Original Research Article

Evaluating Novel Dried Formulations for the Preservation of Human Mesenchymal/Stromal Stem Cells

ABSTRACT

Aim: To overcome the logistical hurdles associated with cryostorage, cell therapy products would benefit from being stored as a (freeze-) dried product. However, there is currently unfortunately not a lot of focus in the field to evaluate formulations for their capabilities to preserve cells, especially not in dried states. At early stages of freeze-dried cell therapy product development an efficient and reliable approach to select suitable formulations is key to enable the development process. It was thus the aim of this study to offer an approach that screens for potentially viable formulations for dried cell therapy products.
Methodology: This multi-step approach comprised of measuring glass transition temperature, residual moisture, cell membrane integrity, morphology, viability, and proliferation to successively narrow the range of potentially applicable formulations. We employed this approach for a selected set of novel and potentially applicable urea- and glucose-based formulations with clinically relevant human mesenchymal/stromal stem cells.
Results: Some evaluated conditions yielded partially dried products that showed cell viability and other conditions yielded high numbers (up to 95%) of membrane intact cells in elegant cakes.
Conclusion: This approach presented here offers an efficient setup to screen formulations for their capabilities to protect cells during freeze-drying. The evaluated formulations yielded promising results, inviting for further investigations.

Keywords: Cell preservation, cell therapy product, drying, formulation, hMSCs, viability

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1. INTRODUCTION

Cell therapy products are a class of advanced medicinal products, composed of live cells or tissues, that show promise in regenerative medicine and in the treatment of severe diseases [1-3]. These products face stability challenges since living components can be fragile and difficult to preserve. Furthermore, cell therapy products must provide sufficient viable and

functional cells to be effective throughout their life cycle while also complying with regulatory guidelines and quality controls to ensure patient safety [4, 5]. Cells can be administered as freshly processed products which limit their shelf live to hours or days [3]. To facilitate long term storage cell therapy products are often cryopreserved (typically in liquid nitrogen or at ≤ - 65 °C [6]). However, cold-chain maintenance, storage, and distribution can lead to substantial logistical hurdles and high financial burdens [7-9], making cryostorage not ideal. In addition, the formulations of such cryopreserved products are often suboptimal from a pharmaceutical and safety perspective, related to the type and quantity of excipients used. To overcome these challenges, cell therapy products may be preferable stored fresh or as dried products at refrigerated temperatures [10].

(Freeze-) Drying of small molecule drugs, antibodies or vaccines is a well-established process [11], however, the drying of cell therapy products faces advanced challenges. The product should be providing stability and retaining efficacy by keeping the cells viable. Despite their relevance, literature on freeze-dried cells relevant for cell therapy products is scarce, likely due to the complexity of process and formulation development of living cells [10]. As pointed out by Merivaara*et al.* [12], recent efforts to select suitable formulations that yield dried cells mostly relied on trial-and-error. This may result in inefficient screening approaches and a lack of translationally applicable standards for the selection of potentially suitable formulations and analyses of cell viability after drying, as recently shown by Rockinger*et al.* [13]. During early development stages, a reliable approach to evaluate a formulations' impact on cell product quality (i.e., viability, cake properties) is vital to streamline the selection of relevant formulations with the desired properties. Multiple works described different approaches (for example, adhesion evaluation [14], colony formation [15, 16], or DNA integrity [17]) to determine parameters such as cell viability after drying. Since cell viability values depend on the choice of assay, these different approaches make inter-experimental comparisons difficult and results might not be directly translational [18], especially when cell line-specific assays are used, such as colony formation or differentiation assays. As Rockinger*et al.*[13] summarized, the most commonly applied assays to measure viability after drying are based on cell membrane integrity principles (i.e., trypan blue). However, in the context of (freeze-) drying, assays solely based on such measurements may not fully represent cell viability as the cell membrane may remain intact while intracellular components are irreversibly damaged during the process [14, 19], resulting in insufficient conclusions about the cellular health status.

It was thus the aim of this study to offer additional framework to assess selected novel formulations for their pertinence and protective capabilities in the context of freezing and subsequent drying of cells. The approach comprises of multiple steps which aid in selecting potentially applicable formulations excipients. Placebo formulations were first evaluated for residual moisture content, glass transition temperature, and cake appearance to select those expected to yield dried, elegant cakes. Then, cell membrane integrity measurements were employed for fast preliminary screens to select formulations with potentially viable cells. Lastly, cellular viability was confirmed via microscopic analysis and by measuring metabolic activity and cell proliferation.

Previously, we showed that urea and glucose synergistically maintain cell viability during freeze-thaw stress in unaltered human mesenchymal stem cells (hMSCs) on comparable levels to conventional dimethyl sulfoxide (DMSO) -based formulations [19]. Building on these findings, urea- and glucose-based formulations were supplemented with additional excipients, specifically, we selected sucrose, DMSO, bovine serum albumin (BSA), mannitol, hydroxyethyl starch (HES) and/or polyethylene glycol (PEG). The excipients used in this study to screen for protection were carefully selected based on previous experiments [9, 12, 19-21]. Glucose serves as a protective agent, while mannitol and sucrose both act as protective and bulking agents [22, 23]. It is hypothesized that these agents provide protection by substituting water molecules during water removal, stabilizing intracellular structures (water replacement hypothesis), or by trapping residual water near biomolecules allowing

them to keep their original structure (water entrapment hypothesis) [24]. DMSO is a known potent cryoprotective agent that readily penetrates the cell membrane [25], providing protection by depressing the freezing point of water and by preventing the formation of damaging ice crystals [26]. Despite intolerances in humans, DMSO is used as a cryoprotective agent in some cell therapy products [3]. HES is a stabilizer for cakes and has favorable properties for drying due to its high glass transition temperature [9]. Furthermore, it can bind extracellular water, promoting its removal from the cell and subsequently shifting ice crystal formation to the extracellular space [27]. BSA can act as a cell membrane stabilizer and as a surrogate for human serum albumin [13, 28] and PEG as a potent cake builder [29]. Furthermore, urea can act as an osmo- and cryoprotectant by stabilizing intracellular structures and by reducing cell shrinkage induced by hyperosmotic stress caused by an up-concentration of solutes during freezing [30]. Freezing-induced intracellular uptake of membrane-impermeable protective agents may be enhanced by urea, as it acts as a penetration enhancer due to its membrane fluidizing and destabilizing properties, as previously shown [31, 32]. In the composite formulations used in this setup it is likely that the different mechanisms act in concert to provide protection.
Although the focus of this manuscript was on formulation screening optimization and process parameters around freeze-drying were out-of-scope, the primary drying times were varied to potentially enhance cake appearance and cell viability.

2. MATERIAL AND METHODS

2.1 Cell Culture

Adherent bone marrow derived human mesenchymal stromal/stem cells (hMSCs) (ATCC) from a single donor were cultured under standard conditions and harvested as described previously [19]. hMSCs were selected as a model cell line because they possess regenerative and immunomodulatory properties and are thus clinically relevant cells at the forefront of clinical research [33-37]. hMSCs were incubated overnight in medium supplemented with 0.2 M trehalose to facilitate intracellular uptake of the protective disaccharide and for potential improvement in cell survival [14, 19].

2.2 Sample Preparation

The following excipients were used as protective and/or scaffolding agents: urea (GE Healthcare), glucose (Sigma-Aldrich), sucrose, trehalose (both Pfanstiehl), mannitol, (Avantor) dimethyl sulfoxide (Arcos), hydroxyethyl starch (Carbosynth), bovine serum albumin (Sigma-Aldrich), polyethylene glycol 400 and 8000 (Arcos, FisherScientific, respectively). Samples were prepared in phosphate buffered saline (PBS) (Gibco). hMSCs were harvested by trypsination and up-concentrated by centrifugation to a target concentration of $1x10^6$ cells per mL. For each condition, 0.5 mL of the cell suspension was added to a 6R TopLyo glass vial (Schott) containing 0.5 mL of the 2-fold concentration solution of protective agents resulting in a total volume of 1 mL and a target concentration of $0.5x10^6$ cells per mL at the target concentration of protective agents. The vials have an inner hydrophobic coating which prevents cells from adhering to the surface. In conditions where the 2-fold concentrated protective agent solution could not be solubilized, the target amount of cells was centrifuged and was then resuspended in solution with the target concentration of protective agents. All vials were immediately placed in a ≤ -65 °C freezer for > 20 hours to minimize osmotic stress. Samples were then loaded into a pilot scale SP Scientific LyoStar 3 freeze-dryer pre-cooled to -65 °C. The samples were equilibrated for 300 minutes. Subsequently, a vacuum was drawn to 35 mTorr and held for 60 minutes, before the temperature was raised to -40 °C, and the vacuum was lowered to 30 mTorr. Samples were dried in this primary drying step for either 60 or 180 hours. Compared to other published

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primary drying times for cells of 18-36 hours [12], this step was increased due to expected slow sublimation rates of water due to the low primary drying temperatures. To avoid loss of viable cells, drying was conducted at the lowest feasible temperature where ice sublimation still occurs while reliably maintaining vacuum. A secondary drying step was omitted to avoid potential loss of viable cells [16]. After this drying step, the samples were allowed to reach room temperature before reconstitution in 6 mL growth medium which was pre-warmed at 37 °C in a water bath. Cells were subsequently transferred to a 96-well plate or a culture flask to allow the evaluation of viability.

2.3 Residual Moisture and Glass Transition Temperature

Residual moisture content was measured with an 851 Karl Fischer titrator (Metrohm). Each sample was reconstituted with 4 mL anhydrous methanol (Sigma-Aldrich), vortexed, and subsequently stored at room temperature overnight to allow for water to dissolve into the methanol. Glass transition temperature of the maximally freeze-concentrated solution (Tg') was measured with a TA Instruments differential scanning calorimeter DSC Q2500. In both cases, measurements were taken in selected placebo (without cells) samples.

2.4 Cell Membrane Integrity and Viability Assessment

Cell membrane integrity was measured after resuspension of the samples using a NucleoCounter NC-200 (Chemometec) which automatically determined cell membrane integrity with an acridine orange/DAPI stain. hMSC viability was qualitatively evaluated by assessing the cells' ability to re-attach to the cell culture vessel plastic surface, their capability to proliferate, and their morphology. Cells were inspected using an Olympus CKX53 inverted microscope at 40x magnification. Viability and relative cell number were also measured by performing alamarBlue assay (Invitrogen) according to the manufacturer`s instructions as described previously [18] for up to nine days after reconstitution and subsequent culturing. The assay is based on the reduction of a resazurin dye into a fluorescent form, a process only facilitated in metabolically active, thus viable, cells. Cells that did not undergo any freezing and partial drying (referred to as "unstressed") served as reference for fully viable cells. Relative numbers of viable cells *(n_{rel})* were determined using the following formula: $n_{rel} = \frac{s_1}{s_0}$ (1), where s_1 is the fluorescence signal from the cells after drying and s_0 is the signal of the unstressed reference cells.

2.5 Statistical Analysis

The software GraphPad Prism (version 10.1.0 for Windows, GraphPad Software, Boston, Massachusetts USA) was used to compare different experimental groups with one-way or two-way ANOVA where applicable and a Bonferroni post hoc test was included to account for multiple comparisons. For all measurements, n is at least 3. Experimental groups were considered statistically different when $P < .05$.

3. RESULTS

Detailed formulation compositions and the results of the measured quality attributes including cell membrane integrity are summarized in Table 1. Measurements of the glass transition temperature of the maximally freeze-concentrated solution (Tg´) in formulations with large amounts of protective agents (F1-F4, F6) were relatively low and ranged from -64 °C to -

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49 °C. In these formulations, high residual moisture contents from 16% up to 42% remained, likely leading to collapsed cakes (see Figure 1). To yield a solid cake with lower residual moisture, the concentrations of urea, glucose and sucrose were successively halved, PEG 8000 and PEG 400 were added as excipients, and the primary drying time was increased (F7-F10). When decreasing the amounts of urea and sugars, residual moisture contents steadily decreased from 38% (1 M urea, 1 M glucose, 0.5 M sucrose) to <1% (no urea, glucose, or sucrose) and Tg´ increased from -49 °C to -24 °C. Formulations comprising of 0.25 M urea, 0.25 M glucose, and 0.125 M sucrose or less, yielded elegant, intact cakes. In formulations with highly concentrated protective agents, an increase in drying time only marginally decreased the residual moisture content, such as from 42% to 38% in the urea, glucose, sucrose, and mannitol samples.

In the evaluated formulations and tested conditions, a large percentage of cells showed intact membranes on comparable levels, 72% in urea/glucose/sucrose and DMSO, 83% in urea/glucose/sucrose and BSA, 80% in urea/glucose/sucrose and mannitol, and 74% in urea/glucose/sucrose and HES. This demonstrated a substantial degree of stabilization of

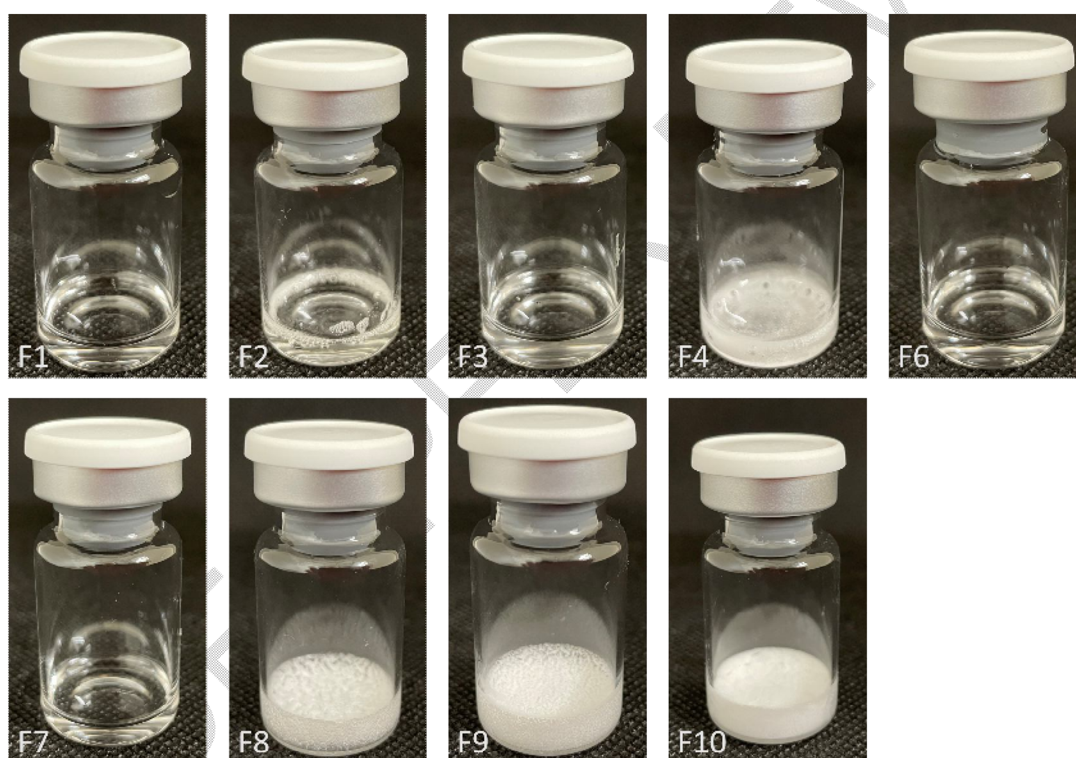

Figure 1: Images of vials after drying
Representative images of vials after drying for 180 hours with placebo formulations (without cells). Detailed formulation compositions are listed in Table 1.

Table 1: Formulation composition, critical attributes, and cell membrane integrity measurements
Formulation composition and drying times of each formulation. Measurements of residual moisture and the glass transition temperature of the maximally freeze-concentrated solution (Tg') where taken in placebo (without cells) samples. Cell membrane integrity measurements were taken after drying and subsequent reconstitution in medium. All formulations were prepared in PBS. n ≥ 3 for all conditions. Values show averages ± standard deviation. n. m.: not measured.

Name	Formulation Composition	Tg' [°C]	Residual Moisture [%]	Cake Appearance	Cell Membrane Integrity [%]	Drying Time [h]
F1	1 M urea, 1 M glucose, 0.5 M sucrose, 4% DMSO	-63.5 ± 0.5	38 ± 3 32 ± 4	collapsed collapsed	72 ± 6 81 ± 2	60 180
F2	1 M urea, 1 M glucose, 0.5 M sucrose, 5% BSA	-57.7 ± 0.8	36 ± 6 33 ± 11	collapsed collapsed	83 ± 8 68 ± 9	60 180
F3	1 M urea, 1 M glucose, 0.5 M sucrose, 10% mannitol	-57.2 ± 0.7	42 ± 1 38 ± 3	collapsed collapsed	80 ± 6 77 ± 3	60 180
F4	1 M urea, 1 M glucose, 0.5 M sucrose, 10% HES	-57.4 ± 0.5	16 ± 4 21 ± 7	collapsed collapsed	74 ± 8 85 ± 11	60 180
F5	PBS	n. m.	n. m.	intact	13 ± 8	60
F6	1 M urea, 1 M glucose, 0.5 M sucrose, 5% PEG 8000, 10% PEG 400	-49.1 ± 0.8	38 ± 5	collapsed	98 ± 1	180
F7	0.5 M urea, 0.5 M glucose, 0.25 M, sucrose, 5% PEG 8000, 10% PEG 400	-49.3 ± 0.5	31 ± 6	collapsed	95 ± 3	180
F8	0.25 M urea, 0.25 M glucose, 0.125 M, sucrose, 5% PEG 8000, 10% PEG 400	-38.6 ± 0.7	14 ± 3	intact	95 ± 5	180
F9	0.125 M urea, 0.125 M glucose, 0.06 M, sucrose, 5% PEG 8000, 10% PEG 400	-23.9 ± 1.0	8 ± 1	intact	87 ± 8	180
F10	5% PEG 8000, 10% PEG 400	-24.7 ± 0.3	0.38 ± 0.33	intact	35 ± 4	180

cells. The highest number of membrane intact cells after freeze-drying was detected in the urea/glucose/sucrose and PEG formulation (98%). Overall, an increase in drying time did not correlate with a decrease in cell membrane integrity, however, a decrease in residual moisture below 1% in a formulation comprising only of PEG resulted in a sharp drop to 35% membrane integrity. Interestingly, the control in PBS showed a subpopulation of dead cells with intact membranes (12%). For this condition no viable cells would also be expected as the formulation lacks cryo- and lyoprotective agents.
To qualitatively confirm viability and proliferation, hMSCs were subsequently cultured for up to nine days and inspected microscopically (see Figure 2). Despite high numbers of membrane intact cells, conditions with decreased amounts of urea, glucose, and sucrose and with added PEG (F7-F10) with intact or collapsed cakes, yielded no viable cells. The control in PBS (F5), as expected, also did not yield viable cells. Some hMSCs dried in formulations with high amounts of protective agents (F1-F4, F6) could re-attach to the culture flasks and had healthy,
regular morphology and increased in number and density over time, indicating that the cells

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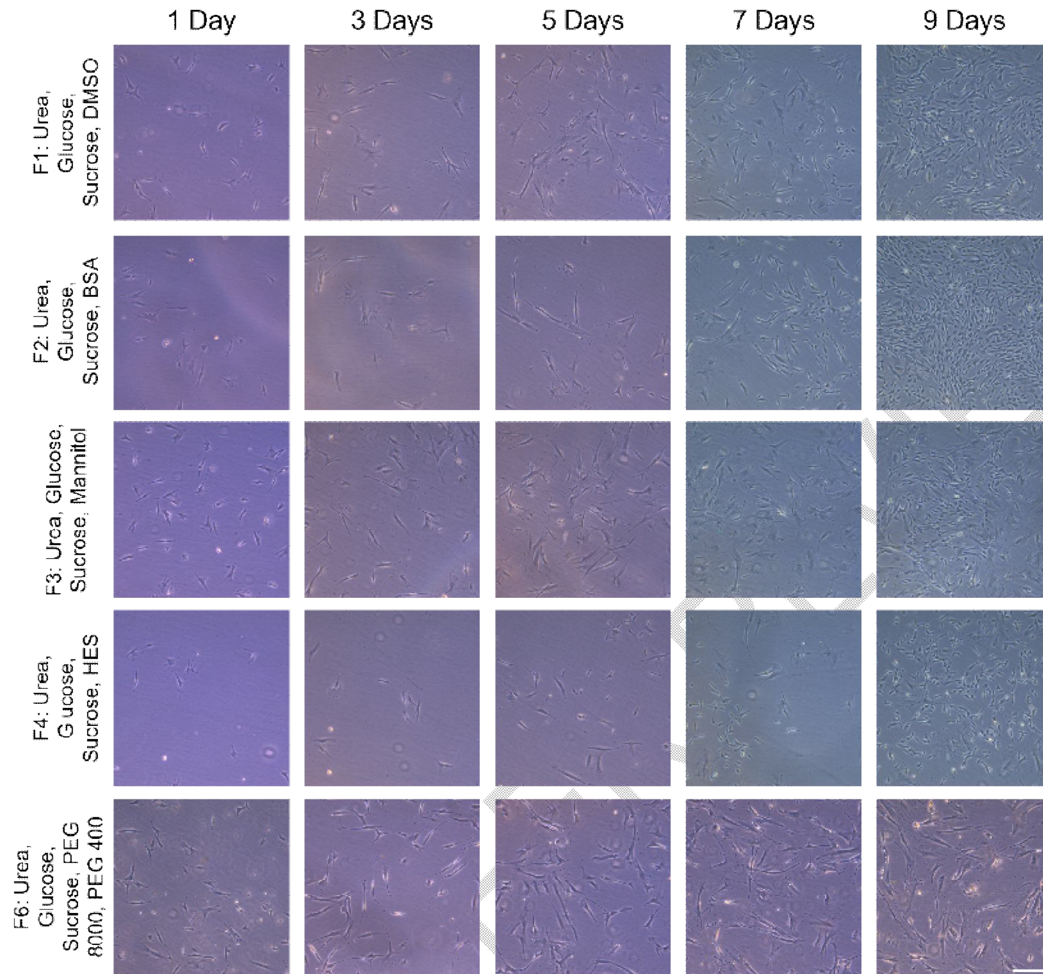
Figure 2: Microscopic images of hMSCs after drying
Representative microscopic images of hMSCs in selected formulations after drying, reconstitution, and subsequent culturing under standard conditions for up to nine days. Formulations F5 and F7-F10 yielded no viable cells (images not shown). Scale bar is 400 µm.



were viable and not in an apoptotic or necrotic stage. hMSCs were also evaluated for their viability and proliferation potential quantitatively using a metabolic assay. The results are shown in Figure 3. Viabilities after overnight incubation (day one) differed depending on the formulation and ranged from 12% (urea, glucose, sucrose, HES) to 38% (urea, glucose, sucrose, mannitol). Formulations with decreased amounts of protective agents did not yield viable cells (data not plotted), highlighting that, in this context, formulations with high percentages of membrane intact cells do not necessarily produce viable cells, such as for F6-F9. Despite relatively high variabilities, for conditions with viable hMSCs (F1-F4, F6), cells proliferated, and their relative cell number at least tripled after nine days in culture. In the urea, glucose, sucrose, mannitol formulation (F3), the relative cell number steadily increased to up to 144% after nine days, whereas in the urea, glucose, sucrose, HES formulation (F4), cell numbers were significantly (P = .008)lower with 37% relative cell numbers. An increase in growth with increasing incubation time was also observed for the other conditions assessed. As expected, unstressed refence samples, which are considered 100% viable on day one, also proliferated steadily and the PBS-only negative control showed neither viable cells nor proliferation (data not shown).

4. DISCUSSION

In this study we evaluated novel excipients in the context of freeze-drying hMSCs. This approach can be employed during early cell product development to screen formulations that could yield stable cakes and viable cells. For freeze-dried products, an intact cake with low residual moisture is key to facilitate stability of a cell-based therapeutic product. Measurements of Tg' and residual moisture can support in predicting cake quality as well as help optimizing drying parameters and subsequently potentially narrow the range of suitable formulations for further experiments and analyses. Alternatively, formulations that yield viable cells can be selected for further optimizations to yield stable, solid cakes by adapting lyophilization processes and excipient choice/concentrations. This approach is less efficient, as a larger

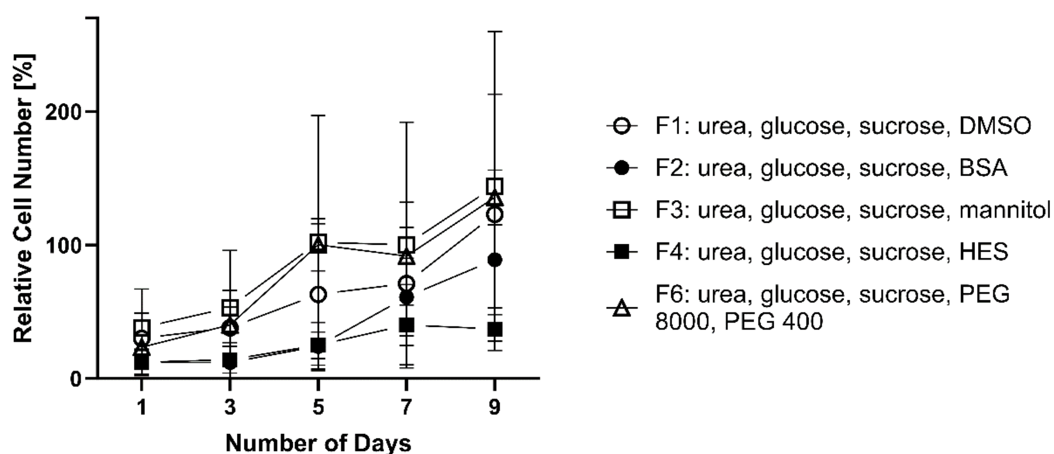


Figure 3: Viability measurements and proliferation of hMSCs after drying
Relative cell number and proliferation of hMSCs, as determined by a metabolic viability assay. hMSCs were dried in selected formulations, reconstituted, and subsequently incubated under standard conditions for up to nine days. 100% relative cell number corresponds to the number of viable cells before drying. Formulations F5 and F7-F10 yielded no viable cells, therefore are not plotted. Points show averages, error bars show standard deviation, $n \geq 3$ per condition.

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number of methods must be employed to confirm harmonization of cake appearance and cell viability; however, it benefits from optimizing formulations with already with viable cells. While some compromises on cake appearances can be made, cell viability cannot be sacrificed as it is impertinent for an effective cell therapy product. As demonstrated, formulations such with urea, glucose, sucrose, and mannitol could yield viable cells and a large percentage of membrane intact cells, however, the high residual moisture contents and low Tg´ would restrict storage potential to sub-zero temperatures.

In promising formulations, membrane integrity measurements can subsequently be employed to obtain preliminary estimates of successfully dried cells using a NucleoCounter, a relatively fast method of quantification. Cell membrane integrity is a key parameter to assess product quality and gives a valuable initial indication of the cell condition since an intact membrane is imperative for regular cell functionality [9]. Compared to a similar study which yielded up to 69% membrane intact hMSCs as a dried product with ~3% residual moisture [14], the approach presented here showed a substantial increase to up to 87% and 95% membrane intact cells in urea, glucose, sucrose, PEG 8000, and PEG 400 with 8% and 14% residual moisture, respectively. Stable lyophilizates with up to 8% residual moisture have been attained previously [38], and in the context of drying cells, an increased residual moisture content may be required to stabilize cells successfully [12, 16]. This is supported by the observation of the sharp decrease to 35% membrane intact cells in samples with residual moisture below 1%. However, the results presented here highlight the limitations of cell membrane integrity-based measurements, as after partial freeze-drying a subpopulation of hMSCs with intact membranes but without metabolic activity is observed with the PBS-only formulation. Such method is unfortunately often employed due to its simplicity and fast turn-around time when evaluating cell viability [13, 39, 40]. However, an assay solely based on cell membrane integrity measurements cannot yield sufficient insight in the cellular status to reliably determine cell viability in the context of freezing and drying of cells. Thus, further analyses are essential to select formulations where cells are viable and can proliferate. Monitoring cell growth for several days after processing is recommended to confirm regular cell functionality, as cells that appear viable immediately after processing may be in an apoptotic or necrotic stage [41]. Cultivating cells after drying and microscopically inspecting morphology, growth, and proliferation can yield quick insights in the cell's health status, as demonstrated in this manuscript. If the cells fail to re-attach to the culture vessel, have abnormal morphology, or fail to proliferate in their usual manner, the drying process has impaired the cells regular functionality, thus reducing the applicability as a medicinal product, as it was the case for formulations with decreased amounts of protective agents (F7-F10). While this approach was straight-forward, it yielded mostly qualitative results. The metabolic assay conducted in this study provided a reliable approach to quantitatively confirm cell viability and proliferation after freeze-drying. For the conditions evaluated, the assay exposed the discrepancy between seemingly viable cells with intact membranes only and cells that were also viable metabolically. Such assay has multiple additional benefits, including its applicability to different cell lines [18], high sensitivity [42], and non-destructive nature [43], allowing for multiple measurements of a single sample at different time points; a valuable advantage in circumstances where cellular material may be scarce [18]. Taken together, a successive array of methods allowed us to comprehensively screen for viable formulations, making this approach translationally applicable, as well as time- and cost efficient.

Novel excipients, screening type, and concentrations should also be carefully considered when developing stable cell products. During the drying process, cells are expected to experience stresses such as (mechanical) damage during freezing, increased osmotic pressure during up-concentration of solutes [44, 45], withdrawal of water, and the applied vacuum, requiring the addition of excipients that protect cells from these stresses [46].

In this study, we could demonstrate viable and proliferating cells were obtained from formulations comprising of well-tolerated pharmaceutical excipients after partial drying (lyophilization). This setup required high concentrations of agents to provide protection, and as the cells were unaltered, these excipients were not expected to penetrate the cell membrane readily and in sufficient quantities [47, 48] without the added extracellular urea [19]. In previous experiments, we showed that urea and glucose synergistically provided cryoprotection at comparable levels to conventional DMSO in hMSCs during freeze-thaw [19], an observation which was exploited in this study. Compared to the partially dried formulation which yielded up to 38% viable cells, the frozen liquid formulation yielded up to 55% viable cells. The ratios of urea to sugars were kept at similar values as previously studied as they had shown to provide optimized protective effect. However, urea has hygroscopic properties [49] which can hamper water sublimation. Indeed, despite long(er) primary drying times, we observed in this study that formulations with high amounts of urea retained relatively high percentages of residual moisture. This property, in combination with the low glass transition temperatures measured in these formulations, likely lead to collapsed cakes. Decreasing the concentration of urea and sugars and adding PEG reduced residual moisture and improved cake formation. However, with decreased amounts of protective agents and residual

moisture, no viable cells were retrieved, indicating that in this setup, hMSCs were only able to withstand partial drying. Secondary drying was not further evaluated; this distinct topic of freeze-drying cycle optimization is out of scope of this manuscript and has been discussed thoroughly elsewhere [12, 23, 50-55].
We applied the screening method described above to several additional preliminary sets of formulation screening experiments not discussed in this study, these can be found in the Supplementary Table 1 (see Appendix). The data shows only pre-selected formulations which yielded solid cakes and associated relative cell viability based on qualitative imaging assessment. These results highlight the need for a comprehensive and sequential screen when developing cell-based formulations for lyopreservation.

5. CONCLUSION

The setup presented here provides an approach for the screening of novel formulations for cell preservation during freeze-drying. Tg´ and residual moisture measurements can aid in selecting formulations predicted to yield stable cakes. Membrane integrity measurements can subsequently be used to obtain fast preliminary estimates of successfully dried cells, and microscopic and metabolic analyses can be employed in promising formulations for confirmation of viability and proliferation. This approach provides a roadmap for initial screening of formulations and would be likely applicable to different cell lines.
In the presented setup, some partially dried hMSCs in collapsed cakes were able to withstand the stresses of primary drying. For other conditions, a large percentage of cells showed intact membranes but no metabolic viability in formulations that yielded elegant cakes. This setup decreases development complexity, is industrially applicable and scalable, and we have shown that it could be a potential starting point for the preservation of cell therapy products. These results invite for further investigation and for the evaluation of novel formulations to address the challenging assessment of the process and formulation variables involved towards developing a robust cell therapy product that can withstand 2-8 °C storage. Formulation composition, excipient concentration ratios, handling, and freezing and drying parameters are expected to have a profound impact on cell viability and need to be carefully adjusted and harmonized to achieve a satisfying outcome.

9. CONSENT

Not applicable.

10. ETHICAL APPROVAL

Not applicable.

11. DISCLAIMER

The authors declare that no generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators were used to write this manuscript.

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13. APPENDIX

Supplementary Table 1: Evaluation of various formulations for preservation of hMSCs
Human mesenchymal stromal/stem cells (hMSCs) were partially dried in a freeze-dryer in various urea and glucose-based formulations and inspected for viable cells using microscopic cell counts. hMSCs were pre-incubated overnight in medium supplemented with trehalose, to facilitate the intracellular update of the protective disaccharide. All formulations were prepared with PBS. n = 1 for each condition.

No.	Urea	Sugar(s)	Additional Excipients	Primary Drying Time [h]	Cake Appearance	Estimates of Relative Cell Viability [%]
1	0.5 M	glucose (0.2 M) mannitol (0.5 M)	hyaluronic acid (10 mg/ml)	16.6	intact, shrunken	none detected
2	0.5 M	glucose (0.5 M), sucrose (0.25 M), mannitol (0.25 M)	hyaluronic acid (10 mg/ml)	38.3	intact, shrunken	none detected
3	0.5 M	glucose (0.5 M), sucrose (0.25 M), mannitol (0.25 M)	hyaluronic acid (20 mg/ml)	38.3	intact, shrunken	none detected
4	1 M	glucose (1 M), mannitol (10%)	0	60.6	intact, minor cracks	none detected
5	1 M	glucose (1 M), mannitol (10%)	hyaluronic acid (10 mg/ml)	60.6	intact	none detected
6	0.75 M	glucose (0.75 M), mannitol (10%)	0	60.6	intact, minor cracks	none detected
7	1 M	glucose (1 M)	0	64	intact	none detected
8	1 M	mannitol (1 M)	DMSO (4%)	64	intact	none detected
9	1 M	mannitol (1 M)	DMSO (4%), hyaluronic acid (5 mg/ml)	64	intact	none detected
10	0	glucose (1 M), mannitol (1 M)	0	64	intact	none detected
11	0	glucose (1 M), mannitol (1 M)	DMSO (4%)	64	intact	none detected
12	0	glucose (1 M), mannitol (1 M)	DMSO (4%), hyaluronic acid (5 mg/ml)	64	intact	none detected
13	1 M	mannitol (10%), trehalose (0.5 M)	0	64	intact,	none detected
14	1 M	glucose (1 M), sucrose (0.5 M), mannitol (10%)	0	181.6	intact, shrunken	none detected
15	1 M	glucose (1 M), sucrose (0.5 M), mannitol (20%)	0	181.6	intact, shrunken	none detected

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16	1 M	glucose (1 M), trehalose (0.5 M),	HES (10%)	181.6	intact, shrunken	none detected
17	0.5 M	glucose (0.5 M), sucrose (0.25 M),	HES (5%), PEG 400 (10%), PEG 8000 (5%)	98	intact, shrunken	none detected
18	0.5 M	glucose (0.5 M), mannitol (5%) trehalose (0.25 M)	PEG 400 (10%), PEG 8000 (5%)	98	intact	none detected
19	0	0	PEG 400 (10%), PEG 8000 (5%)	98	intact	none detected
20	0	0	PEG 400 (20%), PEG 8000 (10%)	98	intact	none detected

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