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The effect of Me_2SO overexposure during cryopreservation on HOS TE85 and hMSC viability, growth and quality

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ABSTRACT

With the cell therapy industry continuing to grow, the ability to preserve clinical grade cells, including mesenchymal stem cells (MSCs), whilst retaining cell viability and function remains critical for the generation of off-the-shelf therapies. Cryopreservation of MSCs, using slow freezing, is an established process at lab scale. However, the cytotoxicity of cryoprotectants, like Me_2SO, raises questions about the impact of prolonged cell exposure to cryoprotectant at temperatures >0 °C during processing of large cell batches for allogenic therapies prior to rapid cooling in a controlled rate freezer or in the clinic prior to administration. Here we show that exposure of human bone marrow derived MSCs to Me_2SO for ≥1 h before freezing, or after thawing, degrades membrane integrity, short-term cell attachment efficiency and alters cell immunophenotype. After 2 h's exposure to Me_2SO at 37 °C post-thaw, membrane integrity dropped to ~70% and only ~50% of cells retained the ability to adhere to tissue culture plastic. Furthermore, only 70% of the recovered MSCs retained an immunophenotype consistent with the ISCT minimal criteria after exposure. We also saw a similar loss of membrane integrity and attachment efficiency after exposing osteoblast (HOS TE85) cells to Me_2SO before, and after, cryopreservation.

Overall, these results show that freezing medium exposure is a critical determinant of product quality as process scale increases. Defining and reporting cell sensitivity to freezing medium exposure, both before and after cryopreservation, enables a fair judgement of how scalable a particular cryopreservation process can be, and consequently whether the therapy has commercial feasibility.

1. Introduction

Cell therapies hold the potential to revolutionise healthcare as regenerative medicines, replicating the success of the human therapeutic protein industry. However, cell therapies are more complex than protein therapeutics, which to the makes the preservation, long-term storage and shipment of cellular therapies a challenging prospect. Cryopreservation methods for long-term storage are described only in brief when reported in cell therapy clinical trial protocols. For example, while a cooling rate may be provided, processing times before freezing or after thawing are rarely given [13].

Components commonly used in lab-based cryopreservation protocols, including animal serum and dimethylsulfoxide introduce commercial, safety and regulatory risks for new therapies [38]. Animal-component free cryopreservation solutions are already available, such as the CryoStor® range from BioLife Solutions and PrimeXV®-FreezIS from Irvine Scientific. However, with cell-based therapeutics approaching the critical Phase III stage of development, there is a clear need to develop cryopreservation processes that operate at meaningful scale, and which integrate with other stages of an overall bioprocess. A well-integrated cryopreservation process enables the decoupling of commercially scaled cell therapy manufacture from final delivery and administration to patients. This in turn allows more cost-effective supply chain strategies for initial cell banking of isolated donor material, as well as the final product. The ideal result is an inventory of well-stored and

Abbreviations: CPA, cryoprotective agent; hMSC, human mesenchymal stem cell; ISCT, International Society for Cellular Therapy; pNNP, p-nitrophenyl phosphate; MoA, mechanism of action.

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consistent cell therapy product, which can be globally distributed and administered at an affordable cost.

Human mesenchymal stem cells (hMSCs) are the most likely candidates for early successful human stem cell therapies, with hundreds of clinical trials evaluating their therapeutic potential [39]. MSCs are multipotent; relatively easy to isolate; have the capacity for self-renewal and proliferation and are undergoing assessment for a number of therapeutic indications including immunological, cardiac and musculoskeletal conditions [17,28]. From a manufacturing perspective, clear definition of critical quality attributes and a link to a therapeutic mechanism of action (MoA) is necessary. Unfortunately, the complexity of MSCs and diversity of patients and individual disease progression has resulted in a lack of clarity around MoA for these therapies [4]. A variety of potency assays have been developed for some therapeutic indications, but at present there is no consensus on which should be used for a given cell type and therapeutic indication. The International Society for Cellular Therapy (ISCT) recommends a minimal hMSC specification as being adherent to culture plastic; positive ($\geq$95%) for the expression of CD73, CD90 and CD105, negative ($\leq$2%) for the expression of CD14, CD19, CD34, CD45 and HLA-DR, and possessing the capacity to differentiate towards the chondrogenic, adipogenic and osteogenic lineages *in vitro* [8]. These guidelines continue to be widely used to specify identity assays for MSCs.

Using cryopreservation to decouple production and delivery of hMSC-based therapies is a popular strategy, as demonstrated by a 2013 survey highlighting that over 80% of MSC-based regulatory submissions used cryopreservation to store and transport the final product [23]. For example, Prochymal® (Mesoblast, USA), which has been conditionally approved for use in children with graft-versus-host disease in New Zealand and Canada, is stored in 15 mL Cryocyte bags with 10% (v/v) Me_2SO [14]. However, recent reports have suggested that cryopreserved cells do not perform as well therapeutically compared with freshly cultured cells [10,25]. Cryopreservation may be one of the reasons, as well as *in vitro* cell age, why several recent hMSC clinical trials have failed to reach their primary end-points, in contrast to earlier academic-led trials using freshly cultured cells. Given the technical nuances of cryopreservation, it is important to determine whether transient loss of cell function is an unavoidable feature of cryopreservation, or whether it is caused by aspects of the process that differ with process scale.

Me_2SO has been used as a cryoprotectant since the 1950s [21], but there is some evidence of rare but significant adverse effects in patients, including strokes, heart attacks and haemorrhages after infusion with bone marrow cryopreserved in 10% (v/v) Me_2SO [6]. Notably, not all adverse reactions can be directly attributed to Me_2SO [34] and the FDA Guidance for Human Somatic Cell Therapy and Gene Therapy [37] does not prohibit the use of Me_2SO as a CPA. However, there is a perception that minimizing exposure of cells to Me_2SO will mitigate infusion-related effects in patient and importantly, Me_2SO is also cytotoxic to human cells above 0 °C and has been linked to changes in differentiation capacity of stem cells [1,9,36,43,44]. The mechanisms of Me_2SO cytotoxicity are not well defined, but may be related to the enhanced diffusion of other molecules, including toxins, across cell membranes [42], or through destabilisation of normal ionic homeostasis. The use of Me_2SO and other permeating cryoprotectants must be managed carefully to minimise the transplantation of poorly functioning cells; dead cells; cell debris or potential cytotoxins into patients.

Cells are often processed in small batches for routine research using passive slow cooling devices (e.g. Mr. Frosty™ or CoolCell®), with modest cell densities around 1×10^6 cell/mL and cells being kept on ice until transfer into their cooling device. However, working cell banks for manufacturing can contains hundreds of vials and allogeneic therapies are expected to require lot sizes of 1×10^9 or 1×10^{10} cells to meet market demands at affordable cost, with each dose containing 5×10^7 - 2.5×10^8 cells [31]. Cryopreservation processes will therefore need to be in place to enable preservation of large numbers of small cryovials for banking and cryobags or larger vials (e.g. Crystal® or Daikyo Crystal Zenith® vials) for cell product packaging. In both instances, processing times will increase compared to lab scale and maintaining chilled temperatures during vial filling and the particulate testing required by USP <790> (Visible particulates in injections) for product vials is an operational burden. This means that the detrimental effects on cell quality of exposure to cryoprotectants like Me_2SO will likely impose limits to the achievable process scales for hMSC therapy manufacturing. For example, it has been reported that for recombinant baby hamster kidney (rBHK) cells, cryopreservation bags must be filled within a 2 h window to avoid adverse effects from Me_2SO exposure [15]. Similarly, Hunt et al. [16] showed that the "recovery index", a measure of viability from membrane integrity and colony-forming-ability after thaw, of CD34 positive cells can be reduced by as much as 50% when exposed to 25% (v/v) Me_2SO for up to an hour at 20 °C. Katkov et al. [18] showed that human embryonic stem cells lose around 50% expression of the vital Oct-4 marker after exposure to Me_2SO using standard protocols. These examples indicate that exposure of MSCs to Me_2SO will need to be limited in order to maintain therapeutically desirable cell characteristics. Furthermore, these tolerances will need to be defined and built into not just manufacturing but also clinical practice. For example, with product thawing and preparation for infusion or injection the implications of failures in a thawing protocol or device (e.g. long-term maintenance of the vial at > ambient temperature) and delays in preparing the patient for treatment must be considered in order to develop preparation guidelines for the product. To that end, we investigated the effect of Me_2SO on cell quality by exposing bone marrow derived hMSCs to this cryoprotectant for varying amounts of time i) without, ii) prior to, and iii) after employing standard freezing protocols. A maximum exposure time of 2 h at ambient temperatures prior to freezing or at 37 °C after freezing was chosen to represent worst case scenarios. Results are discussed in terms of the risk of product failure with cells consider to be 'overexposed' to Me_2SO once cell characteristics such as attachment capability and phenotype fall below pre-defined standards such as those set by the ISCT or FDA. Furthermore, the sensitivity of hMSCs to Me_2SO was compared to that of an osteosarcoma-derived cell line, HOS TE85, to establish whether hMSCs are more or less susceptible to Me_2SO-induced effects than similarly sized human cells.

2. Materials and methods

2.1. Cell culture

Osteosarcoma cells of the line HOS TE85 were acquired at passage 51 (ATCC, USA) and hMSCs derived from bone marrow at two passages post extraction were cultured as adherent monolayers in 25 cm^2 T-flasks (Nunc, UK). hMSCs had been extracted from ethically sourced fresh bone marrow aspirate obtained from Lonza (Lonza, Cologne AG) from a healthy donor after the patient provided informed consent. HOS TE85 cells were incubated in MEM medium (α-MEM, 1 g/l glucose with 10% (v/v) US origin FBS (Performance Plus) and 2 mM L-glutamine, (Thermo-Fisher, UK)), and hMSCs in DMEM medium (made up in the same way with 1 g/L glucose (Thermo-Fisher, UK) in place of α-MEM) in a humidified incubator at 37 °C, 5% CO_2. Passaging was done every 2–3 days (HOS) or 7 days (hMSC). A 100% medium exchange was done for hMSCs on day 4. Spent-medium was collected before passaging for