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Original Article

Reconstitution and post-thaw storage of cryopreserved human mesenchymal stromal cells: Pitfalls and optimizations for clinically compatible formulants

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ABSTRACT

Introduction: The regenerative and immunomodulatory properties of multipotent mesenchymal stromal cells (MSCs) make them an intriguing asset for therapeutic applications. An off-the-shelf approach, using pre-expanded cryopreserved allogenic MSCs, bypasses many practical difficulties of cellular therapy. Reconstitution of a MSC product away from cytotoxic cryoprotectants towards a preferred administration solution might be favorable for several indications. Variations in MSC handling accompanied by a non-standardized use of reconstitution solutions complicate a general clinical standardization of MSC cellular therapies. In this study, we aimed to identify a simple and clinically compatible approach for thawing, reconstitution, and post-thaw storage of cryopreserved MSCs.

Methods: Human adipose tissue-derived MSCs were expanded in human platelet lysate (hPL) supplemented culture medium and cryopreserved using a dimethyl sulfoxide (DMSO)-based cryoprotectant. Isotonic solutions (saline, Ringer's acetate and phosphate buffered saline (PBS)) with or without 2% human serum albumin (HSA) were used as thawing, reconstitution, and storage solutions. MSCs were reconstituted to 5×10^6 MSCs/mL for evaluating MSC stability. Total MSC numbers and viability were determined using 7-aminoactinomycin D (7-AAD) and flow cytometry.

Results: For thawing cryopreserved MSCs the presence of protein was proven to be essential. Up to 50% of MSCs were lost when protein-free thawing solutions were used. Reconstitution and post-thaw storage of MSCs in culture medium and widely used PBS demonstrated poor MSC stability (>40% cell loss) and viability (<80%) after 1 h of storage at room temperature. Reconstitution in simple isotonic saline appeared to be a good alternative for post-thaw storage, ensuring >90% viability with no observed cell loss for at least 4 h. Reconstitution of MSCs to low concentrations was identified as critical. Diluting MSCs to $<10^5$/mL in protein-free vehicles resulted in instant cell loss (>40% cell loss) and lower viability (<80%). Addition of clinical grade HSA could prevent cell loss during thawing and dilution.

Conclusion: This study identified a clinically compatible method for MSC thawing and reconstitution that ensures high MSC yield, viability, and stability. The strength of the method lies within the simplicity of implementation which offers an accessible way to streamline MSC therapies across different laboratories and clinical trials, improving standardization in this field.

1. Introduction

The regenerative and immunomodulatory properties of multipotent mesenchymal stromal cells (MSCs) make these cells an intriguing asset for therapeutic applications. Through their secretome and membrane molecules, MSCs are able to modulate

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immune responses and control inflammation, supporting normal tissue regeneration [1,2].

In 2022, more than 1000 clinical trials applying MSCs were registered at clinicaltrials.gov. The spectrum of conditions targeted by MSC-based therapies is broad, ranging from systemic MSC treatments in type 2 diabetes, solid organ transplantation, and graft versus host disease to localized MSC therapies in osteoarthritis, inflammatory bowel disease, soft tissue regeneration, and degenerative disc disease [3–9]. Both autologous and allogenic MSCs are investigated as a therapeutic entity.

Many autologous therapies have used freshly harvested MSCs for clinical investigation. This approach, however, is logistically challenged by coordination within narrow treatment windows when MSC procurement, operating room availability, and scheduling of treatments needs to be synchronized within short notice. An off-the-shelf approach, using pre-expanded cryopreserved allogenic MSCs bypasses the practical difficulties and increases the indication range to support availability for acute indications. Additionally, allogenic cryopreserved MSCs can be produced in large quantities and may present the only economically sustainable solution for clinical implementation of MSC-based therapies. To cryopreserve MSCs, addition of cryoprotectants is necessary to ensure acceptable cell recovery and viability post thawing.

Depending on the clinical indication, MSCs are administered either systemically by intravascular infusion or locally by injection or topical application. Intravenous infusion is a popular MSC administration route and is used for immunomodulatory indications such as graft versus host disease, solid organ transplantation, and stroke [10–12]. Intravascular administration is often performed by direct infusion of freshly thawed cells in a suspension containing the cryoprotectant [13,14]. Safety studies have shown that intravenous administration of MSC products containing the cryoprotectant can be performed without associated serious adverse events [15]. For local treatments, reconstitution of the MSC product, to remove cryoprotectants, might be preferred to prevent potential cytotoxicity at the site of administration. This is particularly applicable for local administration in poorly vascularized recipient sites such as intraarticular or intradiscal injection [9,16]. The reconstitution can be done on-site, however, this requires additional post-thawing handlings that introduce potential and unwanted product variation.

To optimize and guarantee viability and potency of MSC-based cellular therapies, investigation of the impact of MSC thawing and post-thawing reconstitution conditions have been a primary focus for MSC procurement facilities. Variation in MSC handling has shown to influence both recovery and viability of MSCs delivered for therapeutic applications [17–21]. Current literature, however, has relied on pre-clinical cell handling protocols, using reagents infrequently applied for clinical applications, such as phosphate buffered saline (PBS) or culture medium supplemented with bovine serum. Most studies have focused on investigation of either the cryopreservation and thawing of MSCs or the reformulation and post-thawing storage of MSCs. The entire cell handling process from thawing of cryopreserved MSCs to point of administration by the end-user, however, is rarely covered. The mismatch in reagent usage between pre-clinical studies and clinical trials, combined with large variation in investigated methods, limits the inter-study comparison and may not represent a clinical translational approach [22–24].

To bridge this gap, the aim of this study was to investigate cryopreserved MSC recovery and viability, using clinically relevant solutions after thawing, reconstitution, and post-thaw storage. This covers the cell handling processes from thawing of the MSCs to release of the final formulated MSC product by the MSC procurement facility.

2. Material and methods

The research protocol was approved by The Central Denmark Region Committees on Health Research Ethics, journal number 1-10-72-1-20.

2.1. MSC isolation, expansion and cryopreservation

Culture medium consisted of Minimum Essential Medium (MEMα no nucleosides; Gibco-Thermo Fisher Scientific-22561-021) supplemented with 5% PLTGold Human Platelet Lysate (Mill Creek Life Sciences-PLTGOLD100GMP), 2 mM L-Glutamin (Gibco-Thermo Fisher Scientific-25030-024) and 50 units/mL Penicillin-Streptomycin (Gibco-Thermo Fisher Scientific-15070063). Adipose tissue was collected from adults undergoing cosmetic surgery. Between 10 and 20 mL of lipoaspirate or 5–10 mL minced adipose

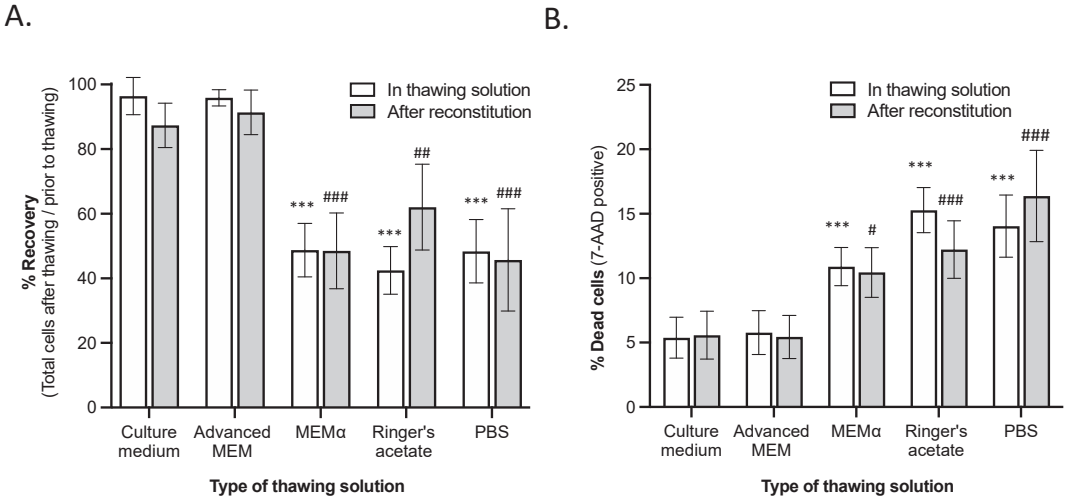

Fig. 1. Effects of different thawing solutions on MSC stability. (A) Percentage recovery (total cells after thawing compared to total cells prior to thawing). (B) Percentage of dead cells (7-AAD positive). Data represents the mean ± standard deviation of five independent experiments using five MSC batches derived from different donors. *p <0.05, **p <0.01, ***p <0.001 versus culture medium (in thawing solution) by one-way ANOVA. #p <0.05, ##p <0.01, ###p <0.001 versus culture medium (after reconstitution) by one-way ANOVA.

tissue was washed twice with 20 mL PBS by using centrifugation at 650 *g* for 10 min. After wash, 4–6 mL adipose tissue was transferred to a 50 mL centrifuge tube, containing 20 mL RPMI supplemented with 6 units of collagenase (Nordmark Biochemicals-N0002779) and incubated horizontally in an orbital shaker at 37 °C at 140 rpm for 40 min. After digestion, 10 mL culture medium was added, and the cell solution was centrifuged at 650 g for 10 min. Pellet (stromal vascular fraction) was resuspended in 25 mL culture medium and filtered over a 70 um cell strainer (Corning, CLS431751). This stromal vascular fraction (SVF) was seeded in T175 culture flasks (Greiner Bio-One-Gr-660175) and incubated at 37 °C in humid air with 5% CO_2. Next day, culture medium was exchanged twice to remove erythrocytes and debris. After 4–5 days, cells were split (passage 0) using 6 mL TrypLE™ Select (Gibco-Thermo Fischer Scientific- A1217701) and centrifugation at 440 *g* for 5 min. Cells were subcultured in 4–8 new T175 culture flasks containing 20 mL culture medium. Culture medium was changed when the culture reached 40–50% confluency and cells were harvested when the culture reached about 90% confluency. For all experiments, MSCs were expanded to a maximum of passage three. After harvest, cells were resuspended to approximately 5×10^6 cells/mL in CryoStor CS10 (Stemcell Technologies-07952) in 1.8 mL cryotubes (Thermo Fisher Scientific-363401) and transferred to a CoolCell™LX Cell Freezing Container (Corning, CLS432002) placed at −80 °C. After freezing, cells were kept at −80 °C until use.

2.2. Multi-color flow cytometric MSC characterization

Isolated and expanded MSCs were observed for plastic adherence and fibroblast-like spindle morphology. For flow cytometric characterization, 100 μl of MSC resuspended in culture medium at a concentration of 1–1,5 $\times$ 10^6 MSC/ml was incubated with 1,5 μl anti-CD73 [PE] (BioLegend cat. no. 344044), 1,5 μl anti-CD90 [APC-Cy7] (BioLegend cat. no. 328132), 5 μl anti-CD105$^+$ [APC] (BioLegend cat. no. 323208), 0,5 μl anti-CD14 [FITC] (BioLegend cat. no. 301804), 0,5 μl anti-CD19 [FITC] (BioLegend cat. no. 302206) 0,5 μl anti-CD31 [FITC] (BioLegend cat. no. 303104), 1,5 μl anti-CD45 [FITC] (BioLegend cat. no. 368508), 0,5 μl anti-HLA-DR [FITC] (BioLegend cat. no. 307604) and 1 μl of 1 mg/mL 7-Aminoactinomycin D (7-AAD, AAT Bioquest, cat. no. ABD-17501) for 30 min at room temperature in dark. After incubation, 3 mL of PBS with 0.1% BSA was added to the cells, followed by centrifugation at 500 *g* for 5 min. The pelleted cells were resuspended in 400 μl of PBS with 0.1% BSA, followed by analysis on a Novocyte 3000 (Agilent). The acquisition rate was kept below 1.000 for all analyses. A fluorescence minus one-guided gating strategy was applied to identify positive and negative events. In all applied MSC batches, ≥90% of the viable cells expressed the positive markers CD73, CD90, and CD105, while ≤2% expressed the negative markers CD14, CD19, CD31, CD45, and HLA-DR.

2.3. MSC absolute quantification and viability determination

MSC viability was measured using 7-AAD and flow cytometry (NovoCyte 3000). Samples with a concentration higher than 1×10^6 cells/mL were diluted to 1×10^6/mL in culture medium prior to analysis. Before addition to the cells, 7-AAD was diluted in culture medium to a concentration of 0.2 mg/mL (from a 1 mg/mL stock in DMSO). In total, 100 μl MSC sample was stained with 5 μl pre-diluted 7-AAD and incubated for 5 min at room temperature in the dark. All samples were stored in 5 mL polypropylene tubes (Beckman Coulter cat. no. 2523749). After incubation, 300 μl of

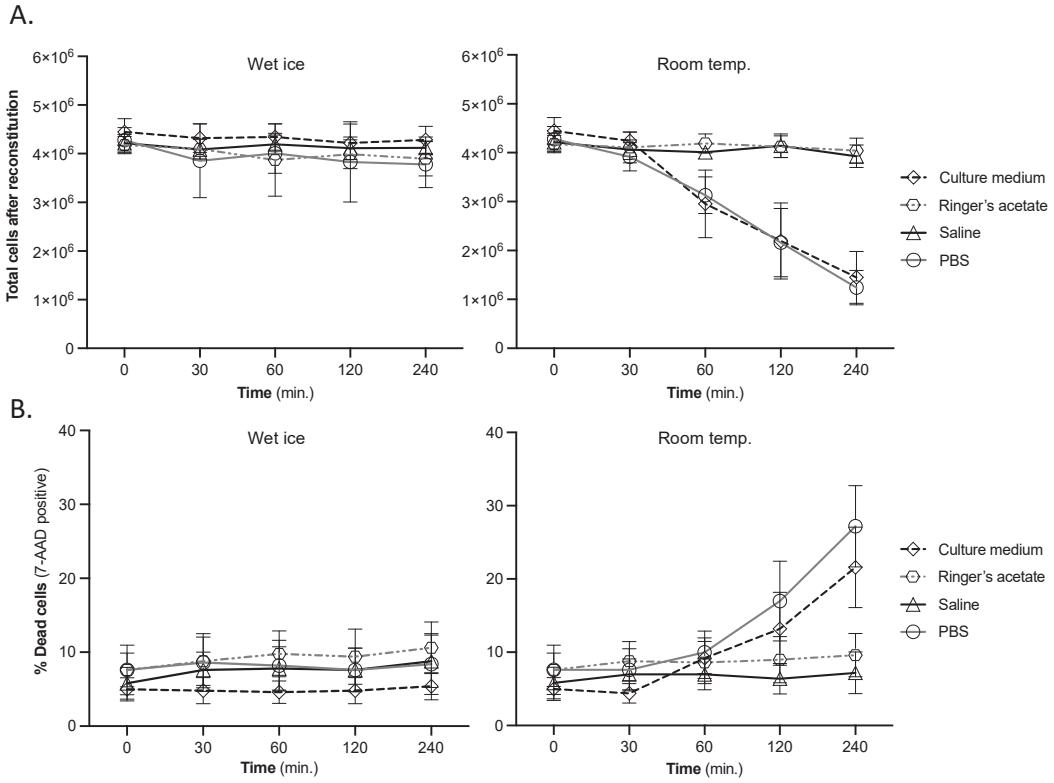

Fig. 2. Effects of different storage solutions and temperature on MSC stability over 4 h. (A) Total cell amount during storage when stored on wet ice or at room temperature. (B) Percentage of dead cells (7-AAD positive) during storage when stored on wet ice or at room temperature. Data represents the mean ± standard deviation of five independent experiments using five MSC batches derived from different donors.

culture medium was added per sample and samples were directly analyzed by flow cytometry, using excitation at 488 nm and an emission filter 667/30 nm. Acquisition rate was kept below 1.000 events/sec for all analysis. Cells were gated based on their forward scatter and sideward scatter and 7-AAD negative cells were considered viable. Each sample was analyzed in triplicate (three individual tubes from same sample) and a mean was used as a data point. Data from Fig. 5 was, in addition to flow cytometry, quantified using a counting chamber (Bürker Türk, Sigma Aldrich), to confirm the physical integrity of the MSCs. Data from Fig. 7 was also quantified by using an NucleoCounter NC-202 (Chemometec) to confirm the results found by flow cytometry.

2.4. Thawing and reconstitution

Cryotubes with 1 mL cryopreserved cells (5×10^6 MSC/mL) were thawed by holding the cryotube in a container with warm water (about 37 °C) under continuous swirling until a pea-sized clump of ice was left. Thawed cells were slowly pipetted to a 50 mL centrifuge tube, containing 20 mL culture medium or other thawing solutions (as stated in the results section). This was followed immediately by centrifugation at 440 *g* for 5 min. To remove residual thawing solution, the cell pellet was washed by carefully adding 20 mL of reconstitution solution on top of the pellet (without resuspending the cell pellet) followed by centrifugation at 440 *g* for 5 min. In the experiments investigating the impact of thawing solutions (Figs. 3 and 7A), all conditions were reconstituted in culture medium prior to analysis. For the experiments investigating the impact of low cell concentration, cells were washed and reconstituted in Ringer's acetate (Figs. 5 and 6) or isotonic saline (Fig. 7C and D) prior to dilution into other solutions. The following solutions were used; Ringer's acetate (Fresenius Kabi-B05BB01), PBS (CTS DPBS, calcium, magnesium; Gibco-Thermo Fisher Scientific-A1285801), isotonic saline (Fresenius Kabi-B05BB01, V07AB), Minimum Essential Medium α (MEM α, no nucleosides Gibco-Thermo Fisher Scientific-22561054), advanced MEM (Gibco-Thermo Fisher Scientific- 12492021), and human serum albumin (Alburex 20, CSL Behring).

2.5. PKH26 staining

MSCs were labeled with the PKH26 membrane stain, using a PKH26 Red Fluorescent Cell Linker Kit, (Sigma Aldrich). In total, 5×10^6 cryopreserved MSCs were thawed as described earlier and washed with 10 mL of plain MEMα. The solution was centrifuged at 440 g for 5 min and the pellet resuspended in 250 μl Diluent C. A PKH26 stain buffer solution was made by mixing 2,5 μl PKH26 with 250 μl Diluent C. The PKH26 stain buffer was then added to the MSC suspension and mixed by pipetting 10 times. After exactly 60 s of incubation, the PKH26 stain was neutralized by addition of 5 mL culture medium. The MSC solution was then centrifuged at 440 g for 5 min and the pellet resuspended in 5 mL culture medium. The final wash step was repeated, and the cells were resuspended in Ringer's acetate to a concentration of 3×10^6 cells/mL.

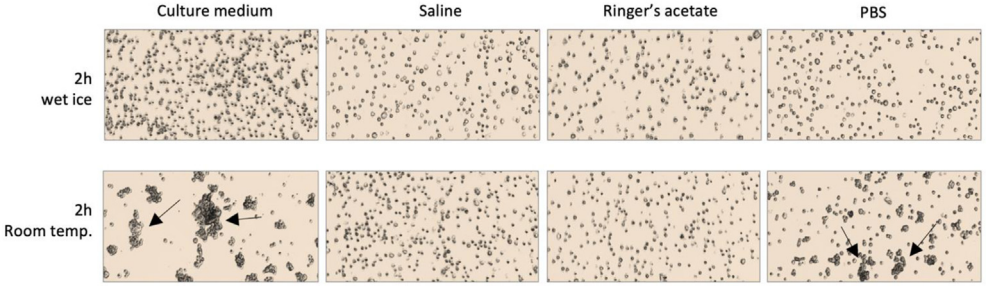

Fig. 3. Light microscopy pictures of MSCs stored on wet ice or at room temperature in different storage solutions. Cell aggregates (arrows) are observed during storage at room temperature in culture medium and in PBS. Images acquired at × 40 magnification.

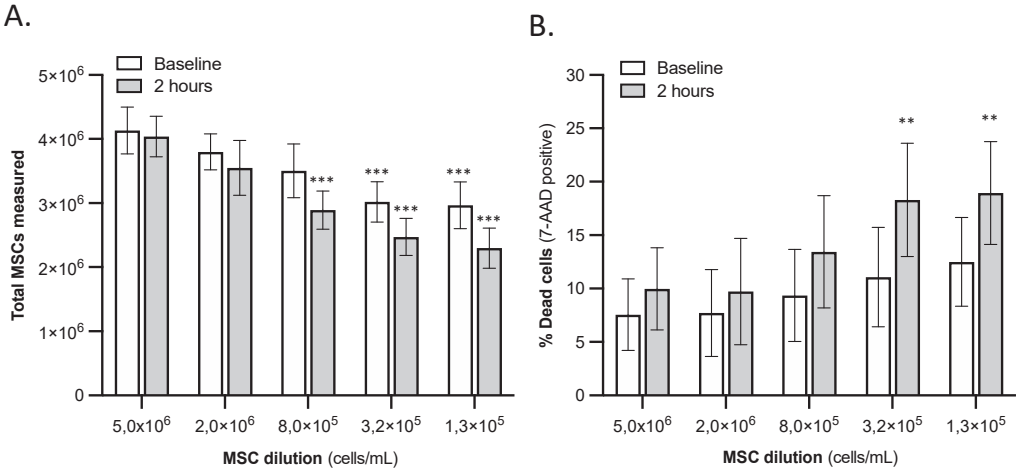

Fig. 4. Effect of MSC concentration on MSC stability. (A) Total cell amount after dilution in Ringer's acetate at baseline and after 2 h of storage. (B) Percentage of dead cells (7-AAD positive) after dilution in Ringer's acetate at baseline and after 2 h of storage. Data represents the mean ± standard deviation of five independent experiments using five MSC batches derived from different donors. *p <0.05, **p <0.01, ***p <0.001 versus 5 × 106 cells/mL samples (baseline) by two-way ANOVA.

2.6. Generation of heat inactivated MSCs (HI-MSCs)

HI-MSCs was generated following a previously published protocol [25]. Briefly, cryo-preserved MSCs were thawed and reconstituted in PBS, as described earlier, to a concentration of 5×10^6 MSC/mL and incubated for 35 min at 50 °C, followed directly by incubation on ice for 5 min. Cells were then centrifuged at 440 g for 5 min and resuspended in either PBS or Ringer's acetate at a concentration of $2{,}5 \times 10^6$ HI-MSC/mL. After 24 h, HI-MSC stained >98% positive for 7-AAD and were not able to adhere to plastic under culture conditions.

2.7. Statistical analysis

For experiments investigating thawing, reconstitution, and post-thaw storage conditions, each dependable variable was investigated in five individual donors. All dependable variables were blocked and investigated together using a single donor at a time. Thus, the data represents five individual experiments. Data were analyzed using two-way and one-way ANOVA with multiple comparison testing. The assumption of normality was assessed with both raw and log transformed data, using QQ-plots. A p value < 0.05 was considered statistically significant. Statistical analysis and graphs were performed and created using GraphPad Prism 9.0.2 Software (Dotmatics).

3. Results

3.1. Protein-free thawing solutions induces cell loss and decreased cell viability

Thawing and reconstitution of cryopreserved cells often includes a first step where the cells, after thawing, are transferred to a protein-containing solution such as culture medium. The reintroduction of culture medium, however, will result in the presence of undefined proteins in the final cell product, which is unwanted in a clinical application. Therefore, it was investigated if a protein-free thawing solution could be used instead. Cryopreserved MSCs were thawed and transferred to three different protein-free thawing solutions: Ringer's acetate, PBS, and plain MEMα. This was compared to transfer of the cells to culture medium and advanced MEM (MEM medium supplemented with albumin). Total cell number and cell viability were measured directly after transfer to thawing solution and again after centrifugation and reconstitution.

Transfer of thawed MSCs to protein-free thawing solutions resulted in significantly decreased total cell numbers and viability. Up to 50% of the MSCs were lost in this step and the fraction of dead cells significantly increased, when compared to thawing in culture medium or advanced MEM. Importantly, cell loss was introduced during the transfer to thawing solution, while the process of

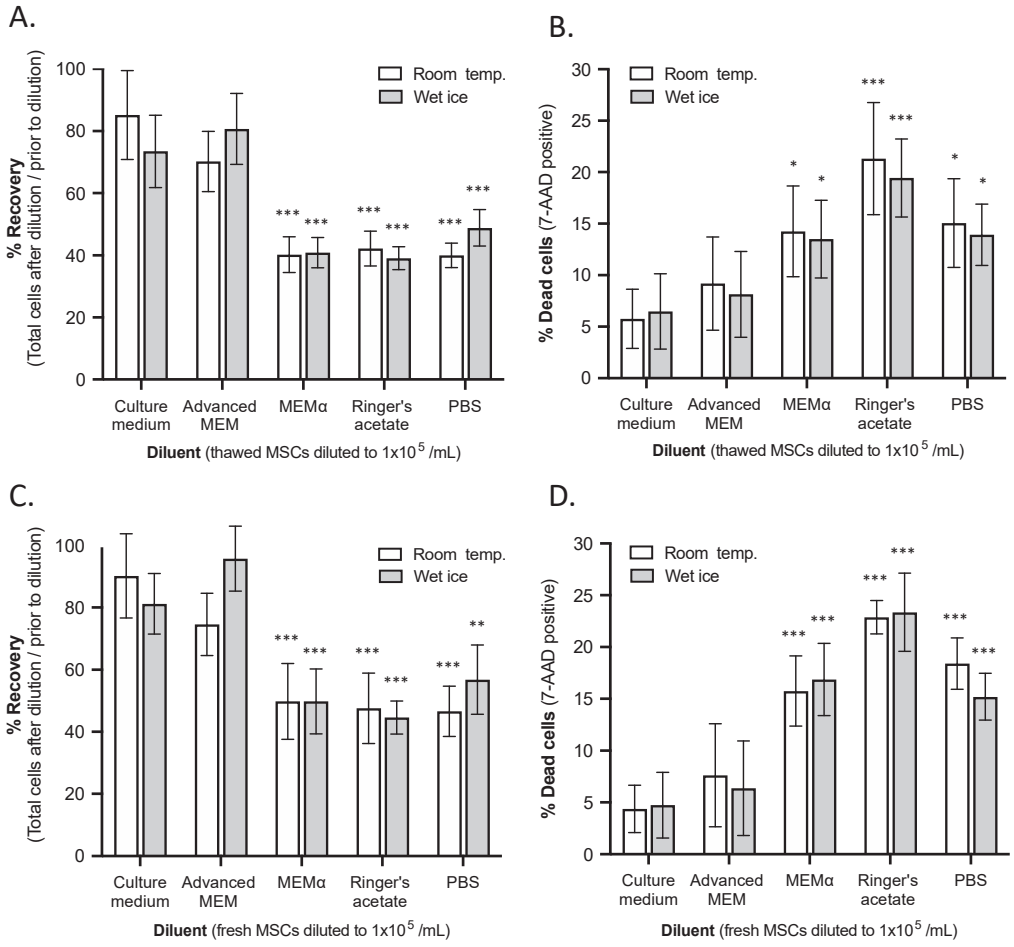

Fig. 5. Effect of temperature and used diluent on MSC stability at a low cell concentration (1×10^5 cells/mL). (A, C) Percentage recovery (total cells after dilution compared to total cells prior to dilution) after dilution of thawed MSCs and freshly harvested MSCs. (B, D) Percentage of dead cells (7-AAD positive) after dilution of thawed MSCs and freshly harvested MSCs. Data represents the mean ± standard deviation of five independent experiments using five MSC batches derived from different donors *p <0.05, **p <0.01, ***p <0.001 versus dilution in culture medium (room temperature) by one-way ANOVA.

centrifugation and reconstitution did not add extra cell loss (Fig. 1A and B).

3.2. Type of reconstitution solution affects MSC stability

Next, MSC stability was monitored after thawing and reconstitution, using different types of reconstitution solutions such as Ringer's acetate, isotonic saline (saline), and PBS. Reconstitution in culture medium was used as a control. Total cell amount (Fig. 2A) and cell viability (Fig. 2B) were measured immediately after reconstitution and during 4 h of storage on wet ice or at room temperature. Saline and Ringer's acetate provided good MSC stability for up to 4 h, when stored at room temperature or on wet ice. Surprisingly, at room temperature, culture medium, but also PBS, showed a strong decline in total cell numbers and cell viability, which was already visible after 1 h (Fig. 2A and B). Where in most conditions MSCs appeared as a clear single cell solution, MSCs reconstituted in PBS or culture medium formed cell aggregates, when stored at room temperature (Fig. 3). During 4 h of storage, both at room temperature and on wet ice, the MSCs maintained their immunophenotype with >90% of the MSCs being CD14-, CD19-, CD31-, CD45-, HLA-DR-, CD73+, CD90+ and CD105+. This applied for MSCs in all reconstitution solutions (3 independent experiments using 3 different donors, data not shown).

3.3. MSCs loss and reduced viability when reconstituted at low concentrations

Using Ringer's acetate as a reconstitution solution and diluent, the impact of final cell concentration after reconstitution was investigated. After thawing, MSCs were reconstituted and diluted to different concentrations, ranging from of 5×10^6 cells/mL to $1{,}3 \times 10^5$ cells/mL. Total cell number and cell viability were measured immediately after dilution and after 2 h of storage at room temperature. MSC suspensions diluted to concentrations lower than 8×10^5 MSCs/ml resulted in a significant cell loss that reached up to 45% with a coinciding decrease in cell viability (Fig. 4A and B). Interestingly, cell loss and decreased viability was observed immediately after dilution.

3.4. MSCs can be diluted to low concentration in protein containing solutions

To investigate if cell loss at low cell concentrations could be prevented, MSCs were thawed and reconstituted at a low cell concentration of 1×10^5 cells/mL in both protein-free solutions (PBS, plain MEMα, Ringer's acetate) and protein-containing solutions, such as culture medium and advanced MEM. Moreover, the impact of temperature was tested by using pre-chilled solutions (wet ice).

The resulting observations confirmed that reconstitution to low cell concentration leads to instant cell loss (up to 50%), when protein-free diluents are used. In contrast, when MSCs were diluted in culture medium or advanced MEM, only minor cell loss was observed (Fig. 5A and B). The temperature had no impact on the observed cell loss and reduced cell viability.

To exclude the possibility that thawed MSCs were more sensitive to the processing steps than freshly harvested MSCs, the experiment was repeated using freshly harvested MSCs. This showed that freshly harvested MSCs are equally unstable at low concentrations, when using protein-free solutions (Fig. 5C and D). To confirm these unexpected observations, a similar set of dilution experiments was performed, where MSCs were counted using a Bürker-Türk cell counting chamber in addition to absolute quantification by flow cytometry. This confirmed the findings of instant cell loss after dilution in protein-free diluents (data not shown).

3.5. Dilution-induced MSC loss can be rescued by cell-to-cell contact

As MSCs in high concentrations were not affected by protein-free diluents, we investigated if MSC stability was dependent on the paracrine milieu or by direct cell-to-cell contact. For this, MSCs were thawed and then diluted in protein-free solutions that were already supplemented with high amounts of MSCs. The fluorescent membrane dye PKH26 was used to pre-label MSCs (PKH-MSCs) prior to dilution to allow tracing and counting the cells after dilution (Fig. 6A).

First, PKH-MSCs were diluted to concentrations of 1×10^5/mL in culture medium, Ringer's acetate or PBS. This confirmed poor MSC stability at low cell concentration, when using protein-free solutions (Fig. 6B). Next, PKH-MSCs were diluted in a similar way, however, dilution occurred in the presence of $2{,}5 \times 10^6$/mL unlabeled MSCs or $2{,}5 \times 10^6$/mL unlabeled secretome-deficient HI-MSCs. This revealed that the presence of high amounts of MSCs, including HI-MSCs, prevented the dilution-induced cell loss of PKH26 pre-labeled MSCs (Fig. 6B).

3.6. Clinically compatible MSC thawing and MSC dilution using saline and human serum albumin

Contemplating the results of the previous series of experiments, a clinical compatible thawing and reconstitution method was formulated, using commonly clinically available reagents like saline and human serum albumin (HSA). Saline supplemented with 2% HSA (saline-HSA) was tested as a thawing solution and compared to the transfer in culture medium and saline only. This showed that saline-HSA as a thawing solution performed equally well compared to culture medium (Fig. 7A and B). Saline-HSA also ensured MSC stability at a low concentration (Fig. 7C and D). Finally, we tested the complete process of thawing, reconstitution and storage using only saline and HSA. MSC were thawed using saline-HSA and reconstituted to either saline or saline-HSA. MSC count and viability was measured directly after reconstitution and after 4 h of storage at wet ice or room temperature. This confirmed that thawing in saline-HSA, combined with reconstitution in saline or saline-HSA, results in a stable reconstituted MSC product.

4. Discussion

In this study series, we investigated different approaches for handling of cryopreserved MSCs, covering thawing, reconstitution, and post-thaw storage of the cells. By evaluating MSC stability, a complete post-thaw MSC handling approach was formulated, relying on two clinically compatible reagents, saline and HSA. The addition of HSA was necessary to prevent cell loss during the thawing process. However, reconstitution of thawed MSCs in saline was well tolerated by the MSCs, allowing a final MSC product free from additional additives. Critically, reconstitution to lower MSC concentrations in protein-free solutions resulted in a significant cell loss and reduced cell viability. The addition of small amounts of HSA, however, prevented this loss of cell stability. MSCs formulated in either pure saline or saline supplemented with HSA proved to be stable for at least 4 h. Thus, relying solely on commonly used clinically compatible reagents, a complete MSC handling approach with flexible storage capabilities was possible.

The results from this study emphasize that even simple changes to MSC thawing and post-thaw MSC conditions can present big

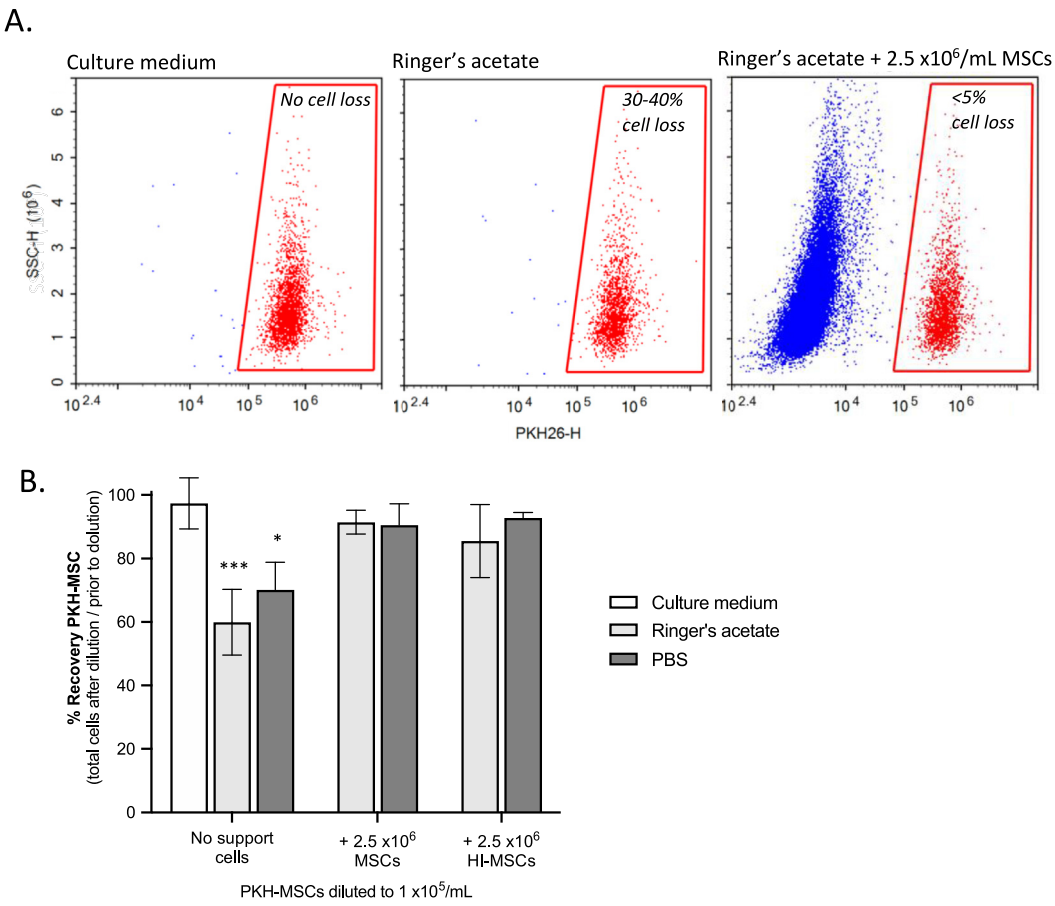

Fig. 6. Effect of the presence of supporting cells on dilution induced MSC loss. (A) Representative scatter plots of PKH26 prelabeled MSCs (PKH-MSCs) diluted in culture medium, Ringer's acetate, or Ringer's acetate supplemented with 2.5 × 10^6/mL unlabeled MSCs. (B) Percentage MSC recovery (total cells after dilution compared to total cells prior to dilution) after dilution in the presence and absence of supporting cells. Data represents the mean ± standard deviation of three independent experiments using three MSC batches derived from different donors. *p <0.05, **p <0.01, ***p <0.001 versus dilution in culture medium by one-way ANOVA.

impacts on the quality of the MSC product. Originating donor variance and batch-to-batch variance from the cell culturing process further introduce extra layers of complexity, hampering standardization of MSC based cellular therapies. To allow better comparability and reproducibility of clinical studies of MSC based therapies, more uniform MSC handling protocols will help to reduce the inter-study variance, accelerating clinical investigation and development. Relying on viability and cell number measures, this study aimed to validate a simple, low cost, and easily accessible approach for post-thaw MSC handling, which would be accessible for the majority of laboratories working with MSCs.

The observation of severe cell loss at low MSC concentrations was unexpected and the exact mechanism remains unknown. Interestingly, the effect was almost instant and could be rescued by direct cell-to-cell contact or protein additives. Importantly, the instant cell loss at low MSC concentrations was not due to a more fragile nature of thawed MSCs as a similar effect was seen when using freshly harvested MSCs. In retrospect, the observed cell loss during the thawing process in protein-free thawing solutions, might be explained by poor cellular stability at low cell concentrations, which are reached during transfer to thawing solution.

Severe cell loss, as a consequence of low MSC concentrations, will not be of relevance for most clinical applications. In clinical trials, MSCs are rarely reconstituted to these low concentrations and, moreover, protein-containing solutions are frequently used. However, examples exist of clinical trials using MSC conditions that according to our presented data are in risk of critical cells loss. In addition, in many animal and *in vitro* studies, MSCs are often used at concentrations identified as suboptimal for MSC stability by this study. This dilution-induced cell loss also pose significant importance for quality assurance and release criteria assays of MSC based products in the case an assay requires dilution of the MSC product.

Surprisingly, reconstitution in widely used PBS resulted in poor MSC stability and cell clotting at room temperature. Reconstitution in culture medium was initially used as a positive control as we expected this to be the most optimal condition for MSCs. Reconstitution in culture medium, however, also led to a lower cell stability and unwanted cell clotting. Importantly, culture medium and PBS are both frequently used in *in vitro* assays and pre-clinical research with PBS further advancing as storage solution in clinical trials [7,26,27]. Cell clotting was only observed at room temperature and might be related to the formation of functional MSC aggregates described in other studies [28].

Commercially available cryoprotectants now exists which eliminates the need for serum and thereby undefined proteins during the cryopreservation process. However, in many standard MSC thawing procedures, protein additives are reintroduced as the thawed cellular product is transferred to thawing solutions supplemented with serum or platelet lysate. Although seen as standard procedures, little information can be found in the literature supporting the need of such additives. Our data indeed supports the need of protein for optimal thawing. However, the use of undefined

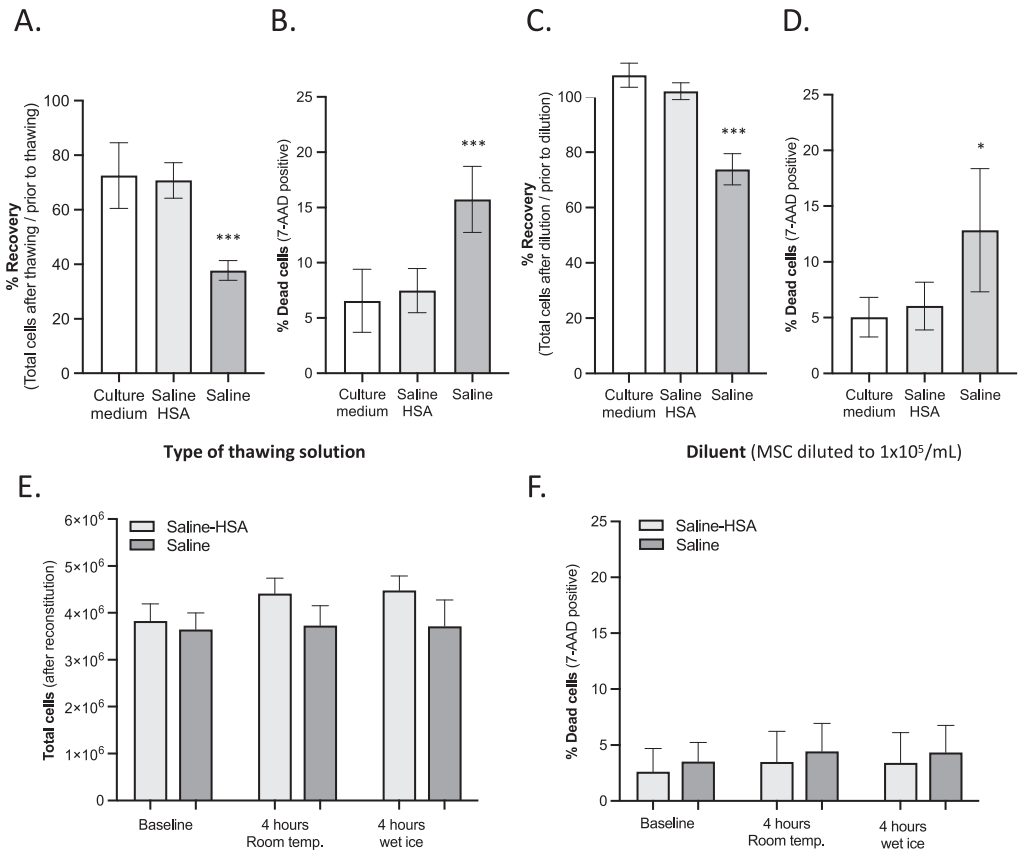

Fig. 7. Thawing, dilution, and storage of MSCs using clinical compatible solutions (saline and saline + HSA). (A) Percentage recovery (total cells after dilution compared to total cells prior to dilution) after thawing in saline-HSA. (B) Percentage of dead cells (7-AAD positive) after thawing in saline-HSA. (C) Percentage recovery (total cells after dilution compared to total cells prior to dilution) after dilution in saline-HSA. (D) Percentage of dead cells (7-AAD positive) after dilution in saline-HSA. (E, F) Total cell amount and percentage of dead cells (7-AAD positive) after 4 h of storage in plain saline or saline-HSA (after thawing in saline-HSA). Data represents the mean ± standard deviation of five independent experiments using five MSC batches derived from different donors. *p <0.05, **p <0.01, ***p <0.001 versus culture medium by one-way ANOVA.

and complex protein mixtures, such as serum or platelet lysate, was avoidable by addition of clinical compatible solution such as HSA. If serum albumin is unwanted in the final product, albumin amounts could be minimized by a simple wash step prior to final reconstitution.

Reviewing the pre-clinical and clinical literature, it is difficult to estimate the impact of the used thawing and reconstitution procedure on the quality of the MSC product and study outcomes. The root cause being poorly or entirely missing descriptions of the thawing and reconstitution procedures. As broadly demonstrated by current literature and by the present study, minor changes in MSC processing can pose significant impact on the MSC stability. We therefore encourage a stringent reporting of thawing and reconstitution procedures, to evaluate and compare results between studies.

Other research groups have also investigated MSC stability after reconstitution. A plethora of combinations of storage vehicles, times, and temperatures together with different types of MSCs have been used [20,29,30]. Notably, current literature has primarily used freshly cultured MSCs in their study setups. For an off-the-shelf MSC-therapy, direct use of thawed or reconstituted cryopreserved MSCs presents the most flexible and feasible approach for MSC therapy. Validation and optimization of this approach has only been investigated in a few studies [17,31].

In the study by Mirabel C et al. [31] the effect of different types of albumins for cryopreservation, thawing, and post-thaw storage of bone marrow MSCs (BM-MSCs) was investigated. The study found yeast-derived albumin to be superior to human serum albumin, when supplemented to both the cryopreservation solution and the thawing solution. Using HSA for cryopreservation and thawing, a viability of only 60% was obtained, which does not directly correlate with the 95% viability observed from our HSA-based thawing solution.

In the study by Pal et al. [17], freshly harvested BM-MSCs were compared to thawed cryopreserved BM-MSCs. The study found a viability of 90% after 4 h of storage at 4 °C, using isotonic saline as vehicle, which is similar to the results presented in our study. The results from Pal et al., however, were obtained using a thawing protocol lacking serum or protein supplements, which in our hands resulted in a significant drop of viability and cell recovery. Importantly, their study clearly demonstrated that removal of cryoprotectant by reconstitution was essential for optimal MSC post-thaw stability. Applying protocols from the present study, reconstitution of cryopreserved MSCs can easily be performed without loss of MSC numbers or viability. Thus, inclusion of a reconstitution step for cryopreserved MSC therapies will not only limit the possible cytotoxic effect of cryoprotectants in patients, but also stabilize and standardize the MSC product.

We encourage the development of a more uniform MSC thawing and reconstitution process between different laboratories to increase comparability between studies and thus clinical development. As a first step towards standardization, we present an approach which supports MSC stability for at least 4 h, conveniently also in protein-free vehicles such as saline and even at room

temperature. A 4-h post-thawing MSC stability provides sufficient flexibility for the MSC product to allow for delays in transport to the department of administration or changes of the administration protocol as consequences of surgical complications.

Other studies have demonstrated MSC stability for significant longer periods with up to 72 h storage at refrigerator temperatures [22,23,29,30,32,33]. MSC stability and viability, however, may not be a representative measure for long-term stored cells function and potency. Studies have found that while long-term refrigerator stored MSCs maintain their viability, the colony forming capacity was greatly reduced even after as short as 1 h of storage [18,23,30]. This indicates that even though MSCs seems to be stable and maintain high viability, additional testing might be relevant regarding MSC potency. Selecting the pertinent potency assays to predict the pharmaceutical dynamics of a cellular product for specific indications, however, is a general and yet unsolved challenge in the field of MSC therapy.

5. Conclusion

This study presents pitfalls and optimizations for thawing, reconstitution, and post-thaw storage of cryopreserved MSCs. From this, a clinically compatible method for MSC thawing and reconstitution was developed that ensures high MSC yield, viability, and stability. The strength of the method lies within the simplicity of implementation which offers an accessible way to streamline MSC therapies across different laboratories and clinical trials, improving standardization in this field.

Disclosure

The authors declare no conflict of interest. All authors have approved the final article.

Declaration of competing interest

All the authors declare no conflict of interest.

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