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Key words

Mesenchymal stem cell, cryopreservation, freezing, thawing, concentration, cell manufacturing.

Abstract

Introduction: For stem cell therapies to be adopted in mainstream healthcare, robust, reliable and cost-effective storage and transport processes must be developed. Cryopreservation currently remains the best current platform for this and freezing cells at high concentration may have many benefits including savings on cost and storage space, facilitating transport logistics and reducing cryoprotectant volume. Cells, such as mesenchymal stem cells, are typically frozen at just one million cells per millilitre (mL) but the aim of this study is to examine the post-thaw attributes of human bone marrow derived mesenchymal stem cells (hBM-MSCs) frozen at one, five and ten million cells per mL.

Methods: Thawed cells were assessed for their morphology, phenotypic marker expression, viability, apoptosis level, metabolic activity, proliferation and osteogenic and adipogenic differentiation.

Results: Here, for the first time, it is shown that all assessed cells expressed the typical MSCs markers (CD90, CD105 and CD73) and lacked the expression of CD14, CD20, CD34, CD45 and HLA-DR. In addition, all cells showed elongated fibroblastic morphology. Post-thaw viability was retained with no difference among the three concentrations. Moreover, no significant statistical difference was observed in post-thaw apoptosis level, metabolic activity, proliferation and osteogenic potential, indicating that these cells are amenable to cryopreservation at higher concentrations.

Conclusion: The results of this study are of paramount importance to the development of manufacturing processes around a useful freezing concentration, when cells are targeted to be stored for short term duration up to six months.

Introduction

As of December 2021, there were 22 cellular and gene therapy products approved by the US Food and Drug Administration [1]. With more than a thousand clinical trials using mesenchymal stem cells (MSCs) aiming to treat a wide variety of diseases (ClinicalTrials.gov), it is expected that many more products will soon reach the market which is predicted to triple in size by 2025 [2]. Improved MSC therapies' manufacturing will allow their broader application [Wobma & Satwani, 2021]. A key process required to facilitate this market growth is product storage. Currently, storing cells at cryogenic temperature is the most common practice. It is more cost-effective than shipping fresh cells and conveys less risk when coordinating between the supply chain, surgical team and the patient [3]. Allogeneic cell therapies require production at a scale that is dependent on the number of patients, the number of doses required to treat a patient and the number of cells per dose [4]. A 2020 - study analysed 914 MSC clinical trials between 2004 and 2018 and concluded that the median dose for intravenous delivery is 100 million cells per patient per dose [5]. The range of annual demand on MSC products is estimated between 10 billion and 10,000 billion cells [4] which means a huge number of doses are to be stored and distributed. Freezing MSCs at high concentration not only contribute to cost savings but is also beneficial clinically and for *in vitro* applications; in particular, bioreactor seeding. From a clinical perspective, increasing the cell concentration at freezing reduces the number of vials to be handled at bedside, the space required for cell storage, the volume of freezing medium and ultimately the volume of cryoprotectant infused into a patient. For *in vitro* applications, freezing MSCs at higher density makes the transition from a working cell bank to seeding bioreactor more feasible since $2x10^7$ is a commonly used starting inoculum for many bioreactor systems [6-8].

Bahsoun *et al*. [9] analysed 41 studies on the cryopreservation of human bone marrow derived mesenchymal stem cells (hBM-MSCs) and concluded that, within a research environment, the most common cell concentration for cryopreservation is $1x10^6$ cells per mL. Only one of the analysed studies briefly assessed the impact of cell concentration at freezing on post-thaw cell function [10]. Dave *et al.* [X] analysed 15 studies on using fresh and cryopreserved MSCs in pre-clinical models and inflammation. Two thirds of the compared studies used MSCs at concentration between $1x10^6$ and $1x10^7$. According to Woods *et al.* [11], "with non-hematopoietic adult stem cells such as mesenchymal stromal cells, no comprehensive study has yet reported the effects of increasing cell concentration during cryopreservation and the resulting clinical outcome".

The novelty of this study lies in investigating the effect of hBM-MSCs concentration at freezing on post-thaw cell morphology, phenotypic marker expression, viability, apoptosis level, metabolic activity, proliferation and differentiation in order to assess the feasibility of freezing at high concentration. To our knowledge, this is the first study to compare these post-thaw attributes across cells frozen down at three different concentrations ($1x10^6$, $5x10^6$ and $10x10^6$). This is an important study that will inform efficient banking strategies for bioprocessing and clinical settings.

Materials and methods

hBM-MSCs culture

hBM-MSCs were isolated from mononuclear cells (purchased from Lonza (USA)) by adhesion-based cell selection process. Isolated hBM-MSCs were cryopreserved at passage 0 (P0). Vials at P0 were thawed immediately in a water bath (40°C) for about 1 minute. Cells were re-suspended in warm complete culture medium

(Dulbecco's Modified Eagle's medium (DMEM) (Gibco, UK) low glucose supplemented with 10% FBS v/v (RMBIO, US)) and centrifuged to remove DMSO. Cells were seeded at 5000 cells per cm^2 in warm complete culture medium. All cultures were maintained at 37°C and 5% CO_2 in a humidified incubator. A complete medium change was done at day 3. At day 6 in culture (when cells reached about 80% confluency), medium was aspirated, and cells washed with phosphate buffer saline (PBS) (Gibco, UK) then detached with 0.25% (w/v) trypsin-EDTA (Gibco,UK), counted and re-seeded at 5000 cells per cm^2. For all assays, cells at Passage 2 or 3 were used.

Cryopreservation and thawing of hBM-MSCs

At the end of P2 or P3, cells were detached, centrifuged and counted. According to cell count, the cells were re-suspended at $1x10^6$, $5x10^6$ or $10x10^6$ cells per mL in FBS supplemented with 10% DMSO (v/v) (Sigma, UK). 1mL of cell suspension was transferred to each vial (Corning cryogenic vials, internal thread, 2mL capacity). Vials were kept in Mr Frosty (Nalgene cryogenic freezing container filled with 100% isopropyl alcohol) in a -80°C freezer for 24h to cool at a rate of -1°C per minute. After 24h, the vials were transferred to liquid Nitrogen (LN_2) for at least six months.

For thawing, vials were removed from LN_2 and immediately placed through a floating mat in a water bath at 40°C for about one minute. Next, cells were added to fresh warm complete medium (9 mL) to dilute DMSO and centrifuged at 200g for 5 minutes at room temperature. The supernatant was discarded, and cells were then re-suspended in complete fresh medium, counted and used for assays.

hBM-MSCs immunophenotyping

The expression of surface markers was assessed by flow cytometry using MSC phenotyping kit (human) (Miltenyi Biotec, UK) according to the manufacturer's instructions. The kit confirms compliance with International Society for Cellular Therapy (ISCT) criteria [12]. Positive markers stained for were CD105 linked to PE, CD90 linked FITC and CD73 linked to APC. Negative markers stained for were CD14, CD20, CD34, CD45 and HLA-DR all linked to PerCP. Analyses for the three cell concentrations were done immediately post-thaw. $5x10^5$ cells were suspended in 100µL flow cytometry buffer with 10µL of Human MSC phenotyping cocktail and 10µL of Human Anti-HLA-DR-PerCP. Cells were incubated for 10 minutes at 5°C in the dark. After that, cells were re-suspended in 1mL buffer, centrifuged, supernatant removed and then re-suspended in fresh 500µL buffer and analysed. All samples were analysed using the BD Accuri C6 (BD Biosciences, UK). 100,000 events were collected for each sample.

Viability and apoptosis assay

The viability and percentage of apoptotic cells were assessed using the FITC Annexin V apoptosis detection kit with 7-AAD (Biolegend, UK) according to the manufacturer's instructions. Analyses of all samples were done immediately after thawing. $3x10^5$ cells were suspended in 100 µL Annexin V binding buffer with 5 µL of FITC annexin V and 5 µL of 7AAD viability staining solution. Cells were incubated at room temperature in the dark for 15 minutes then analysed using the BD Accuri C6. 100,000 events were collected for each sample. All flow cytometry data was analysed using the BD Accuri C6 plus software.

WST-1 assay

30,000 cells per well were seeded in 100µL of complete culture medium in 96-well plates. 10µL of warm WST-1 solution (Sigma, UK) was added to each well at 0h (immediately after seeding) and 24h after seeding. Plates were then incubated for further 2 hours at 37˚C in a humidified incubator. After incubation, the absorbance was measured at 450nm using Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific, UK).

Cell proliferation assay

To determine the post-thaw growth of cells frozen at each concentration, six T25 flasks at a density of 5000 cells per cm^2 were seeded immediately post-thaw for one passage. At day 6 in culture, flasks were removed from the incubator and the cell passage protocol was undertaken to determine the number of live cells per cm^2.

hBM-MSCs differentiation

The assessment of the differentiation potential was conducted via incubation with osteogenic and adipogenic differentiation media for 16 and 19 days respectively. All media were prepared in-house and a complete medium change was performed every three days [13]. The chosen seeding concentrations, type of plates and length of incubation were based on the protocols for the StemPro differentiation kits (Thermo Fisher Scientific, UK) with adjustments to optimise the conditions for the cell line used. The detailed protocols are published in Bahsoun *et al.* [14].

In this specific study, chondrogenesis differentiation was not conducted, due to the big number of cells required for this assay, but the same cell line was used in other studies from our lab where chondrogenesis of the M2 cell line was shown [15]. This confirms the compliance with the ISCT criteria for defining MSCs.

Statistical analyses

All data are presented as mean of triplicates or duplicates from two different experiments ± SD. The study was repeated twice, and for each repeat, the assays were conducted in triplicates except for the proliferation assay which was conducted in duplicates. Shapiro–Wilk test and Levene's test were used to check normality of distribution and homogeneity of variance respectively. For normally distributed data, one-way between groups analysis of variance (ANOVA) was used as a parametric test and Kruskal-Wallis H was used as a non-parametric test when data was not normally distributed. Results were deemed to be significant at $p \leq 0.05$.

Results

The assessment of cell morphology, phenotypic surface marker expression, cell viability and apoptosis level

Figure 1 shows that a fibroblastic elongated shape was observed in all thawed cells meaning that cell concentration at freezing did not influence the post-thaw cell morphology. **Figure 2** shows that all samples expressed the most common MSCs markers (CD90, CD105 and CD73) at a level ≥95% which is the threshold set up by the ISCT for defining an MSCs population [12]. In addition, the percentage of cells expressing CD14, CD20, CD34, CD45 and HLA-DR is ≤2% for all three cell samples (**Figure 2**) thus further confirming compliance with the ISCT criteria.

Immediately post-thaw, no significant statistical differences in viability or apoptosis levels were obtained across the three samples. Viability after thawing was maintained at 95.8%±1.6, 95.8%±2.0 and 97.5%±0.8 for cells frozen at one, five and ten million cells per mL respectively (**Figure 3A**). The percentages of apoptotic cells

for the three cell concentrations were all less than 6.5% with no significant statistical difference observed (**Figure 3B**). Therefore, it can be suggested that cell concentration at freezing has no impact on viability and apoptosis levels post-thaw when a standard freezing medium and protocol were used.

The assessment of cell metabolic activity and proliferation

The metabolic activity of thawed cells was assessed at 0h and 24h post-thaw. At both time points, cells frozen at the three concentrations showed similar level of activity and no significant statistical difference was observed (**Figure 4A**).

From a manufacturing perspective, the ability of MSCs to proliferate is one of the most important characteristics of this type of cell as the aim is to produce a large number of cells, given that the median dose for intravenous delivery is 100 million cells per patient per dose [5]. To assess growth, cells were defrosted and seeded at 5000 cells per cm^2 and incubated for 6 days with medium change at day 3. At day 6, cells were detached and counted. The number of live cells per cm^2 is presented in **Figure 4B** where no significant statistical difference was obtained.

The assessment of cell differentiation capability

The post-thaw adipogenic and osteogenic differentiation potential of the MSCs were assessed qualitatively and quantitatively. **Figures 5 & 6** show that differentiation potentials were preserved with thawed cells able to differentiate into adipocytes and osteoblasts. From the quantitative data, it can be noticed that cells frozen at five and ten million have a higher adipogenic potential compared to those frozen at one

million (**Figure 7A**) while no significant statistical difference was observed for the osteogenic potential (**Figure 7B**).

Discussion

Freezing cells at high concentration is associated with several issues such as increased intracellular ice formation, stresses on intracellular tight junctions, increased cell-cell interaction and subsequently cell clumping [11, 16-18]. Despite this, cells, other than MSCs, are successfully cryopreserved at high concentrations. Hematopoietic stem cells from umbilical cord blood and bone marrow are regularly cryopreserved at 30 – 50x10^6 cells per mL [19-20]. Even higher concentrations, such as 100x10^6, are considered acceptable for freezing hematopoietic progenitor cells from bone marrow or peripheral blood [21]. It has also been shown that freezing nucleated cells from mobilized peripheral blood stem cells at 40, 70, 100, 160 and 200x10^6 for 2-12 months has no detrimental effect on cell viability or stem cell clonogenicity [22]. Even higher concentrations have been reported showing enriched mononuclear cells from mobilized peripheral blood stem cells can be frozen at 2x10^8 without the use of a programmed freezer [23]. These listed studies suggest that freezing cells at high concentrations is therapeutically and practically necessary and is achievable. However, these results cannot be generalised because it is known that cells from different tissue sources do not respond ‘equally’ to the cryopreservation stresses. Biological and physical factors, including the fluidity and antioxidant capacity of the cell membrane, may contribute to the cells’ freezing tolerance (Mizuno et al., 2022). Cells from different sources may exhibit variabilities in their physical and biological properties in response to freezing [11].

Very limited data exists in the literature regarding the cryopreservation of MSCs at high cell concentration. The study herein is the first to assess this aspect of hBM-MSCs cryopreservation in a systematic way using six different assays. From the data presented above, it was determined that freezing hBM-MSCs at five or ten million per mL of standard freezing solution is feasible and comparable to freezing at one million cells per mL. All assessed cells showed markers expression as per the ISCT guidelines and exhibited elongated fibroblastic morphology. Post-thaw viability was retained with no difference among the three samples and this is in line with results in Yuan *et al.* and Thirumala *et al.* [10, 24]. Moreover, no significant statistical difference was observed in post-thaw apoptosis level, metabolic activity, proliferation and osteogenic potential while adipogenic differentiation potential increased with increasing cell concentration at freezing. This shows higher cell commitment to differentiation and potentially the initiation of some cell signalling related to adipogenesis as a result to freezing at a high concentration. From a therapeutic perspective, this observed impact can be evaluated according to the therapy being developed and manufactured.

Freezing cells at high concentration has many benefits in cell manufacturing including reducing the volume of cryoprotectants, such as DMSO, to be infused in patients. In this study, the freezing medium was composed of 10% DMSO in FBS which is the most commonly used in BM-MSCs studies [9]. In fact, DMSO is considered the “default” cryoprotectant for research laboratory and clinical banks [25-26] and it is the most commonly used for the cryopreservation of mammalian cells including stem cells [27]. DMSO is an example of permeating cryoprotectants which have a high solubility and readily diffuse through the plasma membrane. Cryoprotectants reduce solution effect by replacing a fraction of the solutes in the

partly frozen cells [27]. This reduces solutes concentration and consequently affects water transport and ice formation [28]. A drawback on the use of these types of cryoprotectants is the damage they can cause to cells and their adverse effects when infused into patients.

In *in* vitro experiments, DMSO has been shown to impact the epigenetic profile in mouse embryoid body [29], induce embryonic stem cell differentiation [30], down regulate the expression of Oct-4 and Sox-2 in human embryonic stem cells [31-32] and induce apoptosis in SV40-transformed human keratinocytes [33]. In clinical trials, when infused in patients, DMSO has been reported to cause severe respiratory depression [34], nausea, abdominal cramping and flushing [35], cardiac side effects [36-37] and severe neurotoxicity [38-39]. It was suggested that a reduced DMSO toxicity incidence rate was associated when using lower DMSO concentrations or washing cells before infusion [40]. The data in this study demonstrates that freezing five or ten times more hBM-MSCs in the same amount of freezing solution is not harmful to the cells. Thus, in a clinical setting, where cells are to be thawed and injected immediately into patients, this will potentially result in injecting five or ten times less DMSO. Without doubt, a reduction in the amount of DMSO delivered to any individual patient will be clinically beneficial. In addition, cells frozen at higher concentrations will reduce the number of vials to be handled at bedside, save on the cost of space required for cell storage as well as the cost of transportation.

Freezing hBM-MSCs at up to 10 million cells per mL does not seem to incur cell damage and this is valuable information for starting to establish a benchmark of cell density from which damage starts to occur. It is worth noting that this study has some limitations. Since cells were manually cultured by one operator, it was not

practical to freeze MSCs at a concentration higher than ten million cells per mL. The data was collected from one cell line only (due to the large number of cells required) and it is possible that the response to freezing may vary among donors. The freezing solution contained FBS which is not considered therapeutically relevant. Where large volume culture systems such as bioreactors or automatic platforms are available, larger number of cells can be cultured more easily so it would be of great interest to assess freezing cells from multiple donors at larger concentration combined with varying the DMSO and FBS concentrations in the freezing medium or with testing alternatives to DMSO such as sugars, proteins, polymers, amino acids, and other small molecules and osmolytes (Yao & Matosevic 2021) . In addition, this study only assessed the short-term effect of the impact of cell concentration at freezing. This does not rule out the possibility that long-term effects may only be observed after several passages post-thaw. Therefore, it is necessary to assess the impact of cryopreservation beyond immediately post-thaw [14]. Moreover, evaluating the impact on the desired clinical function in the context of a particular treatment is also important.

Despite the mentioned limitations, this study highlights the fact that increasing MSCs concentration at freezing is feasible but can make a difference in some cell attributes and therefore a manufacturing process must be developed around a useful freezing concentration, rather than assuming that what works for one million cells per mL will work the same way for the final product (biological therapy scale-up) [41].

Authors' contributions: S Bahsoun, EC Akam and K Coopman designed the study, S Bahsoun and Mj Brown collected the data, S Bahsoun analysed and wrote the article. EC Akam and K Coopman contributed throughout the preparation and edited the manuscript. All authors read and approved the final manuscript.

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Dave, C., Mei, S.H., McRae, A., Hum, C., Sullivan, K., Champagne, J., Ramsay, T. and McIntyre, L., 2022. Comparison of Freshly-Cultured versus Cryopreserved Mesenchymal Stem Cells in Animal Models of Inflammation: A Pre-Clinical Systematic Review.

Wobma, H. and Satwani, P., 2021. Mesenchymal stromal cells: Getting ready for clinical primetime. *Transfusion and Apheresis Science*, *60*(1), p.103058.

Mizuno, M., Matsuzaki, T., Ozeki, N. *et al.* Cell membrane fluidity and ROS resistance define DMSO tolerance of cryopreserved synovial MSCs and HUVECs. *Stem Cell Res Ther* **13,** 177 (2022). https://doi.org/10.1186/s13287-022-02850-y

Yao, X., Matosevic, S. Cryopreservation of NK and T Cells Without DMSO for Adoptive Cell-Based Immunotherapy. *BioDrugs* **35,** 529–545 (2021). https://doi.org/10.1007/s40259-021-00494-7

References

[1] FDA, US Food and Drug Administration, 'Approved Cellular and Gene Therapy Products', 2019. (Online). Available: https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/approved-cellular-and-gene-therapy-products (Accessed: 06-Dec-2021).

[2] Hargreaves, B. (2019) *Cell therapy market to triple in size by 2025*. (Online). Available at: https://www.biopharma-reporter.com/Article/2019/03/04/Cell-therapy-market-to-triple-in-size-by-2025 (Accessed: 09-Aug-2020).

[3] Harrison, R. P., Medcalf, N. and Rafiq, Q. A. Cell therapy-processing economics: Small-scale microfactories as a stepping stone toward large-scale macrofactories. *Regenerative Medicine* 2018, 13(2):159–173. doi: 10.2217/rme-2017-0103.

[4] Chilima, T. D.P., Moncaubeig, F. and Farid, S. S.Impact of allogeneic stem cell manufacturing decisions on cost of goods, process robustness and reimbursement. *Biochemical Engineering Journal* 2018, 137:132–151. doi: 10.1016/j.bej.2018.04.017.

[5] Kabat, M., Bobkov, I., Kumar, S. *et al.* Trends in mesenchymal stem cell clinical trials 2004-2018: Is efficacy optimal in a narrow dose range? *Stem Cells Translational Medicine* 2020, 9(1):17–27. doi: 10.1002/sctm.19-0202.

[6] Das, R., Roosloot, R., van Pel, M., *et al.* Preparing for cell culture scale-out: establishing parity of bioreactor- and flask-expanded mesenchymal stromal cell cultures. *Journal of translational medicine* 2019, 17:241 doi: 10.1186/s12967-019-1989-x.

[7] Hanley, P.J., Mei, Z., Durett, A.G. *et al.* Efficient manufacturing of therapeutic mesenchymal stromal cells with the use of the Quantum Cell Expansion System. *Cytotherapy* 2014, 16(8): 1048–1058. doi: 10.1016/j.jcyt.2014.01.417.

[8] Lambrechts, T., Papantoniou, I., Rice, B. *et al.* Large-scale progenitor cell expansion for multiple donors in a monitored hollow fibre bioreactor. *Cytotherapy* 2016, 18(9):1219–1233. doi: 10.1016/j.jcyt.2016.05.013.

[9] Bahsoun, S., Coopman, K. and Akam, E. C. The impact of cryopreservation on

bone marrow-derived mesenchymal stem cells: A systematic review. *Journal of Translational Medicine* 2019, 17(1):397. doi: 10.1186/s12967-019-02136-7.

[10] Yuan, Z., Lourenco, S.D.S., Sage, E.K., *et al.* Cryopreservation of human mesenchymal stromal cells expressing TRAIL for human anti-cancer therapy. *Cytotherapy* 2016, 18(7):860–869. doi: 10.1016/j.jcyt.2016.04.005.

[11] Woods, E.J., Thirumala, S., Badhe-Buchanan, S.S. *et al.* Off the shelf cellular therapeutics: Factors to consider during cryopreservation and storage of human cells for clinical use. *Cytotherapy* 2016, 18(6):697–711. doi: 10.1016/j.jcyt.2016.03.295.

[12] Dominici, M.L.B.K., Le Blanc, K., Mueller, I. *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006, 8(4):315–7. doi: 10.1080/14653240600855905.

[13] Ciuffreda, M.C., Malpasso, G., Musarò, P. *et al.* Protocols for in vitro differentiation of human mesenchymal stem cells into osteogenic, chondrogenic and adipogenic lineages. In *Mesenchymal Stem Cells* 2016, Humana Press, New York, NY, 1416:149–158. https://doi.org/10.1007/978-1-4939-3584-0_8

[14] Bahsoun, S., Coopman, K. and Akam, E.C. Quantitative assessment of the impact of cryopreservation on human bone marrow-derived mesenchymal stem cells: up to 24 h post-thaw and beyond. *Stem cell research & therapy* 2020, 11(1):1-15.doi: https://doi.org/10.1186/s13287-020-02054-2.

[15] Hanga, M.P., Murasiewicz, H., Pacek, A.W. *et al.* Expansion of bone marrow-derived human mesenchymal stem/stromal cells (hMSCs) using a two-phase liquid/liquid system. *Journal of Chemical Technology and Biotechnology* 2017,

92(7):1577–1589. doi: 10.1002/jctb.5279.

[16] Mazur, P. and Cole, K. W. Influence of cell concentration on the contribution of unfrozen fraction and salt concentration to the survival of slowly frozen human erythrocytes. *Cryobiology* 1985, 22(6):509–536. doi: 10.1016/0011-2240(85)90029-X.

[17] Pegg, D.E. and Diaper, M. P.The packing effect in erythrocyte freezing, *CryoLetters* 1983, 4(2):129–136.

[18] Rowley, S.D., Bensinger, W.I., Gooley, T.A. *et al.* Effect of cell concentration on bone marrow and peripheral blood stem cell cryopreservation. *Blood* 1994, 83(9):2731-2736. doi: 10.1182/blood.v83.9.2731.bloodjournal8392731.

[19] Fleming, K. K. and Hubel, A. Cryopreservation of hematopoietic and non-hematopoietic stem cells. *Transfusion and Apheresis Science* 2006, 34(3):309–315. doi: 10.1016/j.transci.2005.11.012.

[20] Rowley, S. D. Hematopoietic stem cell processing and cryopreservation. *Journal of clinical apheresis* 1992, 7(3):132–134.

[21] Lecchi, L., Giovanelli, S., Gagliardi, B. *et al.* An update on methods for cryopreservation and thawing of hemopoietic stem cells. *Transfusion and Apheresis Science* 2016, 54(3):324–336. doi: 10.1016/j.transci.2016.05.009.

[22] Perseghin, P., Epis, R., Vigano, M. *et al.* Satisfactory recovery and viability of stem cells cryopreserved at high cell concentration. *Transfusion science* 1997, 18(3):399–403.doi: https://doi.org/10.1016/S0955-3886(97)00037-4.

[23] Kawano, Y., Lee, C.L., Watanabe, T. *et al.* Cryopreservation of mobilized blood stem cells at a higher cell concentration without the use of a programmed freezer. *Annals of Hematology* 2004, 83(1):50–54. doi: 10.1007/s00277-003-0817-8.

[24] Thirumala, S., Fearnot, E., Goebel, W.S. *et al.* A cryopreservation system for direct clinical use of MSC. *Cytotherapy* 2013, 15(4):S46–S47. doi: 10.1016/j.jcyt.2013.01.177.

[25] Svalgaard, J.D., Haastrup, E.K., Reckzeh, K. *et al.* Low-molecular-weight carbohydrate Pentaisomaltose may replace dimethyl sulfoxide as a safer cryoprotectant for cryopreservation of peripheral blood stem cells. *Transfusion* 2016, 56(5):1088–1095. doi: 10.1111/trf.13543.

[26] Thirumala, S., Goebel, W. S. and Woods, E. J. Clinical grade adult stem cell banking. *Organogenesis* 2009, 5(3):143–154. doi: 10.4161/org.5.3.9811.

[27] Hunt, C. J. Cryopreservation: Vitrification and Controlled Rate Cooling. In Stem Cell Banking. *Methods in Molecular Biology* 2017, 1590:41–77. doi: 10.1007/978-1-4939-6921-0_5.

[28] Jang, T.H., Park, S.C., Yang, J.H. *et al.* Cryopreservation and its clinical applications. *Integrative Medicine Research* 2017, 6(1):12–18. doi: 10.1016/j.imr.2016.12.001.

[29] Iwatani, M., Ikegami, K., Kremenska, Y. *et al.* Dimethyl Sulfoxide Has an Impact on Epigenetic Profile in Mouse Embryoid Body. *Stem Cells* 2006, 24(11):2549–2556. doi: 10.1634/stemcells.2005-0427.

[30] Adler, S., Pellizzer, C., Paparella, M. *et al.* The effects of solvents on embryonic stem cell differentiation. *Toxicology in Vitro* 2006, 20(3):265–271. doi: 10.1007/BF01038309.

[31] Katkov, I.I., Kim, M.S., Bajpai, R. *et al.* Cryopreservation by slow cooling with DMSO diminished production of Oct-4 pluripotency marker in human embryonic stem cells. *Cryobiology* 2006, 53(2):194–205. doi: 10.1016/j.cryobiol.2006.05.005.

[32] Pal, R., Mamidi, M.K., Das, A.K. *et al.* Diverse effects of dimethyl sulfoxide (DMSO) on the differentiation potential of human embryonic stem cells. *Archives of Toxicology* 2012, 86(4):651–661. doi: 10.1007/s00204-011-0782-2.

[33] Koike, M., Ishino, K., Kohno, Y. *et al.* DMSO induces apoptosis in SV40-transformed human keratinocytes, but not in normal keratinocytes. *Cancer Letters* 1996, 108(2):185–193. doi: 10.1016/S0304-3835(96)04408-4.

[34] Benekli, M., Anderson, B., Wentling, D. *et al.* Severe respiratory depression after dimethylsulphoxide-containing autologous stem cell infusion in a patient with AL amyloidosis. *Bone Marrow Transplantation* 2000, 25(12):1299–1301. doi: 10.1038/sj.bmt.1702452.

[35] Davis, J., Rowley, S. D. and Santos, G. W. Toxicity of autologous bone marrow graft infusion. *Progress in Clincal Biological Research* 1990, 333:531–540.

[36] Martino, M., Morabito, F., Messina, G. *et al.* Fractionated infusions of cryopreserved stem cells may prevent DMSO- induced major cardiac complications in graft recipients. *Haematologica* 1996, 81(1):59–61.doi: https://doi.org/10.3324/%25x.

[37] Zenhäusern, R., Tobler, A., Leoncini, L. *et al.* Fatal cardiac arrhythmia after infusion of dimethyl sulfoxide-cryopreserved hematopoietic stem cells in a patient with severe primary cardiac amyloidosis and end-stage renal failure. *Annals of Hematology* 2000, 79(9):523–526. doi: 10.1007/s002770000186.

[38] Stamatovic, D., Balint, B., Tukic, L. *et al.* Severe neurotoxicity following peripheral blood stem cell transplantation. *Bone Marrow Transplantation* 2011, 46:1110. doi: http://dx.doi.org/10.1038/bmt.2011.48.

[39] Windrum, P. and Morris, T. C. M. Severe neurotoxicity because of dimethyl

sulphoxide following peripheral blood stem cell transplantation. *Bone Marrow Transplantation* 2003, 31(4):315–315. doi: 10.1038/sj.bmt.1703848.

[40] Windrum, P., Morris, T.C.M., Drake, M.B. *et al.* Variation in dimethyl sulfoxide use in stem cell transplantation: A survey of EBMT centres. *Bone Marrow Transplantation* 2005, 36(7):601–603. doi: 10.1038/sj.bmt.1705100.

[41] Pigeau, G.M., Csaszar, E. and Dulgar-Tulloch, A. Commercial scale manufacturing of allogeneic cell therapy. *Frontiers in medicine* 2018, 5:233. doi: https://doi.org/10.3389/fmed.2018.00233.