Dispersible and dissolvable porous microcarrier tablets enable efficient large scale hMSC expansion

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Abstract:

Mesenchymal stem cells (hMSCs) have wide applications in regenerative medicine but their clinical translation is largely hindered by limited production capacity of current cell expansion regime. This study utilizes novel dispersible and dissolvable porous microcarrier tablets, 3D TableTrix™ in stirred bioreactor to demonstrate a scalable expansion protocol for industrial manufacturing of hMSCs. 3D TableTrix™ are ready-to-use tablets that disperse into tens of thousands porous microcarriers upon contact with culture media, eliminating the need to prepare microcarriers before cell seeding, hence simplifying operation process. We demonstrate over 500 times expansion of adipose-derived hMSCs using serum-free culture medium in 11 days with bead-to-bead transfer for a partial scale-up from laboratory-scale spinner flasks to a 1L bioreactor system. A final yield of 1.05±0.11×10^9 hMSCs was achieved, and yield of over 3×10^9 with an overall expansion factor of 1530 could theoretically be realized with full scale-up. Cells were harvested by dissolving microcarriers with 98.6±0.1% recovery rate. Cells retained their immunophenotypic characteristics, tri-lineage differentiation potential and genome stability with low indications of senescence phenotype. This study illuminates the potential of industrializing clinical-grade hMSC production using 3D TableTrix™ microcarrier tablets and stirred tank bioreactors.
Impact statement

3D TableTrix™ is a newly available microcarrier ingeniously designed as dispersible and dissolvable porous microcarrier tablets for hMSC expansion. This eliminates the need of tedious preparation work usually required for microcarriers and its dissolvable nature allows for high cell recovery rate of 98.6±0.1%. Over 500 times expansion of AD-MSCs in serum-free culture media using a 1L bioreactor system demonstrates its tremendous potential for industrial production of hMSCs.
1. Introduction

Human mesenchymal stem cells (hMSCs) are one of the most preferred adult stem cells in clinical applications, demonstrating their great value and potential for cell therapy and tissue engineering[1]. However, challenges remain for successful clinical translation of hMSCs therapies, and one critical bottleneck issue is scalable and controllable expansion of mesenchymal stem cells (MSCs)[2]. It is anticipated that production of $10^{12}$ cells per batch would be necessary for industrialization and commercialization of hMSCs[3], hence there is a strong drive to develop robust bioprocesses for large-scale cell production. A major challenge for stem cell therapy companies is scaling up of their manufacturing process[4] and stirred-tank bioreactors are one of the most cost-effective bioprocessing systems available[5, 6].

Bioreactors are common large-scale manufacturing systems used in production of biologics like protein drugs and vaccines, with potential to scale from small vessels (a few hundred milliliters) to large vessels (thousand liters)[6, 7]. As hMSCs are adherent cells, identifying a suitable supporting matrix is key to successful expansion of hMSCs in stirred tank bioreactors[4, 6-9]. Researches have been conducted on commercially available microcarriers and bioreactor parameters to optimize hMSCs yield and quality which have been reviewed elsewhere[5, 10]. However, regardless of the characteristics and nature of microcarriers investigated in published literatures, a common feature among all microcarriers is the need for long preparation work before cell inoculation. The process includes weighing out the microcarriers and rehydrating them for hours before sterilizing and then balancing with culture medium, with some microcarriers needing additional protein coating procedures to enable cell adhesion. Such preparation is not only troublesome but also time-consuming, especially when scale-up to vessels of hundreds to thousand-liter capacity is anticipated for commercial manufacturing of hMSCs.

In this article, application of dissolvable macroporous gelatin-based microcarriers in a novel and proprietary dispersible tablet formulation, 3D TableTrix™, for scalable expansion of hMSCs is demonstrated. 3D TableTrix™ are macroporous, highly water-adsorbent and elastic gelatin-based microcarriers packed into weight-defined sterile tablets which, upon
contact with aqueous solution, readily absorb liquid and disperse into individual microcarriers again. Thus, no weighing, rehydration, coating or sterilization are required prior cell inoculation. Furthermore, by using complimentary microcarrier dissolution reagent to fully dissolve these microcarriers, cells can be gently harvested with superior recovery rate, hence integrating expansion and cell harvesting in the same vessel, further simplifying bioprocesses of large-scale hMSC production. 29.8±3.0- and 5.47±0.93-fold expansions were achieved for adipose-derived hMSCs (AD-MSCs) and umbilical cord- derived hMSCs (UC-MSCs) after 4 days of culture in a spinner flask with serum-free medium respectively. With bead-to-bead transfer for a partial scale-up from 125mL spinner flask to a 1L bioreactor system, high yield of 1.06×10⁹ cells in 11 days from 110 tablets (1.2g microcarriers) were obtained. High vitality and quality cells were harvested by dissolving all microcarriers and their phenotypic markers and differentiation potentials were not affected. This study thus indicates the potential of using 3D TableTrix™ microcarrier tablets and stirred tank bioreactors to industrialize clinical-grade hMSC production.

2. Materials & Methods

2.1 Characterization of 3D TableTrix™

3D TableTrix™ were obtained from Beijing CytoNiche Biotechnology Co., Ltd. To analyze particle size, each 3D TableTrix™ tablet was hydrated in 3mL PBS for 10 minutes and analyzed with a particle size analyzer (SCF-105B, Zhuhai OMEC Instruments, China) according to manufacturer’s instruction. 3D TableTrix™ tablets were dispersed in DI water, frozen at -20°C for 2 hours and lyophilized for 5-6 hours to obtain dry microcarriers in powder form for scanning electron microscopy. Microcarriers were coated with gold for 90s and then imaged with a scanning electron microscope (FEI Quanta 200, Thermo Scientific, USA). Pore sizes were analyzed from these scanning electron micrographs using ImageJ software (National Institutes of Health). Atomic force microscope (AFM) was used to measure the local Young’s Modulus of microcarriers that were ground into small pieces according to a method detailed elsewhere[11]. Particle density (number of microcarriers per mg) was analyzed by counting the number of microcarriers adhering to a piece of 3cm
by 3cm transparent tape under the microscope and divided by the weight of microcarriers that adhered to this tape.

2.2 Monolayer Culture of hMSCs

AD-MSCs were isolated as reported elsewhere[12]. 1×10⁶ of AD-MSCs or UC-MSCs (Nuwacell Ltd, China) were inoculated to T75 for monolayer culture with serum-free hMSC culture medium (RP02010, Nuwacell Ltd, China) to 80-90% confluence and harvested with 0.25% Trypsin-EDTA (325-043-EL, Wisent, Canada). P4-P6 cells were used for subsequent experiments.

2.3 Cell inoculation and expansion on 3D TableTrix™ in spinner flask

Spinner flask (SF125, CytoNiche Biotech, China) with magnetic impeller adjusted to appropriate height, according to manufacturer’s instructions, were autoclaved and oven-dried. These spinner flasks were equipped with vented caps for gas exchange. 3D FloTrix™ miniSPIN system (M2, CytoNiche Biotech, China) was set up inside a 37°C, 5% CO₂ incubator (CCL1708-8, ESCO, Singapore). miniSPIN M2 has a stirrer system to sit 4 flasks and two controllers to independently control 2 flasks each (Fig. 5A). The controller allowed for programmable agitation protocols.

5-10 tablets of 3D TableTrix™ (F01, CytoNiche Biotech, China) were introduced to a sterile 125mL-spinner flask with 10mL cell culture medium (serum-free hMSC culture medium (RP02010, Nuwacell Ltd, China), chemically defined hMSC culture medium (NC0103, Yocon, China) and serum-containing medium (M001, Viral Therapy Technologies, China) via the side-arm and fully dispersed by gentle agitation. 1.10×10⁶ AD-MSCs were then added and cell culture medium was topped up to a final volume of 40mL (for 5 tablets) or 60mL (for 10 tablets) immediately. Spinner flasks were then placed on a 3D FloTrix™ miniSPIN system (Fig. 5A) inside the 37°C, 5% CO₂ incubator and different agitation protocols were programmed. Agitation speeds could be set to 0rpm for ‘Delayed’ protocol, 60rpm for ‘Constant’ protocol and 58 cycles of 60rpm×5min and 0rpm×20min for ‘Intermittent’ protocol. Agitation was set to 60rpm after 24h inoculation period. Cell growth was monitored by taking 1mL samples from the side-arm with a pipette and cells were enumerated. To ensure uniform sampling, constant agitation of 60rpm was used
while aliquoting samples of microcarriers. Attachment efficiency was evaluated by determining the number of cells in supernatant of the samples at 2, 6 and 24h after inoculation[13]. All cells were cultured for 4 days under this method.

### 2.4 Bead-to-bead transfer

2×10^6 AD-MSCs (P4) were cultured on 10 tablets of 3D TableTrix™ in 60mL serum-free medium with a 125-mL spinner flask for 4 days according to method described in 2.3, under constant agitation of 60rpm. Microcarriers laden with 2×10^6 cells were aliquoted from the spinner flask (or cryopreserved microtissues were thawed and resuspended in medium) and added to a new 125-mL spinner flask containing fresh 10 tablets of 3D TableTrix™ and topped up with serum-free medium to a final volume of 60mL. Cells were cultured for 4 days and samples were taken according to method described in 2.3.

### 2.5 Cryopreservation and thawing of microtissues

1 tablet worth of microcarriers laden with cells were resuspended in 1mL of cryoprotectant containing 10% DMSO and 90% culture medium and then frozen slowly at -1°C/min to -80°C for 24h. They were then transferred to liquid nitrogen for longer storage.

To thaw, microtissues in cryopreservation vials were removed from liquid nitrogen and immediately submerged in 37°C water bath for 2 minutes with constant swirling to facilitate thawing. When the microtissues were fully thawed, they were transferred to a 15mL centrifugal tube and 10mL of 37°C culture medium was slowly added. Centrifugation at 1000rpm for 5min were performed to sediment the microtissues and supernatant was removed. Cells were harvested from thawed microtissues and assayed for viability according to protocol in section 2.7. Thawed microtissues were then resuspended to appropriate cell density in culture medium and inoculated to new microcarriers according to bead-to-bead transfer protocol in section 2.4.

### 2.6 Scale-up to 1L bioreactor

2×10^6 AD-MSCs were cultured on 10 tablets in 60mL serum-free medium with a 125-mL spinner flask for 4 days according to method described in 2.3, under constant agitation of 60rpm. Then microcarriers laden with 2×10^7 cells were aliquoted from the spinner flask
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for bead-to-bead transfer to 1L bioreactor (Fig. 5B). 50 tablets of fresh 3D TableTrix™ (F01, CytoNiche Biotech, China), cultured microcarriers laden with 2×10^7 cells and 500mL serum-free hMSC culture medium (RP02010, Nuwacell Ltd, China) were added to a sterilized 1L culture vessel of 3D FloTrix™ vivaSPIN bioreactor system (V1, CytoNiche Biotech, China, Fig. 5B) via the vessel’s side-arm. After setting up the culture vessel and medium feeding tubing according to manufacturer’s instructions, a 7 day cell culture protocol was set to: Temperature: 37°C, gas inlet: 20ccm for 5% CO₂ mixed with 95% air, agitation: 60rpm.

Additional 500mL fresh medium was fed into the system at 24h. After which, 50% medium was aspirated and equivalent amount of fresh medium was fed every 48h. 30min settling time (no agitation) was set to allow microcarriers to settle before medium aspiration and agitation was automatically resumed to 60rpm after aspiration was completed. All medium flow was set to 100mL/min. Cell growth was monitored by taking 1mL samples via the sampling port which was connected to a dip-tube at a height corresponding to 50% of the vessel’s working volume.

2.7 Cell harvesting & enumeration

Cell-laden microcarriers were allowed to sediment to the bottom of the sampling tube/EP tube or culture vessel and medium was aspirated carefully to ensure no microcarriers were removed. 3D FloTrix™ Digest (CNR001-500, CytoNiche Biotech, China) were then added at a ratio of 0.15mL/mg microcarrier and incubated at 37°C for 30min. Agitation of 60rpm was used when harvested in culture vessels while gentle pipetting was performed for sampling tubes/EP tubes every 10min to assist dissolution of microcarriers and dissociating cells. Cells number and viability were counted by Trypan Blue exclusion assay using an automatic cell counter (Countstar Biotech, ALIT Life Science, China).

2.8 Fluorescence staining of cells on microcarriers

Cell-laden microcarriers were allowed to sediment to the bottom of the wells of 96-well micro-plate and medium was aspirated carefully to ensure no microcarriers were removed. They were then stained with calcein AM and propidium iodide (PI) (Wako, Japan), diluted in PBS according to manufacturers’ instruction, at 37°C for 15min. Dye solution was aspirated and PBS was added to the wells before imaging with a fluorescence
microscope. Confocal images were maximum projections of Z-stack images taken by Zeiss LSM710 confocal microscope at 10µm step intervals for a total of 150µm.

2.9 Differentiation of AD-MSCs:

Adipogenic (HUXMD-90031), osteogenic (HUXMD-90021) and chondrogenic (HUXMD-9004) differentiation and characterization kits were purchased from Cyagen (US). AD-MSCs harvested from monolayer culture (inoculated P5 cells at 1×10^6 in T75 and cultured for one passage, i.e. harvested as P6) or 3D TableTrix™ expansion (from section 2.6) were plated, cultured, differentiated and characterized for adipogenic, osteogenic and chondrogenic lineages using respective culture and characterization kits. All protocols were performed according to manufacturer’s instructions.

2.10 Flow cytometry

AD-MSCs from monolayer culture (inoculated P5 cells at 1×10^6 in T75 and cultured for one passage, i.e. harvested as P6) or 3D TableTrix™ expansion (from section 2.6) were harvested according to respective protocols and then stained directly for the positive markers CD73-FITC, CD90-FITC, and CD105-FITC as well as negative markers CD45-PE, CD34-FITC, CD14-FITC, CD19-PE, HLA-DR-FITC, and CD11b-PE. With the exception for CD11b-PE (561001, BD Biosciences, US), all antibodies were purchased from Biolegend (US) and were pre-conjugated. More than 5×10^4 total events were acquired on an Aria SORP FACS (BD Biosciences, US) and analyzed using FlowJo Ver. 10.1 (BD Biosciences, US).

2.11 SA-β-gal Staining

AD-MSCs harvested from monolayer culture (inoculated P5 cells at 1×10^6 in T75 and cultured for one passage, i.e. harvested as P6) or 3D TableTrix™ expansion (from section 2.6) were plated at 1×10^4 cells/cm^2 in 6-well microplates and cultured for 3 days before staining with SA-β-gal staining kit (G1580, Solarbio, China) according to manufacturer’s instructions.
2.12 Karyotype Analysis

AD-MSCs harvested from monolayer culture (inoculated P5 cells at 1×10⁶ in T75 and cultured for one passage, i.e. harvested as P6) or 3D TableTrix™ expansion (from section 2.6) were cryopreserved and sent to a certified third-party laboratory (KingMed Diagnostics, China) for standard chromosome analysis at 550-band resolution according to standard procedures.

2.13 Statistical analysis

Data are presented as mean ± standard deviation. ANOVA was used for statistical analysis and specific p values are given. P value > 0.05 is considered not statistically significant and hence not reported.

3. Results

3.1 3D TableTrix™, a novel dispersible porous microcarrier tablet

Unlike most microcarriers which are in powder form, 3D TableTrix™ (Cytoniche, China) is a novel and proprietary formulation of microcarriers. These microcarriers are provided as weight-defined tablets, at 1.58±0.01 mm thick and 7.98±0.06 mm diameter (Fig. 1A, iii-iv show a bottle of 3D TableTrix™ tablets and its enlarged image). These tablets readily disperse into individual microcarriers upon contact with aqueous solution (Fig.1B, Movie S1), and with agitation, microcarriers can be uniformly dispersed within a minute. At a density of 4893±288 microcarriers/mg and a D₁₀-D₉₀ size distribution between 125-273 μm (hydrated, Fig. 1C and insert), 3D TableTrix™ pack over 9×10⁴ microcarriers into each 20mg tablet, offering at least 9000 cm²/g surface area, calculated based on the modest assumption that these microcarriers are perfectly round non-porous solid spheres. Microcarriers packed in 3D TableTrix™, however, are in fact highly porous (Fig. 1D-E) with pore sizes of 20.6±5.7μm (Fig. 1F), hence it is highly possible that much more surface area than theoretical value is available for high cell density culture to realize efficient cell production. The median local Young’s Modulus of these microcarriers (i.e. Young’s Modulus sensed by cells attached to walls in these porous microcarriers) were tested to be...
57.23kPa (Fig. 1G insert) as measured with AFM, with a majority of elasticity of modulus falling between 25-100kPa (Fig. 1G).

### 3.2 Inoculation protocol for ready-to-use 3D TableTrix™ in stirred culture system

Agitation played a significant role in affecting cell attachment to microcarriers. In this work, laboratory-scale stirred systems, i.e. spinner flasks, were used to investigate effects of three different agitation modes, namely delayed, constant and intermittent, on cell attachment within the first 24h of inoculation. Significantly lower attachment rate (80.2±1.2%) was observed for constant agitation compared to delayed or intermittent inoculation protocol (94.6±1.2% and 96.6±0.5% respectively) at 2h post inoculation (Fig. 2A), as attested by fluorescence staining of cells which showed several cells not attached to microcarriers for constant agitation (Fig. 2B).

Another parameter affecting cell attachment rate was the ratio between cells and microcarriers. $1\times10^6$ (ultra-low), $5\times10^6$ (intermediate) and $10\times10^6$ (high) AD-MSCs were inoculated to 5 microcarrier tablets (0.1g) in 40mL medium for this investigation. Even at a constant agitation of 60rpm, more than 80% attachment rates were observed for all inoculation densities at 2hr and 6hr, more than 98% of all cells were attached to microcarriers at 24hr post inoculation (Fig. 2C-D).

### 3.3 Dynamic expansion of hMSCs cultured on 3D TableTrix™ in spinner flasks

Results in Fig. 2 demonstrated that cell attachment to 3D TableTrix™ was highly efficient. Comparison of cell growth for different inoculation density was then analyzed. It was evident from cell enumeration that after the initial cell attachment phase, the log phase expansion begun from day 2 (Fig. 3A). Total cell yield for ultra-low, intermediate and high inoculation density after 4 days of culture were $2.98\pm0.30\times10^7$, $8.48\pm1.75\times10^7$ and $9.92\pm0.63\times10^7$ respectively (Fig. 3A). These corresponded to expansion factors of $29.8\pm3.0$, $17.0\pm3.5$ and $9.92\pm0.63$, where expansion factors for ultra-low density became significantly higher than those of intermediate and high seeding density from day 3 (Fig. 3B). As there had been reports that found seeding MSCs at low densities (e.g. 10–1000 cells/cm$^2$) promoted cell proliferation rate and maintained better tri-lineage potential[14],
seeding density of 2 cells/microcarrier (equivalent to 2×10^6 cells for 10 tablets) was used for subsequent experiments.

As it had been reported that medium compositions, such as serum or growth factor concentrations, may affect cell attachment and expansion on microcarriers [15, 16], we then investigated if medium composition would affect 3D TableTrix™ performance in dynamic expansion of AD-MSCs, specifically we tested three different culture mediums, i.e. chemically defined (CDM), serum-free (SFM) and serum-containing (MSCM). AD-MSCs were able to proliferate well on 3D TableTrix™ in all three cell culture mediums, albeit a slightly lower final yield on day 4 when cultured with CDM (Fig. 3C). Nonetheless, cell viability of higher than 90% is assured in these three culture mediums (Fig. 3D), suggesting successful culture of AD-MSCs on 3D TableTrix™ was not restricted by medium composition, further affirming the potential application of 3D TableTrix™ in large-scale production of clinical-grade hMSCs in which GMP-compliant serum-free or chemically defined mediums are preferred. To demonstrate the compatibility of 3D TableTrix™ to various sources of hMSCs, dynamic expansion of UC-MSCs is also verified. Total yield of 1.09±0.19×10^7 cells (5.47±0.93-fold expansion) were harvested from 10 tablets in 120mL working volume after 4 days of serum-free culture, with 92.7±6.5% cell viability (Fig. 3E). Live/dead staining of cells on microcarriers attested the growth of UC-MSCs on microcarriers (Fig. 3F).

3.4 Bead-to-bead transfer enables continuous expansion

Cells would eventually stop proliferation when the surface area available for growth become limited. To continue expanding cells for higher yield, they would have to be provided with more surface area, which in typical monolayer cultures would mean harvesting them from the culture flasks and inoculating them to more new flasks to increase surface areas. For microcarriers, we could realize continuous expansion without having to harvest cells and re-inoculating, but to supply new microcarriers at adequate time-points of the culture process so that sufficient surface areas were available for the expanding population of cells. This expansion strategy would require bead-to-bead transfer technology which was a process where cells migrate from microcarriers, during
culture and cell division, to fresh microcarriers [6, 17]. The effect of agitation protocols on effective bead-to-bead transfer was first examined in spinner flasks by inoculating microcarriers laden with 3×10^6 cells (‘microtissues’, Fig. 4A) to 10 tablets. Agitation was set to intermittent or constant (agitation protocols investigated for free cell inoculation) for the first 24h and then constant agitation at 60rpm for the rest of the culture period. Confocal imaging attested that cells could migrate from one microcarrier to another as bridges of cells could be seen between microcarriers after fresh microcarriers were inoculated (Fig. 4B). Fluorescence staining of live cells further confirmed that cells could successfully transfer to new microcarriers (Fig. 4C). No significant difference in final cell yield or cell viability were observed in both protocols (Fig. 4D). A unique feature of 3D TableTrix™ is its compatibility with conventional cryopreservation technology, cells remained attached to microcarriers (Fig. 4E) and retained more than 90% viability upon thawing after cryopreservation for 3, 10, 14 and 40 days (Fig. 4F). Cell expansion was also successful using thawed cell-laden microcarriers (microtissues), which was not significantly different from using thawed cells as starting seeds for expansion (Fig. 4G), thus demonstrated the feasibility of storing microtissues as seeds for bead-to-bead transfer in subsequent batches of culture.

3.5 Scalable production of hMSCs on 3D TableTrix™ in stirred tank bioreactor

With the feasibility of bead-to-bead transfer on 3D TableTrix™ verified, scale-up to larger culture volume was performed. Stirred tank bioreactors are scalable, robust and well-controlled bioprocessing systems that facilitates microcarrier-based suspension culture for large-scale production of adherent cells[6, 18]. Hence, a stirred tank bioreactor with a 1L glass vessel with temperature and gas control (Fig. 5B) was used to illustrate scalability of hMSCs production using 3D TableTrix™ microcarriers using microtissues obtained from laboratory-scale spinner flasks (Fig. 5A). Starting with 2×10^6 cells with 10 tablets in a 125-mL spinner flask, 5.82±0.45×10^7 AD-MSCs (29.1±2.2-fold expansion) were yielded after 4 days of culture (Fig. 5C-D). Microtissues (microcarriers laden with cells) containing 2.0×10^7 cells from the spinner flasks were aliquoted and inoculated to 1g (50 tablets) microcarriers in a 1L bioreactor and cultured for another 7 days. Total final yield
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3.6 Cell recovery and cell characterization

3D TableTrix™ were dissolvable microcarriers and hence all cell enumeration and harvesting in this article were accomplished by fully dissolving 3D TableTrix™ microcarriers with its specific dissolution reagent, 3D FloTrix™ Digest (CytoNiche, China). 3D TableTrix™ microcarriers could be fully dissolved within 30min under gentle agitation as observed under microscope (Fig. 6A). 98.6±0.1% cell recovery rate was accomplished and cell viability was maintained at above 90% (Fig. 6B). Harvested cells were then characterized for their immunophenotype, tri-lineage differentiation ability and karyotype stability. 3D TableTrix™-expanded AD-MSCs retained their phenotypic surface markers where expression was >98% and <1% for positive and negative markers (Fig. 6C), meeting the criteria set by International Society of Cell Therapy (ISCT)[1]. Cells also maintained their ability to differentiate into adipogenic, osteogenic, and chondrogenic lineages (Fig. 6D). In comparison with 2D counterparts (population doubling were 2.47 in one passage, 3-4 days culture, harvested at 80-90% confluence), cells harvested from 3D TableTrix™ expanded in the 1L bioreactor scale-up process had a total population doubling of 10.6 in 11 days (4.86 in 4-day spinner flask culture and additional 5.71 in subsequent 7-day bioreactor), thus concerns with excessive expansion leading to genome instability and senescence were raised. Despite the higher population doubling, karyotyping affirmed genome stability (Fig. 6E) of microcarrier-expanded cells and these AD-MSCs did not show increased β-galactosidase activity as characterized with SA-β-gal staining in comparison to their 2D counterparts (Fig.6F).

4. Discussions

Microcarrier selection is one of the most important choice to make for successful large-scale expansion of hMSCs in stirred tank bioreactors[4, 18, 19]. Efficient cell seeding protocol is essential as cell attachment to microcarriers is critical to the success of expansion in stirred tank bioreactors. High percentage and uniform attachment of cells to initiate cell expansion is desired. Important parameters affecting cell attachment include
surface chemistry of microcarriers, agitation protocols and cell and microcarrier concentrations, among others [18]. As 3D TableTrix™ microcarriers are made of gelatin, it is presumed that cell adhesion motifs were inherently available [20, 21] and thus no pre-coating is performed for these microcarriers for cell attachment. Also, since these microcarrier tablets are sterile and readily absorbed liquid to disperse into individual hydrated microcarriers, they can be introduced directly into culture medium together with cell suspensions without weighing, rehydrating, autoclaving or other prior preparations.

Agitation mode is another important parameter investigated. Literatures had recommended a 20hr delay of agitation after cell inoculation to aid cell attachment [22], or intermittent agitation between high and low speed (or intervals of agitated and non-agitated periods) to maximize cell-microcarrier interaction with homogenous distributions facilitated by agitation and cell anchorage to microcarriers during low or no agitation periods [6, 13, 18, 23, 24]. While no agitation achieved the best efficiency of 99.7±0.1% and 99.4±0.8% at 6 and 24h post inoculation respectively, our study did not find significant differences in attachment rate and uniformity for different agitation protocols. Cell inoculation density for microcarriers ranging from 0.6 to 30×10³/cm² (corresponding to less than 1 cell/microcarrier to over a hundred cells/microcarrier ratio) have been reported [4, 8, 9, 13, 17, 19, 23-28], with 6000 cells/cm² being a common choice of density in a number of studies. As inoculation density varied largely in literature, we investigated densities that cover the range of densities reported. By inoculating 1×10⁶, 5×10⁶, and 1×10⁷ cells to 5 tablets of 3D TableTrix™ (0.1g which gave a total of at least 900cm² surface area and nearly 5×10⁵ microcarriers), we were able get inoculation densities of at most 1111 cells/cm² (ultra-low), 5555 cells/cm² (intermediate) and 11111 cells/cm² (high) or about 2, 11, 22 cells per microcarrier respectively. Our study found that these three densities were all suitable cell/microcarrier ratio for efficient inoculation on 3D TableTrix™.

In comparison to most microcarriers investigated thus far, performance of 3D TableTrix™ is unprecedent. In our experiments, we have demonstrated that 3D TableTrix™ could achieve a yield of up to 2.48×10⁶ cells/mL in 4 days from 40mL culture volume in 125-mL spinner flask. Most commercial microcarriers investigated thus far were non-porous spheres made of plastic or dextran (e.g. Cytodex, Hillex, Plastic, Synthemax II),
which typically yielded 1.3×10^{5}/mL for AD-MSCs [13] [29] [26] and 1.8×10^{5}/mL for other hMSCs [13] [30] [31]. Theoretically, use of macroporous microcarriers would provide higher surface area per unit volume to achieve higher cell density, however, investigations found that macroporous CultiSpher did not outperform non-porous microcarriers in cell yield. In one study, only 2.5×10^{5}/mL bone marrow-derived (BM) MSCs were grown on CultiSpher after 144h culture, whilst Plastic or collagen-coated plastic microcarriers more than triple the yield [8]. Other study on fetal MSCs also demonstrated that CultiSpher performance was poorest among the microcarriers investigated, with Cytodex-3 and Plastic been the best, yielding 6.8×10^{5} cells/mL [31]. While a study showed that CultiSpher had higher cell adhesion rate for adipose-derived MSCs (AD-MSCs) than Plastic (88% versus 69%), the eventual yield was not significantly different for these two microcarriers, expanding about 14 times in 7 days to reach an eventual 1.4±0.5×10^{5} cells/mL [13]. Another study also demonstrated that CultiSpher only yielded 4.2×10^{5}/mL bone-marrow derived MSCs [16]. These data (2.5×10^{4} – 4.2×10^{5}/mL) for CultiSpher are mainly obtained from cells cultured in 100-mL spinner flasks for 6-8 days (with culture volume ranging from 50 to 100mL), and in comparison, 3D TableTrix™ yielding 7.45×10^{5} – 2.48×10^{6} cells/mL in 125-mL spinner flask (with 40mL culture volume, and depending on inoculation density) within 4 days show evidently superior performance. As these two microcarriers are of similar chemical composition and macro-structure, the difference in performance between these two microcarriers would require more systematic studies to identify the performance-determining differentiation factor. Nonetheless, our study on 3D TableTrix™ provides some evidence to support the theory that porous microcarriers could yield more cells than non-porous microcarriers, which potentially offer more advanced expansion configuration for stem cell manufacture.

We also further demonstrate that with bead-to-bead transfer technology, scaling up hMSCs expansion on 3D TableTrix™ could be realized without enzymatic dissociation of cells from microcarriers. We demonstrate over 500 times expansion of AD-MSCs in serum-free culture media, starting with 2×10^{6} cells and yielding 1.05×10^{9} cells in 11 days with bead-to-bead transfer from 125-mL spinner flask to 1L bioreactor. As only 34.3% of the cells from the spinner flask were used as starting materials during the 1L bioreactor scale-
up, we anticipate that yield of over $3 \times 10^9$ cells with an overall 1530 times expansion could theoretically be realized with full scale-up.

With bead-to-bead transfer, it was found that cells could migrate from one microcarrier to another microcarrier during bead-to-bead transfer to populate fresh microcarriers added to the culture system, greatly simplifying the process of passaging cells in a scaled-up system. The ability for cells to form cell bridges between microcarriers as they migrate during bead-to-bead transfer raise concerns that large aggregations of microcarriers would form during long-term culture and thus hindering mass transfer and further expansion of cells. We did observe that loose clusters would form as cell population on microcarriers increased, however these clusters could easily break up with gentle pipetting and agitation. No increase of cell deaths were found in these clusters too, as observed by live/dead staining. As these microcarriers are highly porous, we believe that gas and nutrient could still be adequately supplied to cells even when these loose clusters formed. Regardless, as reported by a literature that investigated means to limit aggregation during MSC expansion on microcarriers[25], optimizing the time for fresh microcarrier addition could prevent large clusters from forming, as the increasing cell population would be suitably distributed to more surface areas provided by the new microcarriers.

Besides expansion efficiency, effectively harvesting cells from microcarriers is equally important for bioprocesses that need cells as final products. Currently, cell harvesting process is one of the most challenging step in ensuring hMSC cell quantity and quality[19]. Most commercially available microcarriers are non-biodegradable and require additional filtration steps to separate and harvest cells from microcarriers [23, 32], which cost loss of cells. Hence, some studies have favored biodegradable microcarriers, such as gelatin-based CultiSpher, so that cells could be released by fully dissolving microcarriers, which simplify downstream purification processes without having to physically remove microcarriers from final desired living cell products [9, 16]. Other than CultiSpher, reports of commercially-available biodegradable microcarriers are few, of which Corning dissolvable microcarriers are noteworthy and have been studied for scalable iPSCs expansion[33]. In this study, we not only achieved high expansion efficiency with 3D
TableTrix™, high cell recovery rate of 98.6±0.1% was also realized as 3D TableTrix™ could be fully dissolved using respective dissolution reagent. This eliminates complex downstream processing (i.e. filtration and separation) to ensure higher cell yield as compared to non-biodegradable microcarriers. Cells harvested were of high vitality and retained critical immunophenotypes and functionality.

In addition to using microcarriers for cell expansion, we find that there is increased interest in developing biodegradable microcarriers for both in vitro expansion and in vivo therapy. Some of these promising microcarriers are made of biodegradable polymers such as polycaprolactone (PCL) and poly(lactic-co-glycolic acid) (PGLA). Lam and his colleagues fabricated porous PCL microcarriers coated with extracellular matrix (ECM) proteins for expansion of hMSCs, and they successfully expanded MSCs from bone marrow, cord blood, fetal and Wharton’s jelly, with a yield of 3.6-4.4×10⁵/mL in either 100mL-spinner flask or 1L bioreactor in 5 days, which was comparable to commercial microcarrier Cytodex-3[34].

Besides using these microcarriers for expansion, this research team further explored the potential of these microcarriers as cell delivery tools for tissue engineering purposes. The team were able to induce bone formation when MSC-laden PCL microcarriers were implanted in a mouse model[35]. Another team working on PLGA microcarriers were able to expand 3.2-folds of MSCs in 12 days and then induced these MSCs into smooth-muscle-like cells for potential transplantation into smooth muscle tissues[36]. Other than synthetic microcarriers, natural biomaterial-based carriers had also been used to deliver cells. Li et al. have developed gelatin-based porous microscaffolds as injectable microniches for efficient MSC delivery, thus enabling low-dosage treatment of lower limb ischemia in mice[37]. Commercial gelatin-based microcarriers such as CultiSpher and Spheramine had also been reported as cell delivery agents for tissue repair [38-40]. The advantage of such biodegradable microcarriers is that cells do not have to be detached from their adhesion surface after expansion for transplantation, which would otherwise be detrimental and result in poor clinical outcomes[36]. Other microcarriers that could serve as cell delivery tools for tissue engineering are reviewed extensively elsewhere[10, 41-43]. In this retrospective, gelatin-based 3D TableTrix™, with its possibility of been stored as
Tissue Engineering

Dispersible and dissolvable porous microcarrier tablets enable efficient large scale hMSC expansion (DOI: 10.1089/ten.TEC.2020.0039)

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cryopreserved microtissues (cell-laden microcarriers), has high potential for use as part of cell therapy strategies to deliver cells for enhanced therapeutic effects.

In conclusion, 3D TableTrix™ as a newly available microcarrier option on the market has displayed its tremendous potential for industrial production of hMSCs with its results reported in this study. The ingenious design of 3D TableTrix™ as dispersible and dissolvable porous microcarrier tablets eliminates the need of tedious preparation work usually required for microcarriers, and with bead-to-bead transfer eliminating the need for enzymatic dissociation during passaging, bioprocesses involved in scaling up hMSCs expansion could be greatly simplified. Not only so, cells could be cryopreserved in situ on 3D TableTrix™ microcarriers and be successfully thawed with high vitality and retained the 3D macrostructure, this provides an option to store cell-laden microcarriers (microtissues) as seeds for bead-to-bead transfer in subsequent batches of culture, as well as for use as implantable carriers to aid cell therapy. While our results supersede microcarriers currently reported in literature, more theoretical and practical considerations[1, 22, 44] need to be investigated to realize the full potential of 3D TableTrix™ microcarriers for hMSCs production, including potential risks of aggregation, ease of removing any microcarrier-associated chemical residues in the final cellular products and more comprehensive analysis of cell quality[1]. Nonetheless, with this high performance microcarrier, it is expected that cost-effective, industrial-scale production of quality hMSCs or MSC-derived products, such as exosomes, under a controllable process compliant with GMP practices [5, 6, 10, 19, 44] will soon be possible.

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FIGURE LEGENDS

Fig. 1. Characterization of 3D TableTrix™. (A) Diameter (i) of one 3D TableTrix™ tablet and thickness (ii) of 5 tablets. Images (iii, iv) of one bottle of 3D TableTrix™ tablets. (B) Images of a tablet of 3D TableTrix™ dispersing into individual microcarriers in culture medium at 0s, 10s and after agitation. (C) Relative frequency plot of size distribution of microcarriers dispersed in aqueous solution and insert showed cumulative frequency plot. (D-E) SEM images of microcarriers, scale bar=1mm and 100μm respectively. (F) Relative frequency plot of pore size distribution. (G) Relative frequency plot of local Young’s Modulus of microcarriers analyzed by AFM. Insert shows the median and span of modulus measured.
Fig. 2. Inoculation Protocol for 3D TableTrix™. (A) Attachment rate of AD-MSCs to 3D TableTrix™ microcarriers in spinner flask with no agitation (delayed), constant agitation of 60rpm (constant) or alternation of no agitation for 20min and 60rpm for 5min (intermittent) at 2, 6 and 24h post inoculation. (B) Calcein-AM staining of AD-MSCs and microcarriers mixture at 2, 6 and 24 hours post agitation for respective agitation protocols (scale bar=40μm). (C) Attachment rate of 1×10^6 (ultra-low), 5×10^6 (intermediate) and 10×10^6 (high) AD-MSCs to 5 tablets of 3D TableTrix™ microcarriers in spinner flask with constant agitation of 60rpm at 2h post inoculation. (D) Calcein-AM staining and bright field images of AD-MSCs and microcarriers mixtures at 2h post inoculation for respective densities (scale bar=100μm).
Fig. 3. Expansion of hMSCs on 3D TableTrix™ in spinner flask. (A) 4-day growth curve of AD-MSCs seeded at $1 \times 10^6$ (ultra-low), $5 \times 10^6$ (intermediate) and $10 \times 10^6$ (high) densities on 5 tablets of 3D TableTrix™ microcarriers in 125mL-spinner flask with constant agitation of 60rpm. (B) Comparison of folds in expansion between ultra-low, intermediate and high inoculation densities. (C) 4-day growth curves and (D) viability of AD-MSCs seeded at ultra-low density in serum-containing (MSCM), serum-free (SFM) and chemically defined (CDM) mediums. (E) 4-day growth curve and viability plot of UC-MSCs grown on 3D TableTrix™ microcarriers with serum-free medium and (F) fluorescent images of live/dead staining of cells on day 2 and 4 of culture, scale bar=100μm. For (C)-(F), $2 \times 10^6$ cells were inoculated to 10 tablets of 3D TableTrix™ microcarriers and cultured in 125mL-spinner flask with constant agitation of 60rpm.
Fig. 4. Bead-to-bead transfer with microtissues. (A) Confocal image of cell-laden microcarriers, i.e. microtissues, scale bar=100μm. (B) Confocal image of cells forming bridges between microcarriers to facilitate bead-to-bead transfer, scale bar=100μm. In (A) and (B), live (Calcein-AM) and dead (PI) cells were stained green and red respectively, with pseudo blue colour added to microcarriers (based on auto-fluorescence property of protein-based material) to indicate spatial relation between cells and microcarriers. (C) Images of cell/microcarrier mixture 1 and 3 days after cell-laden microcarriers were inoculated to fresh microcarriers under constant or intermittent agitation. Fluorescent images of calcein-AM stained cells and bright-field images of microcarriers were merged to illustrate population of cells on microcarriers, scale bar=100μm. Fewer microcarriers were populated with cells on day 1 as these are fresh microcarriers, which then became populated with cells on day 3. (D) Growth curve (black) and viability (red) plot of cells expanded using bead-to-bead transfer method under constant or intermittent agitation. (E) Fluorescent images of calcein-AM stained cells on microcarriers prior to cryopreservation (fresh) and thawing after cryopreservation (thawed), scale bar=40μm. (F) Percentage of viable cells retained on microcarriers after cryopreservation of microtissues for 3, 10, 14 and 40 days. (G) Growth curves of AD-MSCs by inoculating thawed cell-laden microcarriers (i.e. microtissues) or thawed cells to fresh microcarriers.
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Fig. 5. Scale-up of AD-MSC expansion to 1L bioreactor. (A) 3D FloTrix™ miniSPIN system consisted of 2 controllers (i, ii), a magnetic stirrer (iii) and spinner flask (iv). (B) 3D FloTrix™ vivaSPIN bioreactor system with 1L culture vessel and parts labeled. (C) Growth curve and viability plot of sequential scale-up of 2×10^6 AD-MSCs on 3D TableTrix™ microcarriers by expanding in 125mL spinner flasks for 4 days and then partial-scale up to 1L bioreactor for additional 7 days culture with bead-to-bead transfer starting with 2×10^6 AD-MSCs-laden microcarriers. (D) Expansion factors achieved during 4-day spinner flask expansion stage, 7-day bioreactor expansion stage and the overall 12-day expansion protocol.
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Fig. 6. Cell recovery and quality assessment. (A) Bright field (BF) and fluorescence images of live/dead staining of cells during microcarrier dissolution process, scale bar=100μm. (B) Recovery rate and viability of cells harvested by complete dissolution of 3D TableTrix™ microcarriers. (C-E). (C) FACS analysis of surface markers of AD-MSCs expanded using 3D TableTrix™ microcarriers and harvested by microcarrier dissolution. (D) Multipotency of monolayer or 3D TableTrix™-expanded AD-MSCs were tested by differentiation along the adipogenic, osteogenic, and chondrogenic pathways, scale bar=100μm. (E) Karyotyping of AD-MSCs harvested form (i) monolayer culture or (ii) 3D TableTrix™ expansion. No chromosomal anomaly was observed. (F) Senescence evaluation by SA-β-gal staining of (i)monolayer, (ii)3D TableTrix™-expanded AD-MSCs at P6, (iii) monolayer-cultured AD-MSCs at P5 as negative control and (iv) monolayer-cultured AD-MSCs at P10 as positive control, scale bar=40μm.