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Alginate-Encapsulation for the Improved Hypothermic Preservation of Human Adipose-Derived Stem Cells

Running title: Alginate-encapsulation for improved cell storage

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ABSTRACT

Despite considerable progress within the cell therapy industry, unmet bioprocessing and logistical challenges associated with the storage and distribution of cells between sites of manufacture and the clinic exist. We examined whether hypothermic (4°C–23°C) preservation of human adipose-derived stem cells could be improved through their encapsulation in 1.2% calcium alginate. Alginate encapsulation improved the recovery of viable cells after 72 hours of storage. Viable cell recovery was highly temperature dependent, with an optimum temperature of 15°C. At this temperature, alginate encapsulation preserved the ability for recovered cells to attach to tissue culture plastic on rewarming, further increasing its effect on total cell recovery. On attachment, the cells were phenotypically normal, displayed normal growth kinetics, and maintained their capacity for trilineage differentiation. The number of cells encapsulated (up to $2 \times 10^6$ cells per milliliter) did not affect viable cell recovery nor did storage of encapsulated cells in a xenofree, serum-free, current Good Manufacturing Practice-grade medium. We present a simple, low-cost system capable of enhancing the preservation of human adipose-derived stem cells stored at hypothermic temperatures, while maintaining their normal function. The storage of cells in this manner has great potential for extending the time windows for quality assurance and efficacy testing, distribution between the sites of manufacture and the clinic, and reducing the wastage associated with the limited shelf life of cells stored in their liquid state.
INTRODUCTION

The medical applications of cell-based therapies have expanded considerably in recent years with an ever-increasing number of clinical trials being registered globally, many of which are in phase III of development. These encompass treatments for a wide range of conditions including cardiovascular disease, neurodegenerative diseases, cancer, liver disease, diabetes, skeletal disorders and eye diseases [1-3]. Accompanying this growing intensity in cell-based clinical research is the emergence of a number of Food and Drug Administration (FDA)- and European Medicines Agency (EMA)- approved cell therapy products (CTPs) to the market [4-9].

Hypothermic storage involves maintaining a living biologic in a suspended state at temperatures above 0°C but below the mammalian normothermic range of 32-37°C [10]. Due to the biological and technical issues associated with cryopreservation, including the required use of cryoprotective agents, such as dimethyl sulfoxide (DMSO), which can be costly to remove [11], and the risk of thawing with a hold time as little as two hours before a loss in cell viability occurs [12], the majority of approved CTPs adopt hypothermic storage. This means that they are manufactured ‘just-in-time’ in relatively small unit numbers rather than being stockpiled; keeping production costs low [13]. It does however mean that storage time is restricted before cells experience considerable loss in viability and function. This could not only affect the therapeutic potency of the CTP, but also introduce variability in the final product: an unfavorable situation for cGMP compliance.

Methods that improve the hypothermic preservation of cell viability, function and therapeutic potency offer a number of benefits throughout the complex CTP supply chain: i) extending the period for quality, safety and efficacy testing; ii) allowing distribution from the donor or bank to sites of processing; and iii) delivery to the clinic with reduced wastage associated with inadequate scheduling between different sites [14-16].
Human adipose-derived stem cells (hASCs) have received much attention as a source of mesenchymal stem cells (MSCs) for cell therapy applications in recent years, with over 130 clinical trials being registered between February 2007 and April 2015 [17]. Despite this growing interest, the effect of hypothermic storage on hASC viability and function has been limited. Here we examine encapsulation in alginate hydrogels as a method to support hASCs during hypothermic storage.

Alginate is a natural polysaccharide derived from seaweed that polymerizes rapidly in the presence of cations to form a biocompatible hydrogel [18], and as such, has gained considerable interest as a biomaterial for cell therapy applications [18-21]. Alginate encapsulation has previously been investigated for its protective effect on the hypothermic storage of rat hepatocytes, recombinant baby hamster kidney cells, human bone marrow-derived mesenchymal stem cells (MSCs), mouse embryonic stem cells (ESCs), and human limbal epithelial stem cells [22-25]. Its potential to heighten the preservation of cells during hypothermic storage has been postulated to be due to its contribution to membrane stabilization and subsequent protection against the osmotic shock and mechanical stress experienced during storage and recovery [23].

Here we describe the encapsulation of hASCs in alginate hydrogels, and investigate the conditions necessary for cell survival and function over short-term periods in hypothermic storage. We demonstrate the effectiveness of alginate-encapsulation in preserving cell viability and reveal the importance of controlling storage temperature for maximal viable cell recovery. Using an optimal storage temperature, we demonstrate the effectiveness of alginate-encapsulation to preserve the ability of hASCs to attach and regain metabolic activity upon return to normothermia, proposing that this should be taken into account upon defining viable cell recovery. We further demonstrate that hypothermic storage maintains the expression of key stem cell markers and does not affect the capacity for cells to proliferate and differentiate.
into osteo-, adipo-, and chondrogenic lineages. Finally, we explore scalability and adaptation to a serum-free, xeno-free storage system.

Taken together, we present alginate-encapsulation as a low-cost, simple method to improve the hypothermic preservation of hASCs, which has considerable potential in extending the shelf life of hypothermically stored CTPs for distribution throughout the cell therapy supply chain.

MATERIALS AND METHODS

hASC culture

hASCs obtained from the subcutaneous fat of three healthy donors (Invitrogen, UK), were used for all experiments. Cells were isolated from both male and female subjects (aged 45-63 years) and purity was determined by the manufacturer as cells being ≥95% positive for CD29, CD44, CD73, CD90, CD105, and CD166 and ≤ 2% positive for CD14, CD31 and CD45 surface antigen expression. Following recovery from cryostorage, cells were seeded at 800 cells/cm² and maintained in reduced-serum (RS) growth medium [MesenPRO™ RS medium containing 2mM GlutaMAX™ and 1% (v/v) antibiotic-antimycotic (all from Life Technologies, UK)] in a humidified incubator at 37°C and 5% CO₂ with medium changes every 3-4 days until approximately 80% confluency was reached. Cells were harvested using TrypLE™ Express enzyme (Life Technologies, UK).

Encapsulation, storage and release of hASCs

Harvested cells were incubated with 1 µM Calcein-AM (eBioscience, UK) for 15 minutes before counting viable (stained) cells using a Countess® II FL automated cell counter (Invitrogen, UK). A 0.25 mL suspension of 0.5–2x10⁶ cells in growth medium was mixed with 0.25 mL 2.4% (w/v) sodium alginate (Sigma, UK) in PBS (Invitrogen, UK) before
casting into calcium alginate discs using 102 mM calcium chloride as described previously [22]. Following a brief wash in PBS, gelled discs were transferred into 2 mL cryovials containing 1 mL growth medium, sealed, and placed in a polystyrene box before transferring to an actively-cooled incubator set to a specified temperature (11-23°C) or refrigerator (4°C). Control (non-encapsulated) cell suspensions (0.25 mL) were added directly to 2 mL cryovials containing 1 mL growth medium before storing in the same manner as encapsulated samples. Following 72 hours of storage (during which time the cells were left undisturbed), cells were allowed to equilibrate to room temperature before dissolving the calcium alginate hydrogel encapsulating them using 3 mL 100 mM trisodium citrate (Sigma, UK) for 5 minutes at room temperature, with control samples being treated in the same manner. The effect of trisodium citrate treatment on control samples was additionally compared against non-treated cells. Trisodium citrate was diluted using PBS before separating cells by centrifugation and dissociating them with TrypLE™ Express enzyme for 5 minutes (necessary due to aggregation of control samples). An equal volume of growth medium was added and cells were thoroughly re-suspended prior to counting. For serum-free conditions, storage was set up as above but growth medium was substituted for either StemPro® MSC SFM (SFM) or HypoThermosol-FRS (H-FRS). For 4°C experiments in H-FRS, cells were added directly to chilled H-FRS and were not allowed to equilibrate to room temperature before release as per the manufacturer’s instructions.

**Assessment of viable cell recovery**

The number of viable cells released from hypothermic storage was enumerated by incubating cells with 1 µM Calcein-AM for 15 minutes at 37°C before counting viable (stained) cells using a Countess® II FL automated cell counter (Invitrogen, UK).
**Assessment of apoptosis and cell death**

Cells recovered from hypothermic storage were assessed for apoptosis and cell death using an Annexin V-FITC Apoptosis Detection Kit (eBioscience, UK) according to the manufacturers instructions. Staining was analyzed using a Canto II flow cytometer (BD Biosciences, UK) and cells were classified as being viable (unstained), early-apoptotic (Annexin V-positive) or late-apoptotic/dead (Annexin V- and/or PI-positive).

**Functional assessment of hASCs before and after storage**

hASCs were examined for their capacity to re-attach, regain metabolic activity, proliferate and differentiate into osteo-, adipo-, and chondrogenic lineages. Stored samples were compared to non-stored control (NSC) samples; obtained from the same cultures harvested prior to storage that were plated, incubated, and assessed using the exact same methodology as detailed below.

**Assessment of attachment and metabolic activity**

Following release from storage, cells were plated at 20,000 cells/cm² in 24-well plates and incubated for 24 hours at 37°C, 5% CO₂ in a humidified incubator. Metabolic activity was then assessed using the alamar blue assay (as described in [26]). After incubation at 37°C for 3 hours, fluorescence was read at an excitation wavelength of 530-560nm and emission wavelength of 590nm using a Flurosakan Ascent™ FL plate reader (Thermo Scientific, UK). The same cultures were then assessed for attached cell number using the methylene blue-based assay for proliferation (as described in [27]). Following elution of the methylene blue stain, absorption was read at 650nm using an Infinite F50 plate reader (Tecan, UK) and attached cell number was determined using a standard curve.
Assessment of proliferative capacity

Cells were plated at 5,000 cells/cm$^2$ in 24-well plates and incubated for 3, 6, 9, 12 and 15 days at 37°C, 5% CO$_2$ in a humidified incubator. Cell number was determined using the methylene blue-based assay for proliferation (as above). Absorption was read at 650nm using an Infinite F50 plate reader (Tecan, UK) and cell number was determined using a standard curve.

Trilineage differentiation induction

For osteo- and adipogenic differentiation, the previously stored cells were plated at 5,000 cells/cm$^2$ in 96-well plates in growth medium for 4 days at 37°C, 5% CO$_2$ in a humidified incubator, changing medium after 2 days, before replacing growth medium with either osteogenic or adipogenic medium [StemPro® Osteogenesis or Adipogenesis Differentiation Kits containing 1% (v/v) antibiotic-antimycotic (all from Life Technologies, UK)] and maintaining for up to 31 days with media changes every 2-3 days. For chondrogenic differentiation, 5 µL of growth medium containing 80,000 cells was seeded into the center of 96-well round-bottomed plate and incubated for 2 hours at 37°C. 200 µL chondrogenic medium [StemPro® Chondrogenesis Differentiation Kit containing 1% (v/v) antibiotic-antimycotic (both from Life Technologies, UK)] was then added and cultures were maintained at 37°C, 5% CO$_2$ in a humidified incubator for up to 35 days with media changes every 2-3 days. Non-induced controls for all cultures were maintained in growth medium for the same time period.

Assessment of differentiation

All differentiation cultures were fixed with 10% (v/v) formaldehyde for 1 hour at room temperature and washed 3 times with ddH$_2$O prior to staining. Osteogenic differentiation was examined by staining mineralized matrices with 1 mg/mL Alizarin Red S (pH 5.5,
Ammonium Hydroxide) as described in [28], whilst adipogenic differentiation was assessed by staining lipid droplets with 1.8mg/mL Oil Red O in isopropanol as described in [29], both at room temperature for 30 minutes with gentle agitation. Excess stain was removed with 4 sequential washes with ddH₂O before capturing images. Alizarin Red S stain was eluted with 150µL 10% (v/v) acetic acid (as adapted from [28]) for 30 minutes at room temperature with gentle agitation before removing 100 µL aliquots and determining absorbance at 405nm. Oil Red O stain was washed for 5 minutes with 60% isopropanol before eluting with 150 µL isopropanol containing 4% IGEPAL CA-630 for 30 minutes at room temperature, removing 100 µL aliquots and determining absorbance at 520nm. Alizarin Red S and Oil Red O staining were quantified using a standard curve of known concentrations and absorbance was read using an Infinite F50 plate reader (Tecan, UK). Chondrogenic differentiation was assessed by staining cartilage-associated glycosaminoglycans with 0.1 mg/mL Alcian Blue 8GX in 6 parts ethanol to 2 parts acetic acid at room temperature overnight in the dark. Non-specific staining was washed 4 times with 6 parts ethanol to 4 parts acetic acid before capturing images. All components for staining and elution were purchased from Sigma Aldrich, UK.

**Assessment of cell surface marker expression**

Following release from storage, the cells were resuspended in sterile PBS and were labeled by incubation with the fluorochrome conjugated primary antibodies for 25 minutes at room temperature in the dark. After the incubation, the samples were washed with sterile PBS and centrifuged at 1500 rpm for 5 minutes. The cells were then resuspended in 300 µL PBS and analyzed with a Canto II flow cytometer (BD Biosciences, UK). The antibody panel used was composed of antibodies against CD14 and CD45 as negative MSC markers, CD90 and CD73 as positive MSC markers, CD29, CD44 and CD166 as adhesion markers present on the
surface of MSC, as well as HLA-ABC and HLA-DR. Samples were run at a medium flow rate (60 μL/minute). Isotype controls were performed with antibodies against IgG1 and 10,000 events, excluding cellular debris, were analyzed for each sample. All antibodies were obtained from BD Biosciences (UK) with the exception of the IgG1 isotype control (Beckman-Coulter, UK) and anti-CD90 (Dako, UK) antibodies.

Statistical analyses

Statistical analysis was carried out using GraphPad Prism (v4.00). Data are expressed as means of values from three separate donors ± standard error of the mean (SEM). Statistical comparisons were made using one-way or two-way repeated measures Analysis of Variance (ANOVA) with Bonferroni post hoc tests, with the exception of comparisons between two single variables where paired two-tailed T-tests were used. P-values < 0.05 were considered significant (*p < 0.05; **p < 0.01; ***p < 0.001).

RESULTS

The effect of storage temperature on viable cell recovery

In order to elucidate the storage temperature that would achieve the greatest recovery of cells, we encapsulated hASCs in 1.2% alginate discs and stored them for 72 hours at various temperatures, comparing viable cell recovery to non-encapsulated controls. We found that hASCs were surprisingly sensitive to deviations in changes in storage temperature over 72 hours. At 4°C, non-encapsulated (control) samples demonstrated a dramatic decrease in viable recovery, yielding only 17.8±15.6% of viable cells initially stored; a significantly lower recovery compared to any other temperature examined (Fig. 1A, 1B). Conversely, encapsulated cells exhibited a 3.7±0.7-fold increase in the number of viable cells recovered (P = 0.0224). Temperature had no significant effect on the viable cell recovery of control
samples at any other temperature tested, but did increase from 11°C reaching an optimum storage temperature at 15°C (63±5% viable recovery) before demonstrating greater variability between 17 and 23°C, with an average viable recovery of approximately 50 percent (Fig. 1A, 1B). Similarly, 15°C was the optimum temperature for storage of alginate-encapsulated cells, achieving a viable cell recovery of 86±6%, a significantly higher recovery compared to control samples ($P = 0.0002$) (Fig. 1A, 1B). At temperatures below or above 15°C, viable cell recovery did decrease slightly following storage in alginate, but only at 23°C was there a significant decrease in cell recovery compared to all other temperatures tested, with a heightened level of variability in the percentage viable recovery (29±29%) (Fig. 1A, 1B). Of note, the storage of encapsulated hASCs at 13-19°C consistently delivered a percentage viable recovery greater than 70 percent, the minimum acceptable viability specification generally set by the FDA for cellular products [30], and this was clearly not achieved without alginate encapsulation (Fig. 1A, 1B). As 15°C storage resulted in the greatest viable cell recovery in both control and encapsulated samples, subsequent experiments were conducted at this storage temperature.

**Assessment of apoptosis and cell death in stored-cell populations**

As well as determining viable cell recovery after 72 hours of hypothermic storage, we aimed to assess the contribution of apoptosis and death in recovered cell populations. Whilst non-encapsulated (control) samples predominantly exhibited a viable population of cells (Fig. 2Ai, 2B), this was variable (77±14.2%) with contribution of propidium iodide-stained dead cells, and propidium iodide and Annexin V co-stained late-apoptotic cells (11.3±7.2% and 8.6±7.1% respectively). Neither control (Fig. 2Ai) nor encapsulated (Fig. 2Aii) samples exhibited a large population of Annexin V-stained early-apoptotic cells (2.7±0.4% and 2.7±0.9% respectively). Encapsulated cells consistently exhibited a high level of viability
(92±2.3%) confirming a healthy population of recovered cells with little contribution of apoptosis or death (Fig. 2Aii, XB). Thus, encapsulation both enhanced viability and reduced variability in the health of cell populations recovered from hypothermic storage.

**Attachment, metabolic activity and total viable recovery of stored cells**

In addition to examining the viability of cells following release from storage, it was also important to perform subsequent tests to ensure cells were functional. We therefore examined the ability for stored cells to re-attach to tissue culture plastic and recover metabolic activity 24 hours after returning to a cell culture environment. Whilst non-encapsulated (control) hASCs exhibited a significant decrease in attached cell number compared to the non-stored control (NSC), encapsulated cells did not (Fig. 3A). A similar trend was observed with the percentage metabolic activity of cultures, although not significant (Fig. 3B). Furthermore, when normalized to cell number and expressed as the fold change relative to the NSC (Fig. 3C), no change in metabolic activity per cell was evident. Utilizing this data, a value can be calculated that represents total percentage recovery, that is the percentage of viable cells able to attach and recover metabolic activity [total % recovery = % viable recovery × (fold change in attachment x fold change in metabolic activity/cell)]. Using this equation, the protective effect of encapsulation in preserving functional cell recovery is highlighted (Fig. 3D), whilst control conditions only yielded a 45±3% recovery, the alginate-encapsulated samples achieved a significantly higher 80±3% recovery ($P = 0.0053$). As trisodium citrate was required for the dissolution of alginate for cell release, and this variable was matched between control and encapsulated samples, it was also important to determine whether citrate itself could have influenced the outcome with regards to the control. We determined that trisodium citrate had no significant effect on cell recovery, attachment, metabolic activity or total percentage recovery compared to non-treated controls (supplement online Fig. 1A-D).
Importantly, both yielded a total percentage recovery similar to the control presented here of 42±1% and 43±4% in citrate-treated and non-treated samples respectively.

**Morphology and proliferative potential of hASCs following storage**

Following their subsequent attachment to tissue culture plastic, stored hASCs exhibited a normal spindle shaped fibroblast-like morphology, indistinguishable between any of the conditions, forming tightly packed confluent monolayers by twelve days in culture (Fig. 4A). The proliferative potential of hASCs was unaffected by storage and, although control samples exhibited a cell number lower than encapsulated samples at every time point, this was not significant (Fig. 4B). Growth kinetics were normal in all samples demonstrating a rapid exponential period of growth up to day 6 which plateaued after this point (Fig. 4C).

**Differentiation potential of cells following storage**

When induced to do so, hASCs have the capacity to differentiate into osteogenic, adipogenic and chondrogenic lineages. In order to investigate whether this differentiation potential was maintained following alginate encapsulation, hypothermic storage and release, hASCs were induced to differentiate to appropriate lineages for up to 35 days. All samples were able to differentiate into osteocytes, adipocytes and chondrocytes as demonstrated by Alizarin Red S, Oil Red O and Alcian Blue staining respectively (Fig. 4D). Although quantification of Alizarin Red S (Fig. 4Ei) and Oil Red O (Fig. 4Eii) staining suggested a slight conservation of differentiation capacity by alginate encapsulation, this was not significant.

**Immunophenotype of cells following storage**

In order to determine whether storage at 15°C for 72 hours affected the immunophenotype of encapsulated hASCs, flow cytometric analysis was performed and compared to non-stored
cells maintained in culture at 37°C. In terms of size (FSC) and granularity (SSC), a similar profile was observed between cells obtained from culture (fig. 5A) and from storage (fig. 5B), with density plots indicating two distinct populations of different cell sizes. Non-stored hASCs exhibited a high level of expression of the MSC-associated markers CD73 and CD90 (fig. 5C) that was maintained following storage, albeit at a slightly lower percentage (fig. 5D). Of the MSC-associated markers that have key roles in cell-cell interaction and cell adhesion, CD29 expression was high in both non-stored and stored cell populations, whilst the expression of CD44 and CD166 were considerably decreased following storage (fig. 5C&D). The expression of negative MSC markers CD14 and CD45 as well as HLA-DR and HLA-ABC remained unchanged (fig. 5C&D).

**Effect of cell density on viable cell recovery**

For the purpose of scalability in the number of cells that could be preserved in calcium alginate, we investigated the viable recovery of cells after storage at up to $2 \times 10^6$ cells/mL, representing a four-fold increase in cell density. We found that encapsulation up to this density ($1 \times 10^9$ cells in a 0.5 mL calcium alginate disc) did not compromise the recovery of cells, as the encapsulated samples consistently yielded an above 70 percent viable recovery whilst control samples resulted in a less than 70 percent viable recovery (Fig. 6). Alginate-encapsulation at the highest cell concentration tested ($2 \times 10^6$ cells/mL) significantly improved viable cell recovery compared to control ($P = 0.0484$).

**Examination of storage in serum-free conditions**

For the purposes of adoption of a cell storage system into cell therapy logistics, it is important to demonstrate that any such system can be adapted to be serum-free, xeno-free and cGMP-compliant. For this reason, we examined hypothermic storage in two commercially available
serum-free, cGMP compliant medium: StemPro MSC SFM medium (SFM), formulated for MSC expansion, and HypoThermosol-FRS, a biopreservation medium designed for hypothermic storage at 4°C (H-FRS). These were compared against storage in the reduced-serum growth medium (RSM). Whilst storage of cells at 15°C in SFM, did not significantly compromise viable cell recovery compared to RSM in either control or encapsulated conditions, storage in H-FRS resulted in a significant decrease in the number of viable cells recovered (Fig. 7A). As H-FRS is formulated for 4°C, we also examined storage at this temperature and again observed a lower viable cell recovery compared to 15°C. This surprising result confirmed the unsuitability of H-FRS for storage of hASCs in suspension. Conversely, storage in SFM resulted in a comparable total percentage recovery to RSM (73±12%) (Fig. 7B) and maintained the cytoprotection elicited by alginate-encapsulation ($P = 0.0188$), confirming its suitability for hASC storage in serum-free, xeno-free conditions.

**DISCUSSION**

Despite extensive progress being made within the cell therapy industry, major logistical complications exist for cell storage and distribution. Here we present a simple, low-cost system capable of heightening the preservation of cells stored at hypothermic temperatures.

Alginate-encapsulation has previously been demonstrated to be efficacious in the preservation of human bone marrow-derived MSCs, mouse ESCs, and human limbal epithelial stem cells at ambient temperature [22, 25], as well as rat hepatocytes and recombinant baby hamster kidney cells at 4°C [23, 24]. Here we explore this system further, elucidating the optimal storage conditions for cell survival and more fully characterizing cells upon release from storage.

The examination of hypothermic preservation of MSCs has previously been reported on cells derived from different tissues and at different storage temperatures including: (i) bone
marrow at 4°C [31-38], 24°C [33, 35], ambient temperature [22, 36], and 37°C [35]; (ii) adipose tissue at 4°C [39, 40], 8°C, 25°C and 37°C [40]; and (iii) umbilical cord at 4°C [41]. Although these studies presented promising findings, the majority had been conducted at 4°C. Where direct comparisons between temperatures other than 4°C have been investigated, it has involved relatively large (greater than 12°C) steps in temperatures studied. Here we have demonstrated that small changes in temperature can have a considerable effect on the viable recovery of alginate-encapsulated cells with an optimal temperature in the range of 13-19°C and the greatest level of recovery at 15°C in both non-encapsulated and encapsulated samples. The importance of temperature on enzyme activity and subsequent metabolic rate of cells is likely to be key to this observation where there is an optimal point in which cell metabolism is slowed sufficiently for relative quiescence, before lower temperatures induce a phase transition in the cell membrane resulting in the segregation of membrane proteins and the uncontrolled entry of ions and subsequent cellular edema [42]. Previously, when protein leakage from liposomes was examined during chilling at a rate of 0.5°C/min, leakage was initiated below 15°C [43], which could go some way to explain why viable cell recovery decreases at temperatures lower than this in our system. Interestingly though, alginate-encapsulation elicited its highest level of cytoprotection at 4°C but did not result in a recovery better than that shown at 13-19°C.

At temperatures as high as 23°C, viable cell recovery decreases considerably, likely due to the temperature being too high to suppress enzyme activity and subsequent metabolism of cells. The result of this being the activation of dormant cells with ensuing metabolic requirements, and a further sensing of the sub-optimal environment for survival. What is more, the combination of higher hypothermic temperatures and alginate encapsulation appears to worsen viable recovery. Alginate is an inert hydrogel in which cells are suspended
and separated from each other resulting in little or no physical cell-cell interaction that may offer protection at higher temperatures. Whilst the effect of higher temperatures being detrimental for hypothermic preservation is perhaps unsurprising, it is pertinent that this occurs at what might be considered as a low ambient temperature of 23°C. This emphasizes the importance of controlling temperature during storage. We have, however, demonstrated that there is a range of suitable temperatures (13-19°C) where viable recovery was consistently above 70% suggesting a degree of tolerance to possible deviations during storage and distribution. This optimal temperature range for hypothermic preservation is consistent with studies on adherent retinal pigment epithelial cells [44] and cultured conjunctival epithelium [45], as well as Chinese hamster ovary (CHO) cells stored in suspension [46]. These studies similarly demonstrated that the storage of cells at 4°C or the upper range of hypothermic temperatures, was highly detrimental for cell survival. The application of non-chilled (above 4°C) hypothermic temperatures for CTP preservation is also reflective of the storage conditions employed by approved CTPs including ChondroCelect® (15-22°C), Epicel® (13-23°C) and Apligraf® (20-23°C) [4, 6, 8]. Taken together, controlled, non-chilled conditions are optimal for cell preservation in this system.

With CTP consistency and lack of variability being an important concern for cell delivery, we examined the contribution of early-apoptotic, late-apoptotic and dead cells in populations released from storage at 15°C. As well as the increased viable cell recovery effected by encapsulation, it also resulted in a consistently high viability in the cell population recovered with little incidence of apoptosis or death. This, on the other hand, was highly variable in non-encapsulated cell populations. With the major cause of cell death during hypothermic preservation being a result of membrane destabilization, ultimately resulting in cellular edema and lysis [42], we were not able to account for all cells present prior to storage, likely due to the nature of cell death resulting in full membrane disintegration. Due to this
common effect of cell preservation, the presentation of percentage viability as a measure of storage success can often be anomalous. These results support the fact that the encapsulation of hASCs is able to yield a consistently high recovery of viable cells, compared to those present prior to storage, and the end product has minimal contribution of death and/or apoptosis.

Whilst it is of great importance to examine viable cell recovery as a quality control measure for CTPs, it is also critical to assess cellular function after a period of rewarming. Such assessment is important due to the possibility of the activation of stress-pathways up to 24 hours post-return to normothermia as previously described following cryopreservation [47], as well as in the reported effects of attenuated attachment of bone marrow-derived MSCs following hypothermic storage [38]. Herein we have demonstrated that encapsulation protected against the decrease in attached cell number exhibited by non-encapsulated samples after 24 hours in culture, with no change in metabolic activity per cell. We believe it important to take these measurements into account when calculating a value for ‘total percentage viability’, i.e. the number of viable cells released from storage that are able to attach and recover normal metabolic activity. Through using this approach (multiplying the viable cell recovery by the fold change in attachment multiplied by the metabolic activity per cell) for every donor, we can take into account all permutations that represent a final recovery of cells upon return to tissue culture. Consequently, any deviations in attachment or metabolic activity are represented. There is also a degree of normalization with those samples that achieving the highest level of viable recovery, also having the lowest level of attachment. This suggests that a population of cells that are counted as viable may already be damaged but this only becomes apparent when cells are rewarmed on return to normal tissue culture conditions. Thus we can exclude those cells that would not survive upon return to
physiological conditions producing a reliable, consistent measure of recovery. This approach has also highlighted the increased functional cell yield effected by alginate.

Upon cell reattachment, morphology was shown to be unaffected compared to non-stored controls moreover the released cells exhibited a normal pattern of growth, an important concern considering the previous reports of attenuated growth of bone marrow-derived MSCs following hypothermic storage [34]. As well as attachment and growth characteristics, one of the most important functional characteristics of MSCs is their ability to differentiate into osteoblasts/osteocytes, adipocytes and chondroblasts/chondrocytes [48], a capacity reported to be diminished after short-term hypothermic storage [36]. However, under the conditions tested here, we observed no difference in the capacity for trilineage differentiation following storage when compared to non-stored controls. Although encapsulation did appear to elicit greater protection against reduced proliferation and differentiation compared to non-encapsulated controls, this was only slight and not significant after 72 hours storage. Thus, alginate-encapsulation elicited a 1.79±0.15-fold increase in the total percentage recovery of hASCs that maintained a normal phenotype and function following release from storage at 15°C for 72 hours.

In support of hypothermic storage in alginate being able to hold cells in an un-altered state, we demonstrated that a high expression of the majority of MSC-associated markers was maintained following storage, importantly CD90 and CD73, albeit at a lower level. We did, however, observe a considerable decrease in the expression of two MSC-markers associated with adhesion (CD44 and CD166). This is perhaps unsurprising as alginate is an inert polymer and, as such, the interaction of encapsulated-cells with their surrounding environment would be minimal, which may have resulted in their expression being lost. Indeed, it has previously been reported that the expression of a number of cell surface markers, including CD44, CD90 and CD166, are diminished during the culture of human
bone marrow-derived MSCs in alginate, but these are recoverable following return to monolayer culture [49]. Thus, certain MSC-associated markers may not be appropriate for assessing cells released directly from alginate.

With the clinical application of MSCs requiring high cell numbers, typically 1-2 × 10^6 cells/kg depending on the treatment [50], it was necessary to examine whether viable cell recovery was compromised at higher cell densities. The cytoprotection offered by alginate encapsulation was maintained, in cell densities of up to 2 × 10^6 cells/mL, resulting in a viable cell recovery consistently above 70 percent. This suggests that scaling up to higher cell numbers is feasible and, accompanied by scalable platforms for cell encapsulation in alginate beads [51], makes scalable process development of large numbers of therapeutic cells achievable.

As fetal bovine serum (FBS) could be a source of prion, viral and zoonotic diseases [52], it was also desirable to examine whether the cytoprotection offered by alginate encapsulation could be recapitulated when hydrogels were placed in serum-free, xeno-free and cGMP-grade storage medium. Whilst HypoThermosol-FRS has demonstrable efficacy in the preservation of adherent bone marrow-derived MSCs at 4°C [32, 37] and offers cytoprotection to hypothermically stored hepatocytes in suspension [53], we found it unsuitable for storage of hASCs in suspension at either 4°C or 15°C. Conversely commercially available StemPro® MSC SFM medium affected no significant difference in viable cell recovery or total percentage recovery of cells when compared to MesenPro reduced serum (RS) medium. This demonstrated that the storage system, described here, could be easily adapted to be clinically safe and cGMP-compliant.

Although we have provided convincing evidence that alginate-encapsulation improves the hypothermic preservation of hASCs, the mechanism by which it exerts its effect remains unclear. It has been suggested, during the hypothermic preservation of hepatocytes, that
alginate encapsulation functions to stabilize the membrane of suspended cells in the absence of an extracellular matrix, and thus minimize the effects of osmotic shock and mechanical stress during cell storage and recovery [23]. Alginate also has a strong influence on ion and solute diffusion throughout the hydrogel matrix [54], as well as having a considerable swelling capacity. This could contribute to modulating the diffusion rates of ions and solutes, and therefore water [42], in the environment surrounding encapsulated cells, further reducing the impact of osmotic shock and cellular edema. Thus, there are a number of mechanisms by which alginate could offer cytoprotection during hypothermic storage.

**CONCLUSION**

In a recent assessment by experts in the field, it was considered that the development of preservation and transport-enabling technologies were critical in the cell therapy industry in order to eliminate GMP-regulated steps of preservation removal and cell thawing associated with cryopreservation [14]. Although liquid-state storage avoids these issues, there still remains concern over limited time-windows associated with the transfer of cells to the clinic due to a restricted shelf life where high levels of material wastage can result [55]. Alginate-encapsulation represents a method whereby the hypothermic preservation of cells can be markedly improved whilst maintaining cell functionality. As a material with demonstrable biocompatibility, application in cell therapy, and scalability, its integration into CTP bioprocessing has considerable potential.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors declare no potential conflicts of interest.

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Figure 1. The effect of storage temperature on viable cell recovery. hASCs were stored at different temperatures either encapsulated (Encaps.) or non-encapsulated (Control) for 72 hours before assessing viable cell number (A) and percentage viable recovery (B). Values are expressed as means ± SEM from 3 separate donors with asterisks representing significance from control values (***, p < 0.001; *, p < 0.05) and symbols representing significance between temperatures ($$ or $$; p > 0.05; $; p < 0.05).
Figure 2. Assessment of apoptosis and cell death in stored cell populations. hASCs were stored at 15°C for 72 hours before assessing the percentage of cells positive for Annexin V, and/or propidium iodide in non-encapsulated control (Ai) and encapsulated (Aii) cell populations. Cells were classified as being viable (unstained), early-apoptotic (Annexin V-positive) or late-apoptotic/dead (Annexin V- and/or PI-positive) cells (B). Values are expressed as means ± SEM from 3 separate donors.
Figure 3. Attachment, metabolic activity and total cell recovery following storage. hASCs were stored at 15°C for 72 hours before returning a cell culture environment. After 24 hours, cell attachment (A) and metabolic activity (B, C) were assessed. These values were used to calculate total percentage recovery [% viable recovery × (fold change in attachment × fold change in metabolic activity/cell)] (D). Values are expressed as means ± SEM from 3 separate donors with asterisks representing significance from control values (***p < 0.01; *p < 0.05). NSC: non-stored control; Control: non-encapsulated; Encaps.: encapsulated.
**Figure 4.** Morphology, proliferation and multilineage differentiation potential following storage. hASCs were stored at 15°C for 72 hours before returning a cell culture environment. Cells were cultured in growth medium with images being captured after one and twelve days, (A) and cell number was assessed every three days for fifteen days (B, C). For differentiation, cells were cultured in osteo-, adipo-, or chondrogenic media for up to 35 days. Cultures were stained with Alizarin Red S (osteogenic), Oil Red O (adipogenic) or Alcian Blue 8GX (chondrogenic) and images were captured (D). Osteogenic (Ei) and adipogenic (Eii) stains were quantified. A: scale bars = 100µm; B, C, E: values are expressed as means ± SEM from three separate donors; D: scale bars = 50µm. NSC: non-stored control; Control: non-encapsulated; Encaps.: encapsulated.
Figure 5. Assessment of size, granularity and immunophenotype of encapsulated cells following storage. hASCs were assessed for size and granularity following no storage (A) and storage for 72 hours at 15°C (B). Similarly, immunophenotype was assessed following no storage (C) and storage for 72 hours at 15°C (D). Size and granularity plots denote representative images and values for the percentage expression of cell surface markers are expressed mean ± SEM from three separate donors.
Figure 6. The effect of cell load on viable cell recovery following storage. hASCs were stored at 15°C for 72 hours at a range of densities before assessing viable cell recovery. Values are expressed as means ± SEM with the level for 70% viable cell recovery indicated by a dotted line. Control: non-encapsulated ($R^2 = 0.9268$); Encaps.: encapsulated ($R^2 = 0.9859$).
Figure 7. Cell storage in serum-free, xeno-free, cGMP-grade medium. hASCs were stored at 15°C for 72 hours in growth medium (reduced-serum medium [RSM]) or serum free medium [SFM] or at 15°C or 4°C in HypoThermosol-FRS (H-FRS) before assessing viable cell recovery (A). Total % Recovery (B) was calculated with attachment and metabolic activity data using the following equation [% viable recovery x (fold change in attachment x fold change in metabolic activity/cell)]. Values are expressed as means ± SEM from 3 separate donors with asterisks representing significance from control values (***p < 0.001; **p < 0.01; *p < 0.05) and symbols representing significance between storage media ($$ or ##: p > 0.05; $#: p < 0.05). Control: non-encapsulated; Encaps.: encapsulated.
Figures 1. The impact of sodium citrate on the viable cell recovery, attachment, metabolic activity and total cell recovery of non-encapsulated cells following storage. hASCs were stored at 15°C for 72 hours before assessing viable cell number (A) and returning a cell culture environment. After 24 hours, cell attachment (B) and metabolic activity (C) were assessed. These values were used to calculate total percentage recovery [% viable recovery x (fold change in attachment x fold change in metabolic activity/cell)] (D). Values are expressed as means ± SEM from 3 separate donors with asterisks representing significance from control values (**p < 0.01; *p < 0.05). NSC: non-stored control.