Strategically Defining Operational Parameters for a Microcarrier-based Mesenchymal Stem Cell Expansion Process within a Single-Use Bioreactor

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BIOPROCESSING

Abstract

Human mesenchymal stem cell (hMSC) therapies have received increased clinical attention over the past decade. The multipotency and immunomodulatory properties of these cells has made them a promising candidate for a variety of therapeutic applications. One of the main challenges limiting the exploration of hMSC treatments are the large quantities of cells required for clinical dosages. Often these quantities are beyond what can be effectively produced using traditional two-dimensional cell culture methods. As hMSC treatments gain further relevance within the clinical space, laboratories are increasingly considering the adoption of three-dimensional, suspension cell culture processes to support the large numbers of hMSCs required for next-generation therapeutic treatments.

The integration of suspension bioreactor system workflows into traditional, two-dimensional cell culture laboratories introduces new variables than can significantly complicate standard expansion protocols. Both agitation and aeration strategies must be appropriately defined to support suitable growth conditions within suspension systems. If parameters are defined too aggressively, excessive energy dissipation can result in poor culture growth and increased cell death. Operational parameters defined too passively can cause the formation of concentration gradients or result in insufficient oxygen transfer within the system. Both of these outcomes can adversely affect culture health and performance. Process knowledge of the nuanced aspects of three-dimensional bioreactor operation is critical for the effective development of cell culture expansion processes within suspension systems.

In this work, we present an elegant and scalable method for the onboarding of a simple, three-dimensional, microcarrier-based hMSC expansion bioreactor process. Results of the study showed an 11.9-fold culture expansion at 96.5% viability after a 6-day culture period. These data demonstrate the suitability of the process for supporting the intricate needs of hMSC cultures. This work can serve as a model for teams looking to successfully onboard three-dimensional hMSC expansion processes onto their own laboratory benches.

Introduction

Over thirty years ago, human mesenchymal stem cells (hMSCs) were first described by Arnold Caplan.[1] These multipotent cells can differentiate into various cell types of mesodermal origin. Methods for the ex-vivo differentiation of hMSCs into adipocytes, osteocytes, and chondrocytes have been documented and are routinely performed within many cell biology laboratories.[2] Such differentiation techniques are commonly utilized as endpoint quality control assays to demonstrate the conservation of multipotency at the culmination of cell culture expansion processes.[3,4]

The trophic and immunosuppressive characteristics of hMSCs make them promising candidates for use across many therapeutic applications.[5] Unlike the use of embryonic pluripotent stem cells (PSC), the use of hMSCs does not challenge the consensus in regard to established ethical standards. The use of hMSCs also does not carry the histocompatibility or teratoma-formation risks associated with the therapeutic usage of other stem cell types.[6]

While the majority of early therapeutics hMSC applications were focused on regenerative medicine, continued work over the past decade has demonstrated that these cells have the potential for more expansive therapeutic utility. Prochyml™

(Osiris Therapeutics) is a bone marrow hMSC therapy that was demonstrated to be highly effective for the treatment of graft-versus-host disease (GvHD).[7] Successful treatments such as Prochyml™ provide increasing evidence regarding the clinical potential of hMSCs as a viable allogeneic cell therapy option. The clinical marketspace has responded to this potential with great interest: 914 clinical trials involving hMSC treatments were identified as ongoing in 2019.[8]

Mesenchymal stem cells are adherent cells which require surface anchorage for growth and proliferation. The *ex vivo* expansion of hMSCs has historically been performed using classical two-dimensional, planar cell culture techniques. These types of cell culture methods have been well described and are considered to be consistent and reliable.[9]

While two-dimensional cell culture processes are relatively established and proven, such techniques ultimately have surface area limitations that constrain overall productivity. Modern process solutions such as HYPERStacks (Corning) and Cell Factory Systems (Thermo Fisher) can offer higher culture outputs as compared to single-layer flasks and dishes. However, cell numbers produced within these newer systems may still not be sufficient to support the quantities required for global allogeneic cell therapy applications. Maximum cell culture growth potential of two-dimensional platforms will always be restricted by the inherent planar design characteristics of these types of systems.

Patient dosages for hMSC treatments have been reported at concentrations of $1 – 10 \times 10^6$ cells / kg.[10,11,12] Future therapeutics may require multiple doses or even greater cell numbers. Dosages as high as 20×10^6 cells / kg have also been reported in preclinical studies involving animal models.[13] Such high concentrations of treatment dosages suggest there will ultimately be a common need for robust, scalable, three-dimensional hMSC expansion processes.

When transitioning adherent cells from two-dimensional to three-dimensional culture expansion systems, scientists and engineers will often consider the integration of microcarriers into their processes.[14] Microcarriers are spherical beads which provide attachment surfaces for adherent cells within suspension systems. The integration of microcarriers into an upstream process can support the successful expansion of anchorage-dependent cell types within three-dimensional bioreactor systems.

The first usage of microcarriers was described in 1967.[15] After their initial inception, microcarriers were used primarily to support vaccine development.[16] However, over the past decade, microcarriers have received heavy consideration for use within three-dimensional hMSC expansion applications.[17] Microcarrier-based hMSC *ex vivo* expansion processes have been reported to yield six to ten-fold increases in cell growth.[18,19,20] To achieve such results, suitable agitation and aerations strategies must be tactically defined during process development.

Agitation Strategies for Microcarrier-Based Cell Culture Expansion Processes

The formation of chemical or thermal gradients within a bioreactor has the potential to adversely affect both culture health and overall product quality. To support suitable mass and thermal transfer throughout the system, most bioreactor processes require the integration of a mixing strategy.[21] For stirred-tank reactor (STR) mammalian cell culture processes, system mixing is typically performed through low-power mechanical agitation. This agitation drives the creation of internal circulation loops, which can help prevent the emergence of harmful gradients.

A negative consequence of bioreactor agitation is the potential for the introduction of hydrodynamic shear forces into the system. The absence of protective cell walls makes mammalian cell cultures vulnerable to these types of forces.[22] Scientists must be conscious of this system characteristic when engineering three-dimensional cell culture expansion processes. Excessive levels of hydrodynamic shear forces can result in the increased induction of apoptotic cascades within cell cultures.[23] For microcarrier-based cell culture processes, minimizing the generation of hydrodynamic forces should be of particular concern when working to optimize culture health and performance.

Excessive agitation within microcarrier-based processes can result in a high frequency of microcarrier-to-microcarrier collisions occurring within bioreactor systems.[24] Such collisions can negatively impact the growth and viability of microcarrier-based cell cultures. To mitigate the effects of these collisions, it is recommended that the agitation parameters be defined so that they produce a just-suspended state (N_{JS}) for the microcarriers within the system.[25]

N_{JS} agitation can be defined as the minimal power input required to achieve a complete suspension of solid particles within a liquid medium.[26] The N_{JS} condition is illustrated in **Figure 1**. During N_{JS} operational conditions, nonuniformity of microcarriers will likely be noted across the vertical axis of system. Heavier concentrations of microcarriers will typically be observed towards the bottom of the vessel. Microcarriers may also briefly drop out of suspension under N_{JS} operational parameters. During such 'drop out' periods, microcarriers should rest no more than a few seconds on the bottom of the vessel before they are lifted back into the circulation loop of the system.[27] Both agitation rate (overall power input) and axial flow direction (upward or downward) should be clearly defined as critical process parameters to adequately describe the N_{JS} for hMSC expansion processes.

After the operational conditions for N_{JS} agitation are determined, a mixing strategy must be defined to support the post-inoculation microcarrier attachment period. These cell attachment periods typically extend between two to six hours post-inoculation. These periods facilitate the adherence of the inoculated cells to the microcarrier surfaces, as cell adhesion molecules (CAMs) attach the cells to the extracellular matrix (ECM) structure of the microcarrier beads.[28] For hMSC expansion protocols, the agitation strategies for the attachment portion of the processes are typically characterized as either delayed, intermittent, or continuous.[29]

A delayed cell attachment strategy involves a static period of no mechanical agitation; cells and microcarriers are permitted to settle for the duration of the process. An intermittent strategy involves pulses of agitation; each agitation pulse lasts approximately one minute and is followed by an extended settling period. A continuous strategy involves constant agitation immediately after inoculation; this method includes no defined microcarrier settling period. The continuous strategy

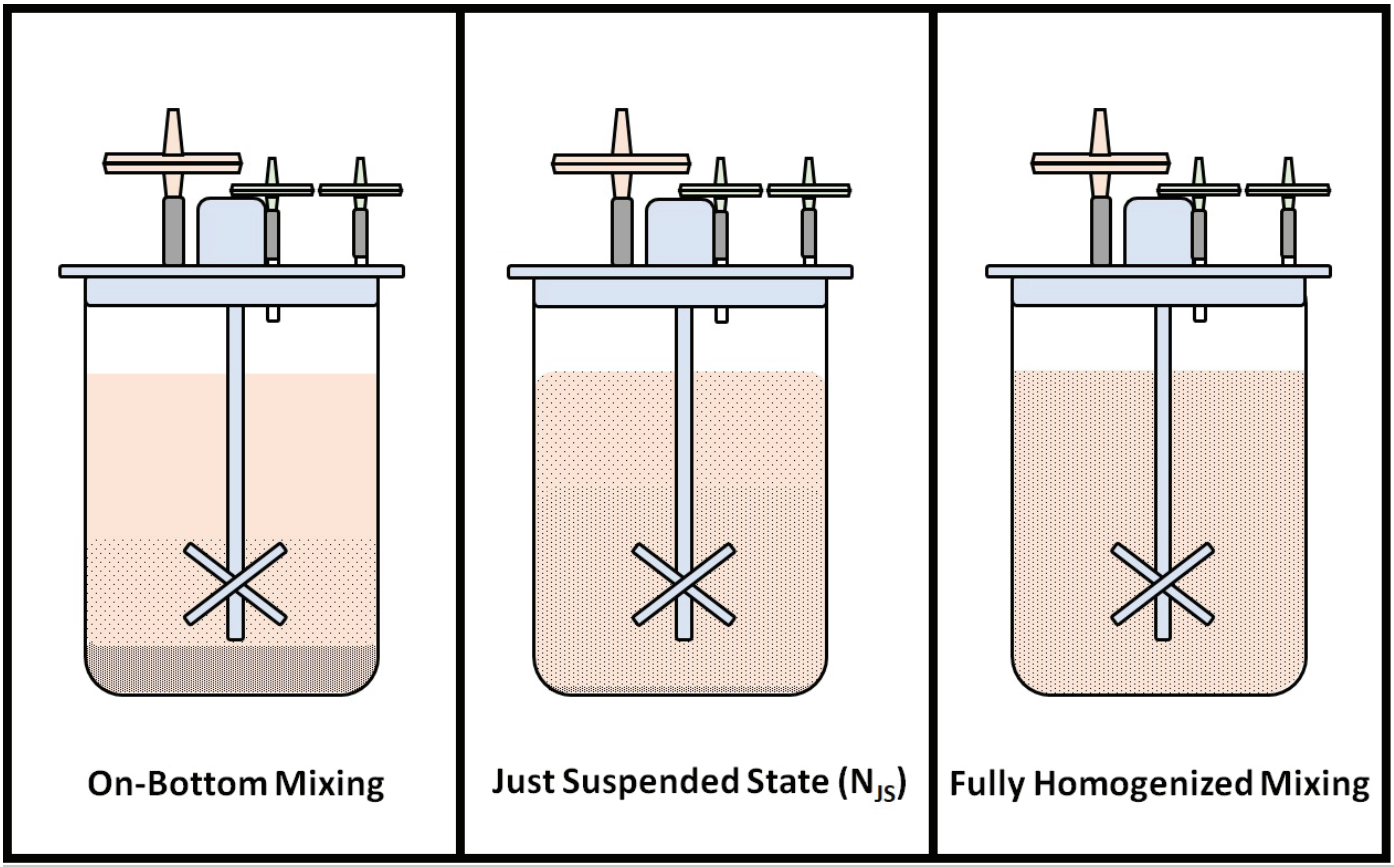
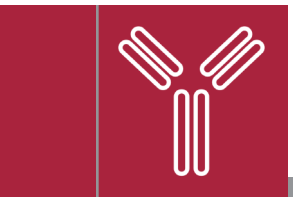


Figure 1: Illustration of different type of solid-liquid mixing strategies. The just suspended state (N_{JS}) condition has been demonstrated to be most appropriate for microcarrier-based hMSC expansion processes.



is the only attachment method which supports the use of temperature control throughout the process. The risk of thermal gradients developing during the static and intermittent strategies requires that these methods are performed at ambient temperature.

It is recognized that multiple process variables such as working volume, agitation rate, microcarrier concentration, and seeding density all contribute to the attachment percentage yielded by the different cell attachment strategies. Due to this multivariate process complexity, there is currently not a consensus in regard to which post-inoculation cell attachment method is the most efficient to maximize the cell attachment percentage. Work performed by Yang et. al., was able to demonstrate a difference in attachment percentage across the three strategies at two hours post-inoculation.[30] However, by twenty-four hours post-inoculation there was no observed difference between the experimental groups. At this time, all three attachment strategies demonstrated greater than 98% microcarrier attachment.

Aeration Strategies for Microcarrier-Based Cell Culture Expansion Processes

A viable aeration gassing strategy must be defined when engineering a three-dimensional hMSC expansion process within a bioreactor system. A suitable gassing strategy is necessary to support the aerobic requirements of the expanding culture and to ensure that an appropriate concentration of dissolved carbon dioxide is present in the media.[31] To meet these requirements, compressed air, oxygen, and carbon dioxide are often introduced to the bioreactor through closed-system flow paths which traverse the bioreactor headplate.

Bioreactor headplates typically have multiple pathways available to support gas transfer. Such options include both sparged and overlay flow paths.[32] As gases are introduced to the system, both oxygen and carbon dioxide diffuse between gaseous and aqueous states. System off-gassing is supported through the integration of an exhaust filter, also located on the bioreactor headplate.

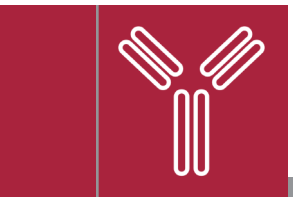
Sparged gas flow paths extend from above the headplate to below the bottom agitator impeller. This pathway introduces gases directly to the culture medium though the creation of bubbles. The size of these bubbles depends on whether a drilled-hole (macro) or frit (micro) sparger is used within the process. With a sparged gas flow path, gases diffuse into the medium as the bubbles are carried within the circulation loops to the surface of the liquid.

Unlike the sparger flow path, the overlay flow path does not extend from the headplate into the culture medium. Rather, the overlay pathway introduces gases to the headspace of the system: the area within the bioreactor located above the surface of the medium. As a result, no bubbles are created during gas transfer through the overlay pathway. The absence of bubbles during overlay gas transfer decreases the amount of interfacial surface area available for gas transfer in the system.

Available interfacial surface area directly correlates with the rate of mass transfer in bioreactor systems.[33] As such, overlay gassing strategies will typically yield reduced oxygen transfer rates (OTRs) when compared to sparged gas processes. However, if the OTR driven by surface aeration from overlay gassing is still sufficient to meet the oxygen consumption rate (OCR) of the culture, overlay gassing can be a highly effective technique within mammalian cell culture processes, due to reduced energy input into the system.

Sparged gas bubbles burst upon reaching the media surface within a bioreactor system. During the bursting process, potential energy from bubble surface tension is released into the bioreactor system. This energy dissipation has been demonstrated to adversely affect the health of mammalian cell cultures.[34] Removing sparged gas bubbles from the process, through the integration of an overlay gassing strategy, can eliminate the risk of bubble energy dissipation harming culture growth. This strategy has been demonstrated to result in improved process performance for microcarrier-based cultures, which have relatively lower overall OCRs, as compared to other traditional suspension mammalian cell culture processes.[35]

In this work, we developed a hMSC expansion process within a 2-L single-use bioreactor (SUB) manufactured by Distek. The power input conditions necessary to achieve N_{JS} were determined through visual analysis of microcarrier suspension at various power inputs. An intermittent agitation strategy was utilized for the microcarrier attachment portion of the process. Aerobic requirements of the culture were supported using an overlay gassing strategy to reduce energy dissipation within the system. The defined operational parameters were demonstrated to be highly appropriate, as offline cell counting results from



the six-day expansion process revealed both a high growth rate and excellent overall culture viability.

Materials and Methods

The hMSCs used for the culture expansion in this study were derived from Wharton's jelly. First described in 1656, Warton's jelly is the connective tissue which surrounds the three vessels in the umbilical cord. This tissue helps ensure the integrity of the blood vessels during the development process.[36] Wharton's jelly is unique compared to other connective tissue, as it does not contain any phagocytic, vascular, or neural elements.[37] The tissue contains only hMSCs, functional myofibroblasts, and precursor cells. This characteristic, combined with the low level of ethical concerns regarding the use of the umbilical cord, make Wharton's jelly a viable source of donor cells for the ex vivo expansion of hMSCs.

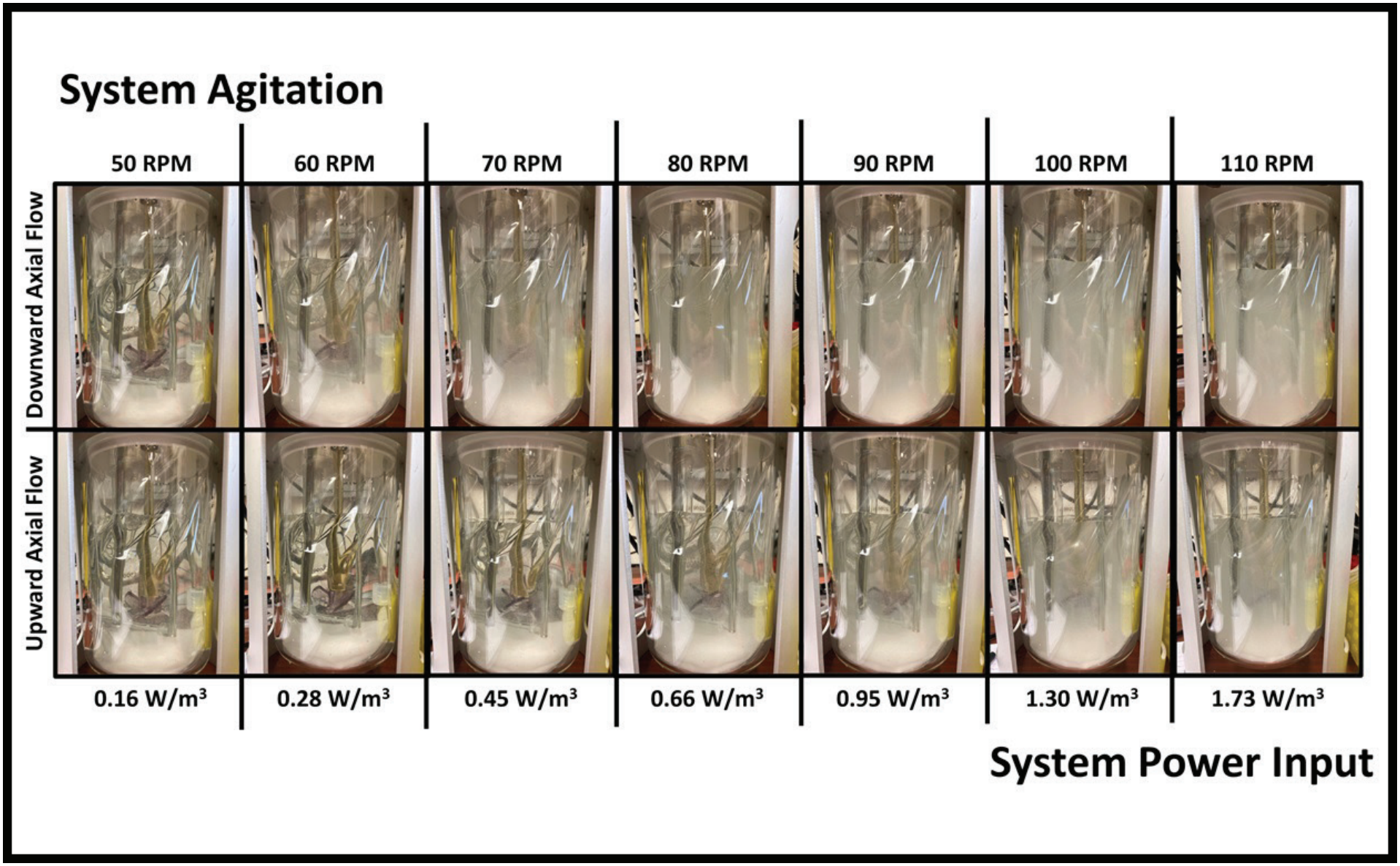
Harvest processes for Wharton's jelly hMSCs have been well described in scientific literature.[38,39] Numerous enzymatic and mechanical harvest methods have been previously documented. The majority of these methods utilize standard tissue and cell culture techniques, making them simple for most laboratories to adopt and put into practice. The cells used for this study were harvested and cryopreserved prior to bioreactor process development. Out-of-thaw (OOT), hMSCs were cultured in two-dimension flasks under standard incubator conditions (37°C / 5% CO_2 / atmospheric O_2). Cells were ultimately expanded to HyperFLASK vessels (Corning) in order to achieve the necessary quantities for stirred-tank bioreactor inoculation.

A 2-L, liner-based, single-use bioreactor (BIOne SUB, Distek) was used as the expansion platform for this study. The bioreactor working volume was defined as 1400 mL for the expansion process (70% of working volume). The operational system aspect ratio at this working volume is 1:1 (liquid height: vessel diameter). It was believed that this aspect ratio would support efficient headspace gas exchange within the system.

The bioreactor system was operated using the BIOne 1250 Bioprocess Control Station (Distek). To appropriately define bioreactor agitation for the culture expansion, mixing parameters required to achieve a just-suspended state (N_{JS}) within the system were determined through a non-sterile process simulation.

To complete the process simulation, an identical 2-L single-use bioreactor model was prepared with the target concentration of microcarriers suspended in 1400 mL of deionized water. Agitation speed was increased stepwise from 50 rpm to 110 rpm at 10 rpm increments. Images of the microcarrier circulation loops were captured at each agitation setpoint. Both upward axial flow (clockwise rotation) and downward axial flow (counterclockwise rotation) circulation loops were evaluated during this simulation. The captured images form this evaluation are shown in Figure 2. These data suggested that the operational parameters required to maintain N_{JS} for the hMSC microcarrier process could be defined as counterclockwise agitation (downward axial flow) at a speed of 90 rpm (0.95 W/m^3).

Figure 2: Experimental determination of just-suspended state (N_{JS}) power input. Results demonstrate that an agitation strategy defined as downward axial flow at 90 RPM could yield optimal microcarrier resuspension conditions with minimal overall system power input.



Previous results with the hMSCs used for this study demonstrated that an intermittent agitation strategy was a viable option to promote efficient cell attachment (data not shown). The pulsed agitation cycle was defined as one minute of 90 rpm downward axial flow mixing followed by twenty-nine minutes of static settling. This process was repeated twelve times for a total duration of six hours. The cell attachment agitation process was automated on the BIOne 1250 Bioprocess Controller via an Open Platform Communication (OPC) connection to an ancillary computer that was running Lucullus software (Securecell). The Lucullus step chain used to automate the intermittent agitation cell attachment process is shown in **Figure 3**.

Due to concerns about cell damage due to the energy dissipation from sparged gas bubbles bursting, an overlay-only gassing strategy was defined for the hMSC expansion process. A 5% CO_2 enriched air supply was introduced to the system headspace through the overlay filter at a gassing supply rate of 140 sccm (0.1 vvm). Oxygen gas was also programmed into the dissolved oxygen (DO) cascade loop for the system. This gas was programmed to be pulsed into the overlay mixture if the online DO value dropped below setpoint. However, the DO concentration within the system never dropped below setpoint (40%) during the culture expansion, so oxygen gas was not introduced into the system during the process.

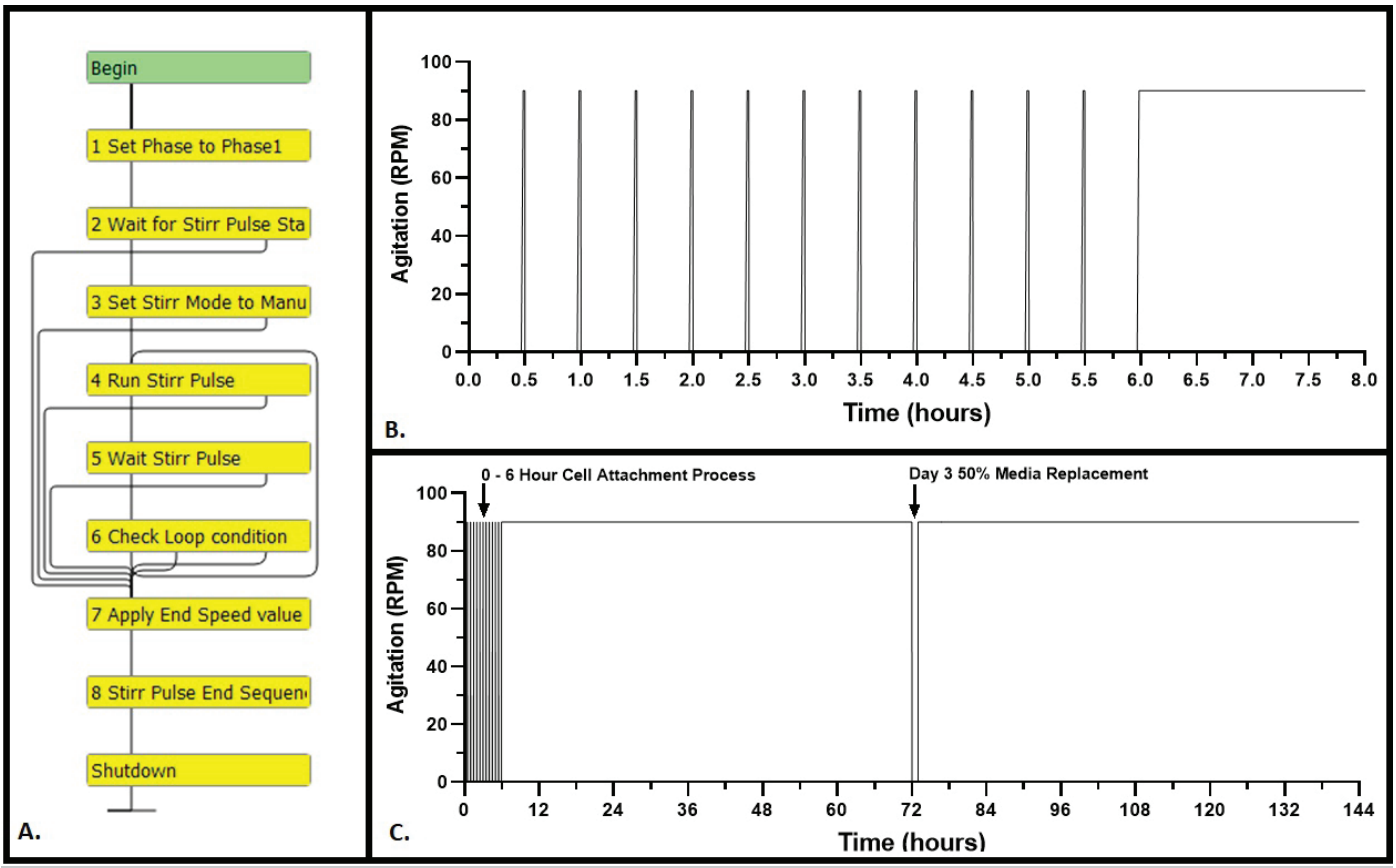


Figure 3: Agitation strategy for hMSC microcarrier expansion in BIOne Single-Use Bioreactor. (A) Lucullus Software (Securecell) step-chain sequence used to automate the microcarrier attachment process post-inoculation. **(B)** Agitation strategy for cell attachment to microcarriers (0 – 6 hours). **(C)** Overall agitation strategy for entire six-day hMSC expansion process (0 – 144 hours).

The hMSC process was propagated for six days. A media replacement was performed on the third day of the process. During the approximately one-hour media replacement process, the agitation, temperature, and dissolved oxygen (DO) control loops on the BIOne 1250 controller were temporarily deactivated, and the microcarriers were allowed to settle. After the microcarrier settling was complete, media was removed via a needleless luer sample port connected to a dip tube on the headplate of the system. Fresh media was then added through an addition port on the headplate of the bioreactor. Upon completion of the media replacement, all control loop modules were reactivated on the BIOne 1250 controller. An overview of all operational definitions for the process parameters used for the BIOne hMSC expansion is presented in **Table 1**.

Table 1: Operational Parameters for hMSC Stem Cell Expansion

Process Phase	Parameter	Setpoint
Bioreactor Inoculation & Microcarrier Attachment (0 - 6 Hours)	Agitation	**Pulsed Agitation Sequence** 90 rpm for 1 Minute (0.95 W/m^3) 0 rpm for 29 Minutes
	Sparged Gas	*Not Activated*
	Overlay Gas	5% CO_2 Enriched Air 140 sccm (0.10 vvm)
	Temperature	*Not Activated*
	pH	*Not Activated*
hMSC Culture Expansion (6 – 144 Hours)	Agitation	90 rpm [1]
	Sparged Gas	O_2 Cascaded for Dissolved Oxygen Control [2]
	Overlay Gas	5% CO_2 Enriched Air 140 sccm (0.10 vvm)
	Temperature	37°C [1]
	pH	7.5% $NaHCO_3$ Cascaded for pH Control [3]

[1] Agitation and temperature control modules were deactivated during Day Three media change process (approximately one hour)
[2] Dissolved oxygen parameter never dropped below setpoint (40%) during process operation
[3] pH never deviated outside setpoint range (7.4 +/- 0.2) during process operation

Results and Discussion

Cell growth results from the hMSC expansion process in the BIOne SUB are shown in **Figure 4**. An 11.9-fold increase was observed across the six-day expansion period. Final culture viability was 96.5%. These results are comparable or superior to 6 – 10-fold increases previously reported for microcarrier-based hMSC suspension processes.[18,19,20]

The presented cell growth data suggests that the defined process parameters resulted in operational conditions that were suitable for the viable expansion of the culture. The results suggest that the combination of N_{JS} agitation and CO_2-enriched overlay aeration likely did not create excessive extracellular stressors within the bioreactor system. The online DO reading for the system remained above setpoint throughout the duration of the culture (data not shown). This result suggests the at the OTR created by the process operational definitions was sufficient to meet the OCR and aerobic requirements of the hMSC culture.

Visible culture aggregates can be observed in the day six microscopy images. Such aggregation has been previously described in hMSC expansion processes in suspension systems.[40] It has been suggested that, within processes that involve media change steps, such aggregation correlates with the end of the exponential growth phase. It has been proposed that a post-media-replacement agitation increase may mitigate aggregate formation. The timing and power input of a post-media replacement agitation shift could be considered as experimental factors for further optimization studies.

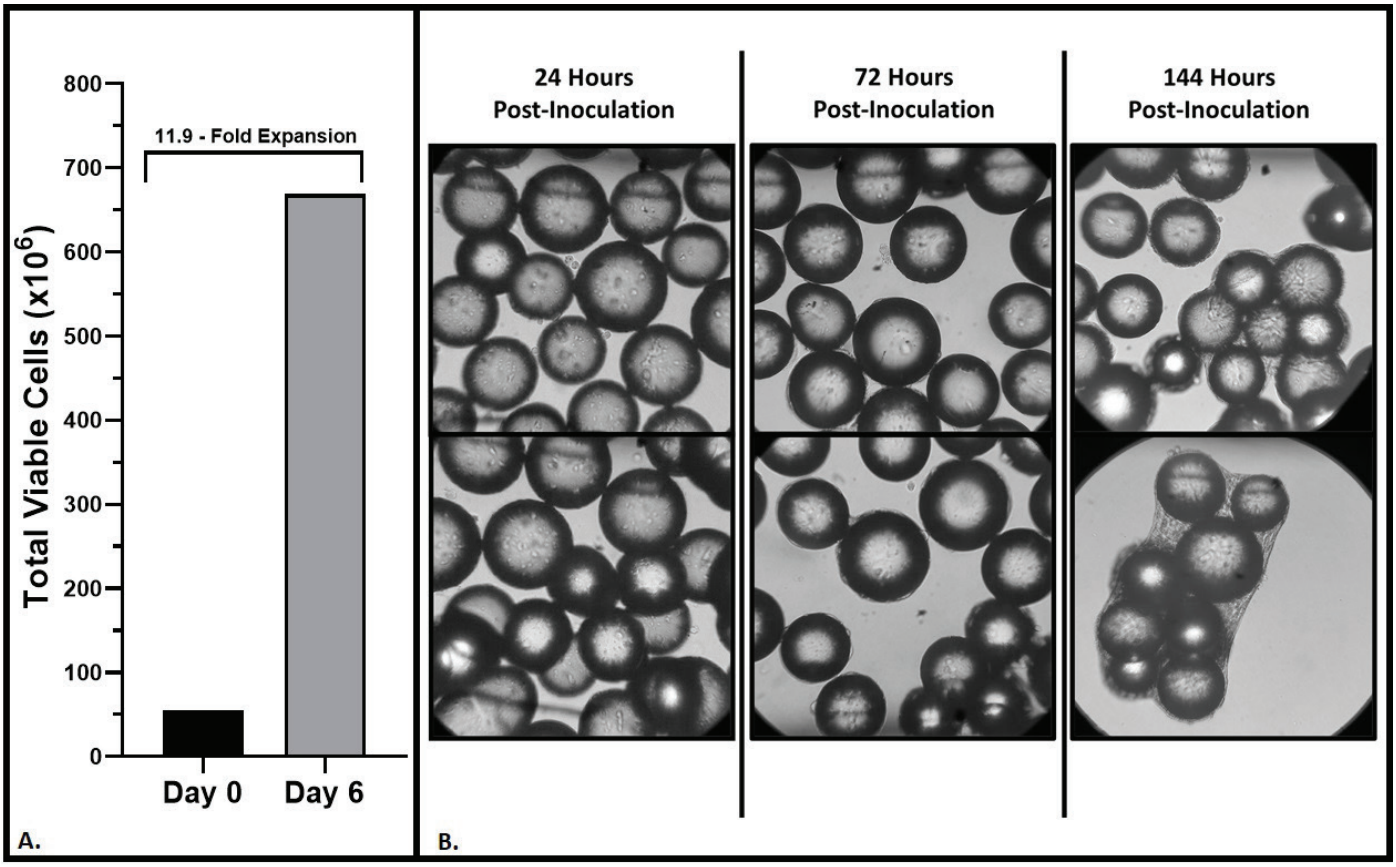


Figure 4: Cell growth demonstrates the suitability of the BIOne Single-Use Bioreactor for microcarrier-based hMSC expansion. (A) Total viable cell data demonstrates 11.9 – fold growth of cultured cells over the six-day cell expansion process. Final culture viability was 96.5%. **(B)** Brightfield microscopy images (10x) show increased cell attachment aggregate formation by Day 6 of hMSC expansion process.

Conclusions

The potential of hMSCs for a variety of therapeutic applications has received increasing attention over the past decade. For hMSCs to succeed as a viable allogeneic treatment option, the relatively high concentrations of cells needed for therapeutic dosages must be addressed. Scalability and productivity limitations of traditional two-dimensional cell culture techniques may limit the utility of such methods for the manufacture of large quantities of hMSCs. The strategic development of three-dimensional suspension processes for hMSC expansion is likely necessary for hMSC-based therapies to be successfully integrated into the clinical setting.

In this work we presented an overview for the engineering of a simple microcarrier-based bioreactor suspension process to expand Wharton's jelly derived hMSCs. By strategically defining operational parameters for agitation and aeration strategies, we were able to create process conditions that were demonstrated to be suitable for the successful expansion of an hMSC culture. The process development technique described in this paper could be of value to upstream development engineers and scientists working to transition similar adherent-cell processes into three-dimensional suspension systems.

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