around the world. These PT test items should have percent viability (an assigned value), calculated after cell thawing. Furthermore, the test items should be homogeneous and stable before and after shipment.

Some early studies have demonstrated how storage[2,3] and cryopreservation[13,19–22] may influence PBMC viability and functionality.[23–26] Previous studies established the practice to cryopreserve PBMCs within the first hours after blood collection[1,14,27] in order to preserve PBMCs functionality for immunological assays. Several studies[28–30] have also focused on handling and

storage of cryopreserved PBMCs addressing the importance of blood shipment conditions in infectious disease studies. The effect of ambient temperature during shipment of fresh PBMCs on subsequent processing and recovery has been evaluated.[31]

However, there are a lack of data cross-investigating cell type, cryopreservation medium, transatlantic shipment conditions, and assessment methods. Our current study is the first to evaluate multiple variables affecting PT specimen integrity: viability (including early stage of apoptosis), functionality of peripheral blood mononuclear cells (PBMCs) and Jurkat cell line (an immortalized line of T lymphocyte cells), preserved in different preservation media (serum with cryoprotectant, commercial cryopreservation solution, and room temperature transport medium), shipped under different conditions (liquid nitrogen [LN], dry ice [DI] for frozen cells) or stored and shipped at ambient temperature.

Usually PBMCs are cryopreserved in LN using 10% DMSO[27] and shipped in LN or DI.[28] In our study, we have additionally assessed the viability and function of cells stored in commercially available preservation media CryoStor® CS10[32,33] and AQIX® RS-I.[34,35] The first medium, CryoStor® CS10, is pre-formulated with 10% DMSO,[33] and provides a protective environment for cells during the freezing, storage, and thawing process. The second medium, AQIX® RS-I, is designed[35] to simulate the composition of human interstitial fluid and thereby afford isolated cells to maintain homeostasis of biophysical and metabolic parameters during periods of both hypothermic and normothermic preservation. It could therefore allow a PT provider to reduce shipment costs while performing transport at ambient temperature.

Stability testing is necessary before implementation of a PT scheme in order to (i) assess the most cost-efficient shipment mode for the PT test items (viable mononuclear cells), and (ii) verify that there is no consequential instability of the test items. Furthermore, since a PT program requires a value assessment after cell thawing, under the same conditions as those employed by the PT participants, the baseline viability values used for normalization were calculated after cell thawing.

MATERIALS AND METHODS

Participating laboratories

Three facilities participated in the study, all operating in compliance with ISO17025, CLIA, and Good Laboratory Practice (GLP) guidelines. Three laboratories were used for this study: one laboratory was located on the West Coast (*Lab 1*) and one on the East Coast (*Lab 2*) of the United States and one in central Europe (*Lab 3*). Each facility was responsible for preparing samples for their testing and shipping to other facilities for testing (*Fig. 1*). Shipping was performed according to standard operating procedures (SOPs) including continuous temperature monitoring. Two shipment rounds were performed.

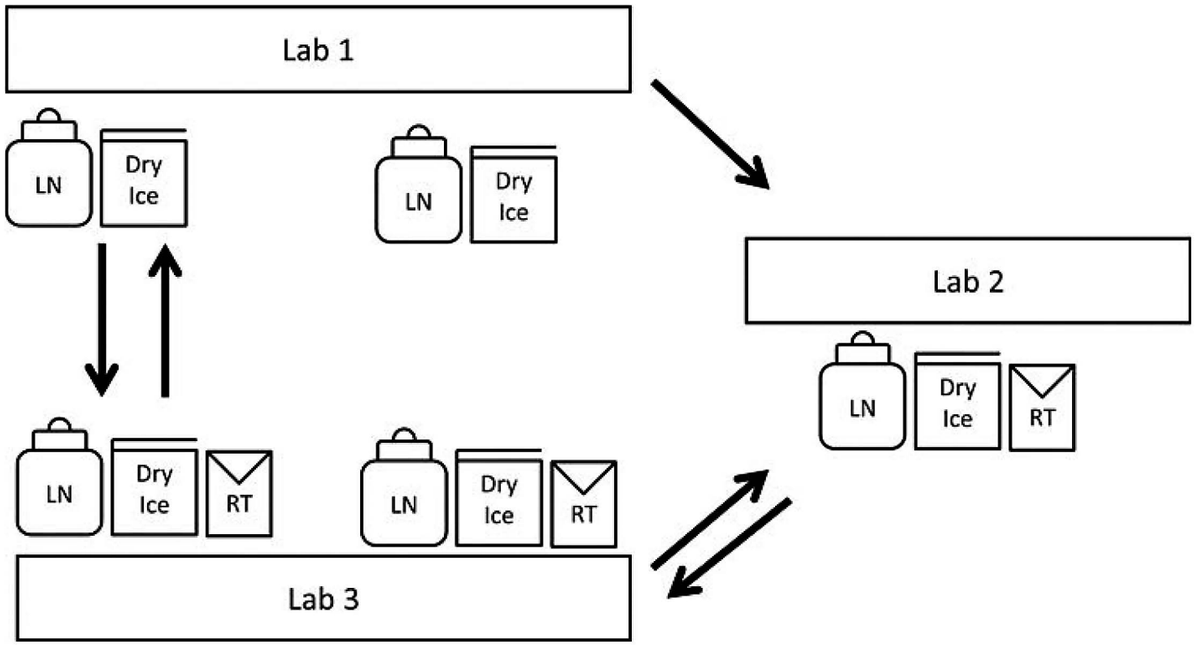

Figure 1. Shipment schema and participating laboratories: Three facilities participated in the study, all operating in compliance with ISO17025, CLIA, and good lab practice (GLP) guidelines: IBBL (Integrated BioBank of Luxembourg), PPD (PPD Vaccines and Biologics Laboratory), UCSF (University of California San Francisco AIDS Specimen Bank).

Samples used

Normal PBMC and Jurkat cell line samples were used and prepared as follows.

PBMC suspension was prepared by pooling blood from 12 8-mL ACD tubes, either from the same or from different HIV-negative donors, in order to obtain a minimum of 10^9 cells. All donors signed informed

consent. PBMCs were separated on Ficoll gradients (EUROBIO, ref. CMSMSL01-01) and washed three times in phosphate-buffered saline (PBS).

Jurkat samples (Clone E6-1) were purchased from the American Tissue and Cell Collection (ATCC, ref. TIB-152). Cells were cultured at 37°C, 5% CO_2 in RPMI 1640 (Invitrogen, ref. RPMI A10491-01) supplemented with 10% fetal bovine serum (FBS, Invitrogen, ref. 26140-079). Routine passage was carried out every 2 or 3 days.

Both cell cultures were divided in two halves. Each half was processed as "high viability suspension" (HV) and as "intermediate viability suspension" (IV) for the first shipment round, or as "high viability suspension" and as "low viability suspension" (LV) for the second shipment round. The HV suspension corresponded to the Jurkat cells incubated according to manufacturer's instructions (cell passage 1–8) or freshly isolated cells from whole blood for PBMCs; the IV suspension was obtained, following incubation of HV suspension of Jurkat cells (cell passage 1–8) or fresh isolated PBMCs for one week at +4°C; the LV suspension was obtained, following incubation of Jurkat cells (cell passage 10–18) for one week at +4°C.

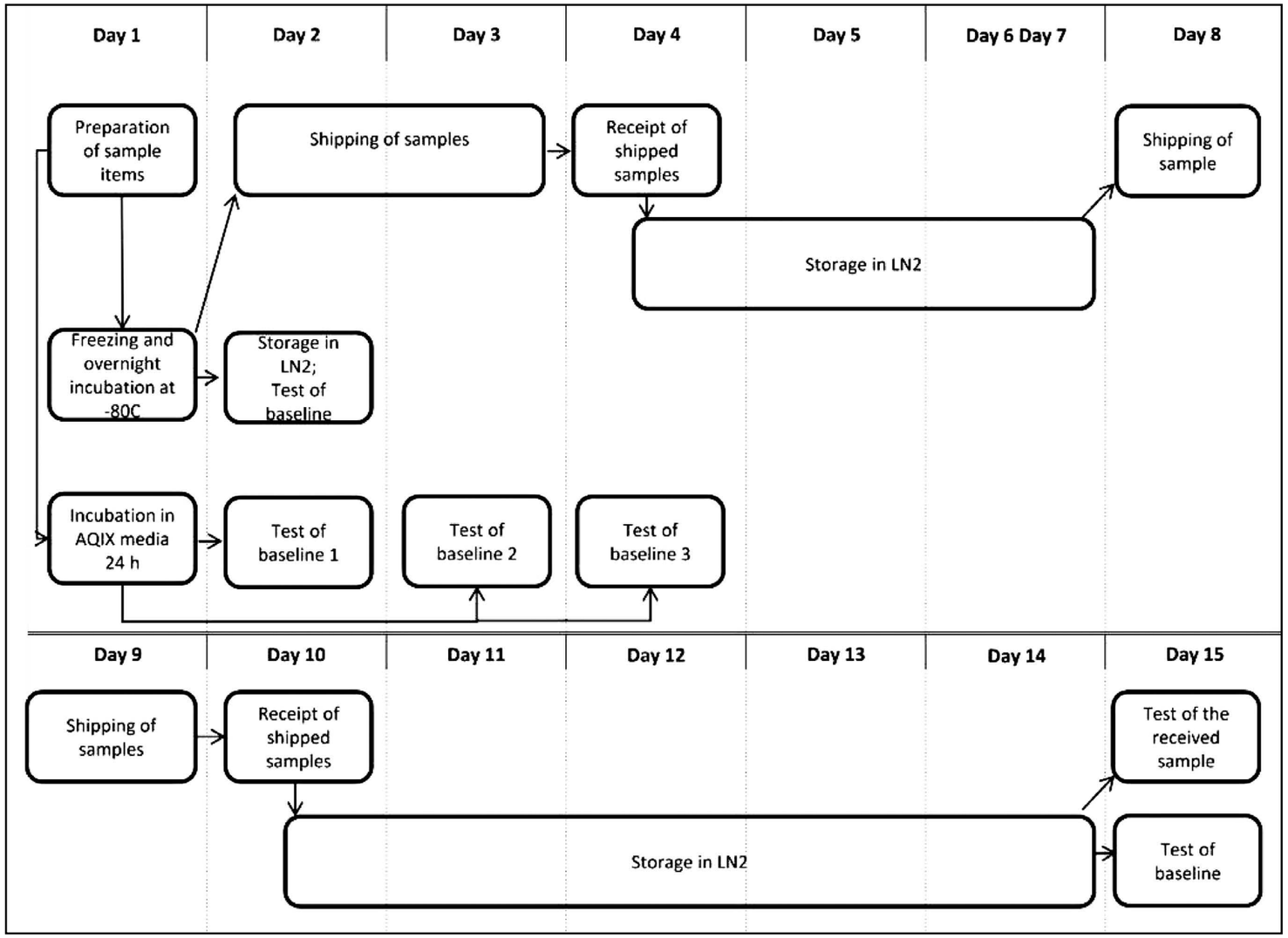

Figure 2. Flow charts illustrating the distribution and testing of specimens for one shipment round. Sample items included HV, IV, or LV cell suspensions, each in duplicates, in order to ship back one duplicate to the original production laboratory.

Each cell suspension was divided into three parts (*Fig. 2*) and culture medium was completely replaced with cold (+2° to –8°C) preservation medium, using either:

- 90% heat inactivated FBS containing 10% DMSO freezing media (Sigma, ref. D2438),
- CryoStor® CS10 freezing media (BioLife Solutions, ref. 210102)
- AQIX® RS-I preservation media (Aqix Ltd, ref. RSI/KIT)

with a final cell concentration of 5×10^6 cells/mL.

One mL aliquots in FBS with 10% DMSO or CryoStor® CS10 were placed in freezing isopropanol containers (Mr Frosty, Nalgene®) for freezing at –80°C, kept 18 h (overnight), and then transferred to LN vapor for storage until shipment. After at least 6 h of LN storage, samples were shipped in LN or dry ice (DI). The aliquots made in AQIX® RS-I, were kept at +4°C for 24 h until shipment. For all study centers, the shipment took place simultaneously for all conditions (*Fig. 2*), while the stock of the same sample aliquots was kept for baseline testing on site.

Table 1. Baseline Values of Viability of Jurkat Cells Cryopreserved with 10% DMSO and Stored in LN_2 Assessed by Various Methods

	Viability (%) of cells cryopreserved in 10% DMSO		
Method	***HV***	***IV***	***LV***
Trypan Blue	**80.0**	**48.1**	**5.8**
CASY	**82.8**	**68.2**	**65.1**
Flow cytometry	**67.8**	**24.8**	**5.0**
	Viability (%) of cells cryopreserved in CryoStor 10		
Method	**HV**	**IV**	**LV**
Trypan Blue	**86.1**	**48.4**	**12.7**
CASY	**85.2**	**77.5**	**68.5**
Flow cytometry	**78.5**	**29.7**	**6.3**

Initial cell suspension quality before storage was set as: HV=high viability, IV=intermediate viability, LV=low viability.

Definition of sample quality value categories

The quality of the initial cell suspension before storage and shipment was set as: HV=high viability, IV=intermediate viability, LV=low viability. The definition of HV, IV, LV was based on Trypan Blue assessment of the Jurkat cell suspensions cryopreserved with 10% DMSO and stored in LN in Lab 3: 0<LV<25%, 25%<IV<80%, 80%<HV<100% (*Table 1*).

Logistics

Logistics of the sample shipments to and from the three study centers is shown in *Figure 1*. Lab 1 participated only in the first shipment round and testing run, while Lab 2 and Lab 3 participated in both shipment rounds and testing runs. All study centers used the same references of consumables and protocols in both cell processing and testing experiments. The shipments were operated by Federal Express. The receiving laboratory was responsible for return shipment or testing of samples (*Fig. 2*).

All ambient and dry ice shipments were sent with a Sensitech Temptale 4 (Sensitech Inc, ref. D4400-01) to monitor the temperature of the package through a shipment. SafTPak packaging (SafTPak Category B insulated shipping carton: for cryo- and dry ice shipments, ref. STP-320; for ambient shipments, ref. STP-309) was used and the Sensitech Template 4 was placed with the samples inside the inner box. Frozen, dry ice shipments had the shipper box filled to capacity with dry ice before being sealed and weighed. Upon receipt of the shipments at their destinations, samples and dataloggers were removed.

All cryogenic shipments were sent utilizing a Chart MVE Cryo Shipper equipped with a Cryolid with Datalogger (Chart MVE, ref. 10508967). The datalogger was started after the cryo shipper was charged with LN and stopped once the samples were removed from the cryo shipper. SafTPak packaging was used to safeguard the samples during transport.

Viability assessment

Viability of cryopreserved PBMCs and Jurkat cells was measured immediately after thawing on the day of shipment and after 1 and 2 weeks of storage (storage baseline values) in the laboratory of origin, simultaneously with samples having been shipped and tested by the recipient laboratory. For ambient transport media, the baseline measures included the measures on the day of shipment and after 1 and 2 days of storage (*Fig.2*). Sample quality was measured by determining the levels of viability and early apoptosis, whereas sample functionality was assessed by ELISpot. The viability parameters were assessed by Trypan Blue dye exclusion test, flow cytometry (by Guava, Guava Technologies and Influx, Becton Dickinson) and CASY cell counter (Cell Analysis System),[36] for all applied conditions as described below. Cell recovery and cell viability were assessed immediately after thawing (within 1 h). Each sample was measured 2–3 times.

Thawing procedure

Upon removal from LN storage, no more than two cryovials were thawed at a time by gentle agitation in a 37°C water bath. When the last crystal dissolved, the contents of the vials were transferred immediately to a 15 mL tube and slowly diluted in 9 mL RPMI 1640 containing 10% FBS. The tubes were centrifuged at 150–200 g for 10 min and gently re-suspended in fresh complete RPMI medium.

Trypan Blue

The Trypan Blue exclusion assay was used to determine cell viability and membrane integrity. The cell suspension was mixed with 0.04% Trypan Blue dye. The live cells (negative for staining) and dead cells (positive for staining) were counted using a hemocytometer. The analytical uncertainty of the method, as previously established in Lab 3, corresponded to a CV% of 6.8% ($p<0.05$).

CASY cell counter

Using the CASY cell counter, an electric field multi-channel cell counting system, cell viability can be assessed based on the integrity of plasma membrane; the living cells have intact plasma membranes, whereas membranes of dead cells are disrupted. The analytical uncertainty of the method, as previously established in Lab 3, corresponded to a CV% of 2.1% ($p<0.05$).

Flow cytometry by Guava

Viability was assessed using the Guava PCA-96 system (Millipore) with the Guava ViaCount™ kit (ref. 4000-0040) utilizing DNA-binding dyes that stain viable and nonviable cells based on permeability. Sample analysis was automatically performed by the CytoSoft software and results were obtained as viable and total cell counts per mL. Apoptosis was also assessed by using the Guava Nexin™ kit (ref. 4500-0450) to identify early and late stages of apoptosis of the cells based on the staining with two dyes: Annexin V-PE and 7-ADD. Sample analysis was automatically performed by the CytoSoft software and results were obtained as viable, early apoptotic, late apoptotic cells, and cell debris. Both assays were performed according to the manufacturer's instructions. The analytical uncertainty of the method, as previously established in Lab 2, corresponded to a CV% of 4% ($p<0.05$).

Flow cytometry by Influx

The percentage of necrotic, early apoptotic and viable cells was determined with a BD Influx flow cytometer[37–39] by staining with Annexin V and Sytox green (Invitrogen, Molecular Probes®, ref. A13202, S7020) according to manufacturer's instructions. For each sample, two measures of up to 50,000 events were acquired and the data were analyzed using the FlowJo software program (Treestar, Ashland, OR). Regions were drawn to identify the percentage of cells in each of the three possible populations: viable, necrotic, or early apoptotic. Cells that were negative for Sytox/Annexin V were considered viable as the membranes were intact enough to exclude the dyes; cells that were bright Sytox/Annexin V positive were considered dead/necrotic as very permeable to the dye; cells undergoing early apoptosis were positive for Annexin V staining.

The analytical uncertainty of the method, as previously established in Lab 3, corresponded to a CV% of 1.27% and 2.56% for viable and early apoptotic cells, respectively ($p<0.05$).

T cell functionality assessment by ELISpot assay

Thawed PBMC cells were cultured overnight in R10 and viable cells were counted and tested for IFN-γ ELISpot responses to a peptide pool of 23 10-mer and 11-mer CD8-restricted viral epitopes derived from influenza viruses (CEF) peptide pool, and phytohemagglutinin (PHA). 96-well plates with PVDF membranes (Millipore) were coated overnight at 4°C with anti-IFN-γ monoclonal antibody (MabTech). Cells were added to the blocked plates at 1×105 and 2×105 viable cells per well with CEF peptide pools diluted to approximately 2.5 µg/mL final concentration per peptide and PHA at a final concentration of 5.0 µg/mL. After overnight incubation, bound IFN-γ was detected with biotinylated anti-IFN-γ antibody (MAbTech), followed by alkaline phosphatase-conjugated anti-biotin antibody (Vector Laboratories) and BCIP/NBT substrate (Pierce). Spots were analyzed using a digital imager and automated counting system (AutoImmun Diagnostika). The assay response can vary by as much as two-fold, as previously established in Lab 2.

Data analysis

The data evaluation was carried out according to the International Harmonized Protocol for the Proficiency Testing of Analytical Laboratories,[40] ISO 13528.[41]

The analysis was based on comparison of the mean values (across cell batches) of all participating laboratories to the mean values of the laboratory producing the test items. The percentage of viability was normalized by calculating the ratio of viable cells to the number of viable cells cryopreserved with 10% DMSO and stored in LN at the laboratory producing the test items. Significant differences were considered to be those exceeding each method's analytical uncertainty.

For the evaluation of the instability in the scope of the PT program results, the assigned value and the PT target standard deviation were calculated. The assigned values corresponded to viability values obtained from cells cryopreserved with 10% DMSO and stored in LN. SigmaPlot 11.0 software was used for calculations of mean and standard deviation values.

According to IUPAC and International Harmonized Protocol for the Proficiency Testing,[40] the instability (Δ) of a test item is not consequential if the difference between the mean assigned value (or baseline) and the post-shipment mean is lower than $0.3\ \sigma_p$ ($\Delta<0.3\ \sigma_p$), where σ_p is the PT standard deviation. The PT coefficient of variations (CV%) were 15%, 20%, and 25% for high viability test items, measured by Trypan Blue, CASY, and flow cytometry, respectively. They were 20%, 25%, and 30% for intermediate and low viability test items, measured by Trypan Blue, CASY, and flow cytometry, respectively. The Proficiency Testing σ_p is obtained by multiplying the CV% by the mean value of viability (assigned value).

RESULTS

Shipping process

All samples were shipped and received within 48 h. Results of the temperature monitoring devices did not differ among testing laboratories and showed similar fluctuations during flight and vehicle travel. The highest temperatures occurred during one shipment, excluding one dry ice shipment that thawed during transit.

Effect of cryopreservation and storage in LN with different cryopreservation media on cell viability

Viability of cryopreserved and stored in LN cell suspensions was measured on the day of shipment (after 1 day of storage) and after 2 weeks of storage in different cryopreservation media (*Fig. 2 & 3*).

Figure 3A summarizes the behaviour of Jurkat cells of the three viability levels (HV, IV, and LV) when stored in LN. The comparison between samples showed certain differences from the initial sample conditions as assessed by either Trypan Blue staining or flow

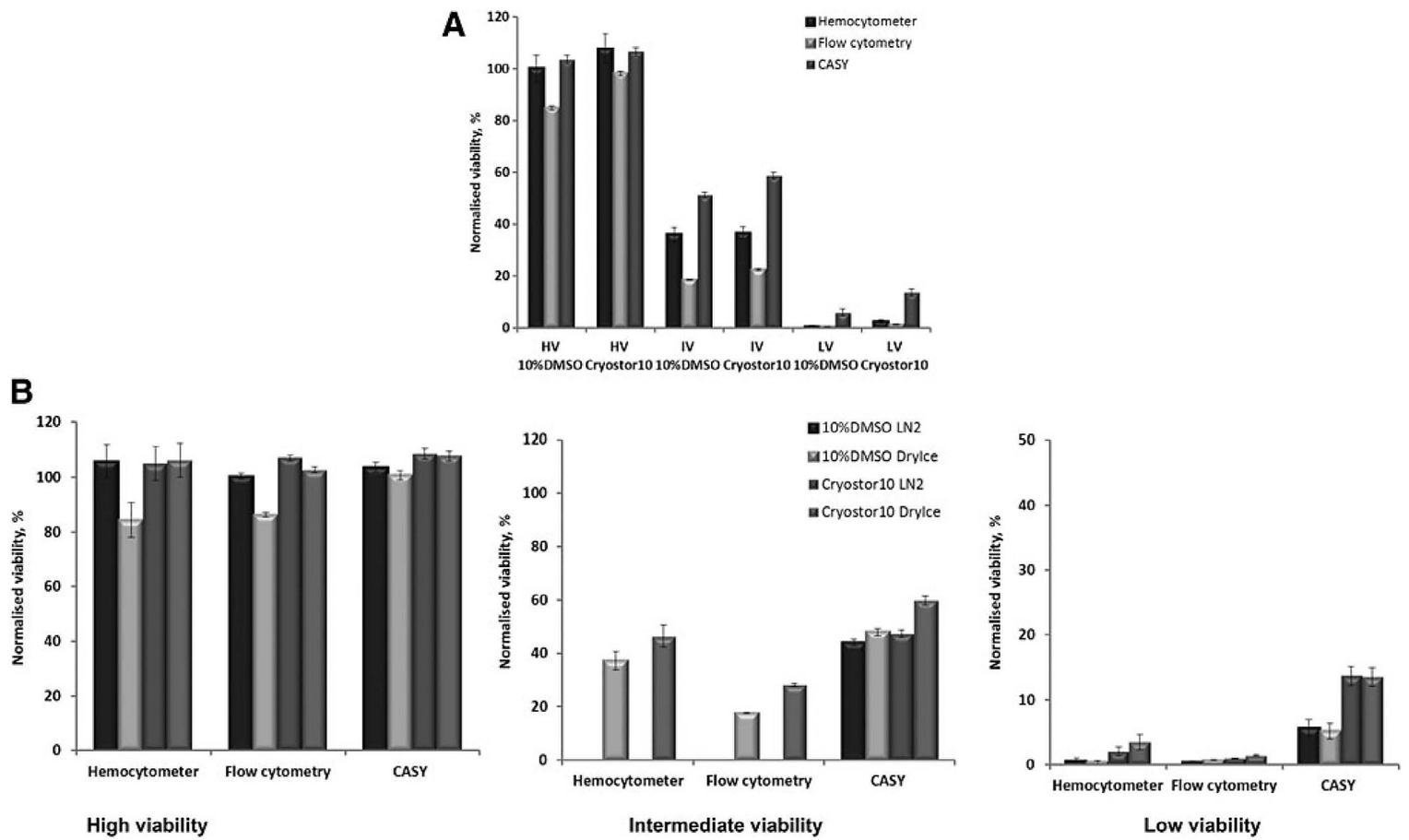

Figure 3. Effect of 2-week-storage in LN2 (<-130°C) in different cryopreservation media on Jurkat cells viability. HV=high viability, IV=intermediate viability, LV=low viability; A=after storage, B=after shipment.

cytometry. Comparison of the viability of the cells cryopreserved in 10% DMSO and CryoStor® CS10 showed no significant differences as measured by Trypan Blue and significant differences as measured by flow cytometry (*Fig. 3A*), with higher viability observed in CryoStor® CS10.

Effect of shipment in LN and Dry Ice with different cryopreservation media on cell viability

Viability of cryopreserved Jurkat cell suspensions shipped in LN and DI was measured after two shipments (*Fig. 3B*). A protective effect of CryoStor® CS10 was observed after shipment. After two shipments, there were differences between LN and DI, especially for Jurkat cells cryopreserved in 10% DMSO, with higher viability in LN. Such differences between LN and DI shipment were not observed in CryoStor® CS10. The difference in viability between cells in DMSO shipped in LN and those shipped in DI was observed by hemocytometry and flow cytometry, but not by CASY.

Figure 4 summarizes the viability results of PBMCs of different viability status. High viability PBMCs reacted similarly to Jurkat cells in the media used. Similar to the Jurkat cells, PBMCs had higher viability when cryopreserved and shipped in CryoStor® CS10 than in DMSO, as assessed by flow cytometry. However, this difference was not significant, when assessed by hemocytometry.

We have also determined if a consequential instability occurred.[40,41] A consequential instability occurs when the viability of the samples, after shipment, is more than $0.3\sigma_p$. *Table 2* shows the results in terms of consequential instability ($\Delta>0.3\sigma_p$) for the shipped Jurkat cells in DI. We showed that consequential instability was dependent on both the initial cell viability status and the assessment method used. Thus, for Jurkat:

- (1) HV cell suspension PT testing by Trypan Blue and flow cytometry, all medium/transport mode combinations were sufficiently stable except for DMSO/DI; for PT testing by CASY, all combinations were sufficiently stable;

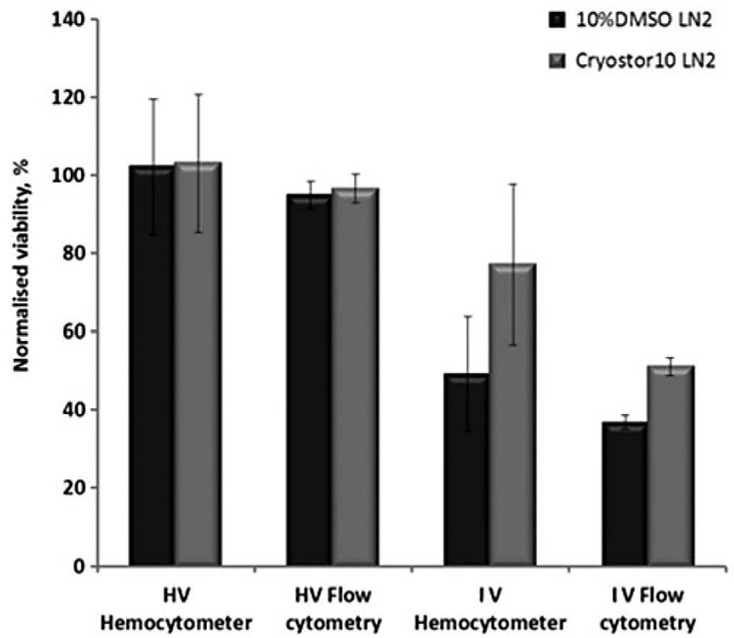

Figure 4. Effect of shipment in LN2 (<-130°C) with different cryopreservation media on PBMCs viability. Initial cell suspension quality before storage was set as: HV=high viability, IV=intermediate viability. Flow cytometry performed by Guava Nexin assay.

- (2) IV cell suspension PT testing by Trypan Blue, flow cytometry, or CASY, cells were sufficiently stable; however flow cytometry showed consequential instability;
- (3) LV cell suspension: only flow cytometry and CASY testing of cells in CryoStor® CS10 showed acceptable stability.

Effect of storage and ambient shipment in AQIX® RS-I preservation medium on cell viability

Viability levels of PBMC and Jurkat cells preserved in room temperature transport medium AQIX® RS-I were compared at different time points. There was a decrease of viability for both types of cells, independent of their initial viability status, during 2–7 day storage in the medium.

There was a significant decrease in viability for Jurkat cells preserved in the AQIX® RS-I on day 2 and 3 (*Fig. 5*) compared to 1 day of storage. A similar decrease in viability was observed after shipment and measurement on day 3 and 4 in the AQIX® RS-I medium (data not shown). An additional limiting

Table 2. Evaluation of Consequential Instability

	Dry Ice			*After shipment*		
DMSO	***0.3 σp***			**Δ**		
Method	***HV***	***IV***	***LV***	***HV***	***IV***	***LV***
Trypan Blue	**3.8**	**2.9**	**0.4**	**16.4***	**1.4**	**2.3***
CASY	**5.1**	**5.1**	**4.9**	**4.4**	**4.4**	**8.6***
Flow cytometry	**5.8**	**2.2**	**0.5**	**8.7***	**1.3**	**3.0***
	DryIce			***After shipment***		
CryoStor	***0.3 σp***			**Δ**		
Method	**HV**	**IV**	**LV**	**HV**	**IV**	**LV**
Trypan Blue	**3.9**	**3.5**	**0.8**	**1.2**	**3.0**	**4.9***
CASY	**5.1**	**5.8**	**5.1**	**1.0**	**1.4**	**0.4**
Flow cytometry	**6.2**	**3.0**	**0.6**	**0.2**	**4.2***	**0.0**

σp, PT standard deviation, expressed as percent viability; Δ, viability percent difference between baseline and post-shipment conditions. *correspond to consequential instability.

factor for experimental measurements was a clotted cell pellet which formed after shipment or after 3 days in the AQIX® RS-I medium (*Fig. 6*).

Functional assessment of cryopreserved and shipped cells

ELISpot assay, which measures specific T cell responses by counting T cells secreting cytokine (IFN-γ) after peptide stimulation, was used to monitor cell functionality. The PBMCs and Jurkat cells showed equivalent functionality in the ELISpot regardless of the preservation media used. The only limitation of the study was the cell quantity in the "low viability" (LV) cell suspension (*Table 3*). Relative to the IV suspension of PBMCs, the IV suspension of Jurkat cells resulted in a more important decrease in the number of IFN-γ producing cells in response to CEF and PHA. Lower spot counts to PHA and to CEF were observed with PBMCs of intermediate viability compared to PBMCs of high viability. PHA-stimulated PBMCs preserved in room temperature transport medium showed a decrease in the number of IFN-γ producing cells during the period of storage. The Jurkat cells had comparable functionality in the ELISpot when stored

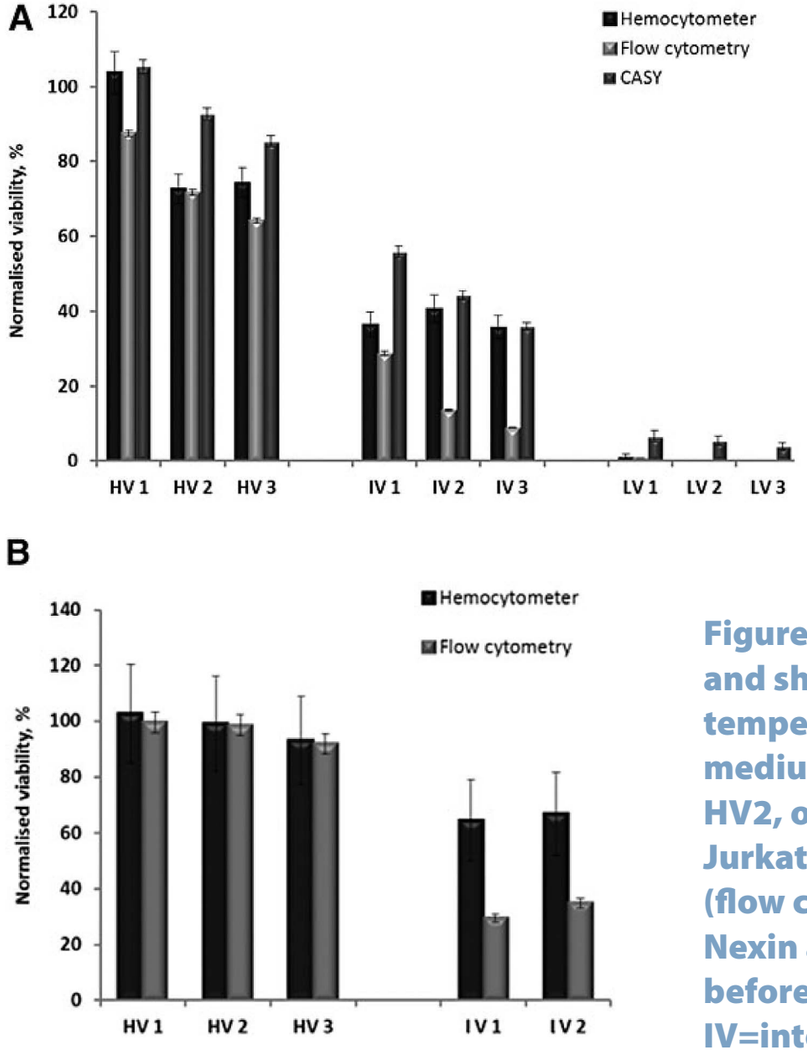

Figure 5. Effect of short-term storage and shipment at RT in AQIX® RS-I room temperature transport medium (in the medium 1, 2, or 3 days, indicated as HV1, HV2, or HV3, etc., respectively) on: (A) Jurkat cells viability, (B) PBMCs viability (flow cytometry, performed by Guava Nexin assay). Initial cell suspension quality before storage was set as: HV=high viability, IV=intermediate viability, LV=low viability. Viability was normalized to the viability of cell suspension cryopreserved with 10% DMSO and stored in LN2 (baseline).

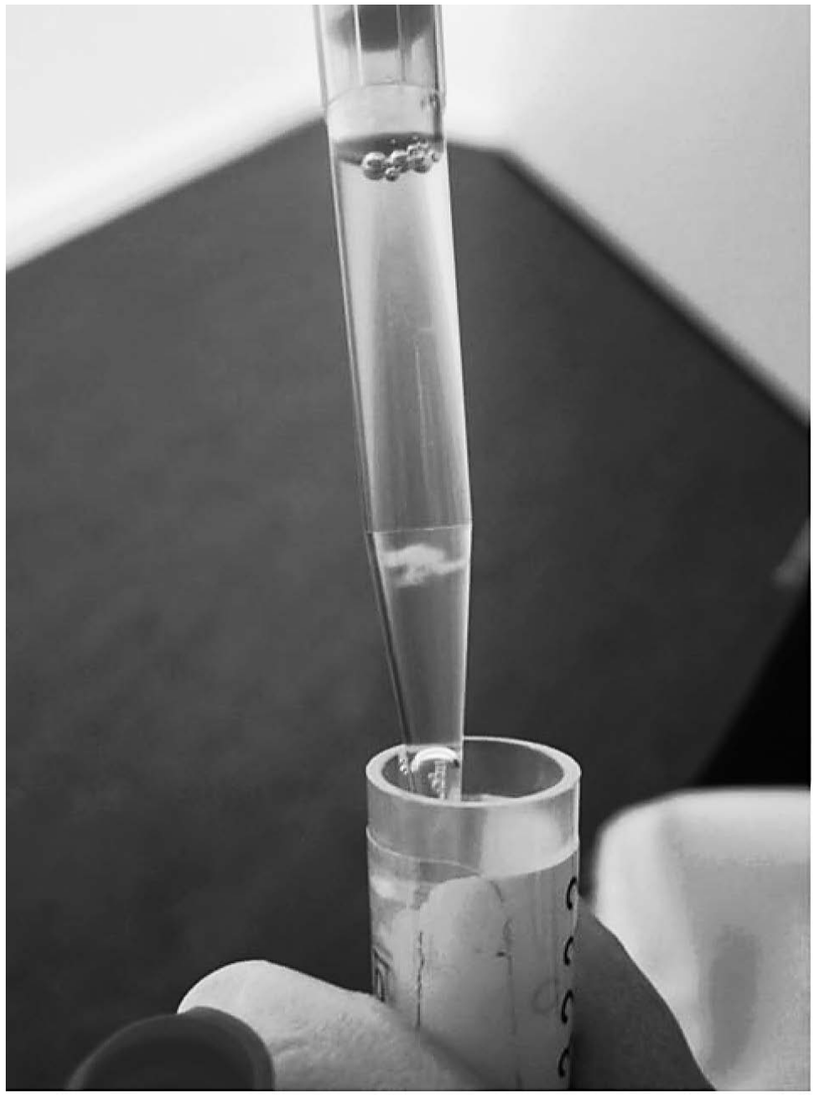

Figure 6. PBMCs after room temperature shipment in AQIX RS-I. The white cloud represents cell pellet/clot.

Table 3. Effects of Cryopreservation Media and Temperature Conditions on IFN-γ ELISPOT Responses of Cell Suspensions of Different Initial Quality

A. PBMCs

ELISPOT Results (mean spots/10^6 cells)

Sample Type	*Storage media*	*Temp condition*	*CEF*	*PHA*
HV PBMC	**Cryostor 10**	**Stored LN2**	**564**	**tmtc**
HV PBMC	**Cryostor 10**	**Shipped LN2**	**643**	**tmtc**
HV PBMC	**10% DMSO +FBS**	**Stored LN2**	**614**	**tmtc**
HV PBMC	**10% DMSO+FBS**	**Shipped LN2**	**433**	**tmtc**
HV PBMC	**AQIX**	**Stored RT 1d**	**703**	**tmtc**
HV PBMC	**AQIX**	**Stored RT 2d**	**663**	**tmtc**
IV PBMC	**Cryostor 10**	**Stored LN2**	**13**	**541**
IV PBMC	**Cryostor 10**	**Shipped LN2**	**10**	**649**
IV PBMC	**10% DMSO+FBS**	**Stored LN2**	**18**	**543**
IV PBMC	**10% DMSO+FBS**	**Shipped LN2**	**6**	**570**
IV PBMC	**AQIX**	**Stored RT 1d**	**11**	**473**
IV PBMC	**AQIX**	**Stored RT 2d**	**28**	**393**

Table 3 continues on next page

in either 10% DMSO or CryoStor® CS10, and a lower response when stored in AQIX® RS-I. The shipment method did not have an impact on the cell function measured by ELISpot. The treatment applied to both types of cells, to produce the LV or IV cell suspensions, had a negative impact on the function measured by ELISpot.

Discussion

Increasingly, biological specimens are transported domestically and internationally in the context of clinical trials or research projects. Many variables can influence the sample integrity during the transport process, including: temperature, packaging, courier, sample type, import/export requirements, seasons, and transit time days.[6]

In our study, we focused mainly on the impact of critical factors, including shipping temperatures, different applied preservation media, different sample types, and different assays to assess their impact on cell viability and function, for the purpose of establishing a mononuclear cell viability PT scheme for bioreposito-ries.[7,42] Our results show the trends of these impacts.

Table 3 continued from previous page

B. Jurkat cells

ELISPOT Results (mean spots/10^6 cells)

Sample type	*Storage media*	*Temp condition*	*CEF*	*PHA*
IV Jurkat	**Cryostor 10**	**Shipped LN2**	**1**	**215**
IV Jurkat	**Cryostor 10**	**Shipped Dry Ice**	**1**	**229**
IV Jurkat	**10% DMSO+FBS**	**Shipped LN2**	**0**	**139**
IV Jurkat	**10% DMSO+FBS**	**Shipped Dry Ice**	**0**	**183**
IV Jurkat	**AQIX**	**Shipped RT**	**1**	**31**
LV Jurkat	**Cryostor 10**	**Shipped LN2**	**QNS**	**43**
LV Jurkat	**Cryostor 10**	**Shipped Dry Ice**	**0**	**10**
LV Jurkat	**10% DMSO+FBS**	**Shipped LN2**	**QNS**	**28**
LV Jurkat	**10% DMSO+FBS**	**Shipped Dry Ice**	**QNS**	**13**

HV=high viability, IV=intermediate viability, LV=low viability; A. PBMCs, B. Jurkat cells;

QNS, quantity not sufficient; tmtc, too many to count.

PT test items have different assigned values of percentage viability, and the assigned values should ideally cover the whole range of the viability measures (from 0 to 100%), with test items of different assigned values being sent to participants from one PT round to the other. Therefore, initial cell suspension viability levels before storage and shipment were set as: HV=high viability, IV=intermediate viability, LV=low viability. Common methods applied for assessing viable cell quality after storage include Trypan Blue dye exclusion[43] and apoptotic assays.[44] In addition, we used CASY cell counting, a method based on the impedance principle. Our results showed differences between different methods. The CASY cell counting method overestimated the viability, especially for intermediate and low viability cell suspensions. The lower the viability, the more important the CASY cell counter mis-estimation was. The most likely explanation is that the impedance-based method can detect only late apoptotic cells, when the cell membrane is already severely compromised. Trypan Blue exclusion assay distinguishes only between cells with intact and disrupted membranes, therefore this method does not give an indication of the cell death state. Our data suggest flow cytometry as an analytical method of choice, because of its higher specificity relative to Trypan Blue assessment and to CASY cell counting, especially in the case of compromised cell viability levels. However, when studying HV cell suspensions, all three methods gave comparable results.

In previously published literature, different procedures have been proposed, including storage in LN or −70°C and shipment of cells in DI or LN.[1,29] Those studies have used −70°C storage and detected bias in viability or function measures due to the difference in the time of storage. Additionally, it has been shown that LN/DI or −70°C/DI storage/shipment of cryopreserved PBMCs is associated with a decrease in viability and viable cell recovery compared with LN/LN. However, those studies did not assess the impact of different preservation media during shipment or ambient temperature shipment. The room temperature transport medium (AQIX® RS-I) has been shown to be very efficient in preserving organs and tissues for several hours.[45] However, its efficiency in maintaining mononuclear cell viability of individual cell suspensions has not been studied. Additionally, if such a medium maintained stable levels of viability at room temperature, it would be very cost efficient for shipment of the PT viable mononuclear cell test items.

Previous studies demonstrated that DMSO-containing freezing medium affects the level of apoptosis,[46] and impacts PBMC function and viability.[47] Other studies reported that storage of the specimens at higher temperatures than LN could increase apoptosis and reduce viability measured by Trypan Blue.[44] The current study has addressed the effect of 10% DMSO in serum and as a component of the commercially available medium CryoStor® CS10 on the viability and function of cryopreserved and shipped PBMCs and Jurkat cells. Earlier publications have reported the cryoprotective and anti-apoptotic effects of CryoStor® CS10 on specific cell types.[23,32] Similarly, our results have confirmed CryoStor® CS10's protective effect on Jurkat and PBMCs; there was higher post-thaw

viability when cells were stored or shipped in CryoStor® CS10 and DI. Medium with serum and 10% DMSO resulted in lower cell viability and AQIX® RS-I resulted in a significant decrease of cell viability. Our results are in concordance with previously published data[27] that reported a deleterious effect of submitting the cryopreserved cells to large temperature variations, all below 0°C.

Our results show that LN storage and LN shipment using cryoshippers ensures higher viability of cryopreserved mononuclear cells and therefore better stability of the shipped biospecimens, applying either serum with 10% DMSO or CryoStor® CS10. Additionally, the main advantage of cryoshippers is that they have a much longer cooling capacity compared to the dry ice. However, the use of cryoshippers significantly increases the shipment costs. First, a cryoshipper charged with liquid nitrogen vapor in its protective shipment packaging weighs around 25 kg, while most dry ice shipments can be done with about 10–15 kg of dry ice. This has a direct impact on the cost of the shipments, with most shipping companies based on standard transport rates. Second, dry ice shipments are usually made in single-use boxes that require no return shipment, while the cryoshippers need to be returned to the station of origin, requiring a return shipment that increases the price of the overall transport of samples by 30%–50%. Third, dry ice polyvinylchloride boxes are cheap compared to a fully equipped cryoshipper that costs about €400–800, and cases of damage during transport are not uncommon. To evaluate correctly the overall cost of cryoshipper-based shipments, one has to add the amortized cost of the cryoshipper to the cost of the shipment. With this in mind, we may conclude that LN shipments are impractical for PT program purposes. LN storage followed by dry ice shipment of viable mononuclear cells, cryopreserved in CryoStor® CS10, was shown to be most cost-efficient solution. AQIX® RS-I and ambient shipment cannot be recommended for the examined sample types. However, more investigations are required for use of AQIX® RS-I on other types of biospecimens and its use for short term preservation (24 h) at ambient temperature could be efficient.

The impact of the type of preservation medium was less obvious on PBMCs than it was on Jurkat cells. The IV PBMCs displayed a significant drop in viability when AQIX® RS-I medium was used. The different impact could be due to the different nature of the cells (multi-passaged/tumor vs. fresh/normal). Flow cytometry showed that the reduction in viability for the HV, IV, and LV cells was due to an increase in apoptotic/dead cells. The results obtained by CASY cell counter showed an overestimation of viable cells.

The baseline values were used for the PT-consequential instability calculations. Instability was not consequential in the scope of the PT program for Jurkat cells of high viability, shipped in CryoStor® CS10/DI, and tested by all methods; for Jurkat cells of intermediate viability, shipped in CryoStor® CS10/DI, and tested by Trypan Blue and CASY; for Jurkat cells of low viability, shipped in CryoStor® CS10/DI and tested by CASY and flow cytometry. For cells shipped in DMSO/DI instability was inconsequential only for the intermediate viability samples.

Good recovery, viability,[48] and consistent IFN-γ ELISpot responses[49] have been reported for PBMCs optimally stored in LN (or below −130°C). However, there is little information on the impact of shipment in different cryopreservation media and at different temperatures on frozen mononuclear cell sample quality, as assessed by IFN-γ ELISpot testing. Therefore, IFN-γ ELISpot responses to CEF and PHA were evaluated. Our data suggest that the initial quality of stored samples is a critical factor for the post-thaw results. Initial low quality of the cell suspensions lead to lower responses in the ELISpot assay, after stimulation with mitogens or T-cell-specific antigens.

Depending on the antigen used, the method showed different sensitivity to the conditions tested. Responses to CEF were only detected in the HV PBMC suspension. PHA stimulation was possible in both HV and IV PBMCs suspensions. Viable PBMCs generated high responses to PHA. Such high PHA responses make it difficult for the automated counter to distinguish individual spots resulting in a "tmtc" (too many to count) read-out (*Table 3*). However, PHA serves as a good qualitative indicator of PMBC functionality, as shown by the fact that the IV PBMCs demonstrated several-fold lower PHA responses as compared to the HV PBMCs. ELISpot also shows that long hypothermic treatment is detrimental to cell functionality. However, this method could not differentiate between cell physiological conditions at different days, as could the CASY cell counting and the flow cytometry methods.

Overall, the results obtained are in accordance with previously published results showing that the function of cryopreserved PBMCs is associated with viability,[30] and indicating that cell viability <70% may introduce a bias in the responses measured by ELISpot.[14,43] Our data also support that the baseline quality of biospecimens before storage and shipment influences their stability. Besides, the use of serum in a medium may have an impact on lymphocyte activation in T cell assays due to the presence of different cytokines in serum.[50] As proposed by Smith et al.,[27] the ELISpot cut-off value should be ≥1000 SFC (spot-forming cells). However, functional assays are variable and require large sample sets to demonstrate significant differences.

Neither we nor others[51] could identify systematic "shipping errors" for cryopreserved cells; however the applied preservation conditions (temperature, time, media, etc.) may be critical factors affecting biospecimen quality and analytical results. We conclude that CryoStor® CS10/Dry Ice combination gives higher viability by every method, for each viability level, and is the most cost-efficient shipment method. Another critical factor is the laboratory/operator performance. Therefore, whenever possible, laboratories should participate in the "inter-laboratory exercises" or Proficiency Testing Programs[7,42] in order to control random or systematic laboratory errors, to establish quality assurance programs and guarantee the quality of cryopreserved and shipped biospecimens, and to increase standardization among biobanks and/or end-user laboratories.

Our study shows the trends of analytical results variations under the influence of different shipment temperatures and different preservation media. It is known that LN/LN storage/shipment

ensures the highest quality of cryopreserved biospecimens, and this was confirmed. However, under limited funding conditions or in the context of large PT programs, LN shipments are impractical. In these cases, we show that alternative high-quality shipment conditions for viable cells are dry ice shipment and CryoStor® CS10 cryopreservation medium. Indeed, for both Jurkat and PBMC cells, it is possible to use CryoStor® CS10 with dry ice shipment or when the initial cell suspension displays suboptimal viability, without dramatic impact on cell viability. Room-temperature transport medium was not fit-for-purpose, it dramatically and adversely affected the integrity of mononuclear cells. Since shipment temperature is so critical, we propose monitoring of shipment temperatures. ■

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Disclosure Statement

No competing financial interests exist.

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