

ARTICLE

Bioprocess development for cord blood mesenchymal stromal cells on microcarriers in Vertical-Wheel bioreactors

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Abstract

Equine mesenchymal stromal cells (MSCs) have been found to be beneficial for the treatment of many ailments, including orthopedic injuries, due to their superior differentiation potential and immunomodulating properties. Cell therapies require large cell numbers, which are not efficiently generated using conventional static expansion methods. Expansion of equine cord blood-derived MSCs (eCB-MSCs) in bioreactors, using microcarriers as an attachment surface, has the potential to generate large numbers of cells with increased reproducibility and homogeneity compared with static T-flask expansion. This study investigated the development of an expansion process using Vertical-Wheel (VW) bioreactors, a single-use bioreactor technology that incorporates a wheel instead of an impeller. Initially, microcarriers were screened at small scale to assess eCB-MSC attachment and growth and then in bioreactors to assess cell expansion and harvesting. The effect of different donors, serial passaging, and batch versus fed batch were all examined in 0.1 L VW bioreactors. The use of VW bioreactors with an appropriate microcarrier was shown to be able to produce cell densities of up to 1E6 cells/mL, while maintaining cell phenotype and functionality, thus demonstrating great potential for the use of these bioreactors to produce large cell numbers for cell therapies.

KEYWORDS

biomanufacturing, bioreactor, mesenchymal stromal cells, microcarrier, scale-up

1 | INTRODUCTION

Orthopedic injury leading to lameness is the most diagnosed ailment for horses. Common injuries include osteoarthritis, tendon and ligament injuries, and other soft tissue damage resulting from overuse, acute trauma, and/or developmental abnormalities. These injuries are the leading cause of retirement as well as death of horses (Ribitsch et al., 2021). Conventional treatments aim to reduce inflammation and encourage endogenous regeneration through surgical and/or nonsurgical means (including pharmacological and

rehabilitation treatments). Unfortunately, current surgical interventions are highly invasive and have a high rate of reinjury, and nonsurgical treatments have been found to have low efficacy (Dyson, 2004; Godwin et al., 2012). Mesenchymal stromal cell (MSC) injections have emerged as a promising treatment alternative to nonsurgical methods, as these cells have potent immunoregulatory abilities through secretion of inflammatory mediators (Carrade et al., 2012), with equine cord blood-derived MSCs (eCB-MSCs) showing the most potential for clinical use. This is due to the cells' high proliferation rates, immunomodulating properties, and

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differentiation potential (Carrade et al., 2012). Studies have shown that effective doses of MSCs are in the range of 10^7 – 10^8 per patient for humans, which is likely to be higher in horses due to the higher body mass (Kabat et al., 2020). Conventional methods of expanding MSCs involve static methods using culture vessels such as T-flasks or hyperflasks. However, this method of expansion is labor-intensive, expensive, has an increased risk of contamination, and can lead to an increase in heterogeneity in MSC populations, which has the potential to impact the effectiveness of MSC treatments. Expansion of MSCs using dynamic bioreactor culture with microcarriers as the attachment surface is a promising alternative to static culture to generate clinically relevant numbers of cells with increased reproducibility while limiting costs.

As MSCs are anchorage-dependent cells, microcarriers are utilized in stirred suspension bioreactor processes to provide a growth attachment surface. The choice of microcarrier in a bioreactor process is critical to ensure adequate attachment, expansion, and efficient detachment of cells. Several different types of microcarriers are commercially available, varying in porosity, matrix composition, as well as coating material. These parameters can affect cell attachment, expansion, and detachment and have been found to be specific to the cell line used (Martin et al., 2011). We have previously conducted the only known study investigating the attachment of equine-derived MSCs on microcarriers (Roberts et al., 2019). When comparing Cytodex 3, Cytodex 1, Cultispher S, Enhanced Attachment, and Synthemax microcarriers, it was found that Cytodex 3 yielded the highest cell attachment as well as expansion potential (Roberts et al., 2019). Conversely, there have been multiple studies examining the large-scale screening microcarriers for the expansion of various sources of human MSCs. Schop et al. (2010) screened nine different microcarriers for attachment potential for bone marrow-derived human MSCs (hBM-MSCs) and found that Cytodex 1 allowed for the highest attachment (Schop et al., 2010). Similarly, Loubière et al. (2019) screened five different microcarriers for the attachment of Wharton Jelly-derived human MSCs and found that Cytodex 1 and Plastic Plus microcarriers allowed for the greatest attachment and expansion (Loubière et al., 2019). Rafiq et al. (2016) performed a very extensive microcarrier screening of 13 different microcarriers with three different donors of hBM-MSCs and found that across all three donors, Collagen-Coated, Plastic, or Pronectin-F microcarriers from Sartorius allowed for the greatest expansion. Few studies have compared the harvesting efficiency from several different microcarriers (Rafiq et al., 2016). Weber et al. (2007) compared harvesting human immortalized BM-MSCs (hMSC-TERT) using different enzymes and several different microcarriers (Weber et al., 2007). They found that regardless of microcarrier type, Cytodex 1 and Cytodex 3 had the lowest harvesting efficiencies (Weber et al., 2007). Furthermore, Loubière et al. (2019) found that there was a low detachment of WJ-MSCs from Cytodex 1 microcarriers as well as low cell viabilities when detaching cells from Hillex microcarriers with TrypLE as the enzyme used (Loubière et al., 2019). These results show that cell detachment and attachment are cell type dependent and need to be optimized for a given process. Downstream separations are also a

challenge; however, this is outside the scope of the current study (reviewed in Mawji et al. 2022).

Along with different microcarriers, various dynamic bioreactor geometries have been employed. The majority of MSC microcarrier expansion processes use stirred-suspension horizontal blade impeller-type mixing, with various impellers such as marine, pitched-blade, or Rushton-type impellers. However, a new bioreactor technology developed by PBS Biotech has recently emerged utilizing a vertical wheel that combines radial and axial flow components to provide a uniform distribution of hydrodynamic forces. These bioreactors are single use, which simplifies the bioprocess due to a reduced risk of contamination, decreased turnaround time between runs, and no sterilization requirement. A study by Sousa et al. (2015) compared the use of a 3 L Vertical-Wheel (VW) bioreactor to a stirred tank bioreactor with a pitch blade impeller for expansion of hBM-MSCs and found no significant differences in cell expansion or in quality of final cell product (Sousa et al., 2015). While the VW bioreactor is a relatively new technology, several other studies have utilized this reactor type in their MSC scale-up processes, achieving final cell densities between 2 and 6×10^5 cells/mL (de Sousa Pinto et al., 2019; Lembong et al., 2020).

Other important considerations when developing a robust bioprocess include donor-to-donor variability as well as the effect of serial passaging, as both these factors will greatly affect the expansion potential of the process. Several studies have shown proliferation differences between donors in static culture in both human (Heathman et al., 2016; Phinney et al., 1999) and equine MSCs (Carter-Arnold et al., 2014). Also, a study by Panchalingam et al. compared the expansion of human BM-MSCs from three different donors and found that while different donors grew comparably in static culture, there was variability in expansion between the different donors in bioreactor expansion. This was hypothesized to be due to the bioreactor process being optimized for a single donor, indicating a need for various donors to be incorporated into bioprocess development (Panchalingam et al., 2015). Few studies have investigated serial passaging of MSCs in bioreactors. This is important because if serial passaging cannot be performed, the maximum cell expansion is severely limited, and this can also be an indication of the process being detrimental to the cells.

Using this new VW bioreactor geometry, we sought to expand eCB-MSCs on microcarriers. The objective of the study was to find an appropriate microcarrier that would facilitate attachment and detachment of the cells grown in VW bioreactors while achieving high cell densities over multiple passages and maintaining cell phenotype and functionality.

2 | MATERIALS AND METHODS

2.1 | Study design

Figure 1 shows the experimental plan of this study. The study was performed in four stages: (1) 11 different microcarriers were

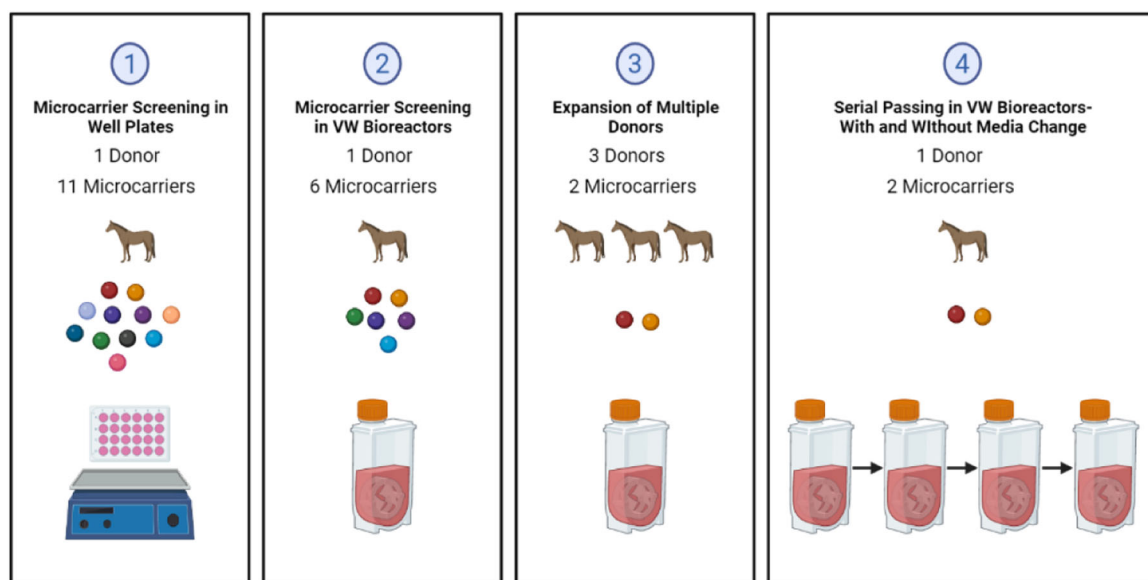


FIGURE 1 Experimental design for the process development. (1) Eleven microcarriers were screened in a 24-well plate on an orbital shaker for attachment and expansion of one donor of eCB-MSCs, (2) The top 6 performing microcarriers from (1) were screened in 0.1 L bioreactors for expansion and harvesting potential of one donor of eCB-MSCs, (3) The top 2 performing microcarriers from (2) were used to expand three eCB-MSCs donors in 0.1 L VW bioreactors, comparing expansion and harvesting potential, (4) the same two microcarriers from (3) were used to expand one donor of eCB-MSCs over four serial passages, comparing batch culture to fed-batch culture. eCB-MSC, equine cord blood-derived mesenchymal stromal cell.

screened for attachment and expansion potential of eCB-MSCs in 24-well plates on an orbital shaker, (2) 6 different microcarriers were screened for expansion and harvesting potential in 0.1 L VW bioreactors, (3) 2 different microcarriers were then used to expand three different donors in 0.1 L VW bioreactors, and (4) 2 different microcarriers were used with one donor to serial passage eCB-MSCs through four passages in 0.1 L VW bioreactors.

2.2 | Static culture of eCB-MSCs

Cord blood from three different foals was collected immediately after birth and the eCB-MSCs were isolated as previously described (Koch et al., 2009). The donors are referred to as Donor 1801, collected from a female thoroughbred, Donor 1810, collected from a male warmblood, and Donor 1811, collected from a male warmblood. Donor 1811 was used for all experiments, other than the expansion of multiple donors in 0.1 L VW bioreactors, in which all three donors were used. A cell bank of passage four cells was established using culture media containing Dulbecco's Modified Eagle Medium (Multicell Cat#319-313-CL), 10% fetal bovine serum (FBS) (Multicell Cat#090150), 200mM L-glutamine (Multicell Cat#609-065-EL), and 5 ng/mL bFGF (Sigma Aldrich Ca#F0291). Cells were seeded in 75 cm² T-flasks (Thermo Fisher Ca#156499) at a density of 5000 cells/cm² and expanded for 5 days with a full media change performed on Day 3. Cells were detached using TrypLE (Gibco Ca#12605-208) and centrifuged for 5 min at 300g. The same culture

media was used throughout this study for both static and bioreactor culture.

2.3 | Microcarrier preparation

All microcarriers were prepared using the same protocol independent of whether hydration of the microcarrier was required. Microcarrier density corresponding to 5.4 cm²/mL was weighed and transported to a siliconized Erlenmeyer flask with 50.0 mL 1X PBS (without calcium and magnesium) with 50 U/mL penicillin/streptomycin for 24 h. The microcarriers were then sterilized by autoclaving before inoculation into the bioreactors. The microcarriers were inoculated into the bioreactors with culture media and incubated at 37°C and 5% CO₂ for 24 h before cells were added to condition the microcarriers for cell attachment.

2.4 | Microcarrier screening in 24-well plates

Microcarrier screening was performed in 24-well plates to investigate cell attachment on 11 different microcarriers (as shown in Table 1). eCB-MSCs and microcarriers were inoculated into 24-well plates at a density of 5000 cells/cm² in 1.0 mL of media, with four wells per condition (two wells for two different time points). The well plates were placed on a shaking platform (Scientific Excella e5) at 100 rpm, at 37°C and 5% CO₂. At Days 3 and 5, the microcarrier-media suspension was removed from the wells, rinsed with 1.0 mL 1X PBS,

TABLE 1 Properties of the different commercial microcarriers used in this study.

Microcarrier	Diameter (μm)	Surface Area (g/cm ²)	Matrix	Density (g/mL)	Coating
Cytodex 1	147–248	4400 (dry g/wet cm ²)	DEAE-dextran	1.03	None (+charge)
Cytodex 3	141–211	2700 (dry g/wet cm ²)	DEAE-dextran	1.04	Porcine denatured collagen
Cytopore 1	200–280	11,000	Porous DEAE- cellulose	1.03	None (1.1 meq/g + charge)
Cytopore 2	200–280	11,000	Porous DEAE- cellulose	1.03	None (1.8 meq/g + charge)
Cultispher S	130–380	7500	Porous gelatin	N/A	None
Plastic	125–212	360	Cross-linked polystyrene	1.02–1.03	None
Plastic +	125–212	360	Cross-linked polystyrene	1.02–1.03	None (+charge)
Hillex II	160–200	515	Modified polystyrene	1.08–1.15	None (+charge)
Star +	125–212	360	Modified polystyrene	1.02–1.03	None (+charge)
Fact III	125–212	360	Cross-linked polystyrene	1.02–1.03	Type 1 porcine collagen (+charge)
Collagen Coated	125–212	360	Cross-linked polystyrene	1.022–1.03	Type 1 porcine collagen

and stained with SYTO 24 (Thermo Fisher Ca#S7559) to visualize the attachment of cells on the microcarriers.

2.5 | Bioreactor culture of eCB-MSCs

This study used 0.1 L single-use VW bioreactors (PBS Biotech). Bioreactors were initiated with media and microcarriers at 37°C and 5% CO₂ at 55% of working volume, 1 day before cell inoculation to allow for conditioning of microcarriers. Cells were then added with more media and the volume was increased to 70% working volume. After 24 h, media was added to increase the volume to 110.0 mL. For all microcarriers other than Hillex II, the bioreactors were run at 30 rpm. The bioreactors using Hillex II were run at 60 rpm due to the increased density of the microcarrier requiring a higher agitation to adequately suspend them. To determine an attached cell density, 2.0 mL samples were removed from the middle of the bioreactors while the bioreactors were under agitation. The samples were rinsed 2X with 1.0 mL PBS, and 0.1% crystal violet (CV) with 0.1 M citric acid was added to lyse the cells and dye the nuclei, which were then counted on a hemocytometer, where the opening of the hemocytometer is small enough to prevent microcarriers from entering the counting field of view. Attached cell densities were then calculated based on the number of nuclei counted, and the surface area of microcarriers within the bioreactor was determined based on the manufacturer's data of the microcarriers. This method is referred to as the CV staining method. Additional 1.0 mL samples were taken from the bioreactors, rinsed with 1.0 mL 1X PBS, and stained with SYTO 24 (Thermo Fisher Ca#S7559) to visualize the attachment of cells on the microcarriers.

The effect of media change was investigated in this study, where batch conditions (no media change) were compared with fed-batch conditions. In the fed-batch conditions, a 50% media change was performed on Day 3 of culture. Additionally, for the Hillex II microcarriers fed-batch condition, folic acid at a concentration of

10 μg/mL was added daily, as it has been reported that Hillex II microcarriers absorb folic acid.

2.6 | Harvesting of eCB-MSCs from microcarriers

To harvest the 0.1 L VW bioreactor, for all microcarriers except Cultispher S, agitation was stopped and the microcarriers were allowed to settle. Next, 80.0 mL culture media was removed, and the microcarriers were rinsed with 50.0 mL 1X PBS. The PBS was removed and 50.0 mL TrypLE was added. The bioreactor was then incubated at 37°C and 5% CO₂ for 10 min at 100 rpm. The cell suspension was then filtered through a 70 μm sieve and centrifuged for 5 min at 300g. To harvest Cultispher S microcarrier, the manufacturer's protocol for dissolving the microcarrier was used. Dispase (Sigma Aldrich Ca#D4693) was dissolved in PBS at a concentration of 5 mg/mL, and 8 mg Dispase per mg of Cultispher S microcarrier was added to the bioreactor. The bioreactor was then incubated at 37°C and 5% CO₂ at 100 rpm for 30 min until the microcarriers had dissolved. The cell suspension was then centrifuged. To determine harvesting efficiencies, Equation 1 was used.

$$\text{Harvesting Eff. (\%)} = \frac{\text{Cells recovered after detachment and filtering}}{\text{Total cells in bioreactor based on CV staining method}} \quad (1)$$

2.7 | Cell characterization

2.7.1 | Peripheral blood mononuclear cell (PBMC) proliferation assay

PBMCs from five unrelated equine donors were isolated and frozen as described in Lepage et al. (2019). To assess the effect of eCB-MSC coculture with PBMCs on their proliferation, frozen PBMCs from all five donors were thawed, pooled in equal ratios, then incubated in

complete RPMI medium (RPMI 1640, 100 IU penicillin-streptomycin, 2 mM L-glutamine, and 10% horse serum) overnight. eCB-MSCs from each condition (Bioreactor expanded: Hillex fed batch, Hillex batch, collagen-coated fed batch, and collagen-coated batch; Static expanded: static fed batch and static batch) were seeded at 10,000 cells/well in a 48-well plate in MSC culture medium and incubated overnight.

The next day, PBMCs were labeled using the CellTrace™ CFSE Cell Proliferation Kit (ThermoFisher) according to the manufacturer's instructions. PBMCs were then activated with Concanavalin A mitogen (Sigma; final concentration: 5 µg/mL); negative control PBMCs were not activated with mitogen. MSCs were washed 1X with PBS before adding activated PBMCs in complete RPMI medium at a ratio of 10:1 (PBMC:MSC). After 5 days, PBMCs were assessed via flow cytometry (BD Accuri) to determine their level of proliferation. PBMCs were washed 1X in PBS before resuspending in the flow buffer. Dead cells were excluded from the analysis via the addition of 7-AAD dye.

2.7.2 | Flow cytometry

eCB-MSCs from each condition were assessed via flow cytometry to determine levels of surface marker expression. Flow was performed as previously described in Lepage et al. (2019) using antibodies described in Table 2. Isotype controls and secondary antibodies are described in Lepage et al. (2019).

2.8 | Statistical analysis

Statistical analysis was performed using GraphPad Prism (v9.3.1). A two-way analysis of variance (ANOVA) with the Tukey multiple comparison test was performed for the microcarrier screening, expansion of multiple donors' experiment, as well as the serial passing experiments. For the flow cytometry analysis, a one-way ANOVA with the Tukey multiple comparisons test was done. For the microcarrier screening experiments and multiple donor experiments, only $n = 1$ for bioreactor and $n = 2$ for static were performed, as these

were preliminary screening tests. Two samples were taken from each vessel (either bioreactor or static) and each was counted twice. For the serial passing experiments, $n = 2$ for the bioreactor and $n = 4$ for static were performed. For the flow cytometry and the PBMC assay, $n = 1$ was done. Significance was assigned as $p < 0.05$. All graphs are presented with error bars representing the standard deviation.

3 | RESULTS

3.1 | Microcarrier screening in 24-well plates

Figure 2 shows images of attached eCB-MSCs to the 11 different microcarriers after 5 days in well plates. Star +, Plastic +, and Plastic microcarriers all had very low cell attachment and expansion. Fact II and Cytodex 1 microcarriers both supported some cell expansion; however, the distribution of cells on the microcarriers was very clumpy. Cell expansion appeared to be highest on Hillex II, Cytodex 1 and 2, Cytodex 3, Cultispher S, and Collagen Coated. Using these results, six microcarriers were then selected to be screened using the 0.1 L VW bioreactors. The five microcarriers that were eliminated at this step were Star +, Plastic +, and Plastic as they did not support cell attachment or growth, as well as Cytodex 1 and Cytodex 2, as both these microcarriers are typically used for applications where the cells are not the desired product as it is difficult to harvest the cells from these microcarriers. While Fact III and Cytodex 1 had a very clumpy distribution of cells, the hydrodynamic forces in a well plate are not representative of the forces in a bioreactor; therefore, these microcarriers were also selected to be tested in the 100 mL bioreactor to investigate whether the clump distribution was still observed.

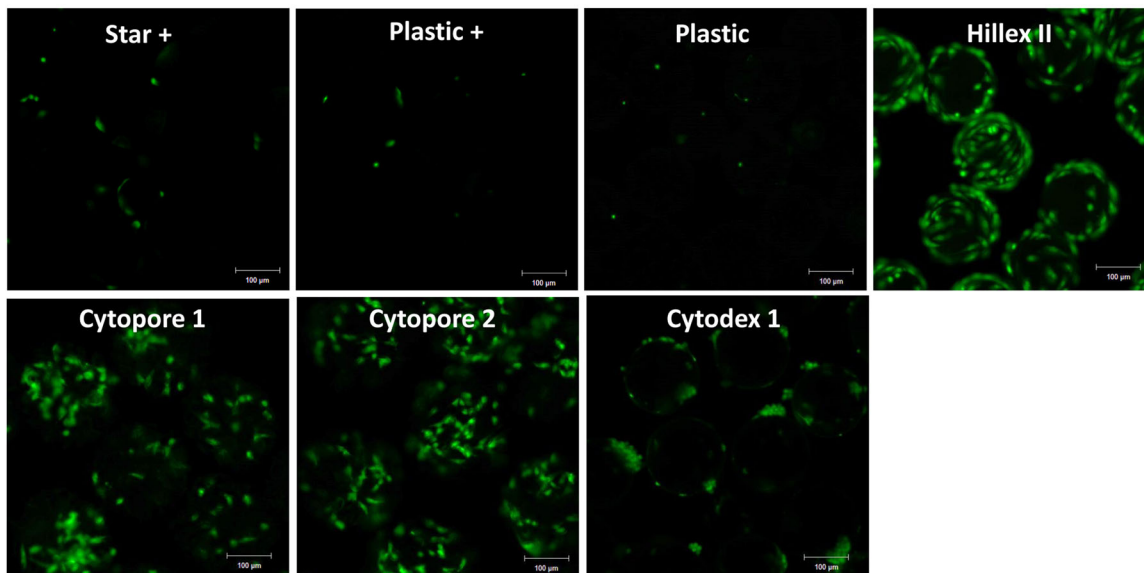
3.2 | Microcarrier screening in 0.1 L VW bioreactors

Cytodex 1 and 3, Hillex II, Cultispher S, Fact III, and Collagen-Coated microcarriers were used to expand eCB-MSCs in 0.1 L VW bioreactors as shown in Figure 3. Figure 3a shows the attached cell densities on the different microcarriers throughout a 5-day culture period in the

TABLE 2 Antibodies used for flow cytometry experiments.

Antibody	Reactivity	Company	Conjugate	Clone	Reference
CD29	Horse	BioLegend	APC	TS2/16	Esteves et al. (2017)
CD44	Horse	BioRad	Purified	CVS18	Esteves et al. (2017)
CD90	Rat	BioRad	FITC	OX7	Esteves et al. (2017)
CD105	Horse	BioRad	FITC	5F/B9	Esteves et al. (2017)
CD73	Human	BD	PE	AD2	N/A
CD146	Human	BioRad	FITC	OJ79c	Esteves et al. (2017)
MHC I	Horse	BioRad	FITC	CVS22	Tessier et al. (2015)
MHC II	Horse	BioRad	FITC	CVS20	Tessier et al. (2015)

Animal Component Free Microcarriers



Animal Component Containing Microcarriers

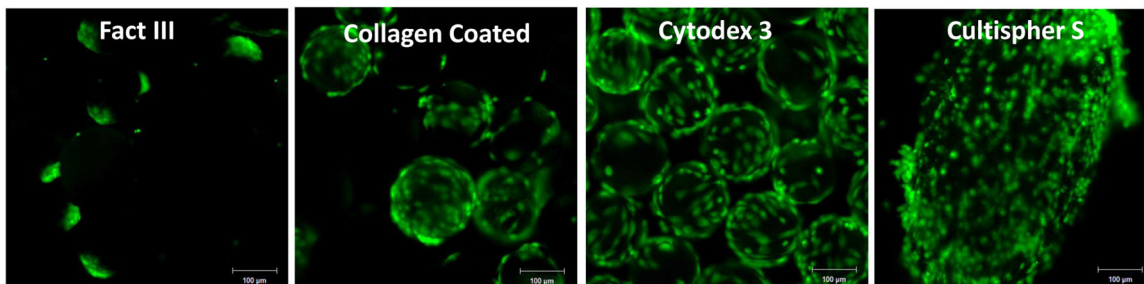


FIGURE 2 Images of eCB-MSCs expanded on 11 different microcarriers in 24-well plates on an orbital shaker after 5 days. Cells are stained with SYTO 24 nuclei stain. eCB-MSC, equine cord blood-derived mesenchymal stromal cell.

bioreactors. After 5 days in culture, the cells were harvested from the microcarriers to determine harvesting efficiencies, as seen in Figure 3b. For Cultispher S, Dispase was used to digest the microcarriers, while for the other four microcarriers, TrypLE was used to remove the cells from the surface of the microcarriers. Fact III microcarriers were not harvested as the expansion was too low. The harvesting efficiency was significantly higher from Hillex than the other four microcarriers. Both Cytodex 3 and Cytodex 1 had extremely low harvesting efficiencies (<15%), consistent with previous experiments performed by our group with Cytodex 3, where even enzyme incubation times of up to 1 h did not detach the cells. Figure 3c shows images of eCB-MSCs on the different microcarriers. Both Hillex II and Collagen-Coated microcarriers supported significantly higher attached cell densities than the other four microcarriers.

3.3 | Expansion of multiple donors in 0.1 L VW bioreactors

To further assess the expansion potential of eCB-MSCs in VW bioreactors, as well as determine which microcarrier to choose for

the process, eCB-MSCs from three different donors were expanded in 0.1 L VW bioreactors with either Collagen-Coated or Hillex II microcarriers as seen in Figure 4. The bioreactors were run for 5 days and then harvested to evaluate differences in both expansion and harvesting from donor to donor and to compare to the same three donor cells expanded in static T-flasks. Figure 4a shows the cell densities over the 5 days for all cells in both VW bioreactors and static culture. The bioreactors outperformed the static cultures for all donors. Figure 4b shows the fold increase of the different donors on the two microcarriers as well as in static. For all three donors, significantly higher attached cell densities were achieved on Hillex II microcarriers when compared with Collagen Coated or static. Additionally, all donors had significantly improved expansion on microcarriers when compared with static. This is also evident in the photomicrographs, which show extensive coverage of microcarriers with cells for all three donors (Figure 4c). Figure 4d shows the harvesting efficiency of removing eCB-MSCs from different microcarriers. The only statistically significant difference was with donor 1801, in which the harvesting efficiency was greater using Hillex II when compared with Collagen-Coated microcarriers.

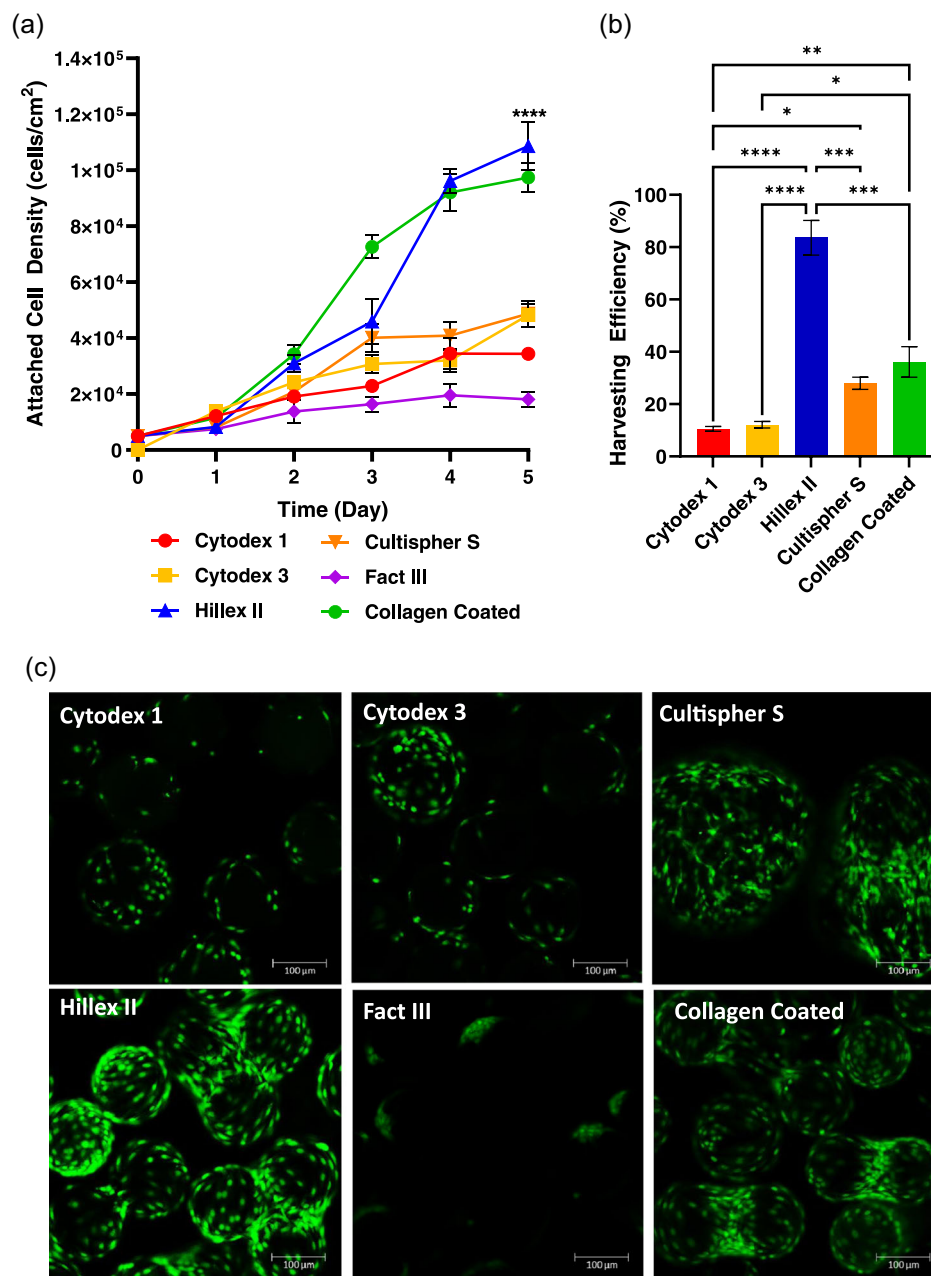


FIGURE 3 (a) Attached cell densities of eCB-MSCs on six different microcarriers through 5 days of culture in 0.1 L VW bioreactors. (b) Harvesting efficiency of removing the eCB-MSCs from the six different microcarriers on Day 5 in 0.1 L VW bioreactors. (c) Images of eCB-MSCs on the six different microcarriers on Day 5 of culture in 0.1 L VW bioreactors. Cells are stained with SYTO 24 nuclei stain. Errors bars represent standard deviation. eCB-MSC, equine cord blood-derived mesenchymal stromal cell; VW, Vertical-Wheel.

3.4 | Serial passaging in 0.1 L VW bioreactors

To assess whether both microcarriers allowed for long-term expansion of eCB-MSCs, a serial passaging experiment was performed with donor 1811. Both Hillex II and Collagen-Coated microcarriers were used in VW bioreactors and batch and fed batch (full media change on Day 3) conditions were compared over four serial passages. Figure 5a shows the attached cell densities over the 20 days in culture, and Figure 5b shows the fold increase

over the four passages for the different conditions. For cells grown on Hillex in batch culture conditions, there were significant differences in expansion between the four different passages. Between the first, second, and third passages, there was a significant decrease in the growth of eCB-MSCs on Hillex, while during the fourth passage, the expansion increased slightly. However, for cells grown on Hillex under fed-batch conditions, there was no significant difference in fold increase between the first three passages, and the only significant differences occurred

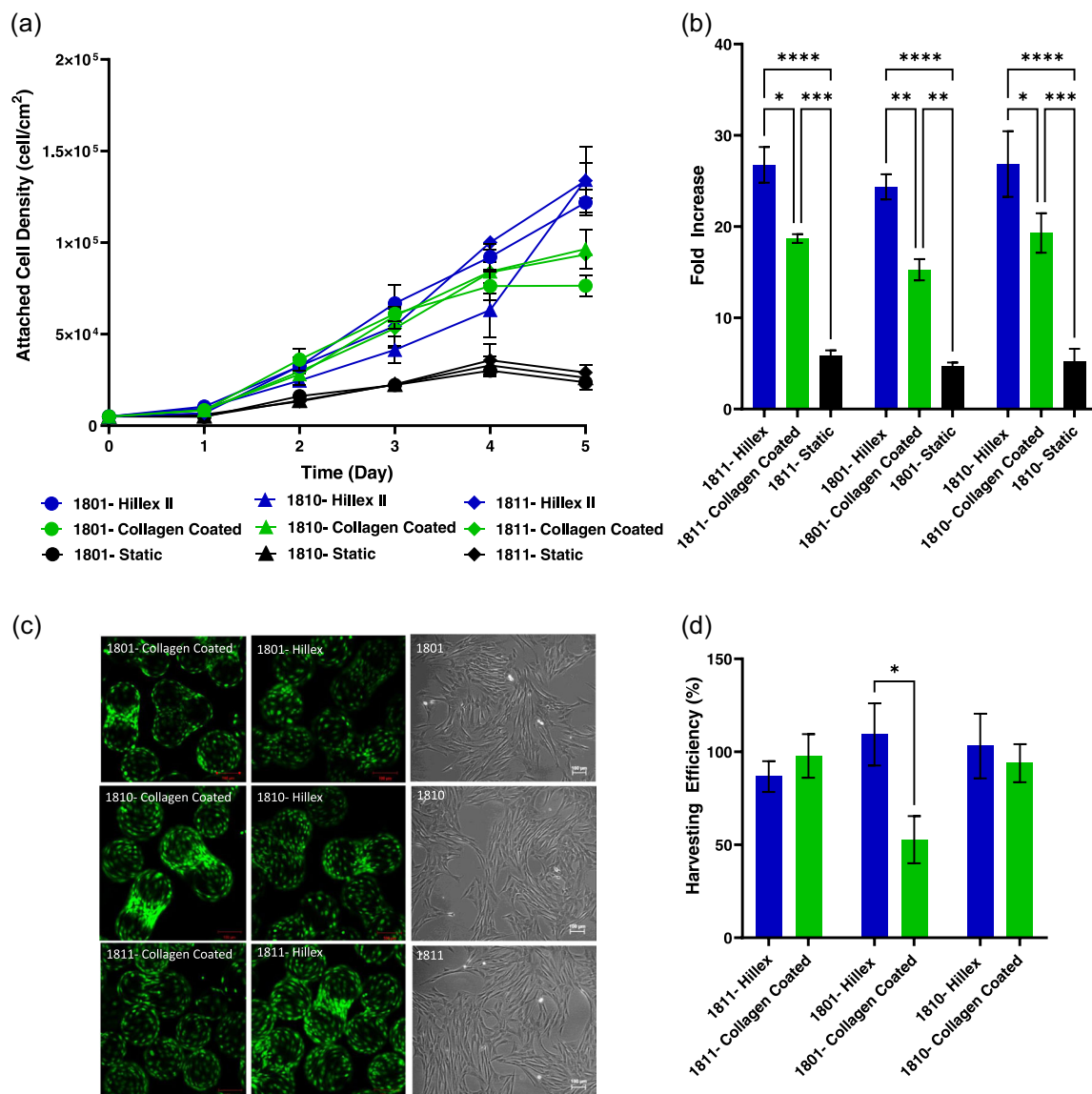


FIGURE 4 (a) Attached cell densities of three different donors of eCB-MSCs on Hillex II and Collagen-Coated microcarriers in 0.1 L VW bioreactors as well as in static T-flask culture. (b) Fold increase over 5 days in the culture of three different donors of eCB-MSCs on Hillex II and Collagen-Coated microcarriers in 0.1 L VW bioreactors as well as in static T-flasks. (c) Images of three different eCB-MSCs donors on Hillex II and Collagen-Coated microcarriers in 0.1 L VW bioreactors as well as static T-flasks on Day 5 of culture. Cells are stained with SYTO 24 nuclei stain. (d) Harvesting efficiency of removing three different eCB-MSC donors from Hillex II and Collagen-Coated microcarriers from 0.1 L VW bioreactors of Day 5 of cell culture. Error bars represent standard deviation. eCB-MSC, equine cord blood-derived mesenchymal stromal cell; VW, Vertical-Wheel.

between passage 1 and 4 and passage 2 and 4 (Figure 5b). For the Collagen-Coated fed-batch condition, the only significant differences were between the first and third passage and the first and fourth passage. There were no differences between any of the four passages for the Collagen-Coated batch condition or either of the static conditions. Figure 5c shows the harvesting efficiency of the four different bioreactor conditions at Day 5 of each passage. There was a significant decrease in the harvesting potential of eCB-MSCs on Hillex II microcarriers expanded in batch culture between the first passage and the second and third passage.

3.5 | Functional and phenotypic testing

To assess whether different culture conditions affect the immunosuppressive capabilities of the cells, expanded eCB-MSCs from donor 1811 were harvested from bioreactors or T-flasks and then cocultured with pooled PBMCs from five donors that were stimulated to proliferate with mitogen. All treatment groups were capable of suppressing PBMC proliferation after 5 days of coculture compared with positive control (activated PBMCs only) as measured by CFSE staining intensity via flow cytometry (Figure 6a).

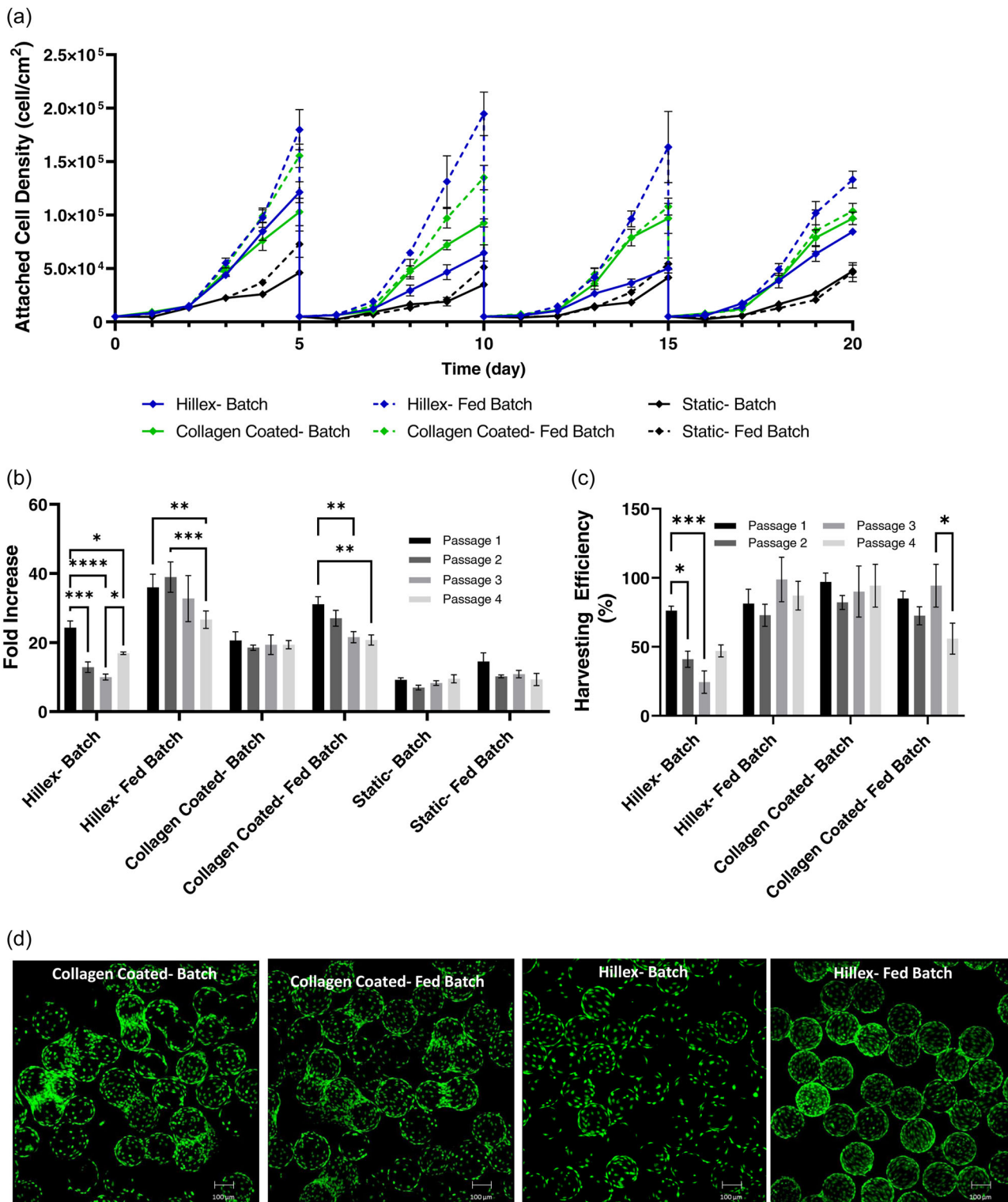


FIGURE 5 (a) Attached cell densities of eCB-MSCs growing on Hillex and Collagen-Coated microcarriers in 0.1 L VW bioreactors as well as static T-flasks in both batch and fed-batch culture over four different serial passages. (b) Fold increase over four passages of eCB-MSCs growing on Hillex and Collagen-Coated microcarriers in 0.1 L VW bioreactors as well as static T-flasks in both batch and fed-batch culture over four serial passages. (c) Harvesting efficiency of removing eCB-MSCs from Hillex II and Collagen-Coated microcarriers on Day 5 of each serial passage. (d) Images of eCB-MSCs growing on Hillex II and Collagen-Coated microcarriers in batch and fed-batch conditions on Day 5 of passage 3. Cells are stained with SYTO 24 nuclei stain. eCB-MSC, equine cord blood-derived mesenchymal stromal cell; VW, Vertical-Wheel.

Expanded eCB-MSCs were also examined for expression of equine MSC markers using flow cytometry (Figure 6b). Cells expanded in all conditions showed high expression of equine MSC markers CD29, CD44, and CD90 and no expression of hematopoietic/endothelial markers CD45 and CD34. When measured using flow cytometry, MSC markers CD73 and CD105 typically show variable expression on equine MSCs (de Schauwer et al., 2012; Esteves et al., 2017; Lepage et al., 2019; Spaas et al., 2013; Tessier et al., 2015). Our preliminary data on donor 1811 is consistent with these reports; however, equine MSCs grown in static culture may display a higher expression of CD73 and CD105 compared with MSCs grown in bioreactors. We also detected moderately high MHC I expression across all groups and no MHC II expression, which is consistent with the literature on MHC expression in equines (Kamm et al., 2019). CD146, a pericyte marker, was expressed at low levels

on MSCs grown in static culture and on Collagen-Coated microcarriers in bioreactors but was expressed at moderate levels when grown on Hillex-coated microcarriers. However, it is not yet known whether these differences are consistent among different MSC donors.

4 | DISCUSSION

In this study, a bioprocess was developed to expand eCB-MSCs in VW bioreactors. Typically the first step in a MSC-based bioreactor process is a series of experiments to determine an optimal microcarrier to maximize cell attachment and growth while allowing for detachment from the microcarrier at the end of the process.

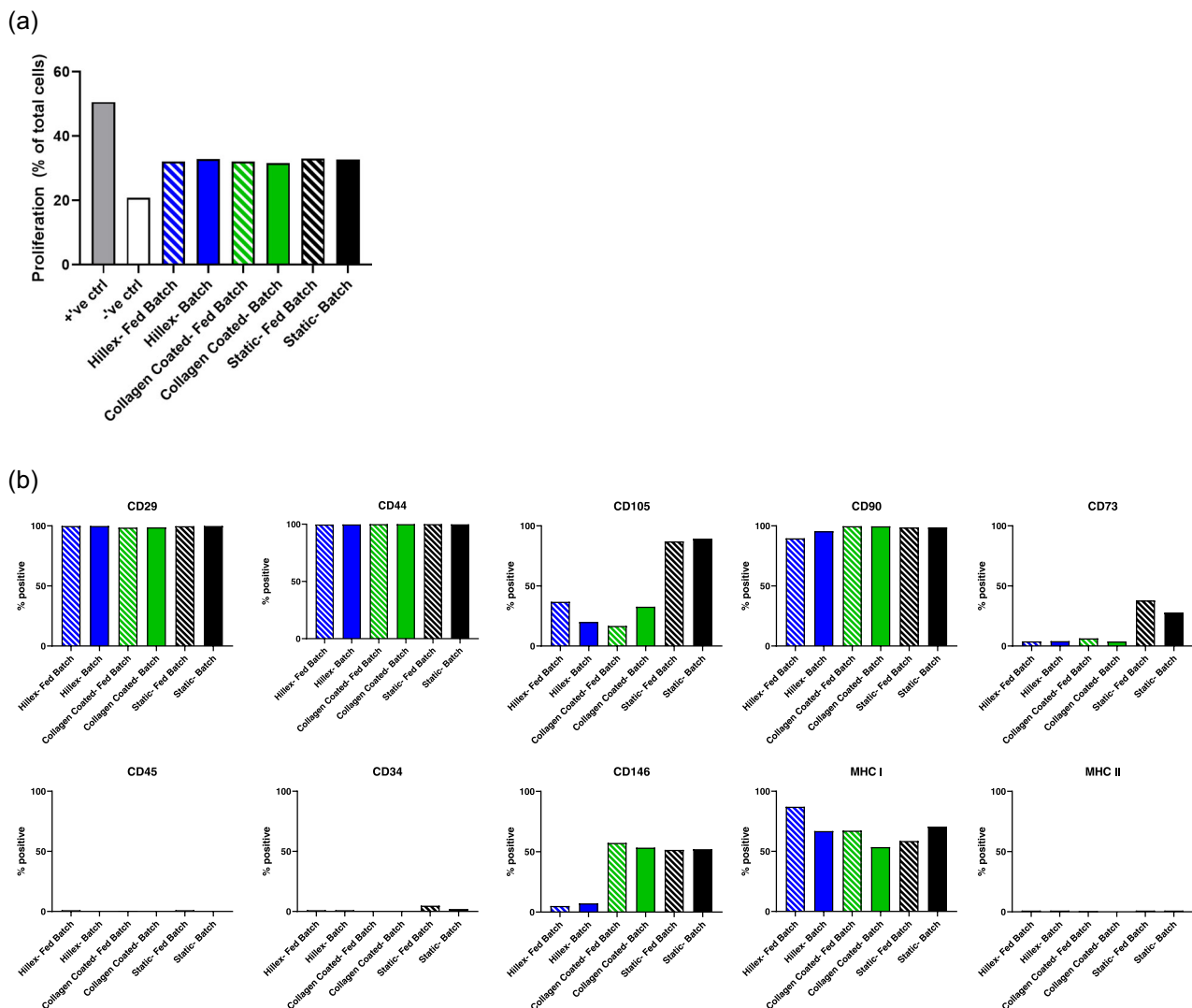


FIGURE 6 Phenotypic and functional characteristics of eCB-MSCs on Hillex II and Collagen-Coated microcarriers in 0.1 L VW bioreactors as well as in static T-flask culture. (a) PBMC proliferation assay. PBMCs stained with CFSE and activated with ConA were cocultured with eCB-MSCs expanded under different conditions. Positive control (+ve ctrl): Activated PBMCs without MSC coculture. Negative control (-ve ctrl): Inactivated PBMCs without MSC coculture. (b) Surface marker analysis of eCB-MSCs via flow cytometry. eCB-MSC, equine cord blood-derived mesenchymal stromal cell; MSC, mesenchymal stromal cell; PBMC, peripheral blood mononuclear cell; VW, Vertical-Wheel.

The first microcarrier screening experiment in this study consisted of screening 11 different microcarriers using well plates on an orbital shaker. Seven microcarriers were animal component-free and four contained animal components. Of the animal component-free microcarriers, Hillex II, Cytopore 1, and Cytopore 2 performed the best, all of which have positively charged surfaces, important for cell attachment when animal-derived components are not used. Both Plastic and Plastic Plus microcarriers had very poor cell attachment, contradicting the findings of Loubière et al. (2019) and Rafiq et al. (2016), which found enhanced attachment and expansion of cells using these microcarriers. However, both these studies used human MSCs from different sources (Loubière et al., 2019; Rafiq et al., 2016). All of the animal component-free microcarriers allowed for cell attachment; however, cells were poorly distributed on Fact II microcarriers compared with the other microcarriers. The superior attachment and expansion of eCB-MSCs on Collagen Coated is similar to the results found by Rafiq et al. (2016) for the expansion of hBM-MSCs.

Based on this preliminary microcarrier screening experiment, several microcarriers were eliminated from the process development study. Star, Plastic, and Plastic Plus were all eliminated due to the very low attachment of cells to the microcarriers. Cytopore 1 and Cytopore 2 were both eliminated due to the porous, nonbiodegradable nature of the microcarriers, as this can make it difficult to harvest cells from these microcarriers. The remaining six microcarriers: Hillex II, Cytodex 1, Cytodex 3, Cultispher S, Collagen Coated, and Fact III, were then tested for expansion and harvesting potential in 0.1 L VW bioreactors. While microcarrier screening in shaken 24-well plates provides a high-throughput method of screening the ability for cells to attach to a certain substrate, there are different shear forces present in an orbiting well plate compared with a stirred bioreactor; therefore, these results need to be further validated. Expansion of eCB-MSCs in 0.1L VW bioreactors was significantly higher on Collagen-Coated and Hillex II microcarriers over the other four microcarriers with cell densities of 2×10^5 cells/mL and 5.9×10^5 cells/mL after 5 days. These cell densities were significantly higher than studies by Rafiq et al. (2016) and Loubière et al. (2019) but slightly lower than a study by Lawson et al. (2017) who used Collagen-Coated microcarriers to expand human BM-MSCs. Specifically, Loubière et al. (2019) had very low cell expansion on Hillex II; however, they were using serum-free media, and as Hillex II is an uncoated microcarrier, this likely affected cell attachment and expansion. Cell harvesting from different microcarriers was also examined. The bioreactor containing Hillex II had the highest harvesting efficiency; however, as cells were also dislodged from Collagen-Coated microcarriers with the basic cell removal protocol, it is predicted that optimizing this protocol with respect to time of exposure to the enzyme as well as agitation rate could result in a higher efficiency. Dispase was used to degrade the gelatinous matrix of Cultispher S. The concentration of dispase and time of exposure were based on the manufacturers' recommended protocol. However, there appeared to still be a small amount of gelatin remaining in the culture, with large cell clumps,

indicating that the microcarriers had not completely been degraded. Therefore, if the protocol was optimized, likely a higher harvesting efficiency could be achieved. The bioreactors containing both Cytodex 1 and Cytodex 3 had very low harvesting efficiencies consistent with other studies, limiting their use in processes that require cells to be released from the microcarriers (Loubière et al., 2019; Weber et al., 2007). Based on significantly higher cell expansion when using Hillex II and Collagen-Coated microcarriers, these two were selected to test further in process development.

As numerous studies have reported significant variation in expansion between different MSC donors in static culture (Burk et al., 2013; Phinney et al., 1999; Ranera et al., 2012), the next step in the study was to compare three different donors for expansion potential using both Collagen-Coated and Hillex II microcarriers. There was very little variability between cells from multiple donors in the process, indicating that this is a robust process that can be used to expand a variety of MSCs from donor horses of different sex and breed. While there were no known studies of variability between donors in harvesting efficiencies, there have been reports of variability in expansion potential between donors; therefore, this is also an important parameter to investigate. Specifically, donor 1801 did have significant differences when harvested on Hillex II and Collagen-Coated microcarriers likely due to the heterogeneity between different donors. As Hillex II and Collagen-Coated microcarriers both achieved high cell densities, significantly higher than static culture, both microcarriers were selected to continue in the process development.

Serial passaging experiments are important to determine if a process is sufficient for expansion through multiple passages, as multiple passages are often required to achieve large cell quantities from small cell aliquots. Cells in the Collagen-Coated batch condition and both static conditions showed consistent cell expansion (fold increase) over multiple passages; this was likely due to the lower expansion at each passage; therefore, the cells experienced fewer population doublings than other conditions. Cells cultured on Hillex microcarriers, both in batch and fed-batch conditions, as well as Collagen-Coated fed batch, showed a decrease in fold expansion in later passages. Since cells that had higher fold increases (FI) per passage would have gone through more doublings, a decrease in later passages is most likely due to cells reaching a cell-division limit. After four passages, the theoretical cumulative fold increase (CFI) was higher in all the bioreactor conditions (Hillex batch: $CFI = 5.28 \times 10^4$, Hillex fed batch: $CFI = 1.22 \times 10^6$, Collagen-Coated batch: $CFI = 1.41 \times 10^5$, Collagen-Coated fed batch: $CFI = 3.75 \times 10^5$) than static conditions (static batch: $CFI = 5.04 \times 10^3$, static fed batch: $CFI = 1.50 \times 10^4$).

The harvesting efficiency from microcarriers showed some variability between the different conditions and over passages. Efficiencies were similar across all microcarriers after the first passage, but then the Hillex batch condition significantly decreased in future passages. Hillex II microcarriers are known to absorb certain components, noticeably the phenol red in the media, causing the media to become clear and the microcarriers to turn from clear to a

red color. The manufacturer reported that folic acid is the only compound that Hillex II absorbs that is significant to cell growth, which was why folic acid was added daily in the fed-batch condition. However, it is unknown if there could be other compounds, specific to certain cells, that Hillex absorbs. The decrease in expansion and harvesting potential after passage 1 could be attributed to important components being absorbed by Hillex II, causing decreased expansion, as well as potentially altering cell properties or selecting for a subpopulation of cells. This was not the case in fed-batch Hillex where the components would be replaced. However, as the three other bioreactor conditions did not experience this, this serial passaging experiment displayed that eCB-MSCs could be expanded to high cell densities over several passages and that a media change enhanced the growth.

In addition to evaluating harvest efficiency and expansion of the equine CB-MSCs on different microcarriers, we also sought to analyze MSC identity via surface marker expression and *in vitro* function as a proxy for their proposed immunomodulatory capabilities *in vivo*. MSC identity through surface marker expression profiling has been challenging in the horse due to the variability of expression across cell populations. This variability is consistent with the heterogeneous nature of MSC populations derived from one donor, so we investigated whether eCB-MSC expansion on Hillex and Collagen-Coated microcarriers affected surface marker expression compared with traditional static culture. Unsurprisingly, commonly expressed surface markers associated with equine MSCs (CD29, CD90, and CD44) were found on nearly 100% of cells in all treatment groups and are consistent with MSCs grown in spinner flask bioreactors (Roberts et al., 2019). CD73 expression, while commonly found on human MSCs, typically displays zero to low expression on equine MSCs (de Schauwer et al., 2012; Esteves et al., 2017; Lepage et al., 2019; Tessier et al., 2015). Our preliminary data appears to follow this trend, with expression levels ranging from 3.8% to 37.9%, with static-expanded cells displaying higher levels of expression. As CD73 is expressed on a multitude of other cell types, including cancer cells (Wang et al., 2013), it is difficult to ascertain whether this variability has any functional correlation.

CD105 and CD146 expression also displayed variability among treatment groups. CD105 expression on equine MSCs has been controversial in the literature (de Schauwer et al., 2012; Esteves et al., 2017; Spaas et al., 2013; Tessier et al., 2015), but our group has recently shown that cord-derived equine MSCs grown in conventional flasks express CD105 (Lepage et al., 2019). Our preliminary data suggests that eCB-MSCs grown on Hillex and Collagen-Coated microcarriers express less CD105 than their static-expanded counterparts. While unconfirmed, CD105 may play a role in MSC adhesion and/or proliferation (Cleary et al., 2016), and thus the surface properties of the microcarriers versus tissue culture plastic may influence the expression of CD105.

CD146+ MSCs have been shown to have superior proliferative ability and functional activity compared with CD146- MSCs (Zhang et al., 2022). We have previously demonstrated that equine CB-MSCs expanded in traditional static culture express CD146 at higher levels

than donor-matched cord tissue-derived MSCs, and this correlated with superior differentiation potential (Lepage et al., 2019). Our current data suggests that eCB-MSCs grown in static culture or Collagen-Coated bioreactor culture express moderate levels of CD146, but Hillex-expanded cells express low levels of CD146. This observation may be linked to the decreased growth with passaging seen with the Hillex II microcarrier, but further investigation with additional eCB-MSCs donors to determine whether such a correlation exists and whether the link is causal is required.

eCB-MSCs expanded under all conditions showed similar immunosuppressive potential in a PBMC suppression assay. We have previously observed that despite differences in differentiation capacity and proliferation, equine MSCs from different donors and/or tissues show remarkable consistency during *in vitro* evaluation of their immunomodulatory potential (Lepage et al., 2019). This preliminary data indicates that VW bioreactor expansion of equine CB-MSCs may not significantly impact their immunosuppressive ability. As therapeutic indications for MSCs continue to point to their effectiveness in immune modulation, this functional feature is encouraging as we continue to develop better cell expansion strategies and dosing regimens.

In a broader context, this study outlined a systematic approach to design a microcarrier-based stirred suspension bioreactor process with specific detail in the choice of microcarrier with respect to cell attachment, expansion, and harvesting over several passages. The results from this study can serve as a starting point for other microcarrier-based stirred suspension bioreactor processes, including processes using human-derived MSCs. The use of equine-derived MSCs reduces the cost compared with the development of human MSC bioreactor processes where the cell source is scarcer and more valuable and often expensive serum-free media is used. Additionally, the use of the equine MSCs in this study can be applied to human health, as several publications have discussed the translation between companion animal models and humans (Arzi et al., 2021; Kol et al., 2015). The pipeline for approval for animal treatments is much more streamlined, requiring significantly less time and money (Füüdös et al., 2015). Therefore, MSC treatments can be used initially on animals as preliminary studies, which can then be applied to human health.

5 | CONCLUSIONS

This is the first known study that has investigated the expansion of equine mesenchymal stem cells from any source in VW bioreactors, as well as only the second study using any bioreactor type, which was also performed by our group. The use of equine mesenchymal stem cells has been shown to be a potential treatment alternative for orthopedic injuries in horses. However, very little research has been done to develop robust bioreactor protocols to produce the required cell numbers for treatment. This study demonstrated the potential to generate large numbers of phenotypically normal, functional cells within a bioreactor system. Additionally, as the bioreactor type and

microcarrier types used in this study are commercially available, the adoption of this process to veterinarians and researchers worldwide would be possible, therefore having the potential to impact research broadly in the field of equine regenerative medicine.

AUTHOR CONTRIBUTIONS

Design of experiments, execution of experiments, acquisition of data, analysis and interpretation of data, manuscript writing: Erin L. Roberts. *Acquisition of data, analysis and interpretation of data, manuscript writing and editing:* Sarah I. M. Lepage. *Data analysis, manuscript editing and revision:* Thomas G. Koch. *Design of experiments, data analysis, manuscript revision, project coordination:* Michael S. Kallos.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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