Equine mesenchymal stem cell–derived extracellular vesicle productivity but not overall yield is improved via 3-D culture with chemically defined media

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OBJECTIVE
Mesenchymal stem cell (MSC) extracellular vesicles (EVs) have emerged as a biotherapeutic for osteoarthritis; however, manufacturing large quantities is not practical using traditional monolayer (2-D) culture. We aimed to examine the effects of 3-D and 2-D culture 2 types of media: Dulbecco modified Eagle medium and a commercially available medium (CM) on EV yield.

ANIMALS
Banked bone marrow–derived MSCs (BM-MSCs) from 6 healthy, young horses were used.

METHODS
4 microcarriers (collagen-coated polystyrene, uncoated polystyrene, collagen-coated dextran, and uncoated dextran) were tested in static and bioreactor cultures, and the optimal microcarrier was chosen. The BM-MSCs were inoculated into a bioreactor with collagen-coated dextran microcarriers at 5,000 cells/cm² or onto culture dishes at 4,000 cells/cm² in either Dulbecco modified Eagle medium or CM media. Supernatants were obtained for metabolite and pH analysis. The BM-MSCs were expanded until confluent (2-D) or for 7 days (3-D) when the 48-hour EV collection period commenced using EV-depleted media. Extracellular vesicles were isolated and characterized via nanoparticle tracking analysis, Western blot, transmission electron microscopy, and protein quantification. The BM-MSCs were harvested, quantified, and immunophenotyped.

RESULTS
The number of EVs isolated was not improved by 3-D culture or CM media, however, the CM 3-D condition improved the number of EVs produced per BM-MSC over the CM 2-D condition (mean ± SD: 306 ± 99 vs 37 ± 22, respectively). Glucose decreased and lactate and ammonium accumulated in 3-D culture. Surface markers of stemness exhibited reduced expression in 3-D culture.

CLINICAL RELEVANCE
Optimization of our 3-D culture methods could improve BM-MSC expansion and thus EV yield.

Keywords: 3-D culture, microcarrier, equine, mesenchymal stem cell, extracellular vesicle

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Osteoarthritis (OA) is the most common joint disease affecting horses and is the leading cause of lameness and humane euthanasia.1 Currently, there is no definitive or effective treatment to halt the progression of OA in horses or reverse the negative effects of the disease. Thus, a targeted therapy that could mitigate the inflammatory processes within the joint to prevent damage in the early stages of disease would be very beneficial. Recent studies have suggested that extracellular vesicles (EVs) released from mesenchymal stem cells (MSCs) are responsible for many of the bioactive properties of the cells. These EVs could play a significant role in decreasing inflammation within the joint and reducing the negative effects of inflammatory cytokines on cartilage homeostasis, therefore, making them an excellent candidate for the treatment of OA.2 Extracellular vesicles isolated from human bone marrow–derived mesenchymal stem cells (BM-MSCs) reduce the adverse effects of inflammatory cytokines on
chondrocytes in vitro. A recent study using equine fetal bone marrow–derived EVs corroborated these findings in the horse. Additionally, in several rat models of OA, EVs derived from MSCs (MSC-EVs) promoted cartilage regeneration in vivo.

If EVs prove beneficial for OA treatment, they may be an ideal candidate for use as an allogeneic off-the-shelf cell-free product due to their decreased immunogenicity compared to BM-MSCs. However, the dose of MSC-EVs likely needed for therapeutic effect in larger volume equine joints may be impractical and expensive to produce using traditional monolayer culture. Thus, methods to scale up MSC-EV production will be necessary. Several techniques have been utilized to expand MSC culture including the use of multilayered flasks, 3-D scaffold systems, and 3-D microcarrier-based systems. The use of microcarrier-based 3-D culture has been demonstrated to improve MSC expansion efficiency in equine MSCs.

In addition to improving MSC culture expansion efficiency, 3-D culture systems have shown promise for scaling up the production of MSC-EVs. One study found that using a microcarrier stirred-bioreactor (3-D) system to expand human umbilical cord–derived MSCs resulted in a 20-fold increase in EV yield and improved EV biological activity compared to monolayer culture. Another found that a microcarrier-based stirred-bioreactor system not only increased the overall yield of EVs by 5.7-fold but also increased the number of EVs produced per cell by 3-fold indicating that the higher yield is not merely due to an increase in the number of MSCs present in culture. While 1 study demonstrated improvements in EV yield of equine embryo-derived MSCs using hollow fiber bioreactors and monolayer bioreactor flasks, our knowledge there are no published studies in the horse investigating the use of microcarrier-based 3-D culture to improve EV yield.

The objective of this study was to compare the production of EVs from equine BM-MSCs in a microcarrier-based stirred bioreactor (3-D) with traditional monolayer culture (2-D). We also aimed to compare the effect of 2 types of media on MSC expansion efficiency and EV production. We hypothesized that 3-D culture would increase MSC-EV production and that a commercially available media designed for bioreactor culture would improve MSC expansion and EV production over traditional media used for 2-D culture.

Methods

Animals

Banked BM-MSCs, previously isolated from 6 healthy young horses, were used in the study, with 3 horses used in the microcarrier compatibility trial (2 to 11 years of age) and another 3 horses used in the remainder of the experiments (3 to 8 years of age). The study protocol was approved by the University of Pennsylvania’s Institutional Animal Care and Use Committee.

Microcarrier compatibility trial

Four types of microcarriers were tested in static culture to determine their compatibility with equine BM-MSCs: collagen-coated polystyrene (CPS); SoloHill Collagen-coated APC; Sartorius), uncoated polystyrene (PS; SoloHill Plastic ACF; Sartorius), collagen-coated dextran (CDX; Cytodex 3; Cytiva), and uncoated dextran (DX; Cytodex 1; Cytiva). Experiments were performed in 6-well ultra-low attachment plates (Nunclon Sphera; ThermoFisher Scientific). A separate 6-well polystyrene plate was used for monolayer (2-D) culture (Falcon; polystyrene microplates; Fisher Scientific). Microcarriers were sterilized according to manufacturer instructions. For all conditions, a 30-cm² surface area was used. The seeding density was 10,000 cells/cm² for microcarrier culture and 5,000 cells/cm² for 2-D culture.

Banked passage 2 (P2) BM-MSCs (n = 3) were thawed and inoculated into each of the 5 conditions (CPS, PS, CDX, DX, and 2-D) in duplicate. For the 2-D wells, 5 mL of complete media were added (10% fetal bovine serum [FBS]; Dulbecco modified Eagle medium [DMEM] with 1 g/L of d-glucose, 2 mM l-glutamine, and 1 mM sodium pyruvate [ThermoFisher Scientific]; penicillin (100 U/mL)-streptomycin (100 μg/mL) solution (Invitrogen); HEPES [ThermoFisher Scientific], and human basic fibroblast growth factor [1 ng/mL; Invitrogen]). For the microcarrier cultures, a 4-hour attachment period was initiated with a reduced volume (2 mL) and reduced FBS concentration (0.05%). A plate rocker was used for 2 minutes every hour to encourage the distribution of BM-MSCs on the microcarriers. After 4 hours, the volume was increased to 5 mL and the FBS concentration was increased to 10%.

A 60% media change was performed at 48 hours for the microcarrier wells, and a complete media change was performed for the 2-D wells. Fluorescent microscopy was performed at 24, 48, and 96 hours to monitor cell attachment. A 300-μL sample of the microcarrier suspension was obtained and rinsed with PBS and then stained with 3 μM calcein AM (eBioscience; Invitrogen) and 3 μM ethidium homodimer-1 (Invitrogen) for 30 minutes. Microcarriers were rinsed with PBS, placed on a glass slide, and covered with a glass coverslip. Images were obtained using fluorescent microscopy with an inverted microscope at 5X magnification (DM IRBE microscope equipped with an IHRC RF4mott fluorescence illuminator [Leica Microsystems] and Axiocam 305s color camera [Zeiss]).

After 96 hours, cells were harvested using a cell detachment solution (Gibco trypsin-EDTA [0.25%]; ThermoFisher Scientific). Cell number and viability were determined using an automated cell counter (Cellometer Auto 2,000 Cell Viability Counter; Nexcelom Bioscience) and AOPI staining solution (ViaStain; Nexcelom Bioscience). Microcarriers were considered compatible with equine BM-MSCs if they had greater than 50% viability at the end of the culture period when examined via microscopy.
Compatible microcarriers were then tested under agitation in a 250-mL stirred bioreactor with a seeding density of 5,000 cells per cm² and a volume of 150 mL. Freshly harvested P3 BM-MSCs were inoculated onto microcarriers. A 4-hour attachment period was initiated using intermittent stirring, with stirring on for 2 minutes every 30 minutes at 40 rpm. Fluorescent microscopy (as described above) was then performed daily for 5 to 7 days to monitor BM-MSC attachment and proliferation. The microcarrier with the best performance, defined as the ability of BM-MSCs to attach and proliferate under agitation, was then chosen for the remaining experiments.

Comparison of EV production in 2-D and 3-D culture

Extracellular vesicle production was compared using 2-D and 3-D culture methods. Additionally, 2 types of media were compared: traditional, DMEM-based media supplemented with FBS, and a commercially available media (CM). Banked passage 1 (P1) BM-MSCs from young, healthy horses (n = 3) were thawed and inoculated onto polystyrene petri dishes in DMEM media and expanded until 90% confluency was achieved. The BM-MSCs were then harvested using a cell detachment solution and inoculated into the following conditions: 2-D DMEM, 2-D CM, 3-D DMEM, and 3-D CM (Figure 1).

Media formulations for BM-MSC expansion

DMEM media formulation was the same as that used in the microcarrier selection trial, with the addition of 0.05% poloxamer 188 (Corning). For CM, RoosterNourish-MSC (RoosterBio) was used. For 3-D CM, a bioreactor feed was also used (RoosterReplenish MSC-XF, RoosterBio).

2-D culture

BM-MSCs were inoculated onto polystyrene petri dishes (Falcon Tissue Culture Dish with Grid; Fisher Scientific), with a total surface area of 1,500 cm², a surface-area-to-volume ratio of 7.5 cm²/mL, and a seeding density of 4,000 cells/cm². For the DMEM condition, a complete media change was performed after 72 hours. For the CM condition, no media change was performed during the cell expansion period as per manufacturer instructions. Cells were expanded to 90% confluency, after which the MSC-EV collection period commenced.

3-D culture

CDX microcarriers, the optimal microcarrier for 3-D culture as determined in the above experiments, were rehydrated and sterilized according to the manufacturer’s instructions. The microcarrier surface area was 3,200 cm² per condition. The bioreactor

Figure 1—Study design for the comparison of 2-D and 3-D cultures using 2 different mediums. CM = Commercially available media. DMEM = Dulbecco modified Eagle medium. EV = Extracellular vesicle. Figure created with BioRender.com.
consisted of a 250-mL internal impeller spinner flask (Chemglass Life Sciences), precoated with a siliconizing reagent (SigmaAldrich, Sigma Aldrich), and autoclaved. Microcarriers were rinsed in media (DMEM or CM) before transfer into the spinner flask with a total volume of 100 mL. Flasks were placed into an incubator (37 °C, 5% CO₂) on a magnetic stir plate at 40 rpm for 1 hour. Freshly harvested P2 BM-MSCs were inoculated at a seeding density of 5,000 cells/cm². A higher seeding density was used for 3-D culture to account for the greater anticipated loss of BM-MSCs during the attachment period. During the initial 4-hour attachment period, intermittent stirring was used (stirring on for 2 minutes every 30 minutes at 40 rpm). After the attachment period, additional media were added for a total volume of 200 mL, with a final surface area-to-volume ratio of 16 cm²/mL. For 3-D DMEM, a 75% media change was performed at 24 hours postinoculation, and then every 48 hours thereafter. On days when the DMEM media were not changed, 1 ng/mL basic fibroblast growth factor was added. For 3-D CM, the media were not changed according to the manufacturer’s instructions; however, a bioreactor feed was added every 3 days. The BM-MSCs were allowed to expand for 7 days, after which the MSC-EV collection period commenced.

**Microscopy**

For the 2-D cultures, light microscopy was used to monitor BM-MSC expansion using an inverted microscope (Eclipse Ts2, equipped with a camera; Nikon). Images were obtained every 24 to 48 hours. For the 3-D cultures, a 1-mL sample of the microcarrier suspension was obtained at 24 hours and on days 7 and 9. The microcarriers were rinsed with PBS and then stained with 3 μM calcein AM (eBioscience; Invitrogen) and 3 μM ethidium homodimer-1 (Invitrogen) for 30 minutes. Microcarriers were rinsed with PBS, placed on a glass slide, and covered with a glass coverslip. Images were obtained using fluorescence microscopy with an inverted microscope at 5X magnification (DM IRBE microscope equipped with an IHRC RF4mot fluorescence illuminator [Leica Microsystems] and Axiocam 305 color camera [Zeiss]).

**MSC-EV collection period**

Once 90% confluency was achieved for 2-D cultures or on day 7 for 3-D cultures, which will be referred to as the Pre-EV time point, EV-depleted media were used for the MSC-EV collection period. For the DMEM conditions, PBS was filtered to remove bovine EVs. PBS was passed through a centrifugal filter unit with a 100-kDa molecular weight cut-off filter (Amicon Ultra-5, UltraCelt-100K; Millipore Sigma) by centrifugation at 3,000 g for 5 minutes. The remainder of the DMEM media components remained the same, with 10% EV-depleted PBS added. For the CM conditions, RoosterCollect-EV media (RoosterBio) was used. After 48 hours, which will be referred to as the Post-EV time point, the cell culture supernatant was collected, centrifuged at 2,000 X g for 20 minutes to remove cellular debris, and frozen at −80 °C until MSC-EV isolation.

**Supernatant metabolite analysis**

Metabolite analysis (pH, glucose, glutamine, glutamate, lactate, ammonium, sodium, potassium, and calcium) was performed on cell culture supernatant samples throughout the culture period. Supernatant samples were obtained from 2-D cultures immediately after inoculation, after 24 hours, before the addition of EV-depleted media (Pre-EV), and at the end of the EV collection period (Post-EV). For the 3-D cultures, supernatant samples were obtained immediately after inoculation and on days 1, 7 (Pre-EV), and 9 (Post-EV). Metabolite concentrations were obtained using an automated cell culture analyzer (BioProfile FLEX2; Nova Biomedical). pH was measured using a benchtop pH meter (SympHony B10P; VWR).

**BM-MSC characterization**

For BM-MSC harvest, a cell detachment solution (Gibco Trypsin-EDTA [0.25%; ThermoFisher Scientific]) was used. The 3-D cultures, after removal of the cell culture supernatant, microcarriers were transferred to 50-mL conical tubes, washed with PBS, and centrifuged at 450 X g for 5 minutes. After removal of the PBS supernatant, the cell detachment solution was added. The tubes were vortexed for 15 seconds, placed in an incubator at 37 °C for 7 minutes, then vortexed again for 15 seconds. The microcarriers were separated from the cell suspension using a disposable vacuum filtration system with a 100-μM nylon net filter (Sterilip; Millipore Sigma), then filtered through a 70-μM cell strainer to remove any remaining microcarrier debris. The cell suspension was then centrifuged for 5 minutes at 450 X g. Cell number and viability were determined using an automated cell counter and AOPI staining solution. Image analysis on day 9 was used to estimate the quantity. Image analysis software (ImageJ) was used to count the number of live and dead cells per microcarrier on 2 images per condition.

The immunophenotype of the BM-MSCs was evaluated using cryopreserved passage 3 (P3) BM-MSCs from all 4 conditions by flow cytometry analysis using specific markers for stemness. Briefly, cells were thawed, resuspended in PBS, and then centrifuged at 450 X g for 5 minutes. Cell pellets were first resuspended in PBS containing 10% normal goat serum and incubated at 4 °C for 20 minutes. Cells were then incubated with the primary antibodies at 4 °C for 45 minutes, rinsed twice with PBS, resuspended in the secondary antibody when appropriate, and incubated at 4 °C for 45 minutes. After the final rinse, the pellets were resuspended in 200 μL of PBS before analysis. Cells were stained with anti-CD29, CD44, CD90, CD105, CD45, CD-79α, major histocompatibility complex I (MHC I), and major histocompatibility complex II (MHC II) antibodies, and isotype controls were used to establish fluorescent gates based on previously reported data (Supplementary Table S1). A 13-color, 4-laser Flow Cytometer instrument was used (CytoFLEX S; Beckman Coulter), and subsequent analyses were performed using CytExpert v2.4 (Beckman Coulter).
MSC-EV isolation

The MSC-EVs were isolated from the cell culture supernatants via differential ultracentrifugation (Optima L-90k; Beckman Coulter) using a fixed angle rotor (Type 45 Ti; Beckman Coulter). Supernatants were centrifuged for 30 minutes at 20,000 X g. The pellet was discarded, and the supernatant was centrifuged for 90 minutes at 100,000 X g. The pellets were resuspended in PBS, combined, and centrifuged again for 90 minutes at 100,000 X g. The final pellet was resuspended in 300 to 350 μL PBS.

MSC-EV characterization

Nanoparticle tracking analysis was performed to measure hydrodynamic particle size and concentration (ZetaView PMX220 Twin; Particle Metrix). For each sample, 11 positions were scanned, and 30 frames were captured per position. The following settings were used: camera sensitivity: 70; shutter: 100; cell temperature: 25°C. The videos were analyzed by ZetaView Software version 8.05.12 with a maximum particle size of 1,000, a minimum particle size of 10, and a minimum particle brightness of 30. The laser wavelength was 488 nm.

Protein quantification of MSC-EV samples was performed. Extracellular vesicle samples were prepared using a protein assay kit (Qubit Protein Assay Kit; Invitrogen), and protein concentration was measured via fluorometry (Qubit 3.0 Fluorometer; Invitrogen). The laser wavelength was 488 nm.

Western blot was used to detect EV marker proteins. Extracellular vesicle samples (6 X 10^7 particles) containing 2X Laemmli loading buffer (Bio-Rad; Cat. No. 161-0737) were loaded into a 4% to 12% gel precasted gel (NuPAGE; Invitrogen; total volume of 20 to 30 mL per well) in a nonreducing condition. Precision Plus Protein Kaleidoscope Standards (Bio-Rad; No. 1610375, 5 mL/well) and PageRuler Prestained Protein Ladder (Thermo Scientific; No. 26616; 5 mL/well) were used as molecular weight markers. The electrophoresis was initially run at 80 V for 20 minutes and then at 150 V for 60 minutes. The proteins were transferred from the gel to a polyvinylidene difluoride membrane (Immobilon-P; Millipore; No. IPVH00010) at 70 V for 2 hours. After transfer, the membrane was briefly rinsed in Tris-buffered saline solution and blocked with 5% dry skim milk for 2 hours at room temperature before incubation with a primary antibody overnight at 4°C. Primary antibodies included anti-CD9 (Biolegend; No. 312102), anti-CD81 (Santa Cruz Biotechnology; sc-166029 horseradish peroxidase (HRP)), anti-TSG101 (Millipore Sigma, No. AV38773), and anti-calnexin (Millipore Sigma; No. AB2301). Then the membrane was washed with TBS + Tween 20 at room temperature (3 times for 5 minutes each) and then incubated with HRP secondary antibody for 1 hour at room temperature. Secondary antibodies included goat anti-mouse for CD9 and CD81 (Biolegend; No. 405306) and donkey anti-rabbit for TSG101 and Calnexin (Biolegend; No. 406401). The membrane was washed with TBS + Tween 20 for 2 hours. The membrane was washed every 15 to 20 minutes, and then incubated with Immobilon Forte Western HRP Substrate (Millipore; No. WBLUF0100) for 2 minutes at room temperature for chemiluminescence detection. Detection was performed with Cytiva Amersham ImageQuant 800 with 30 to 60 seconds of exposure time.

Transmission electron microscopy was used to evaluate MSC-EV morphology. A 5-μL volume of sample was applied to a thin carbon grid that was glow discharged for 2 minutes using a Pelco Easygloow instrument. Then, 5 μL of freshly made 2% uranyl acetate stain solution was applied to the sample holding grid and incubated with the sample for 2 minutes. Excess samples and stains were blotted away with Whatman filter paper leaving a thin layer of stained particles on the grid. The staining process was repeated one more time and the grid was left to dry until imaged. TEM micrographs were collected using a Tecnai T12 TEM microscope at 100 keV. The images were recorded on a Gatan Oneview 4 K X 4 K camera. Each image was collected by exposing the sample for 4 seconds and a total of 100 dose-fractionated images were collected and combined into a single micrograph. The data were collected at −1.5 to 2 μm under focus at 30 K to 40 K magnification.

Statistical analysis

Data were tested for normality using a Shapiro-Wilk test. Parametric quantitative data are presented as mean ± SD. Nonparametric quantitative data are presented as median (range). A mixed effects model was used to analyze continuous variables with horse as a random effect, and day, media, and culture method as fixed effects, with the interaction of all 3 probed. This was followed by Tukey multiple comparison test for the comparison of 3 or more variables, and a Student t test for the comparison of 2 variables. Statistical analysis was performed with JMP 17 (SAS Institute Inc) software and the level of significance was set at P<less than or equal to.05.

Results

Microcarrier compatibility trial

Equine BM-MSCs were cultured on 4 types of microcarriers (CPS, PS, CDX, and DX) in static culture for 4 days, and then BM-MSCs were imaged via fluorescence microscopy, harvested, and quantified. The BM-MSCs were able to attach to all 4 microcarriers. There were no significant differences between harvested cell quantity or viability (Supplementary Figure S1). However, it was noted on microscopy that DX microcarriers had greater numbers of dead cells. CPS, PS, and CDX microcarriers were tested under agitated conditions. While the initial attachment of CPS, PS, and CDX microcarriers were tested under agitated conditions. While the initial attachment of BM-MSCs to CPS, PS, and CDX microcarriers was good, the cells began to detach in large numbers after 48 hours. The BM-MSCs remained attached and were able to proliferate on CDX microcarriers; therefore, they were chosen for the remaining experiments.

BM-MSC microscopy

The BM-MSCs were expanded on tissue culture dishes for 2-D and on CDX microcarriers in a stirred bioreactor for 3-D. Two different mediums (DMEM
and CM) were compared in 2-D and 3-D cultures. The BM-MSC expansion was monitored via light microscopy for 2-D cultures. For 3-D cultures, fluorescence microscopy was used after staining with ethidium homodimer-1 and calcein AM. Images were obtained on day 1, Pre-EV, and Post-EV. In all conditions, BM-MSCs exhibited the expected spindle-shaped morphology (Figure 2). For the 3-D CM condition, microcarriers tended to form clumps, with BM-MSCs bridging across multiple microcarriers.

**Supernatant metabolite analysis**

Cell culture supernatants were obtained from 4 culture conditions (DMEM 3-D, CM 3-D, DMEM 2-D, and CM 2-D) immediately after BM-MSC inoculation (day 0), 24 hours after inoculation (day 1), before the change to EV-depleted media (Pre-EV), and after the 48-hour EV collection period (Post-EV). Measurements included pH, glutamine, glutamate, ammonium, lactate, glucose, sodium, potassium, and calcium concentrations (Figure 3).

**Figure 2**—Light microscopy images of bone marrow-derived mesenchymal stem cells (BM-MSCs) for 2-D culture are shown (A), while fluorescence microscopy images of BM-MSCs cultured on microcarriers (3-D) are shown (B). For 3-D culture, live cells were stained with calcein AM (green) while dead cells were stained with ethidium homodimer-1 (red). The BM-MSC expansion in 2 types of media (Dulbecco modified Eagle medium [DMEM] and commercially available media [CM]) were compared. Images were obtained on day 1, before adding extracellular vesicle (EV)-depleted media (Pre-EV), and after the 48-hour EV collection period (Post-EV).
The pH of CM 3-D supernatants decreased significantly from day 0 and day 1 to the Pre-EV time point. The CM 3-D Pre-EV pH was significantly lower than the CM 3-D Post-EV pH. The Pre-EV pH for CM 3-D was significantly lower than that of all other conditions at every time point, and the Post-EV pH for CM 3-D was significantly lower than that of the other conditions at that time point (Figure 3). Glutamine concentrations did not differ between groups on day 0, day 1, or Pre-EV (Figure 3). At the
Post-EV time point, CM 2-D supernatants had significantly lower glutamine concentrations than CM 2-D supernatants at day 0 and Pre-EV. The Post-EV CM 2-D glutamine concentration was not significantly different than CM 2-D at day 1, or CM 3-D at the Pre-EV time point. However, it was significantly lower than all other conditions at all time points.

Glutamate concentrations in CM media were generally higher than those of DMEM media. On day 0, DMEM 2-D had significantly lower glutamate

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**Figure 4**—Bone marrow–derived mesenchymal stem cell (BM-MSC) characterization of 4 culture conditions (DMEM 3-D, CM 3-D, DMEM 2-D, and CM 2-D). A—Mean ± SD of final BM-MSC quantity. Solid circles represent individual data points. Differing letters denote significant differences between groups. B—Median number of BM-MSCs per milliliter of supernatant. Solid circles represent individual data points. Differing letters denote significant differences between groups. C—Median BM-MSC viability. Solid circles represent individual data points. No significant differences were detected. D—Immunophenotyping of BM-MSCs as determined by flow cytometry. Expression of inclusion markers in BM-MSC populations (top) and expression of exclusion markers in BM-MSC populations (bottom). The gray histograms represent isotype controls, and the white histograms represent respective cell surface marker staining. The mean (±SD) percentage of positive cells, obtained from 3 horses with each horse providing 2 experimental replicates, is in the top right corner of the histogram. Differing letters denote significant differences between groups within each surface marker. CM = Commercially available media. DMEM = Dulbecco modified Eagle medium. MHC = Major histocompatibility complex.
concentrations than CM 3-D and CM 2-D but was not significantly different than DMEM 3-D (Figure 3). On day 1, DMEM 3-D had significantly lower glutamate concentrations than CM 3-D. The glutamate concentrations of CM 2-D decreased over time, such that the concentration was significantly lower at the Pre-EV and Post-EV time points than day 0. The glutamate concentration of CM 3-D supernatants decreased significantly from day 1 to Pre-EV. However, the Post-EV glutamate concentration of CM 3-D supernatants was not significantly different than day 0.

Ammonium concentrations were similar among groups until the Post-EV time point, where the CM 3-D and CM 2-D had significantly lower concentrations than DMEM 3-D and DMEM 2-D (Figure 3). Lactate concentrations did not differ significantly until the Pre-EV time point, where CM 3-D had significantly higher lactate concentrations than other conditions. This difference persisted at the Post-EV time point. Additionally, at the Post-EV time point, the lactate concentrations of DMEM 2-D were significantly higher than that of CM 2-D.

Glucose concentrations of CM media were generally higher than those of DMEM media. At day 0 and day 1, CM 3-D and CM 2-D had significantly higher glucose concentrations than those of DMEM 3-D and DMEM 2-D (Figure 3). For CM 3-D, the glucose decreased significantly from day 1 to Pre-EV and remained at a similar concentration from Pre-EV to Post-EV. The Post-EV glucose concentrations of CM 2-D were significantly lower than those of CM 2-D at day 0. At day 1, sodium, potassium, and calcium concentrations were significantly lower in CM media than DMEM 3-D and DMEM 2-D at Day 1, the Pre-EV, and Post-EV time points.

BM-MSC characterization

After the 48-hour EV collection period, BM-MSCs were quantified (Figure 4). For 2-D cultures, a cell detachment solution was applied and an automated cell counter was used to determine the cell concentration and viability. For 3-D cultures, image analysis was used to estimate the quantity and viability of BM-MSCs on microcarriers. DMEM 2-D had the highest number of BM-MSCs, followed by CM 2-D. There were no significant differences between DMEM 3-D and CM 3-D for BM-MSC quantity. In 2-D cultures, the number of BM-MSCs per milliliter of conditioned supernatant was significantly higher than that of 3-D cultures. There were no significant differences between groups for BM-MSC viability.

Harvested BM-MSCs from each condition underwent immunophenotyping analysis to determine the expression of BM-MSC markers of inclusion (CD29, CD44, CD90, MHC I, and CD105) and exclusion (CD45RB, CD79a, and MHC II) (Figure 4). For CD29, CM 3-D BM-MSCs had significantly fewer cells expressing CD29 than both 2-D conditions. For CD90, CM 3-D BM-MSCs had significantly fewer cells expressing CD90 than DMEM 2-D BM-MSCs. For MHC I, DMEM 3-D BM-MSCs had the lowest number of cells expressing MHC I, followed by CM 3-D BM-MSCs. For the remaining markers, no significant differences were noted between groups.

MSC-EV characterization

Nanoparticle tracking analysis was used to determine the number of EVs in each sample. Extracellular vesicle productivity was determined by dividing the number of EVs in each condition by the number of live cells. Extracellular vesicle productivity was determined by dividing the number of EVs in each condition by the number of live cells. Extracellular vesicle productivity was determined by dividing the number of EVs in each condition by the number of live cells. Extracellular vesicle productivity was determined by dividing the number of EVs in each condition by the number of live cells. Extracellular vesicle productivity was determined by dividing the number of EVs in each condition by the number of live cells. Extracellular vesicle productivity was determined by dividing the number of EVs in each condition by the number of live cells.

Figure 5—A—Mean ± SD of extracellular vesicle (EV) quantity per group. B—Mean ± SD of EVs per live cell. C—Median proportion of particles sized 0 to 100, 101 to 1,000, and 1,001 to 2,000 nm. D—Mean ± SD of EV particles per microgram protein. Differing letters denote significant differences between groups. Solid circles represent individual data points. CM = Commercially available media. DMEM = Dulbecco modified Eagle medium.
BM-MSCs at the Post-EV time point. The DMEM 2-D condition produced significantly more EVs (mean ± SD: 1.07 X 10^10 ± 2.66 X 10^9) than DMEM 3-D (mean ± SD: 5.86 X 10^9 ± 4.6 X 10^9), CM 3-D (mean ± SD: 5.5 X 10^9 ± 6.83 X 10^8), and CM 2-D (mean ± SD: 1.09 X 10^9 ± 4.97 X 10^8) (Figure 5).

Extracellular vesicle productivity was significantly lower in the CM 2-D condition (mean ± SD: 37 ± 22 EVs per cell) than in CM 3-D (mean ± SD: 306 ± 99), DMEM 3-D (mean ± SD: 390 ± 174), and DMEM 2-D (mean ± SD: 260 ± 48) conditions.

Nanoparticle tracking analysis was also used to determine the particle size distribution of each sample. The proportion of particles that were 0 to 100, 101 to 1,000, and 1,001 to 2,000 nm was calculated. There were no significant differences between groups (Figure 5).

The amount of protein in each EV sample was quantified, and the number of EVs per μg protein was calculated and compared. There were no significant differences among groups (Figure 5).

Western blot was used to detect EV markers. Extracellular vesicles should express CD9, CD81, and TSG101 and should not express calnexin. Extracellular vesicles from all conditions expressed CD9 and CD81. TSG101 was detected in all samples but 1 CM 2-D sample (Figure 6). Only 1 sample from the DMEM 3-D condition expressed calnexin. Transmission electron microscopy was used to image EVs. Samples from all groups exhibited cup-shaped particles, with visible lipid bilayers (Figure 6).

**Discussion**

While MSC-derived EVs have shown some promise in vitro as a therapeutic for the treatment of OA, producing EVs in large enough quantities for use in vivo is limited using traditional monolayer culture. In this study, we compared the EV production by equine BM-MSCs expanded via 3-D culture with microcarriers in a stirred bioreactor with that of BM-MSCs expanded via traditional monolayer (2-D) culture. We also examined the effects of 2 types of media: traditional DMEM-based media supplemented with FBS and commercially available media designed for bioreactor culture. Our first hypothesis, which was that 3-D culture would improve EV production, was rejected. While the number of EVs per live BM-MSC (EV productivity) was significantly higher using 3-D culture with CM compared to 2-D culture with CM, there was no difference when DMEM 3-D and DMEM...
2-D were compared. Additionally, 2-D DMEM had the highest EV yield overall, outperforming CM 2-D and CM 3-D; therefore, we also rejected our second hypothesis that using CM would improve EV yield.

While our 3-D culture protocols did not enhance EV yield, we identified changes in metabolite concentrations and pH that could be used to improve these protocols to support further equine BM-MSC expansion on microcarriers. In DMEM and CM 3-D conditions, BM-MSCs were not confluent on the majority of microcarriers. One advantage of 3-D culture is an increased surface area-to-volume ratio, allowing for a greater density of BM-MSCs per milliliter of supernatant. In our experiments, despite the increased surface area available in 3-D culture, the number of BM-MSCs was not significantly different than that of 2-D culture. It is possible that if BM-MSCs were able to achieve confluency on the majority of microcarriers, the increased cell density would result in higher EV yields.

Factors such as glucose and glutamine concentrations as well as lactate and ammonia accumulation can greatly affect MSC expansion efficiency in 3-D culture. We found that for 3-D CM culture, lactate and ammonium accumulated and pH decreased while glucose was depleted over time. The CM media were designed to maintain the bioreactor culture of human MSCs without media changes, with a nutrient supplement added every 3 days. This feeding regimen may not be appropriate for equine BM-MSCs, considering in the CM 3-D condition we observed decreases in glucose and accumulation of lactate and ammonium, which can inhibit BM-MSC expansion. It is possible that if we had performed regular media changes for CM as we did for DMEM, BM-MSC expansion could have improved. Species can differ widely in MSC metabolism in culture; therefore, further studies examining feeding regimens for 3-D culture of equine BM-MSCs are warranted.

We also found that 3-D culture reduced the frequency of expression of some markers of stemness, including CD29 and MHCI. This phenomenon has been demonstrated previously. For example, human adipose-derived stem cells exhibited reduced expression of CD105 after expansion on microcarriers. The change in surface marker expression of CD105 after expansion on microcarriers in 3-D culture tended to plateau after 7 days whether they reached confluency or not; therefore, we chose a specific day (day 7) as the endpoint rather than percent confluency since confluency was quite variable. Confluency has been shown to alter MSC properties because of contact inhibition and can alter autocrine factors and EV productivity. In our study, 3-D cultures were typically less confluent than 2-D cultures at the Pre-EV time point, which could have affected the number and type of EVs released.

One limitation of our study is that we did not examine BM-MSC differentiation capacity; therefore we cannot determine if these changes in markers of stemness represented true changes in the multipotency of the cells.

Another limitation is that we did not perform any functional comparisons of the EVs produced by different culture conditions. 3-D culture can alter EV cargo, including miRNA content, and can improve EV function and therapeutic efficacy. Future studies could examine the effects of culture method and medium on EV cargo via miRNA sequencing, lipidomics, and proteomics. Finally, for the CM condition, the 3-D culture media included the addition of a biofeeder feed on days 3, 6, and 9 to replenish some nutrients. This could have altered the EV output by supporting the nutrition of cells, while these nutrients may have been lacking in the 2-D condition.

In conclusion, while 3-D culture improved the number of EVs produced per BM-MSC using CM media, we were not able to increase the overall yield of EVs. Further optimization of the protocol, including improving feeding regimens, could enhance the expansion efficiency of BM-MSCs to increase the EV yield over 2-D culture.

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None to report.

Disclosures

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Supplementary Materials

Supplementary materials are posted online at the journal website: avmajournals.avma.org