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Comparability exercise of critical quality attributes of clinical-grade human mesenchymal stromal cells from the Wharton's jelly: single-use stirred tank bioreactors versus planar culture systems

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ABSTRACT

Background aims: The increasing demand of clinical-grade mesenchymal stromal cells (MSCs) for use in advanced therapy medicinal products (ATMPs) require a re-evaluation of manufacturing strategies, ensuring scalability from two-dimensional (2D) surfaces to volumetric (3D) productivities. Herein we describe the design and validation of a Good Manufacturing Practice–compliant 3D culture methodology using microcarriers and 3-L single-use stirred tank bioreactors (STRs) for the expansion of Wharton's jelly (WJ)-derived MSCs in accordance to current regulatory and quality requirements.
Methods: MSC,WJ were successfully expanded in 3D and final product characterization was in conformity with Critical Quality Attributes and product specifications previously established for 2D expansion conditions.
Results: After 6 days of culture, cell yields in the final product from the 3D cultures (mean $9.48 \times 10^8 \pm 1.07 \times 10^7$ cells) were slightly lower but comparable with those obtained from 2D surfaces (mean $9.73 \times 10^8 \pm 2.36 \times 10^8$ cells) after 8 days. In all analyzed batches, viability was >90%. Immunophenotype of MSC,WJ was highly positive for CD90 and CD73 markers and lacked of expression of CD31, CD45 and HLA-DR. Compared with 2D expansions, CD105 was detected at lower levels in 3D cultures due to the harvesting procedure from microcarriers involving trypsin at high concentration, and this had no impact on multipotency. Cells presented normal karyotype and strong immunomodulatory potential *in vitro*. Sterility, *Mycoplasma*, endotoxin and adventitious virus were negative in both batches produced.
Conclusions: In summary, we demonstrated the establishment of a feasible and reproducible 3D bioprocess using single-use STR for clinical-grade MSC,WJ production and provide evidence supporting comparability of 3D versus 2D production strategies. This comparability exercise evaluates the direct implementation of using single-use STR for the scale-up production of MSC,WJ and, by extension, other cell types intended for allogeneic therapies.

Introduction

The development of advanced therapy medicinal products (ATMPs) has increased exponentially in the recent years to address complex clinical conditions [1]. Consequently, the global demand of clinical-grade mesenchymal stromal cells (MSCs) is constantly increasing because of their promising regenerative and immunomodulatory properties as well as their solid safety profile [2]. However, most strategies for cell expansion still rely on manual handling of two-dimensional (2D) culture systems, which are highly operator-demanding, time-consuming, prone to variability and with low throughput. There are commercial integrated automated solutions that may offer solutions in clinical manufacturing, such as Lonza's Cocoon (Lonza, Basel, Switzerland), Terumo BCT's Quantum (Terumo

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BCT, Lakewood, CO, USA) or Miltenyi's CliniMACS Prodigy (Miltenyi Biotec, San Diego, CA, USA), but they are intended for autologous product manufacturing or scale-out strategies rather than scale-up production. Therefore, a re-evaluation of manufacturing strategies is desirable to meet scalable production needs, with special interest for allogeneic "off-the-shelf" therapies from cryopreserved batches of MSCs. Marketed products based on MSCs for different indications require high amounts of cells in each treatment, ranging from "low" (2×10^6 cells/dose) to "high" doses (1.5×10^9 cells/dose) [3,4]. For this reason, traditional culture systems based on 2D planar surfaces used in Good Manufacturing Practice (GMP) environments intended to achieve billions of cells per batch are no longer considered cost-effective platforms. Transition toward a volumetric scalable production based on three-dimensional (3D) cultures using microcarriers and bioreactors in automated platforms may offer a more convenient setting for both academic and industrial allogeneic MSC manufacturing while maintaining compliance with current regulations and quality standards [5,6].

Among all the commercially available scale-up strategies, stirred tank bioreactors (STRs) have been successfully used for decades in the industry, becoming the "gold standard" platform for large-scale production of monoclonal antibodies and viral vectors [7], and, more recently, they are also considered a versatile platform that can accommodate the bioprocessing needs of cell and gene therapy products. Indeed, STRs offer significant advantages over 2D conventional systems, as they provide closed, automated, GMP-compatible solutions allowing in-process real-time monitoring and control, thus contributing to the development of more-consistent products, a reduction of cost-of-goods and a decrease in batch-to-batch variability and contamination risks [8]. Although several works have already reported scale-up strategies for MSC-derived products from distinct sources (i.e., bone marrow, adipose tissue or umbilical) using benchtop STR of different volumes from 1 L [9–11], 2L [12–16], 3L [17–19], up to 5 L [20,21], few of them describe a fully GMP-compliant methodology [15,19] and, remarkably, formal comparability exercises with respect to existing 2D systems are missing in the scientific literature.

Herein, we describe and validate a GMP-compliant 3D methodology using microcarriers and single-use STR for the expansion of Wharton's jelly (WJ)-derived MSC in accordance with regulatory and quality requirements and demonstrate comparability with the current production methodology using planar surfaces. Quality controls were defined in order to assess the critical quality attributes (CQAs) of the MSC,WJ obtained with a 3D bioprocess and compared with the established CQA for MSC,WJ produced in 2D standard conditions reported previously [22,23].

Methods

MSC,WJ initial cell culture and intermediate product production

MSC,WJ were derived from donated umbilical cord tissue with appropriate donor informed consent following GMP-compliant procedures in the classified facilities of the Banc de Sang i Teixits (Barcelona, Spain) as reported elsewhere [22]. To summarize, MSC,WJ were isolated, expanded and cyopreserved in order to establish a clinical-grade Master Cell Bank with 2.5×10^6 viable cells/unit.

Single Master Cell Bank units were thawed for each batch of drug product (DP) manufacturing. Each cryovial was thawed and plated onto 2-layer CellSTACK (Corning, Corning, NY, USA) at a cell density of 1.6×10^3–2.1×10^3 cells/cm^2 using high-glucose (4.5 g/L) GlutaMAX Dulbecco's Modified Eagle Medium (DMEM-HG; Gibco, Life Technologies, Waltham, MA, USA) and supplemented with 10% human serum B (hSerB; Banc de Sang i Teixits). All cultures were maintained in incubators at 37°C, 5% CO_2 and 95% relative humidity. The medium was changed 24 h after thawing and then every 3–4 days. When 70–90% confluence was reached, cells were recovered by trypsinization with 0.05% trypsin–ethylenediaminetetraacetic acid (EDTA; Gibco, Life Technologies) as intermediate products. Cell number and viability was determined and intermediate products used as inoculums were prepared for large-scale expansion.

MSC,WJ large-scale expansion in 2D cultures using planar surfaces

MSC,WJ intermediate products were replated with DMEM-HG supplemented with 10% hSerB onto 22 CellSTACK layers (total surface corresponding to 13 992 cm^2) at a cell density of 1.7×10^3–5.7×10^3 cells/cm^2 (total cell number range 2.4×10^7–8.0×10^7). All cultures were maintained in incubators at 37°C, 5% CO_2 and 95% relative humidity until 70–90% confluence for a mean duration of 8 days. Then, trypsinization was performed using 0.05% trypsin–EDTA and cells were harvested, washed and concentrated before cryopreservation. Then, pellets were resuspended in saline solution (Plasmalyte 148; Baxter, Chicago, IL, USA) with 2% (w/v) human serum albumin (HSA; Grifols, Barcelona, Spain) and cryopreserved with a 10% dimethyl sulfoxide (WAK-Chemie Medical GmbH, Steinbach [Taunus], Germant) solution in clinical doses of $2.5 \times 10^7 \pm 20\%$ cells/cryotube. Expanded MSC,WJ met quality and safety standards with regard to cell number and viability, phenotype, microbiological testing, genomic stability and immunopotency, which were examined according to the investigational medicinal product dossier approved by the Spanish Agency of Medicines and Medical Devices (AEMPS; PEI16-017). Table 1 summarizes the acceptance criteria for all the in-process controls considered during clinical-grade MSC,WJ manufacturing in 2D planar surfaces.

Customized single-use gamma-irradiated consumable kits were prepared in our manufacturing unit specifically for each cell culture manipulation step to ensure a semi-closed culture bioprocess [24]. According to current regulations [25], equipment qualification, microbiological monitoring and environmental controls (particles and air pressure) were continuously monitored in critical equipment and classified facilities during the manufacturing process.

MSC,WJ large-scale expansion in 3D cultures using microcarriers and 3-L single-use STR

For 3D volumetric large-scale expansion, a 3-L single-use STR (Applikon AppliFlex ST for cell culture; Getinge, Gothenburg, Sweden) equipped with three pitched-blade impeller (cellular configuration) and temperature, pH and dissolved oxygen sensors (AppliSens; Applikon, Sunnyvale, CA, USA) was used with a homemade closed-

Table 1
Clinical-grade MSC,WJ specifications in compliance with pharmaceutical standards.[a]

Parameter	Acceptance criteria
Dose	$\geq 2.5 \times 10^7 \pm 20\%$ viable cells/cryotube
Cell viability	≥70%
Phenotype	
CD105$^+$/CD45$^-$	≥95%
CD73$^+$/CD31$^-$	≥95%
CD90$^+$	≥95%
HLA-DR$^-$	Informative
Sterility	Sterile
Mycoplasma	Negative
Endotoxin	≤1 EU/mL
Adventitious virus	Negative
Karyotype	Non-chromosomal abnormalities
Immunopotency	>30% inhibition of PBMC proliferation

EU, endotoxin units; MSC,WJ, mesenchymal stromal cells derived from Wharton's jelly; PBMC, peripheral blood mononuclear cell.
[a]According to approved investigational medicinal product dossiers for conducting clinical trials listed next: Clinicaltrials.gov Id. NCT03003364, NCT03798353 and NCT05054803.

system configuration. MSC,WJ intermediate products were concentrated and resuspended in DMEM-HG supplemented with 10% hSerB. Before inoculation, GMP-grade polystyrene *Enhanced Attachment* microcarriers (Corning) were hydrated with basal DMEM-HG for 30–45 min at room temperature (RT). Then, 9×10^8 total cells were seeded at a cell density of 4.5×10^3–4.8×10^3 cells/cm^2 on 50 g of microcarriers (total surface corresponding to 18 000 cm^2 and a concentration of 25 g/L). Please note that seeding density is a critical process parameter that differs from traditional planar systems because the surface area available is "fragmented" into each individual microcarrier. Therefore, seeding density was increased to ensure all microcarriers were effectively loaded with cells. Cell adhesion onto microcarriers was performed for the first 4 h under intermittent agitation, stirring 1 min at 45 rpm, followed by a rest phase of 30 min at 0 rpm, in the minimum working volume (0.65–0.7 L in DMEM-HG supplemented with 10% hSerB). Then, working volume was adjusted to 2 L with downward agitation at 45–50 rpm that was gradually increased throughout the culture duration of 6 days to avoid cell–microcarrier aggregates formation and sedimentation. Dissolved oxygen concentration was maintained by headspace flushing a mixture of air and set to 70% air saturation and pH was adjusted to 7.4 and controlled by CO_2 headspace injection. Temperature was set at 37°C and controlled with a heating blanket. During cell culture, microcarrier suspension samples were collected for culture monitoring of cell number, morphology and distribution and supernatant metabolite analysis. Cell number and viability were determined by flow cytometry after sample cell harvesting. Cell morphology and distribution onto the microcarrier surface was assessed by fluorescent staining (Live/Dead and Hoechst). At days 4 and 5, 50% (v/v) media exchange was performed. To summarize, agitation was stopped to allow microcarrier settling and 1 L was removed and replaced with fresh medium with subsequent agitation restart.

For harvesting procedure, agitation was stopped for microcarrier sedimentation, supernatant was removed and MSC,WJ attached to the microcarriers were washed with 500 mL of saline solution (Plasmalyte 148; Baxter). Then, 500 mL of pre-warmed 0.25% trypsin/EDTA solution (Gibco, Life Technologies) was added and the suspension was stirred at 150 rpm, 37°C for 2 min. Then, the enzymatic reaction was quenched by adding 500 mL of saline solution (Plasmalyte 148) supplemented with 10% HSA and the cell suspension was collected into transfer bags (Fresenius Kabi, Bad Homburg, Germany). An additional 500 mL of saline solution (Plasmalyte 148) supplemented with 2% HSA was added in order to recover the remaining detached cells in the vessel. Separation of cells from microcarriers was performed using a filtration bag containing two coupled meshes with decreasing pore sizes of 175 and 40 μm (Miltenyi Biotec). Cells were then washed with saline solution (Plasmalyte 148) supplemented with 2% HSA and concentrated in order to cryopreserve the final products generated for subsequent assays.

Two batches of clinical-grade DP were produced in classified facilities for ATMP production setting and compared with 11 batches of clinical-grade DP produced from 2D standard conditions. Acceptance criteria were defined based on relevant CQA and product specifications of current batches expanded in 2D standard conditions (Table 1). Additional evaluations, namely differentiation assays, human telomerase reverse transcriptase (hTERT) activity determination, senescence and cumulative population doublings (CPD) calculation were performed to ensure safety and batch consistency from 3D cultures. According to GMP regulations, microbiological contamination differential pressure and environmental particle levels were continuously monitored in critical equipment and clean room facilities during the bioprocess.

Cell counting and viability analyses

Cells were enumerated at the end of harvesting processes and after final product concentration of 2D and 3D cultures by flow cytometry using Flow-Count Fluorospheres (Beckman Coulter, Brea, CA, USA) or Perfect-Count Microspheres (Cytognos, Salamanca, Spain) with a Navios EX device (Beckman Coulter). The percentage of cell viability was determined by 7-aminoactinomycin D (Beckman Coulter) staining, which binds specifically to DNA when the cell membrane is damaged by necrotic processes. Cells were incubated with 15 μL of 7-aminoactinomycin D for 5 min at RT in the dark and then washed with 3 mL of Dulbecco's Phosphate-Buffered Saline (DPBS, Lonza) by centrifugation at 340*g*, 5 min, RT, before cytometric acquisition. Acquired data were analyzed with Navios EX software (version 2.0; Beckman Coulter). Fold increase was calculated as the ratio of total harvested cell yield to initial cell number inoculated.

Immunophenotype analysis

Harvested cells from the 2D and 3D cultures were analyzed by flow cytometry with a Navios EX device to assess cell surface marker expression. Each cell suspension at a concentration of 1×10^6 cells/mL was incubated with the following antibodies (all from Beckman Coulter) for 15 min at RT: CD105-PC7 (B43293), CD90-FITC (IM1839U), CD73-PE (B68176), CD45-APC-Alexa Fluor 750 (A79392), CD31-Pacific Blue (B13035) and HLA-DR-APC (IM3635). Fluorescence Minus One samples were used as negative controls. Acquired data were analyzed with Navios EX software. This panel was consistent with previously approved Investigational Medicinal Product Dossiers for clinical trials listed next: Clinicaltrials.gov Id. NCT03003364, NCT03798353 and NCT05054803.

Live/Dead assays

Microcarrier suspension samples collected from 3D cultures were stained using Live/Dead Viability/Cytotoxicity kit (Calcein AM/Ethidium Homodimer; Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) and Hoechst 33342 (Invitrogen, Thermo Fisher Scientific). One milliliter of cell-microcarrier suspension was washed with DPBS and stained with Live/Dead at 37°C for 30 min, then cell nuclei was counterstained with Hoechst at 1/10 000 dilution at RT for 10 min. After washing with DPBS, cell–microcarrier suspension was visualized under inverted fluorescence microscope (Leica DFC450 and Leica DMIL LED; Leica Microsystems, Wetzlar, Germany) in order to determine qualitatively cell morphology and distribution onto microcarrier surface. Microcarrier colonization by MSC,WJ was performed by imaging manual counting of microcarriers with at least one living cell attached using the ImageJ Software (National Institutes of Health, Bethesda, MD, USA) with the Cell Counter tool.

Metabolite analysis

Supernatants collected from 3D culture samples were analyzed for glucose and lactate concentrations on an Accutrend Plus meter (Roche, Basel, Switzerland) according to manufacturer's instructions. Fresh DMEM-HG supplemented with 10% hSerB was used as baseline control for both metabolites.

Microbiological, endotoxin adventitious virus testing and Mycoplasma *detection*

Sterility test, endotoxin and *Mycoplasma* detection were performed according to European Pharmacopeia (Eu.Ph., 10th Edition) on finished products from 2D and 3D cultures. Absence of microbial contamination was verified by inoculation of 1-mL samples in iFA Plus and iFN Plus media bottles (bioMérieux Industries, Marcy-l'Étoile, France). Products comply with the test for sterility if no evidence of microbial growth was found. Endotoxin quantification was performed with the Endosafe-PTS system (Charles River Laboratories, Wilmington, MA, USA) following the manufacturer's instructions.

The Venor qEP Kit (Minerva Biolabs, Hillsborough, NJ, USA) was used for the amplification of a mycoplasma-specific 16S rRNA gene region and detection by real time polymerase chain reaction (PCR) according to the manufacturer's instructions. *In vitro* detection of adventitious virus in MSC,WJ was performed on co-culture with MRC5 and Vero cells. If cytopathic effect was observed after 14–28 days of culture, PCR and immunofluorescence techniques were used to identify contamination agents.

Immunomodulation assay

Semiquantitative immunopotency assays were performed from cryopreserved finished products from 2D and 3D cultures following methods reported previously [26,27]. MSC,WJ were thawed and seeded at a density of 5×10^4 cells/cm^2 in cell culture-treated 24-well plates (SPL Life Sciences, Gyeonggi-do, Korea) on the day before starting the experiment to allow cell attachment on the surface of the plastic. On the next day, 2.5×10^6 peripheral blood mononuclear cells (PBMCs)/mL were labelled with 0.625 μmol/L carboxy fluorescein diacetate succinimidyl ester (CellTrace CFSE Cell Proliferation Kit; Molecular Probes, Eugene, OR, USA) for 10 min. Cells were washed, $1–2 \times 10^7$ cells/mL were incubated for 12 min at 37°C, washed again and co-cultured onto 24-well plates at 5:1 PBMC:MSC,WJ ratio. Lymphocytes were activated with 25 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO, USA) and 0.5 μmol/L ionomycin (Sigma-Aldrich) in a final volume of 0.5 mL/well of low glucose (1 g/L), 2 mmol/L glutamine, DMEM supplemented with 10% hSerB. Proliferation of PBMC was determined by measuring the reduction of fluorescence intensity at day 5 by flow cytometry with a Navios EX device. Acquired data were analyzed with Navios EX software.

Differentiation assays

To verify multipotency, cryopreserved finished products from 3D cultures were thawed, seeded in 24-well plates and subjected to tissue-specific differentiation inducing conditions *in vitro* using StemPro Differentiation Media toward the adipogenic, chondrogenic and osteogenic lineages, as described elsewhere [28]. On days 34–36 after induction, specific stainings were performed for each differentiation condition. Oil Red O (Sigma), Safranin O (Sigma) and Alizarin Red (Merck Millipore, Burlington, MA, USA) stainings were performed to determine the outcome of the differentiation assays and images were captured using an inverted microscope (Leica DMIL LED).

hTERT activity

Cellular extracts from cryopreserved finished products obtained from at least 2×10^5 3D pelleted cells were initially used for hTERT activity determination, which was performed using the Telo*TAGGG* Telomerase PCR ELISA^{PLUS} kit (Roche) following the manufacturer's instructions. Quantitative determination of telomerase activity was assessed and compared with the telomerase activity observed in transformed human embryonic kidney 293 cells (HEK293T; ATCC: CRL-3216) that was used as positive control.

Senescence assay

The occurrence of senescence in cultured MSC,WJ was detected by determining senescence-associated-β-galactosidase activity. At least 1×10^6 cells from cryopreserved finished products from 3D cultures and two 2D cultures representative batches were thawed and seeded in duplicates at 2×10^4 cells/cm^2 in 24-well plates and further incubated at 37°C, 5% CO_2 for 24 h. The assay was performed using the Cellular Senescence Assay Kit (Merck Millipore) following the manufacturer's instructions. After staining, an inverted microscope (Leica DMIL LED) was used to capture images for further evaluation of blue stained cells.

Karyotype

Metaphase chromosome spreads were prepared from thawed samples of cryopreserved finished products resulting from 2D and 3D culture strategies. In brief, MSC,WJ were thawed, seeded at 2×10^3 cells/cm^2 in 25-cm^2 T-flasks and cultured until the exponential phase of growth is reached. Twenty-five microliters of colcemid was added and incubated for 4 h to the cultures for arresting the cells in metaphase. After trypsinization, cells were fixed with Carnoy solution (methanol-acetic acid) and stained with Wright's for chromosomes banding. Twenty metaphase spreads per sample were captured and karyotyped using an automated imaging system for cytogenetics (CytoVision; Applied Imaging Corporation, Grand Rapids, MI, USA). Karyotypes were described following the International System for Human and Cytogenetic Nomenclature (2020).

Cumulative Population Doublings

Total cell counts from harvested cells of 2D and 3D cultures were used for calculating population doublings (PD) and CPD. These values were determined as PD = [ln (initial cell number/final cell number)]/ln 2 and CPD = PD(0) + PD(1) + PD(2) + . . . + PD(n).

Data analysis

Data collection and analysis were performed using Excel (Microsoft Office, Microsoft Corp., Redmond, WA, USA). GraphPad Prism (version 5.03; GraphPad Software, Inc., San Diego, CA, USA) was employed for statistical analysis and graphics using. Results are presented as mean ± standard deviation. An unpaired Student's *t*-test was conducted for group comparisons, applying Welch's correction when needed. Statistical significance was set at $P < 0.05$ (*).

Results

A GMP-compliant bioprocess using microcarriers and 3-L single-use STRs in a closed-system was validated in a class D manufacturing room at Banc de Sang i Teixits. Only those actions that implied an open process (e.g., sensor mounting, cryovial opening, etc.), were performed in a laminar airflow cabinet (Grade A cleanliness level) in Grade B cleanroom. Two batches from 3D bioprocesses were analyzed and compared with 11 batches from 2D productions.

MSC,WJ scale-up in a 3-L single-use STR

MSC,WJ were successfully expanded on microcarriers and a 3-L single-use STR and DP characterization was in accordance with CQA and product specifications defined for previous 2D standard planar conditions. Cell yields from 3D cultures (mean $9.48 \times 10^8 \pm 1.07 \times 10^7$ cells) in the final product after 6-day culture were slightly lower but comparable with those from planar surfaces (mean $9.73 \times 10^9 \pm 2.36 \times 10^8$ cells) after an average of 8 days culture (6–10 days) (Figure 1A), representing a significant difference ($P = 0.0002$) in fold increase of 10.9 ± 0.7 in 3D cultures versus 32.5 ± 13 in 2D planar surfaces (Figure 1C). Concentrated final products were also comparable, resulting in mean cell yields of $9.01 \times 10^8 \pm 2.37 \times 10^8$ and $7.92 \times 10^8 \pm 8.86 \times 10^7$ cells for 2D and 3D cultures, respectively (Figure 1B). In both processes, cell viability from harvested and concentrated cells was greater than 90%. Total CDP numbers after the expansion were similar in both cultures (Figure 1D).

In the case of 3D cultures, the percentage of colonized microcarriers with at least one single living cell was greater than 90% from day 4 to day 6, being 95% ± 0.2% at day 6 (Figure 2A). MSC,WJ

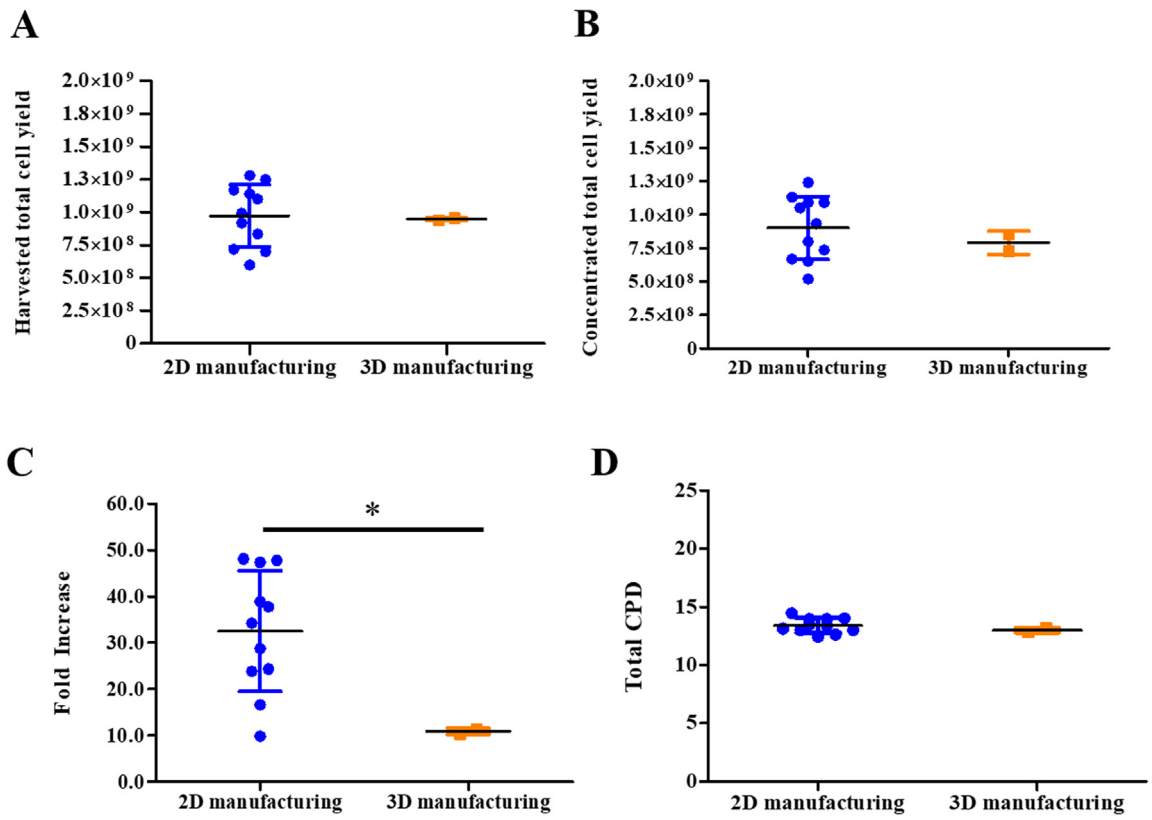

Figure 1. Comparison of 2D versus 3D culture strategies. Comparison of MSC,WJ large-scale expansion in 2D (n = 11) and 3D cultures (n = 2). (A) Total cell yield of MSC,WJ after cell harvesting; (B) total cell yield of MSC,WJ after final product concentration; (C) fold increase (*$P < 0.05$); (D) total CPD. (Color version of figure is available online.)

morphology onto the microcarriers was found to be spindle-shaped and they readily covered the surface of the microcarriers homogeneously and increasingly as the culture progressed (Figure 2B). High cell viability was observed by Live/Dead fluorescent staining, which was further confirmed by flow cytometric viability determination (>90% cell viability). We also observed formation of cell-microcarrier aggregates of different sizes at the end of the culture as cell density increased.

Glucose measurement confirmed there was no depletion at the end of the culture and lactate production increased in the culture time (Figure 3).

CQA assessment and comparability exercise

Finished products from 3D culture batches fulfilled CQA and specifications established for 2D cultures in terms of cell dose, viability, karyotype and immunomodulation characterization, as well as the sterility assessment. In both batches, final products were cryopreserved in clinical doses with a cell viability greater than 70%. Cells maintained their immunophenotype highly positive for CD90 and CD73 markers and lacked expression of CD31 and CD45 and low levels of HLA-DR. However, detection of CD105 showed lower values than expected. Importantly, this change in the specifications did not impact on the rest of parameters assessed in the identity panel and the potency assay and reflects the particularities of 3D systems, which require greater concentrations of trypsin for effectively retrieve cells from the surface of the microcarriers. Moreover, sterility, *Mycoplasma* and adventitious virus tests were negative or undetectable and endotoxin levels were ≤1 endotoxin units/mL for all the finished products, thus meeting the established safety product specifications. Environmental microbial controls, differential pressure levels between cleanrooms and particle count fell within the accepted operational range. No other relevant incidences were reported. Importantly, no genetic abnormalities were found as cells presented normal karyotype. Immunomodulatory potential was measured as the capacity of MSC,WJ expanded in 3D cultures to inhibit the proliferation of activated lymphocytes *in vitro*. Results from co-cultures of MSC,WJ and lymphocytes of both batches were above 30% of inhibition potential, so cells retained their T-cell immunosuppression capacity as established in the acceptance criteria (Table 2).

Further characterization of MSC,WJ after 3D expansion were evaluated and compared with a historical series of 2D manufacturing processes using MSC,WJ derived from two different umbilical cord donors. To evaluate genetic stability of MSC,WJ after 3D cultivation, hTERT activity was determined and compared with a transformed cell line (HEK293T) used as control, highlighting the nearly undetectable levels of hTERT activity in both batches of MSC,WJ (Figure 4A). Occurrence of senescence after cultivation in 3D cultures was measured by senescence-associated-β-galactosidase activity (Figure 4B). The percentage of senescent cells was 0.0005% ± 0.0004% in 3D cultures and 0.0018% ± 0.0019% in 2D cultures, showing no statistical differences ($P > 0.05$). Karyotype analysis show no chromosomal abnormalities in 3D cultures (Figure 4C). Thus, MSC,WJ cultured in dynamic expansion in microcarriers did not display genetic aberrations. Effect of the 3D culture strategy on the preservation of multilineage differentiation potential of MSC,WJ was assessed *in vitro*. Remarkably, osteogenic, chondrogenic and adipogenic differentiation capacity of MSC,WJ was maintained in all cases. Specifically, cells stained positive with Alizarin Red, Safranin O, and Oil Red O, confirming the presence of calcium deposits, glycosaminoglycans and intracellular lipid droplets, respectively (Figure 4D).

Discussion

Cell and gene therapies are complex due to the living nature of the active ingredient. Indeed, the substantial manipulation of substances

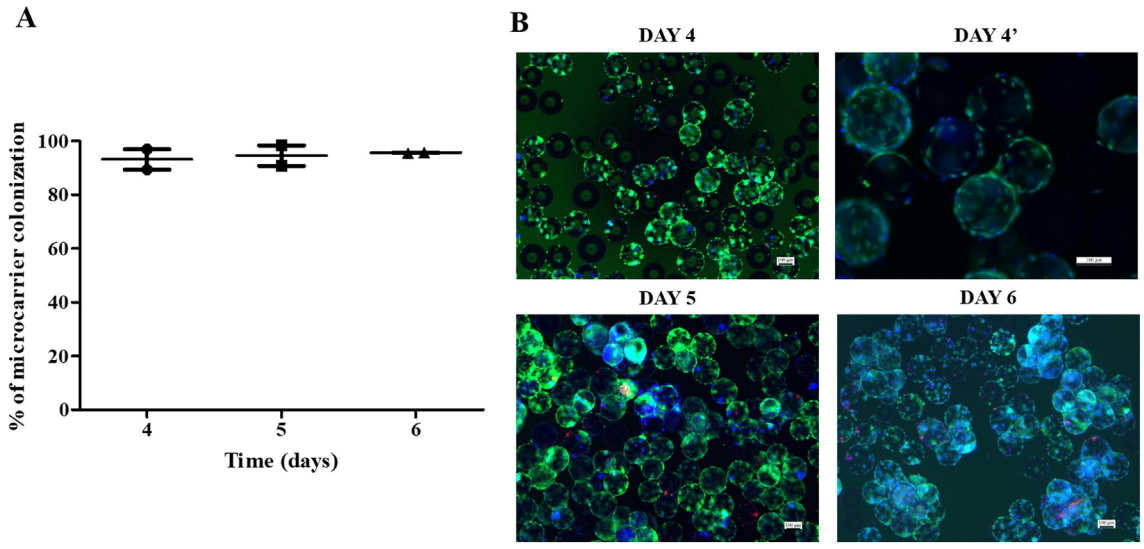

Figure 2. Monitoring of 3D cell culture on microcarriers. (A) Percentage of microcarrier colonization throughout the 3D culture; (B) representative images of MSC,WJ cultured on microcarriers in 3D cultures after Live (green)/Dead (red) and Hoechst (blue) stainings showing distribution and cell morphology at days 4, 5 and 6 (scale bar 100 μm). (Color version of figure is available online.)

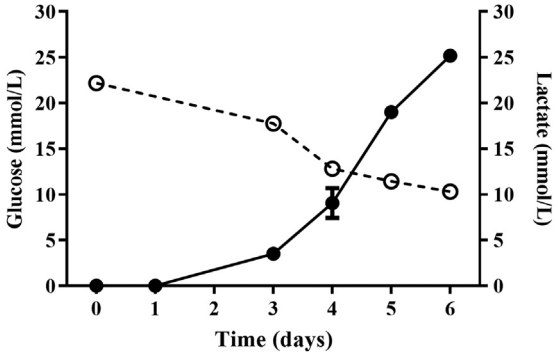

Figure 3. Concentration profiles of glucose and lactate within 6 days in 3D cultures. Solid circles and lines showing lactate production profile, whereas dashed lines and empty circles indicate glucose consumption.

of human origin to transform them into ATMP requires the combination of biologically active reagents and bioprocess designs that need careful consideration to ensure that the drug product remains within established specifications. However, the development program involves multiple preclinical and clinical phases, and changes of reagents, equipment and workflows are often necessary. The impact on the final product of any of such changes in the manufacturing bioprocesses must be assessed by conducting a "comparability exercise" according to "ICH Topic Q5E Comparability of Biotechnological/Biological Products" [6] and the comparability considerations for ATMP established by the Committee for Advanced Therapies [5]. In the present study, conducted in the context of a GMP-compliant validation, we evaluated the CQA of two real scale batches of MSC,WJ produced using microcarriers in 3-L single-use STR and compared them with our previously established 2D manufacturing bioprocess using historical data. First, we demonstrated the feasibility of switching from 2D to 3D bioprocess strategies for the expansion of MSC,WJ in clean room facilities with controlled environment and process controls. Although there exist other types of bioreactors (comprehensively reviewed by García-Fernández *et al.* [3]), we chose using STR for its versatility, easiness of in-process and real-time monitoring and previous experience reported in the literature with MSC. There are well-described manufacturing processes in which MSC production complies with current-standard GMP and ATMP regulations in terms of consistency, reproducibility of the bioprocess and safety of final products obtained. However, most of these processes are described in 2D planar systems, which fulfil dose requirements for small clinical trials or hospital exemption treatments in either autologous or allogeneic cell therapy. In this context, our previous experience in this field includes a description of quality controls and key technical aspects on standardization and quality assurance of ATMP based on MSC that may offer a landmark [22,23]. These manufacturing experiences are improved with the introduction of process automation with continuous closed-system culture control and monitoring that STR can offer.

Table 2
Critical quality attributes of the two validation batches of MSC,WJ produced in 3-L single-use STR.

	Acceptance criteria	Batch 1	Batch 2
Final cell viability	>70%	72.8%	84.2%
$CD45^-/CD105^+$	≥95%	35.8%	67.7%
$CD31^-/CD73^+$	≥95%	99.8%	99.8%
$CD90^+$	≥95%	99.6%	99.7%
$HLA\text{-}DR^-$	≥95%	97.3%	97.3%
Endotoxin	≤1 EU/mL	≤1 EU/mL	≤1 EU/mL
Mycoplasma	Negative	Negative	Negative
Adventitial virus	Negative	Negative	Negative
Karyotype	Non-chromosomal abnormalities	Non-chromosomal abnormalities	Non-chromosomal abnormalities
Sterility	Sterile	Sterile	Sterile
Immunomodulation	>30% inhibition of PBMC proliferation	84.3%	53.0%

EU, endotoxin units; MSC,WJ, mesenchymal stromal cells derived from Wharton's jelly; PBMC, peripheral blood mononuclear cell; STR, stirred tank bioreactor.

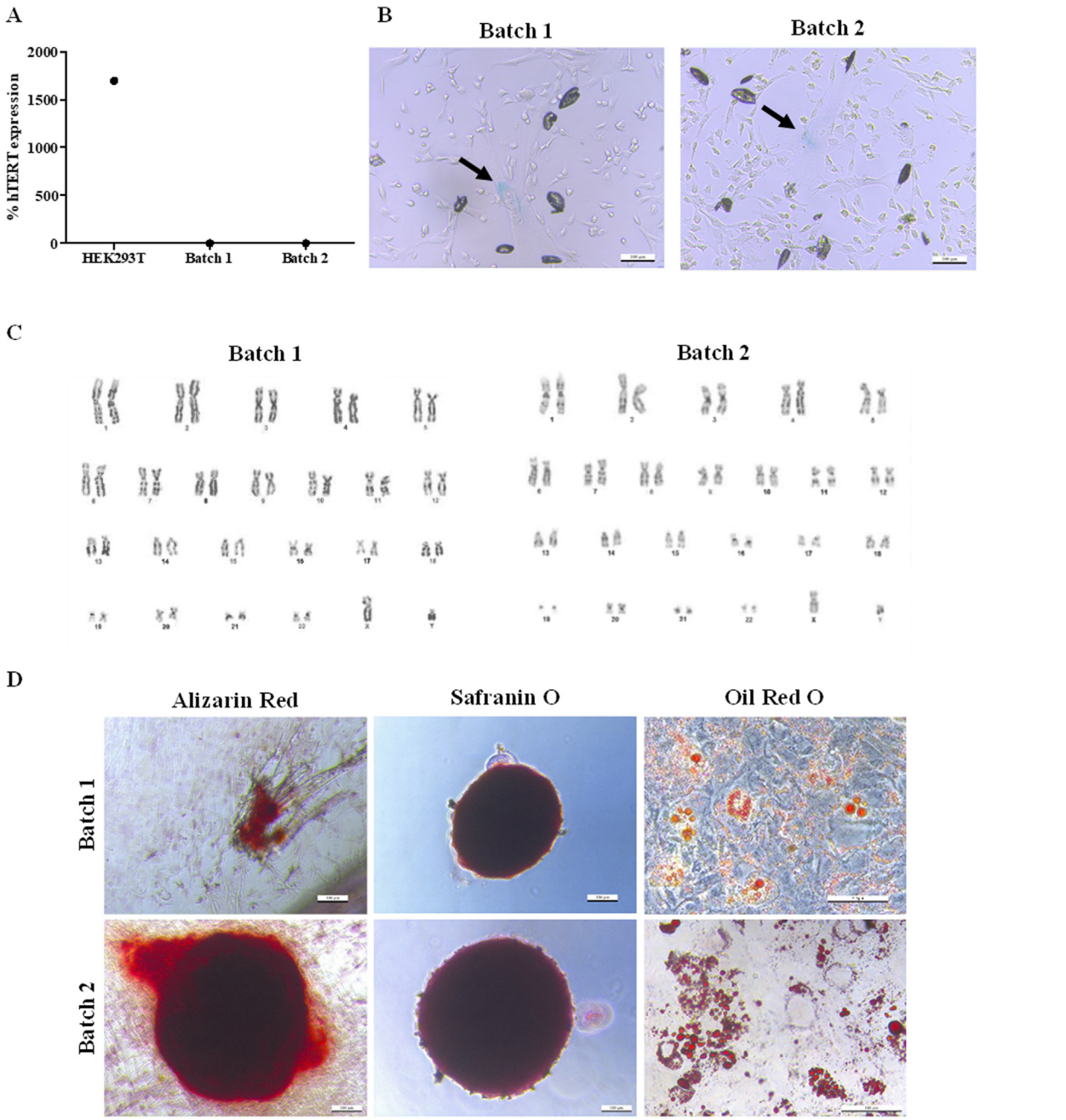

Figure 4. Characterization of expanded MSC,WJ in 3D cultures from finished products from two GMP-grade batches. (A) hTERT expression compared with the control cell line (HEK293T); (B) senescent cells stained blue (black arrow) in representative images (scale bar 100 μm); (C) representative normal karyotypes; (D) differentiation potential *in vitro* after 3D manufacturing upon induction into osteogenic (Alizarin Red staining), chondrogenic (Safranin O staining) and adipogenic (Oil Red O staining) lineages on days 34–36 (scale bar 100 μm). (Color version of figure is available online.)

A risk-based approach was performed to accomplish the regulatory requirements with the standard process (2D cultures) already applied in clinical indications that should be transferred to the modified process (3D cultures). CQAs with regard to quality, purity, potency, safety and stability of the final product, as well as its associated methods and acceptance criteria, were defined and compared with the current manufactured product. Two batches of MSC,WJ were scaled-up in 3D cultures with similar results in terms of cell productions and CQA equivalence. We performed a comparability exercise in terms of final product preservation of identity, purity, potency and safety and its comparability with current product specifications defined for 2D conditions.

Our results of expanded MSC,WJ are in line with other published studies using STR. Elseberg *et al.* [17] described similarly a total cell yield and fold increase of bone marrow–derived MSCs after 6 days of 7.4×10^8 and 7.15, respectively, in 13 650 cm^2 of total microcarrier surface. Other studies reported fold increases for umbilical cord-derived MSC in smaller STR systems of 5.6 ± 1.6 after 5 days [13], 8.9 ± 1.0 after 7 days [14] or 7.5 ± 2.0 after 5 days [29], and in all cases MSC retained their differentiation ability and immunomodulatory potential. No glucose deprivation was observed because of using high glucose concentration medium (4.5 g/L) and constantly monitored lactate concentration never reached inhibitory values greater than 35 mmol/L [30]. Harvesting efficiencies were highly variable between

both batches, mainly due to the formation of cell–microcarrier aggregates and the challenging cell retrieval from microcarriers in the downstream process. Other authors also report similar numbers accounting the same reasons [9,14,29,31]. The change in manufacturing methodology did not alter CPD, as both cultures reached similar numbers and were fewer than the safety limit of 40 CPD [32].

Although the comparability exercise of final product CQA supports a similarity of both GMP-compliant expansion methodologies, it should be assessed if all the product specifications need to be maintained. We need to take into account if the traditional criteria of MSC evaluation in accordance with the ISCT [33]. should be considered in all bioprocessing methodologies. As a matter of fact, downstream bioprocessing from 3D cultures using microcarriers has an impact in MSC immunophenotype, particularly in CD105 marker detection. The detachment of cells from microcarriers using high concentration of enzymatic reagents (i.e., 0.25% trypsin instead of 0.05% trypsin concentration traditionally used in 2D planar cultures) to increase cell recovery from cell-microcarrier aggregates is most likely the reason for reduced CD105 detection when assessed by flow cytometry. This observation has been also reported in similar studies [10,13–15,29,34,35]. Furthermore, a previous study conducted by Mendicino *et al.* [36] already stated the diversity in phenotypic marker expression in proposed MSC-based products for Investigational New Drugs to the U.S. Food and Drug Administration. However, it is noteworthy that MSC,WJ expanded in our 3D culture system preserve CD73 and CD90 marker expression, as well as differentiation and immunomodulatory potential in accordance with CQA and product specifications originally established for 2D planar culture settings. In our opinion, this situation highlights the need to redefine CQA and specifications in the 3D volumetric productivity setting due to the particularities applying to the growth of MSC on microcarriers.

This scalable closed-system bioprocess approach represents advantages in the operational and cost-efficiency of the 3D manufacturing strategy introducing benefits from product quality to economics factors. Process automation reduces labor requirements in critical manipulations and in-process variation, thus increasing operational efficiency and process stability and safety. Also, a reduction in critical equipment (i.e., humidified incubators), manufacture in Grade C or D processing areas with reduced footprint (i.e., less labor requirements for environmental and microbiological monitoring in critical manipulations) and the possibility of creating a shared versatile infrastructure across multiple cell and gene therapy products (i.e., extracellular vesicles from MSC, iPSC and derived products, T-cell based therapies) has an impact in operational costs [31,37]. Taking together all these benefits, they can represent an improvement in manufacturing cost-efficiency of ATMP.

Conclusions

Fully GMP-compliant bioprocess is feasible with single-use STR for the consistent and reproducible volumetric production of clinical-grade MSC,WJ. By conducting a formal comparability exercise in compliance with pharmaceutical guidelines, we demonstrated the successful introduction of a major manufacturing change that offers further scalability of MSC,WJ manufacture in bioreactors.

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Author Contributions

Conception and design of the study: AL-F and JV. Acquisition of data: AL-F, MC, MIC, CP-V, JC, ST and GA. Analysis and interpretation of data: AL-F, MC, CP-V, LR, JV. Drafting or revising the manuscript: AL-F, MC, CP-V, LR, SQ, and JV. All authors have approved the final article.

Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

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