Optimization of Mesenchymal Stromal Cell Culture Methodologies Towards the Development of a Cell Therapy for Autoimmune Diseases

Ana Lúcia Bastos Tiago[1]

Abstract: The optimization of large-scale manufacturing of Mesenchymal Stromal Cells (MSC) towards the development of cell therapy products for the treatment of Graft Versus Host Disease (GVHD) and autoimmune diseases, among others, is an area of growing interest. In this work, cryopreservation using different media was assessed and a post-thawing cell viability of 86.1±1.3% was obtained for 1-month cryopreservation in Cryostor CS5, outperforming the standard culture medium with 10% DMSO (77.4±2.2% for FBS-supplemented medium and 80.3±3.9% for HPL-supplemented medium). Post-thawing immunophenotype characterization revealed no significant alterations. Cells thawed after 2-months cryopreservation showed similar results. Short-term transport solutions, namely fresh culture medium, conditioned culture medium and commercially available media, were also evaluated as a possible alternative to cryopreservation and 2-8 Cellsius was found to be effective in maintaining cell viability above 70% for 5-7 days. Comparatively, both HPL and FBS-supplemented culture medium maintained cell viability above 70% for 3-4 days and the use of conditioned medium was not found particularly beneficial. Alginate encapsulation effectively maintained MSC at RT for 11 days with post-release cell viability of 80.3±1.3%. Additionally, post-encapsulation MSC were able to successfully support hematopoietic stem progenitor cells (HSPC) expansion. Cells retrieved from all transport solutions maintained normal immunophenotype, plastic adherence and multilineage differentiation potential throughout the assay. The Cost of Goods (COG) analysis of the production of an MSC-based product revealed that the number of cryopreservation steps performed and the number of cell passages between them influences the final product's cost/dose. The impact of cryopreservation and transport solutions on MSC quality and functionality, presented in this work will contribute to accelerate clinical translation of these cell products.

Key Words: Human Mesenchymal Stromal Cells, Cell Cryopreservation, Cell Transport, Alginate Encapsulation, Cost of Goods.

1 - Instituto Superior Técnico

1. Introduction

Mesenchymal Stromal Cells (MSC) are a heterogeneous population of fibroblast-like adherent cells that can be isolated, among others, from bone marrow (BM), adipose tissue (AT) and umbilical cord matrix (UCM)[1]. The International Society of Cellular Therapy (ISCT) defined a set of standards to clarify and harmonize the fundamental characteristics of this cell population: adherence to plastic, expression of a panel of surface antigens (more than 95% expression of CD105, CD73 and CD90 and less than 2% expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR), and multipotent differentiation potential into osteoblasts, chondrocytes and adipocytes, under *in vitro* differentiation conditions[2]. MSCs have become the subject of increased research interest due to their particular therapeutic potential, attributed to the secretion of bioactive factors with regenerative and immunomodulatory properties, and the fact that they can be easily obtained with few ethical issues associated[3]. Graft versus host disease[4], autoimmune diseases[5,6], Chron's disease[7] and COVID-19-induced acute respiratory distress syndrome[8] are some of the conditions being explored as therapeutic targets of MSC, with an increasing number of late stage clinical trials and approved cell therapy products on the market[9].

The large-scale production of allogenic MSC for clinical application requires logistical decisions regarding the scale-up platforms to be used for isolation, expansion, recovery, storage and quality control of the cells, as the whole process must comply with Good Manufacturing Practices (GMP)[10]. Cryopreservation is an important step of MSCs biomanufacturing as it constitutes an economically and logistically feasible way of having cell therapies as an off-the-shelf readily available product, storing cells in controlled conditions until administration[11]. Generally, cryopreservation involves the use of Dimethyl Sulfoxide (DMSO), a cryoprotective agent, and a slow cooling rate (1°C/min)[12]. However, cryopreservation and thawing procedures are known to negatively affect the stability and therapeutic efficacy of MSC-based products, which raises economical and regulatory issues[13]. In order to ensure that thawed cells maintain their properties and are ready for patient infusion, it is necessary to optimize cryopreservation and thawing to be less aggressive to cells.

2. Materials and Methods

Cell Culture: AT and UCM-MSC were recovered from cryostorage and cultured in T-Flasks (Corning) with DMEM culture medium (31600-091 Gibco) supplemented with either 10% (v/v) FBS (12662-029 Gibco) or 5% (v/v)

HPL (HPCHXCGLS50 AventaCell) and 1% (v/v) Antibiotic-Antimycotic (Anti/Anti) (15250-062 Gibco).

Cryopreservation Solution Testing: AT and UCM-MSC expanded in FBS and HPL-supplemented medium were cryopreserved in five different cryopreservation solutions: 90% (v/v) FBS or HPL-supplemented Culture Medium+10% (v/v) DMSO, 90% (v/v) Serum or Lysate+10% (v/v) DMSO, 90% (v/v) 2-8 CELLsius (PP338-100 Protide Pharmaceuticals) +10% (v/v) DMSO, CryoStor CS5 (C2999-100ml Biolife Solutions) and PZerve (5720 Protide Pharmaceuticals). For each condition, three cryovials, for three timepoints (1 month, 2 months and 3 months of cryopreservation), were frozen. After thawing, the immunophenotype of cells retrieved was characterized.

Transport Solution Testing: AT and UCM-MSC expanded in FBS and HPL-supplemented medium were suspended at a density of 1M cells/mL in three different transport solutions: Fresh Culture Medium (FM) at Room Temperature (RT) and 4°C, glucose-supplemented Conditioned Culture Medium (CM) at RT and 4°C and 2-8 CELLsius at 4°C. Cells were counted every 24 hours and characterization assays (immunophenotype, multilineage differentiation) were performed every 48 hours until cell viability was lower than 70% and/or cell concentration reached half of its initial value.

Alginate Encapsulation Testing: 6M AT-MSC expanded in FBS and HPL-supplemented medium were resuspended in either FM or CM and encapsulated in alginate beads using the BeadReady kit (BR-MNS-01 Atelerix) and stored at RT (between 10°C and 20°C). Each kit was divided in two to account for two different timepoints. Beads were dissociated at days 5 and 11, released cells were counted, and characterization (immunophenotype, multilineage differentiation) and functional assays (hematopoietic support) were performed.

Characterization Assays:

Immunophenotype: Immunophenotype of cells was characterized via Flow cytometry analysis using a FACSCalibur cytometer (BD Biosciences). Per condition, approximately 100K cells resuspended in 100 µL PBS were placed in three 5 mL round bottom polystyrene tubes (352235 Falcon). Cells were stained with LIVE/DEAD™ Fixable Far Red Dead Cell Stain Kit (L34974 Invitrogen) for 15 minutes in the dark, washed with PBS, stained for 15 minutes in the dark with mouse anti-human monoclonal antibodies, and washed again. The antibody panel used was: CD73 FITC, CD90 PE, CD44 PerCP-Cy5.5, CD105 APC or FITC, CD80 PE, CD45 PerCP-Cy5.5, CD11b APC, CD19 FITC or APC, CD14 PE, CD34 PerCP-Cy5.5 and HLA-DR FITC (BioLegend, Biosciences or abcam). An unstained control was prepared as a negative control in every acquisition. Data analysis was performed using the FlowJo V.10 software (BD Biosciences).

Multilineage Differentiation: For the adipogenic and osteogenic differentiation, cells were plated in 12-well culture plate (Falcon) and allowed to reach 70-80% confluency. Differentiation was induced by adding osteocyte or adipocyte complete differentiation medium containing osteocyte/ chondrocyte differentiation basal medium (A10069-01 Gibco) or adipocyte differentiation basal medium (A10410-01 Gibco), supplemented with osteogenesis supplement (A10066-01 Gibco) or adipogenesis supplement (A10065-01 Gibco) and 1% Anti/Anti. Differentiation medium was changed every 3 days for 14 days. For the chondrogenic differentiation, 30 µL droplets containing 50K cells each were placed on the inner side of a petri dish lid and the hanging drops were left to incubate overnight at 37°C, 5% CO_2 and in a humidified atmosphere. For each condition, 2-3 droplets were placed in one well of a 24-well ultra-low attachment culture plate (3473 Corning) and differentiation was induced with the addition of chondrocyte complete differentiation medium, composed of either MSCgo chondrogenic basal medium (05-220-1B Biological Industries), MSCgo chondrogenic supplement mix (05-221-1D Biological Industries) and 1% Anti/Anti, or MesenCult ACF chondrogenic differentiation basal medium (05456 Stemcell Technologies), MesenCult ACF chondrogenic differentiation supplement (05457 Stemcell Technologies) and 1% Anti/Anti. Differentiation medium was changed every 4 days for 14 days.

To assess differentiation, cells were fixed with 4% (v/v) paraformaldehyde (158127 Sigma Aldrich) for 30 minutes at RT and washed with PBS. For adipogenesis, cells were stained with 0.2% Oil Red O (O0625-25G Sigma Aldrich) in a 60% isopropanol (P/7507/17 Fisher Chemicals) solution (responsible for staining lipidic vesicles) for 1 hour at RT. For osteogenesis, cells were subjected to an alkaline phosphatase stain (ALP, to detect alkaline phosphatase activity) by incubation for 40 minutes at RT in a solution of Fast Violet (99-21-8 Sigma Aldrich) and Naphthol (855-

20ML Sigma Aldrich), being washed with distilled water afterwards, and to a Von Kossa stain (detection of calcium deposits), by incubating cells for 30 minutes with silver nitrate (85193-100ML Sigma Aldrich). For chondrogenesis, cells were incubated for 1 hour in an 1% alcian blue (A5268-10G Sigma Aldrich) solution (responsible for staining glycosaminoglycans). Following incubation, all cells were washed three times with distilled water and kept in PBS. They were then observed under the microscope (Leica DMI3000 B) and pictures were taken (Nikon Digital Camera DXM1200F).

Hematopoietic Support: Following alginate encapsulation release at days 0, 5 and 11, MSC were plated in 2 wells of a 12-well culture plate at a density of 100K cells/cm^2. One of the wells contained FM and the other CM. Cells were left to incubate overnight at 37°C, 5% CO_2 in a humidified atmosphere in order to originate confluent feeder layers. CD34$^+$ enriched cells through Magnetic Activated Cell Sorting (MACS) using the CD34 MicroBead Kit (130-046-702 Miltenyi Biotec) were cultured over the previously prepared feeder layers (FM stroma and CM stroma) and in the absence of MSC for seven days in StemSpan Serum-Free Expansion Medium (SFEM) II (09655 Stemcell Technologies) supplemented with 1% (v/v) Anti/Anti, at a density of 30K cells/mL (2 mL per 12-well) and in the presence of the following human cytokines: stem cell factor (64 ng/mL for wells without a feeder layer and 90 ng/mL for MSC-containing wells), FMS-like tyrosine kinase 3 ligand (61 ng/mL for wells without a feeder layer and 82 ng/mL for MSC-containing wells), thrombopoietin (80 ng/mL for wells without a feeder layer and 77ng/ml for MSC-containing wells) and basic fibroblast growth factor (5 ng/mL only for MSC-containing wells), all from PeproTech. After seven days, hematopoietic stem progenitor cells (HSPC) in each well were counted to assess proliferation. Fold increase (FI) was calculated by dividing the number of HSPCs counted in each well by the number of HSPCs originally seeded. These results were normalized by dividing each FI by the FI of the No MSC well, used as a negative control.

The immunophenotype of HSPCs was analyzed before and after expansion by flow cytometry using previously titrated CD45RA FITC (Biosciences), CD90 PE and CD34 PerCP-Cy5.5 mouse anti-human monoclonal antibodies.

The clonogenic potential of HSPC was evaluated before and after expansion. At day 0, 1K CD34$^+$-enriched cells in 100 µL were resuspended in 2 mL of MethoCult Classic (04434 Stemcell Technologies) medium, plated in a 24-well culture plate and left for 14 days to incubate at 37°C, 5% CO2 in a humidified atmosphere. After expansion, the procedure was repeated with 2,5K cells from each condition. After 14 days, multilineage colony-forming unit (CFU-Mix), burst-forming unit-erythroid (BFU-E), and colony-forming unit-granulocyte, macrophage (CFU-GM) colonies were counted using a brightfield microscope (Olympus CK40). Colony number was divided by the number of seeded cells to obtain the number of colonies per seeded cell. This was multiplied by the number of HSPC harvested at day 7. FI in the number of colonies was calculated by dividing the total colony number after expansion by the total colony number before it.

Statistical Analysis: When more than one experimental replicates were considered, data was analyzed using GraphPad Prism 8 software (GraphPad Software) and presented as mean ± SEM.

3. Results and Discussion

3.1. Cryopreservation Solutions

Cell Viability and Recovery: Approximately one month after each donor was cryopreserved, cells were thawed, and cell viability and recovery were calculated (Figure 1). In general, although there was no significant difference between the post-thaw viability and recovery percentages obtained for each of the cryopreservation solutions, it is possible to observe that the Cryostor condition, a commercial xenogeneic-free cryopreservation medium with 5% (v/v) DMSO, presents the highest cell viability (mean value of 86.1±1.3%, N=8). This result is in line with previously reported results regarding this medium[14–16] and indicates that Cryostor CS5 does not have a particularly detrimental effect on the viability of cryopreserved cells.

Medium, which is considered the standard cryopreservation solution, being often used as control in these studies, and 2-8 Cellsius conditions follow in the best post-thawing viability (mean values of 77.4±2.2% for FBS-supplemented medium, 80.3±3.9% for HPL-supplemented medium (N=4 in both) and 78.3±1.7% for Cellsius (N=8)). 2-8 Cellsius is a commercially available protein-free transport medium that serves as cryopreservation medium when supplemented with 10% (v/v) DMSO. Its use in the cryopreservation of PBMC

resulted in a post-thawing viability of approximately 80%[17], which is concordant with these results.

Mean cell viability values obtained in the Serum condition were 76.3±1.4% for FBS and 58.9±1.6% for HPL. The results obtained are in line with what has been reported for cryopreservation using FBS[18,19]. Regarding HPL, Chinnadurai and colleagues[20] reported cell viability and recovery of approximately 90% after cryopreservation with 90% (v/v) HPL supplemented with 10% (v/v) DMSO, which differs from the obtained value for viability. This may be due to an overestimation of dead cells caused by Trypan Blue staining of debris in suspension in HPL. In comparison with the commonly used Medium condition, Serum results, in general, in smaller viability percentages, although recovery rates are similar or even greater than medium for both FBS and HPL conditions.

The PZerve condition, corresponding to a commercially available DMSO and animal protein-free cryopreservation medium, presents the lowest percentages for both viability (59.9±3.3%) and recovery (67.7±5.4%), performing worse than standard culture medium. No published data regarding this cryopreservation solution could be found.

After two months of the initial cryopreservation, three donors were thawed to assess the influence of longer cryopreservation times in cell viability and recovery. No significant difference was found between the obtained cell numbers and the same pattern of viability and recovery is present in both months (data not shown), which shows that one month may not constitute a relevant timeframe to observe alterations in these parameters.

Immunophenotype: MSCs were considered to meet the ISCT minimal criteria if the percentage of positive cells was higher than 95% for CD44, CD73, CD90 and CD105, and less than 2% for HLA-DR, CD11b, CD14, CD19, CD34, CD44 and CD80. The expression (Figure 2) of the positive markers CD73, CD90 and CD44 remained generally unaltered by cryopreservation for all cryopreservation solutions, which is in line with studies reporting that cryopreservation does not affect the expression of surface markers by MSCs[19,21–24]. The expression of CD105 appears to be the most affected. This was also identified by Antebi and colleagues[18] and may be attributable to the documented heterogeneity of this marker, known to be sensitive to longer exposure times to enzymatic dissociation agents and/or to higher agitation rates and consequent shear stress[25,26].

The expression pattern of the negative cell markers remained unaltered, except for an occasional increase (of approximately 10%) in the expression of CD11b, CD19, CD14 or CD80 in some cases. Some limitations may be pointed out to the flow cytometry analysis which may help to explain the results obtained. Firstly, the analysis was limited by a reduced number of thawed cells in each condition, which resulted in a small number of events acquired. Secondly, only one of the three tubes analyzed per condition contained the viability dye that allowed the exclusion of dead and apoptotic cells. This means that the non-viable cells in tubes 1 and 2 may have contributed with false positives due to unspecific staining which may have been considered in the analysis, skewing the results. Thirdly, the panel of antibodies used had not been optimized for MSCs which may have resulted in excess antibody in some cases, causing an aberrant fluorescence intensity of negative populations. Finally, the only negative control utilized was the unstained sample of MSCs. The use of a fluorescence minus one (FMO) control would have resulted in a more precise gating, by considering fluorescence interference of such a multi-color panel.

None of the donors present any relevant differences between the flow cytometry results from the first and second months, which indicates that 2 months might not be enough to observe changes in MSC immunophenotype due to effects of cryopreservation. This result is in line with the normal immunophenotype obtained by Ginis and colleagues following five months cryopreservation[16].

3.2. Transport Solutions

Cell Viability and Recovery: Regarding solutions for short term storage of cell products, these should be able to retain a satisfactory viability (the FDA defines a cell viability higher than 70% as a release criterion for cell therapy products[27]) for as long as possible so that cells can be transported during their production pipeline. Several hypothermic preservation solutions are already available and their efficacy in MSC transport has been previously compared [16,28,29]. In this experiment, the transport capacity of the 2-8 Cellsius solution was compared with MSC storage in culture medium at 4°C and at RT. Storage of MSC in CM was also evaluated, in order

to determine if the bioactive molecules secreted by MSC could improve cell maintenance at RT and 4°C, in comparison with FM.

For both AT and UCM HPL (Figure 3), cell viability was best preserved by the 2-8 Cellsius solution, which was able to maintain MSCs above the minimum criteria for five and eight days, respectively. The same was not verified for AT FBS, where cells presented viability and recovery lower than 50% on day 1. Nevertheless, the lack of replicates does not allow for sound conclusions. Since no published data could be found describing the use of 2-8 Cellsius for the storage of any cell type at 4°C, experimental results could not be compared. FM proved to be the best transport solution following 2-8 Cellsius, particularly at 4°C, where it was able to maintain MSCs with a satisfactory viability for five days, in case of AT HPL, and four days in case of UCM HPL, outlasting CM 4°C in both cases. In AT FBS, both solutions performed similarly. As expected, both RT solutions were not particularly effective in maintaining MSCs, lasting only until day 1 or 2. These results are superior to those reported by Nofianti and colleagues[30] who observed the viability of AT-MSCs stored in DMEM (basal medium only) at 4°C for four days and obtained a percentage lower than 70% on day 2. In another study, Veronesi and colleagues[31] performed an 18h transport assay of BM-MSCs at 4°C using maintenance medium (composed of αMEM supplemented with 8% HPL) and obtained a viability of approximately 87.87%, which agrees with the results obtained for the FM 4°C condition at day 1. No studies examining the hypothermic storage of MSCs in CM could be found.

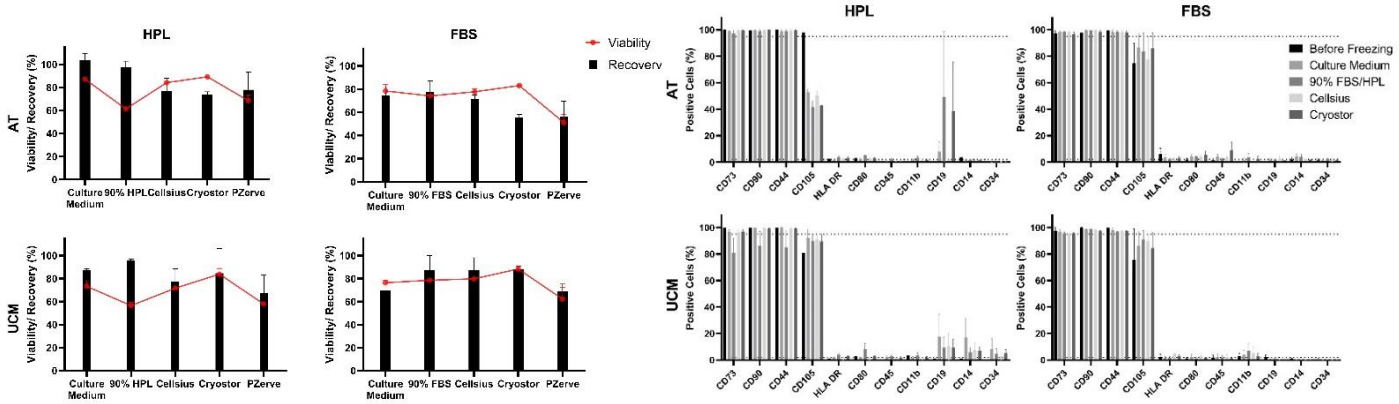

Figure 1 – (Left) Cell viability and cell recovery post-thawing at the 1-month time point per source and growth medium (N=2). Data is represented by the mean and error bars display the SEM.
Figure 2 – (Right) Percentage of surface marker expression, before freezing and post-thawing after 1 month (N=2). Data is represented by the mean and error bars represent the SEM.

Immunophenotype: Except for some exceptions attributable to the previously discussed limitations of the flow cytometry analysis, the expression of surface markers remained within normal levels throughout the assay (data not shown), which indicates the use of transport solutions does not cause great alterations in MSC immunophenotype. This result agrees with the several 4°C transport studies, with durations ranging from six hours to seven days, using different solutions, which report no immunophenotype changes[16,28,29,32].

Plastic Adherence: All cells retrieved from transport solutions were able to adhere and proliferate while maintaining normal cell morphology throughout the duration of the assay. Similar results were obtained by Veronesi and colleagues[31] who found MSC maintained plastic adherence following an 18-hour incubation in αMEM supplemented with 8% HPL at 4°C.

Multilineage Differentiation: After undergoing 14 days of differentiation, MSC from both sources and culture media were stained by Oil Red O, Alcian Blue and ALP-Von Kossa, which indicates differentiation towards the adipogenic, chondrogenic and osteogenic lineages, respectively (Figure 4) . This was more accentuated in AT-MSC-derived differentiations than UCM. No significant difference between cells from the two culture media was identified. Given that the differentiation potential of all conditions throughout the assay appears similar among them and to day 0, it is possible to conclude that the performed transport assay did not negatively affect MSC multilineage differentiation potential. Veronesi and colleagues[31] verified that MSCs maintained osteogenic potential after 18h in storage in FM at 4°C, which is in line with the results obtained here. Other works on MSC hypothermic storage using different transport solutions

also confirm that the differentiation potential is not affected following transports for as long as 4 days[16,28,29].

3.3. Alginate Encapsulation

Cell Viability and Recovery: Alginate encapsulation constitutes an interesting alternative to the transport solutions with short shelf lives and to cryopreservation. For both culture media, encapsulation was able to maintain high cell viability percentages (mean value of 80.3±1.3%, N=8), achieving similar results for both time-points, regardless of whether FM or CM was used (

Figure 5). This suggests that longer encapsulation periods do not affect cell viability, nor does the use of CM influence the results. In comparison with the remaining transport solutions, alginate encapsulation performs similarly to 2-8 Cellsius and is superior to CM and FM in preserving cell viability (Figure 3). Other studies involving MSC alginate encapsulation obtained similar results for post-release viability[33–36]. Results for cell recovery were also similar in both culture media, remaining below 50% for both time points (mean value of 39.7±1.1%, N=8). Since the cell recovery percentage did not change from day 5 to day 11, it suggests that that encapsulation time has no influence on cell recovery. A fifth kit divided in two was used to determine the cell recovery percentage immediately after encapsulation and following 5 days. A cell viability of 66.3% and a cell recovery of 52.1% were obtained after encapsulation. After 5 days, cell viability was 87.7% and cell recovery was 56.3%. Knowing that the number of cells at day 5 was approximately the number of cells encapsulated at day 0, the encapsulation efficiency was calculated (N=5): 58.0±4.8%. Thus, the low cell recovery percentage obtained was due to low encapsulation efficiency and not to the ability of the alginate beads to maintain MSCs.

Immunophenotype: The expression (data not shown) of the positive markers CD90, CD73 and CD44 was not affected by encapsulation in any of the release days for any of the conditions. Similarly, the negative markers HLA-DR, CD80, CD45, CD34 and CD14 remained generally unaltered by the assay. This result agrees with what was obtained by Al-Jaibaji and colleagues[33], who found alginate encapsulation did not affect MSC immunophenotype. The expression of CD105 decreases with encapsulation time, fact that may be related with the previously described heterogeneity of CD105.

Plastic Adherence: AT-MSCs from both culture media were able to adhere, proliferate and maintain morphology after beads dissolution. This was verified in all time-points and agrees with the results obtained in other studies[33–36], which implies that alginate encapsulation does not compromise MSC proliferation potential.

Multilineage Differentiation: AT-MSCs from both growth media presented phenotypic characteristics indicative of differentiation into each of the three lineages: lipidic vesicles stained by Oil Red O, glycosaminoglycans stained by Alcian Blue and calcium deposits and ALP staining activity, corresponding to adipo, chondro and osteogenic differentiation, respectively. No significant difference was found between AT-MSCs cultured in FBS or HPL-supplemented medium, nor between cells encapsulated in FM or CM. Since the differentiation potential of cells released at days 5 and 11 was found to be similar to that of fresh cells (data not shown), alginate encapsulation was proven to not affect the multipotency of AT-MSCs. This is concordant with the result obtained by Swioklo and colleagues[36].

Hematopoietic Support: In Figure 6 it is possible to observe that, following 7 days of coculture, $CD34^+$ enriched cells were able to proliferate more efficiently in the presence of an MSC feeder layer, as the fold increase in $CD34^+$ enriched cell number is always more elevated in FM and CM conditions than it is for No MSC. This is true for MSCs cultured both in HPL and FBS-supplemented medium and for the three time points of the transport assay. It is also clear that, once again, no significant difference can be found between CM and FM conditions in any of the assays. The fold increase of each condition was normalized by dividing its value by the No MSC fold increase. A reduction in the ability to support HSPC expansion in post-encapsulation MSCs was observed for AT HPL, when compared with fresh cells. However, there is no difference between performances of MSCs from day 5 and day 11 of the transport assay, which suggests that even though this function is affected by encapsulation, the duration of the encapsulation does not have an influence. In the case of AT FBS, there is no significant difference between the expansion of HSPCs on days 0 and 5. Furthermore, the fold increase of HSPC number on day 11 is closer to that of the negative control than in the remaining days, hinting to a loss of support capability by MSCs. The fact that all hematopoietic support assays

were performed using $CD34^+$ enriched cells from the same umbilical cord donor except AT HPL on day 0, may help to explain the abovementioned results.

Flow cytometry analysis of cells from all hematopoietic support assays performed showed that fold increase in $CD34^+$, $CD34^+CD45RA^-$ and $CD34^+CD45RA^-CD90^+$ populations was always more significant in the FM and CM conditions, suggesting the presence of MSC feeder layers allowed for a more effective maintenance of primitive populations during HSPC expansion (data not shown).

Regarding the clonogenic potential of HSPC, the fold increase in total number of colonies, showed no significant difference between the results obtained for the No MSC condition and the stroma containing conditions of FM and CM, in all time points and in both culture media. Given that other studies report higher ability to maintain clonogenic potential in HSPCs expanded in the presence of MSCs[37], it is possible that alginate encapsulation may cripple this capacity.

4. Cost of Goods Analysis

The development and manufacturing of MSC-based products is money consuming due to the high costs of the required GMP compliant facility and quality controls, as well as the expensive reagents and high labor costs[38]. Despite benefiting from economies of scale, as the manufacturing process may be scaled up[39,40], allogeneic cell therapies are associated with elevated cost of goods (COG) which may result in a final price that is prohibitive for broad adoption. COG analysis and optimization in the context of cell therapy products aims to minimize the cost per dose, while maintaining product quality[39]. Additionally, it identifies main cost drivers, helps to recognize necessary process alterations in early developmental phases and highlights possible room for optimization, in an effort to ensure maximum efficiency and optimal resource allocation[41]. A COG analysis was performed with the goal of analyzing the costs of each production step and estimating cost per dose of an MSC-based product being developed by Stemlab SA.

Model Assumptions: *1*- The manufacturing process involves two cryopreservation steps (Master and Working Cell Stock) with intermediate passages and quality control between them. Three different bioprocessing scenarios varying in the number of passages before each freezing step were considered. *2*- The formula $NCPD=3.322*\log(N_f/N_i)$[42] was used to calculate the number of cell population doublings (NCPD) obtained during expansion. An initial cell density of 3K cells/cm^2 and a final cell density of 75K cells/cm^2 were assumed. *3*- It is assumed that cryopreservation and thawing represent loss of 25% of cells. *4*- The costs of production of each cell stock were considered separately and the cost of one unit of the final product corresponds to the sum of the costs of one MCS unit and one WCS unit. Five individual cost categories were considered: reagents (concerning expenses with bottled reagents necessary to perform the passaging and freezing steps), materials (expansion platforms and lab disposables), facilities (cost of occupying the GMP clean room during production), human resources (wages of lab operators) and quality control. *5*- The final dose of the product is 100M cells.

Model Results:

Scenario 1: Three passages, corresponding to 12.5 NCPD and only one cryopreservation step. A total of 14 doses are produced in this scenario at a final cost/dose of 907.17€.

Scenario 2: Two passages before cryopreservation in the MCS and two passages before cryopreservation in WCS which represents 16.5 population doublings. The MCS originates 37 doses and each of these doses is able to originate 14 doses in the WCS. The total number of doses produced is 518 and the final cost/dose is 972.74€.

Scenario 3: Two passages before cryopreservation in MCS and one before WCS, representing a total NCPD of 13.5. The MCS originates 37 doses and four of these doses originate 7 doses in the WCS. The total number of doses produced is 63 and the final cost/dose is 1141.69€.

Scenario 2 has the second lowest cost/dose of the final product because of the increment on the number of doses caused by the increased NCPD (the highest of the three scenarios). This is against the notion that, to minimize the risks of cell ageing and senescence effects on the cell product, the best practice is to reduce the NCPD[43], which increase with passage number. Thus, it is important to consider if the reduction in cost/dose obtained in scenario 2 is worth the extra passage and the increased NCPD. Scenario 3 includes two freezing steps but only one passage before the second cryopreservation, in a total of 3 passages. When

compared with scenario 1, which includes the same 3 passages, this scenario yields a considerable larger number of doses with only one extra population doubling, which may be advantageous. The cost per dose of scenario 3 is higher than that of scenario 2 as less doses are produced but the NCPD is kept considerably lower. This means that scenario 3 may be a safer approach which will compensate for the more elevated cost per dose.

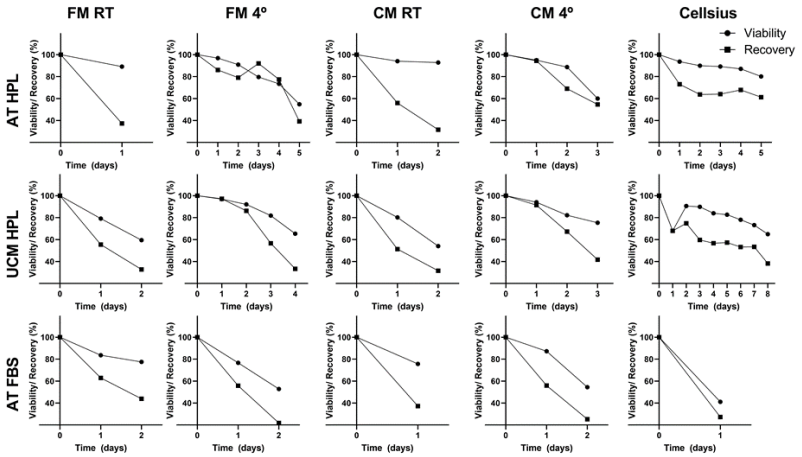

Figure 3 Cell viability and cell density recovery calculated for each donor in each of the transport solutions every 24h until viability (calculated by dividing number of live cells by the total number of cells) was found to be lower than 70% and/or recovery (calculated by dividing the cell concentration by its initial value) was lower than 50% (N=1).

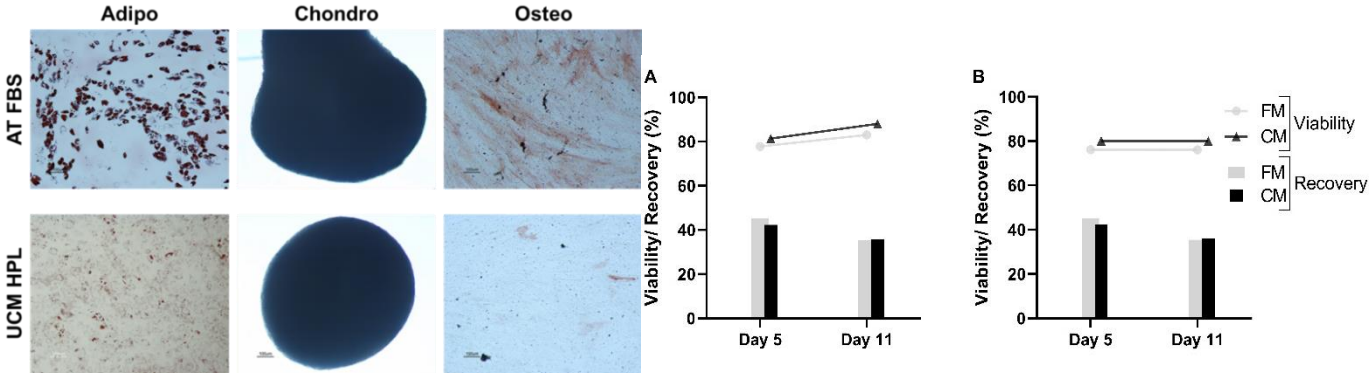

Figure 4 – (Left) Brightfield microscopic pictures (amplification of x100) of AT and UCM-MSCs corresponding to the FM 4°C condition on day 1, after 14 days of differetiation conditions to assess their multipotency. Oil Red O staining of lipidic vesicles demostrates adipogenic differentiation; Alcian Blue staining of glycosaminoglycans in MSC aggregates denotes the presence of chondrogenic phenotype; ALP activity staining highlights the presence of osteogenic progenitor cells and Von Kossa staining of calcium deposits confirms differentiation into the osteogenic lineage.
Figure 5 – (Right) Percentage of cell viability and recovery calculated per timepoint and per encapsulation condition (N=1). (A) AT HPL. (B) AT FBS.

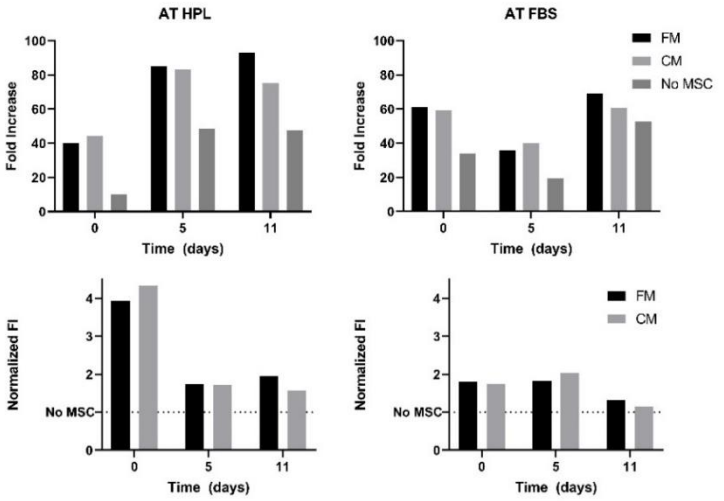

Figure 6 Fold increase in HSPC number after 7 days culture with and without MSCs retrieved from encapsulation at days 0, 5 and 11. The experimental conditions considered were MSCs expanded in FM and CM and No MSCs. Normalized FI was obtained by dividing the FI of each condition by the respective No MSC FI.

5. Conclusion

The manufacturing of MSC towards the development of cell therapies is a logistically complex process that still requires optimization. Namely, the number of cell passages and cryopreservation steps was seen to have a relevant influence on the final cost of a product. The optimization of the cryopreservation and thawing procedures will also reflect of the COG given that cell loses will be minimized and cell dose may, eventually, be reduced due to improved function. In this work, it was established that different cryopreservation media achieve different results and that cryopreservation using Cryostor CS5 represents a viable alternative to the standard culture medium with 10% DMSO. Future studies should include more post-thawing characterization assays as well as functional assays, and a re-assessment of the cells following an acclimation period in culture to evaluate if cryopreservation effects on cells are reversible. Transport solutions may prove to be useful in MSC manufacturing pipeline, either as an alternative to cryopreservation capable of maintaining cell fitness in the case of short periods between manufacturing and patient infusion, or as a vehicle to transfer cells from the manufacturing site, where they are thawed, to the patient, without the need for further manipulation and quality control. This work demonstrated the efficacy of short-term storage in 2-8 Cellsius and the ability of alginate encapsulation to maintain cell viability and function for 11 days. Future work should assess the biocompatibility of these transport solutions and possible regulatory issues regarding their inclusion in MSC production pipeline. Furthermore, it would be interesting to re-plate cells after transport and characterize them to investigate alterations. Optimization of cryopreservation and transport of MSC will contribute to accelerate clinical translation of these cell products.

6. Acknowledgements

I would like to thank Professor Joaquim Sampaio Cabral allowing me to develop the experimental work of my thesis at the Stem Cell Bioengineering Research Group. Thank you also to my supervisors, Dr. Ana Fernandes-Platzgummer, Dr. Francisco dos Santos and Professor Cláudia Lobato da Silva, for all their guidance, encouragement and trust. Furthermore, I would like to acknowledge all my colleagues at SCERG for their availability and help. In particular, thank you to Raquel Cunha, Sara Bucar and André Branco. Regarding the last section of the work, I would like to thank Stemlab S.A. for supplying the manufacturing data I used in my analysis, on the scope of the Stemlab S.A.-IST collaboration. Finally, thank you to my family and friends for their support.

7. References

1. Keating A. Mesenchymal stromal cells: New directions. *Cell Stem Cell*. 2012;10(6):709-716. doi:10.1016/j.stem.2012.05.015
2. Dominici M, 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):315-317. doi:10.1080/14653240600855905
3. Hoogduijn MJ, Lombardo E. Mesenchymal Stromal Cells Anno 2019 : Dawn of the Therapeutic Era ? Concise Review. *Stem Cell Transl Med*. 2019;8:1126-1134. doi:10.1002/sctm.19-0073
4. Le Blanc K, Rasmusson I, Sundberg B, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet*. 2004;363:1439-1441. doi:https://doi.org/10.1016/S0140-6736(04)16104-7
5. Maria ATJ, Toupet K, Maumus M, et al. Human adipose mesenchymal stem cells as potent anti-fibrosis therapy for systemic sclerosis. *J Autoimmun*. 2016;70:31-39. doi:10.1016/j.jaut.2016.03.013
6. Sun L, Akiyama K, Zhang H, et al. Mesenchymal stem cell transplantation reverses multiorgan dysfunction in systemic lupus erythematosus mice and humans. *Stem Cells*. 2009;27(6):1421-1432. doi:10.1002/stem.68
7. EMA: Alofisel. https://www.ema.europa.eu/en/medicines/human/EPAR/alofisel#product-information-section. Published 2020. Accessed June 15, 2020.
8. Leng Z, Zhu R, Hou W, et al. Transplantation of ACE2-Mesenchymal stem cells improves the outcome of patients with covid-19 pneumonia. *Aging Dis*. 2020;11(2):216-228. doi:10.14336/AD.2020.0228
9. Galipeau J, Sensébé L. Mesenchymal Stromal Cells : Clinical Challenges and Therapeutic Opportunities. *Cell Stem Cell*. 2018;680(22):824-833. doi:10.1016/j.stem.2018.05.004
10. Sensebé L, Gadelorge M, Fleury-Cappellesso S. Production of mesenchymal stromal/stem cells according to good manufacturing practices: A review. *Stem Cell Res Ther*. 2013;4(3). doi:10.1186/scrt217
11. Thirumala S, Goebel WS, Woods EJ. Manufacturing and banking of mesenchymal stem cells. *Expert Opin Biol Ther*. 2013;13(5):673-691. doi:10.1517/14712598.2013.763925
12. Aijaz A, Li M, Smith D, et al. Biomanufacturing for clinically advanced cell therapies. *Nat Biomed Eng*. 2018;2(6):362-376. doi:10.1038/s41551-018-0246-6.
13. Jossen V, van den Bos C, Eibl R, Eibl D. Manufacturing human mesenchymal stem cells at clinical scale: process and regulatory challenges. *Appl Microbiol Biotechnol*. 2018;102(9):3981-3994. doi:10.1007/s00253-018-8912-x
14. Svalgaard JD, Munthe-Fog L, Ballesteros OR, et al. Cryopreservation of adipose-derived stromal/stem cells using 1–2% Me2SO (DMSO) in combination with pentaisomaltose: An effective and less toxic alternative to comparable freezing media. *Cryobiology*. 2020;(May). doi:10.1016/j.cryobiol.2020.05.014
15. Gramlich OW, Burand AJ, Brown AJ, Deutsch RJ, Kuehn MH, Ankrum JA. Cryopreserved Mesenchymal Stromal Cells Maintain Potency in a Retinal Ischemia / Reperfusion Injury Model : Toward an off-the- shelf Therapy. *Nat Publ Gr*. 2016;6(26463). doi:10.1038/srep26463
16. Ginis I, Grinblat B, Shirvan MH. Evaluation of bone marrow-derived mesenchymal stem cells after cryopreservation and

hypothermic storage in clinically safe medium. *Tissue Eng - Part C Methods*. 2012;18(6):453-463. doi:10.1089/ten.tec.2011.0395
17. Wegener C, Thompson K. Automated leukapheresis cryopreparation using fully-defined synthetic solutions. *Cytotherapy*. 2020;22(5):S162-S163. doi:10.1016/j.jcyt.2020.03.340
18. Antebi B, Asher AM, Ii LAR, Moore RK, Mohammadipoor A. Cryopreserved mesenchymal stem cells regain functional potency following a 24 - h acclimation period. *J Transl Med*. 2019:1-13. doi:10.1186/s12967-019-2038-5
19. Bahsoun S, Coopman K, Akam EC. 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 Res Ther*. 2020;2:1-15.
20. Chinnadurai R, Copland IB, Garcia MA, et al. Cryopreserved Mesenchymal Stromal Cells Are Susceptible to T-Cell Mediated Apoptosis Which Is Partly Rescued by IFNc Licensing. *Transl Clin Res*. 2016;34:2429–2442. doi:http://dx.doi.org/ 10.1002/stem.2415
21. Oja S, Kaartinen T, Ahti M, Korhonen M, Laitinen A. The Utilization of Freezing Steps in Mesenchymal Stromal Cell (MSC) Manufacturing : Potential Impact on Quality and Cell Functionality Attributes. *Front Immunol*. 2019;10(1627). doi:10.3389/fimmu.2019.01627
22. Yuan Z, Lourenco SDS, Sage EK, Kolluri KK, Lowdell MW, Janes SM. 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
23. Al-Saqi SH, Saliem M, Quezada HC, et al. Defined serum- and xeno-free cryopreservation of mesenchymal stem cells. *Cell Tissue Bank*. 2015;16(2):181-193. doi:10.1007/s10561-014-9463-8
24. Bahsoun S, Coopman K, Akam EC. The impact of cryopreservation on bone marrow-derived mesenchymal stem cells: A systematic review. *J Transl Med*. 2019;17(1). doi:10.1186/s12967-019-02136-7
25. Dos Santos F, Campbell A, Fernandes-Platzgummer A, et al. A xenogeneic-free bioreactor system for the clinical-scale expansion of human mesenchymal stem/stromal cells. *Biotechnol Bioeng*. 2014;111(6):1116-1127. doi:10.1002/bit.25187
26. Carmelo JG, Fernandes-Platzgummer A, Diogo MM, da Silva CL, Cabral JMS. A xeno-free microcarrier-based stirred culture system for the scalable expansion of human mesenchymal stem/stromal cells isolated from bone marrow and adipose tissue. *Biotechnol J*. 2015;10(8):1235-1247. doi:10.1002/biot.201400586
27. FDA. *Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs)*.; 2008.
28. Freitas-Ribeiro S, Carvalho AF, Costa M, et al. Strategies for the hypothermic preservation of cell sheets of human adipose stem cells. *PLoS One*. 2019;14(10):1-16. doi:10.1371/journal.pone.0222597
29. Petrenko Y, Chudickova M, Vackova I, et al. Clinically relevant solution for the hypothermic storage and transportation of human multipotent mesenchymal stromal cells. *Stem Cells Int*. 2019;2019. doi:10.1155/2019/5909524
30. Nofianti CE, Sari IN, Marlina, Novialdi, Pawitan JA. Temporary storage solution for adipose derived mesenchymal stem cells. *Stem Cell Investig*. 2018;5(June):1-6. doi:10.21037/sci.2018.05.04
31. Veronesi E, Murgia A, Caselli A, et al. Transportation conditions for prompt use of Ex Vivo expanded and freshly harvested clinical-grade bone marrow mesenchymal stromal/stem cells for bone regeneration. *Tissue Eng - Part C Methods*. 2014;20(3):239-251. doi:10.1089/ten.tec.2013.0250
32. Celikkan FT, Mungan C, Sucu M, et al. Optimizing the transport and storage conditions of current Good Manufacturing Practice –grade human umbilical cord mesenchymal stromal cells for transplantation (HUC-HEART Trial). *Cytotherapy*. 2019;21(1):64-75. doi:10.1016/j.jcyt.2018.10.010
33. Al-Jaibaji O, Swioklo S, Shortt A, Figueiredo FC, Connon CJ. Hypothermically stored adipose-derived mesenchymal stromal cell alginate bandages facilitate use of paracrine molecules for corneal wound healing. *Int J Mol Sci*. 2020;21(16):1-22. doi:10.3390/ijms21165849
34. Al-Jaibaji O, Swioklo S, Gijbels K, Vaes B, Figueiredo FC, Connon CJ. Alginate encapsulated multipotent adult progenitor cells promote corneal stromal cell activation via release of soluble factors. *PLoS One*. 2018;13(9):1-15. doi:10.1371/journal.pone.0202118
35. Damala M, Swioklo S, Koduri MA, et al. Encapsulation of human limbus- derived stromal / mesenchymal stem cells for biological preservation and transportation in extreme Indian conditions for clinical use. *Sci Rep*. 2019;9(16950). doi:10.1038/s41598-019-53315-x
36. Swioklo S, Constantinescu A, Connon CJ. Alginate-Encapsulation for the Improved Hypothermic Preservation of Human Adipose-Derived Stem Cells. *Stem Cells Transl Med*. 2016;5:339–349. doi:http://dx.doi.org/ 10.5966/sctm.2015-0131
37. Da Silva CL, Gonçalves R, Crapnell KB, Cabral JMS, Zanjani ED, Almeida-Porada G. A human stromal-based serum-free culture system supports the ex vivo expansion/maintenance of bone marrow and cord blood hematopoietic stem/progenitor cells. *Exp Hematol*. 2005;33(7):828-835. doi:10.1016/j.exphem.2005.03.017
38. Bandeiras C, Cabral JM, Finkelstein SN, Ferreira FC. Modeling biological and economic uncertainty on cell therapy manufacturing: The choice of culture media supplementation. *Regen Med*. 2018;13(8):917-933. doi:10.2217/rme-2018-0034
39. Lipsitz YY, Milligan WD, Fitzpatrick IAN, et al. A roadmap for cost-of-goods planning to guide economic production of cell therapy products. *Cytotherapy*. 2017;19(12):1383-1391. doi:10.1016/j.jcyt.2017.06.009
40. Harrison RP, Medcalf N, Rafiq QA. Cell therapy-processing economics : small-scale microfactories as a stepping stone toward large-scale macrofactories. *Regen Med*. 2018;13(2):159-173.
41. Hassan S, Simaria AS, Varadaraju H, Gupta S, Warren K, Farid SS. Allogeneic cell therapy bioprocess economics and optimization: Downstream processing decisions. *Regen Med*. 2015;10(5):591-609. doi:10.2217/rme.15.29
42. Lechanteur C, Briquet A, Giet O, Delloye O, Baudoux E, Beguin Y. Clinical-scale expansion of mesenchymal stromal cells: A large banking experience. *J Transl Med*. 2016;14(1):1-15. doi:10.1186/s12967-016-0892-y
43. Liu J, Ding Y, Liu Z, Liang X. Senescence in Mesenchymal Stem Cells: Functional Alterations, Molecular Mechanisms, and Rejuvenation Strategies. *Front Cell Dev Biol*. 2020;8(May). doi:10.3389/fcell.2020.00258