Delineation of optimal culture conditions for the production of therapeutic human mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) in static cultures

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November 2021

ABSTRACT: Mesenchymal stromal cells (MSCs) have been studied for therapeutic applications due to their promising properties. Recently, MSC therapeutic effects were mostly associated to the release of extracellular vesicles (EVs) as paracrine signaling. MSC-EV therapeutic potential has already been reported as beneficial for a vast number of diseases and seem to recapitulate the therapeutic effects of MSCs, creating expectations to a promising, safe, and effective cell-free-based therapy. However, a considerable amount of EVs is needed for therapeutic applications and thus, cell culture parameters need optimization to ensure high productivity, combined with quality, consistency, reproducibility, and safety. In this manner, this project involves the study of different cell culture parameters to obtain an optimized product of human MSC-EVs based on productivity. Overall, the MSC source and the medium change seemed not to imply significant variability in EV production and productivity, and the time point after MSC expansion and medium conditioning seemed the best one for EV collection. Regarding the removal of culture medium particles that could have contaminated the MSC-EV samples, a filtration with 0.1 µm filter seemed inefficient. The temperature of 33ºC applied during conditioning medium seemed to increase the EV productivity, contrarily to hypoxia, but further studies are necessary to confirm if the increase observed is statistically significant. In sum, besides the necessary further studies, the present work provides insights regarding the influence of different cell culture parameters in MSC-EV production that can contribute to reach in the future the best quality/potency and productivity of EV products.

Keywords: extracellular vesicles; mesenchymal stromal cells; cell culture parameters; EV productivity.

INTRODUCTION

Mesenchymal stromal cells (MSCs) exhibit multilineage differentiation ability, as well as intrinsic immunomodulatory, trophic and homing properties (Dennis and Caplan 2004; Le Blanc and Mougiakakos 2012; Kallmeyer and Pepper 2015), thus MSC have the potential to be used as a cell-based therapy for human diseases. MSCs are able to prevent apoptosis, to promote proliferation, migration and angiogenesis, to suppress fibrosis and scar formation, and have supportive function (Lin et al. 2011). The secretion of paracrine bioactive molecules by MSCs is an important mechanism for tissue repair and to stimulate host cells.

Extracellular vesicles (EVs) released by MSCs are partially responsible for their paracrine action (Zhang et al. 2016; Matei et al. 2019). EVs, including exosomes and microvesicles, are composed of a lipid bilayer membrane and the enclosing cytosolic specific cargo of biomolecules (proteins, RNA, etc), mediating intercellular communication on physiological and pathological processes (Lee et al. 2012; Kalra et al. 2016). Due to their small size and identical structure to the cell membrane, EVs can cross biological barriers and have high biocompatibility to target cells. Therefore, it is expected that EV-based therapeutic products are safer to administer when compared to the delivery of cellular therapies. (Kordelas et al. 2014; Xu et al. 2019). Furthermore, MSC-EV therapeutic potential has already been reported as beneficial for diverse diseases, and they seem to recapitulate the MSC therapeutic effects(Kim et al. 2016).

However, as in cell therapy context, in which large cell numbers per dose are required, very large numbers of EVs are expected to be required for clinical use (Kordelas et al. 2014). Furthermore, several differences have been reported in terms of the EV cargo, between EVs isolated from cell culture supernatants of MSC expanded under different culture conditions, stressing the importance of controlling all culture process parameters to obtain high productivity, combined with quality, consistency, reproducibility, and safety of EV product.

It is important to choose the appropriate cell culture medium that should be ideally serum-/xenogeneic-free to avoid the contribution of EV contaminants. Factors related to the culture techniques also affect MSC-EV production, namely, cell passaging, culture conditions (temperature, oxygen tension, shear stress, stiffness, preconditioning with proteins or small molecules), isolation methods (ultracentrifugation, chromatography, precipitation) and culture system (adherent in 2D or 3D, or spheroids). Additionally, MSC-EV characteristics are also dependent of the MSC donor characteristics, including donor age and sex, cell source, healthy donor or diseased patient and the presence of trauma or systemic diseases (Pountos et al. 2007).

In this work, three experiments were designed to evaluate the effect of different parameters on MSC-EV production based on productivity, namely, cell source, donor variability, time of EV collection, medium change, culture medium composition, oxygen tension and temperature. MSC-EV production comprised a step of MSC expansion and a step of cell conditioning after which the conditioned medium (CM) was collected and used for MSC-EV isolation. Alongside, it was investigated the presence of particles in the culture medium used and their possible impact in the quantification of MSC-EVs produced, filtered and not filtered culture medium were compared. The possible particle degradation, adhesion to plastic and MSC-EV uptake/production profile throughout cell culture were also assessed.

MATERIALS AND METHODS

MSC Isolation From Human Samples

Human primary samples were obtained from healthy donors or patients following ethical and legal guidelines and were obtained under established collaboration agreements (bone marrow (BM)

from IPO; umbilical cord matrix (UCM) from HSFX; adipose tissue (AT) from Clínica de Todos-os-Santos). Human MSC are part of the iBB-IST cell bank and were previously isolated by plastic adherence according to established protocols.

MSC Expansion in Static Conditions

The cryopreserved MSC from the different cell sources and cell donors were thawed and plated in CELLstart™CTS™ pre-coated T-flasks at 3,000 cells/cm^2. If the culture medium used in cells freezing was not StemPro® MSC SFM XenoFree (SP), the cells were plated using the culture medium employed in their freezing and after one passage the medium was changed to SP. Cells were incubated at 37ºC, 5% CO_2 and >95% humidity and the culture medium was replaced every 3-4 days. When reaching 70-80% cell confluence, cells were detached from T-flasks with TrypLE™ 1X, for 5 min at 37°C. Cell number and viability were determined using the Trypan Blue exclusion method. MSC were passaged at least once using SP medium before the final inoculation in T-flasks for EV production under static conditions.

MSC-EV Production Under Static Conditions

MSC-EV production comprised a stage of MSC expansion followed by a stage of medium conditioning.

For the “time point experiment”, three independent donors from each MSC source (BM, AT and UCM) were used to study the optimal time point for MSC-EV collection and the impact of different percentages of medium change (MC). Cells were cultured in T-175 flasks in the same conditions described before for 8 days. At day 3, culture medium was fully renewed, and at day 6 three different percentages of MC (0%, 25% and 100%) were applied. Samples of CM were recovered at days 3, 6 and 8.

In a second experiment, to evaluate if the particles present in the SP medium could affect the quantification of MSC-EVs produced, the culture medium was filtered with 0.1 µm filter, then MSC from BM M79A15 donor were cultured in T-75 flasks in the same conditions described before for 7 days, using SP or SP filtered medium (SPf). The medium was totally changed at day 3 and two percentages of MC were studied at day 5, 0% and 100%. Samples of CM were recovered at day 7.

In a final experiment, BM MSC M78A15 donor was cultured in T-175 flasks during 7 days. The medium was not change during MSC culture. Using SP vs SPf, hypoxia (5% O_2) was compared with normoxia (21% O_2) at 37ºC along culture time, and a temperature of 33ºC was compared to 37ºC only during medium conditioning, both under normoxia. Samples of CM were recovered from T-flasks at day 1, 3 and 5. At day 7, the CM was entirely recovered. Culture medium was used as a control and underwent the same treatment as the MSC-CM.

For all the experiments, the samples of CM recovered were filtered with 0.45 µm filter to remove cell debris and stored at -80ºC until further analysis. At the end of the experiment, the cells were detached from the flasks and cell number was determined as previously described.

Isolation of EVs From MSC Cultures

EVs were isolated from the MSC-CM by precipitation using the Total Exosome Isolation reagent, according to the manufacturer instructions (PEG addition, incubation overnight at 4°C, centrifugation for 1 h at 10000 × *g* and 4°C). The maximum volume of supernatant was removed and the EV fraction was resuspended with DNase/RNase-Free PBS 1x. EV samples were frozen at −80°C in aliquots to nanoparticle tracking analysis (NTA) analysis (40 µL of sample + 1960 µL of DNase/RNase-Free PBS 1x), protein quantification (24 µL of sample + 216 µL DNase/RNase-Free PBS 1x), and the rest of EV samples were stored in 100 µL aliquots.

Characterization of MSC-EVs

Protein Quantification

The MicroBCA™ Protein Assay Kit (Thermo Scientific) was used to quantify total protein in CM and EV samples from all experiments according to the microplate procedure provided by the manufacturer. Duplicates were quantified for each standard or sample. The absorbance was measured at 562nm on a plate reader (Tecan i-control) after incubation at 37°C for 1h for CM samples and 1h30min for EV samples. Protein concentration of the samples was determined by a linear fit of the BSA standards.

Nanoparticle Tracking Analysis

EV size distribution profiles and concentration measurements were determined by NTA, using a NanoSight LM14c instrument equipped with a 405 nm laser (Malvern) and NTA software version 3.1 (Malvern). Samples from the time points experiment were measured using a camera level between 10 and 13. Samples from the other experiments were analyzed with a camera level of 13. Each sample was recorded 15 times for 15 s, using fresh sample injection for each acquisition. PBS was used to thoroughly wash between samples. A threshold level of 7 was applied for video processing.

Western Blot

Western blotting was performed as previously described (de Almeida Fuzeta et al. 2020). Whole cell lysates were obtained by lysis and protein concentration determined by MicroBCA as described before (1h of incubation at 37ºC for WCL samples).

Western blotting was performed in reducing conditions. Samples with 15–60 µg of total protein were loaded in Bolt™ 4–12% Bis–Tris polyacrylamide gels (Thermo Fisher Scientific), in equal protein content for each gel, and subjected to electrophoresis. Proteins were transferred nitrocellulose membranes. Subsequently, the membranes were blocked, incubated with primary and secondary antibodies and revealed. Primary antibodies included anti-Calnexin (1:1000), anti-Synthenin (1:1000) and anti-CD63 (1:1000). Secondary antibodies included Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, HRP (1:5000) and Goat anti-Rabbit IgG HRP-conjugated (1:1000).

Fourier-transform infrared spectroscopy

For the analysis of the CM molecular composition by Fourier-transform infrared spectroscopy (FTIR), the samples were thawed and added in triplicates of 25 µL to a 96 FTIR well plate. The cryopreserved cells were thawed, diluted in SP medium, centrifuged, resuspended in PBS and triplicates of about 70,000 total cells per well were added to the 384 FTIR well plate (in 5-8 µL per well). Furthermore, triplicates of SP medium, PBS and internal FTIR controls were added as controls. FTIR analysis was performed by a technician at Instituto Superior de Engenharia de Lisboa.

Transmission electron microscopy

EVs were thawed and imaged by transmission electron microscopy (TEM), by a technician at Instituto Gulbenkian da Ciência. It was used the Tecnai G2 Spirit BioTWIN Transmission Electron Microscope (FEI) operating at 120kv, and data was collected with an Olympus-SIS Veleta CCD Camera. Imaging was performed following negative staining protocol.

MSC characterization

MSC were immunophenotypic characterized using flow cytometry. MSC multilineage differentiation was also tested using StemPro® Osteogenesis/ Adipogenesis/ Chondrogenesis Differentiation Kits as previously described (Santos et al. 2011).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9.2.0 Software. Results are presented as mean ± standard error of the mean (SEM) of the values obtained from different MSC donors (i.e., biological replicates) or as mean ± standard deviation (SD) of the values from technical replicates.

RESULTS AND DISCUSSION

SP culture medium, a well-defined xeno/serum-free product specially formulated for the expansion of human MSCs, was used in this work to avoid the disadvantages of using animal derived products which are associated with safety concerns. Regarding the cell culture platform, static culture systems (i.e., T-flasks) were chosen to study different cell culture parameters and select the optimized ones (cell source, donor, time of EV collection, medium change, culture medium composition, oxygen tension and temperature) prior to the translation to dynamic conditions.

Time point experiment

MSC Expansion and Medium Conditioning for MSC-EV Production from Three Different Human Sources (BM, AT and UCM) under static conditions

MSCs isolated from three different human tissue sources, BM, AT and UCM, were used to study the optimal time point for EV collection and the influence of different percentages of MC at day 6 of culture in MSC-EV production, 0%, 25 % and 100 % MC. For each cell source, MSCs from three independent donors (n = 3) were successfully expanded under static conditions in T-flasks using SP culture medium. Plated cells were counted at the end of experiment (day 8). UCM MSCs showed relatively higher number of cells (**Figure 1**), ranging between 19.3 ± 0.6 × 10^6 and 33.8 ± 3.2 × 10^6 depending on MC percentage, comparing to BM and AT MSC, which showed similar numbers, ranging between 12.9 ± 2.9 × 10^6 and 20.1 ± 2.2 × 10^6, and between 13.1 ± 2.7 × 10^6 and 18.6 ± 1.2 × 10^6, respectively. The higher proliferative capacity of UCM MSCs could potentially be explained by a more immature state of this cell source and smaller cell size, when compared to the adult BM and AT sources. Furthermore, the MC at day 6 seemed to have influence in the final cell number, since as higher was the percentage of MC, higher was the total cell number at day 8 for the 3 cell sources. However, the differences in cell number between percentages of MC did not seem statistically significant. The lower cell number for the 0% MC, and even for the 25% MC condition, could be explained by higher cell death or lower cell replication due to the metabolite concentration in the culture medium. In fact, the glucose levels were exhausted at day 8 for all BM and UCM MSCs for 0 and 25% MC and for AT MSCs the glucose concentration was also quite low (data not shown). Similarly, higher levels of lactate were produced for most of the donors from the 3 cell sources for 0 and 25% MC, compared to 100% MC (data not shown). Despite of the high levels of lactate, it did not reach the inhibitory concentration for MSC culture (35.4 mM or 3.19 g/L) (Schop et al. 2009).

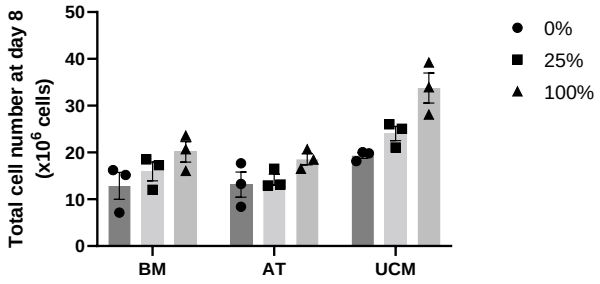

Figure 1. Total cell number counted at day 8 for MSCs from 3 human tissue sources (bone marrow (BM), adipose tissue (AT), and umbilical cord matrix (UCM)). MSCs from 3 different donors were used per tissue source and 3 different percentages of MC at day 6 were investigated, which are represented in three different shades of gray. The measurements are plotted as individual points (0 % (circle), 25% (square) and 100 % (triangle) of MC) and the average of these measurements is also shown. Results are presented as mean ± SEM of cell count for each percentage of MC.

Immunophenotypic analysis of the cells was performed to evaluate if the different percentages of MC affected the expression of MSC surface markers. Despite an unexpected increase in CD34 for AT MSC and despite of a decrease in CD105 expression of BM MSC, which might be due to the antibody, to longer times of exposure to the cell detachment reagent (de Sousa Pinto et al. 2019) or to the several passages (Dominici et al. 2006), the percentage of MC did not greatly affect the expression of the markers (**Figure 2A**). Both CD73 and CD90 biomarkers are expressed in ≥95% of the cells, which is in agreement with the minimal criteria for defining multipotent MSC (Dominici et al. 2006). Additionally, BM, AT and UCM MSCs retained their multilineage differentiation ability toward the osteogenic (**Figure 2B**), adipogenic (**Figure 2C**), and chondrogenic (**Figure 2D**) lineages, for all the different percentage of MC studied.

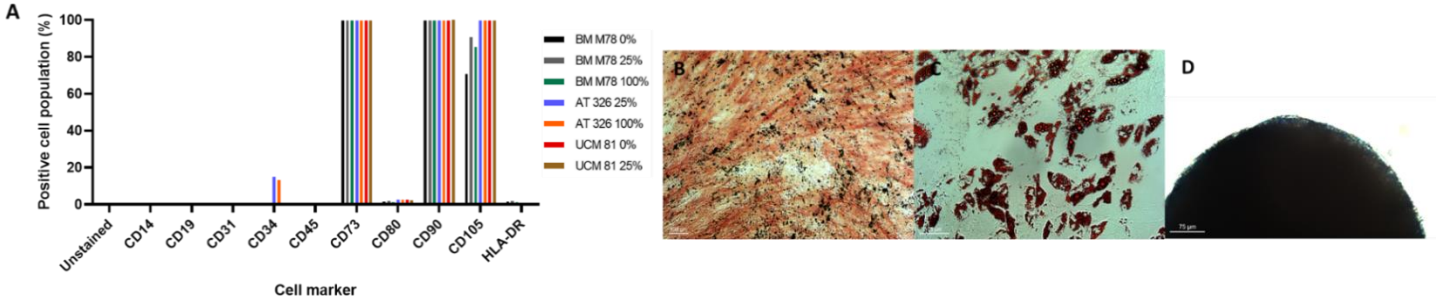

Figure 2. Analysis of MSCs obtained after the time point experiment. **(A)** Immunophenotypic characterization of BM, AT and UCM MSC after expansion and medium conditioning for MSC-EV production (total of 8 days) in static culture, analyzed by flow cytometry. One donor was used for each cell tissue source, corresponding to all percentage of MC studied (0%, 25% and 100%) for BM MSC, to 25% and 100% of MC for AT MSC and to 0% and 25% of MC for UCM MSC. Representative images of multipotency characterization of BM, AT and UCM MSC cultured in static system through multilineage differentiation assays, upon 14 days under **(B)** osteogenic, **(C)** adipogenic, and **(D)** chondrogenic differentiating condition.

Characterization of MSC-EVs produced throughout experiment

MSC-EVs were successfully isolated from samples of CM harvested at different time points of MSC culture, at day 3, 6 and 8.

The presence of EVs secreted from MSCs from the 3 different sources was identified by TEM imaging (**Figure 3A**, **B** and **C**). Several individual EVs were identified showing different sizes around 100 nm, which is within the range of values obtained by NTA analysis (typically between 80-250 nm, **Figure 7B**). However, the sample processing techniques, requiring fixation and dehydration, affected the EV size (thus it is not possible to accurately determine EV size) and EV morphology, (EVs presented a cup shaped with a divot in their center) (Doyle, L. M., & Wang 2019; Twain 2020). Furthermore, the samples seemed to contain other type of particles and filament-like structures beyond the EVs identified, indicating that EV samples were not pure (as confirmed by purity assessment).

Western blotting analysis also confirmed the production of EVs (**Figure 3D**). The EV protein markers CD63 and synthenin were successfully detected in EV samples as well as in WCL, as transmembrane and cytosolic protein EV markers, respectively. Calnexin, a negative EV protein marker for being an intracellular protein from the endoplasmic reticulum, was identified in cells and not detected in EV samples, as expected (Théry et al. 2018; de Almeida Fuzeta et al. 2020). These results are from samples at day 8when 100% MC was applied, but similar results were obtained for 0% MC (data not shown). Thus, it is plausible to assume that also for the 25% MC using the same isolation method, it is possible to isolate MSC-EVs with the expected markers.

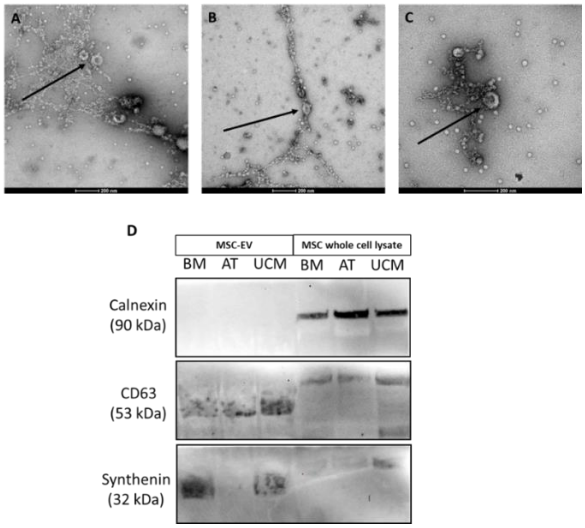

Figure 3. Characterization of MSC-EVs. Representative close-up TEM images of MSC-EVs obtained in static culture (samples from day 8), using MSCs from 3 different human tissue sources (**(A)** bone marrow, **(B)** adipose tissue, and **(C)** umbilical cord matrix). EVs are pointed with black arrows. **(D)** Representative Western blotting detection of calnexin, CD63 and synthenin in MSC-EV samples and corresponding WCL, obtained from BM, AT and UCM MSCs after EV production in static culture. BM, bone marrow; AT, adipose tissue; UCM, umbilical cord matrix.

The molecular composition of CM and cell samples from the time point experiment were analyzed by FTIR, and a PCA analysis was performed to compare the effect of the day of CM sample harvesting, the cell source and the percentage of MC applied. Firstly, the molecular profile of the SP medium that was not in contact with cells shows a more significant difference comparing to the CM (**Figure 4A**), indicating that the cells consume molecules from the culture medium and secrete other elements to it. It was also observed significant differences in the composition of CM obtained at different days of culture (day 3 *vs* day 6 *vs* day 8), regardless of the cell source (**Figure 4A**). Curiously, the differences between the culture media obtained on different days (dispersion of points from different colors) is greater than the differences observed between media from different sources (dispersion of points from the same day, same color), indicating that there was an evolution in the molecular composition of CM throughout the experiment, possibly related to the cell growth and replication, cell metabolism and cell communication (EVs, growth factors, cytokines and other soluble factors produced by MSCs).

The PCA obtained for cell samples from the different tissue sources at day 8 of experiment (**Figure 4C**) showed a distinct molecular profile tendency from AT MSC (red circles) comparing with BM and UCM MSC samples (blue squares and green triangles, respectively), which was according to the observed in CM samples (**Figure 4B**). The differences in the molecular profile of CM and cells from the three cell sources were expected since MSCs have inherent differences associated to the tissue of origin and are conditioned by environment in which they are originated from. In this manner, it is also expected that EVs obtained from cells derived from each MSC source will have different molecular profile and functional characteristics (Ribeiro et al. 2013; de Almeida Fuzeta et al. 2020), reflecting the differences observed in the molecular composition of CM.

Analyzing the molecular profile of CM from day 8 for each percentage of MC (**Figure 4G**, **Figure 4H** and **Figure 4I**), it is noticed an alteration in the molecular composition from the three cell tissue sources when 100% of medium was changed at day 6 (green triangles), less evident in the case of UCM MSC. In the case of the molecular profile from cell samples (**Figure 4D**, **Figure 4E** and **Figure 4F**), it was observed different molecular profiles from BM and UCM MSC also for 100% of MC (green triangles). This noticed impact on the molecular composition for CM and cell samples once the medium was completely changed at day 6 of experiment, can be related with a more significant presence of vesicles added from the cultured medium, with a higher level of cellular communication (number of secreted EVs and other soluble factors higher than the internalized ones), with a higher viability and ”healthier“ state of the cells (due to higher availability of glucose and lower lactate amount), or with the higher cell number.

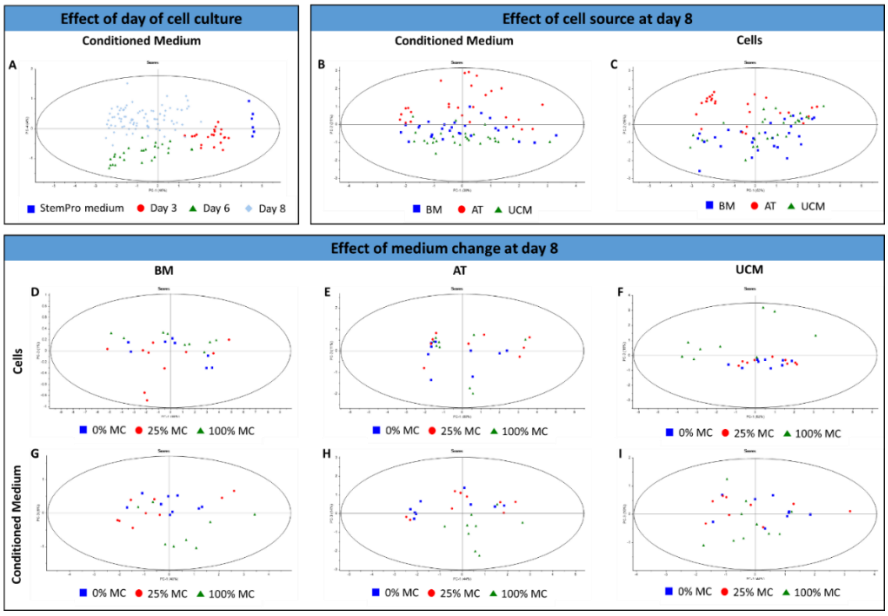

Figure 4. Comparing the molecular composition of CM and cell samples obtained in the time point experiment depending on different parameters, analyzed by FTIR. This representation of the results derives from PCA analysis of FTIR spectra. **(A)** Effect of day of sample harvesting at the MSC CM molecular composition, including comparation with StemPro medium composition. Variation of **(B)** CM and **(C)** cell sample molecular composition at day 8 of experiment depending on cell tissue source (BM, AT and UCM). Influence of the different percentage of MC (0%, 25% and 100%) in the molecular composition of **(D)**, **(E), (F)** cell samples and **(G), (H), (I)** CM from MSC BM, AT and UCM, respectively. BM, bone marrow; AT, adipose tissue; UCM, umbilical cord matrix; MC, medium change).

MSC-EV Quantification

MSC-EVs produced were quantified by NTA after EV isolation and the EV production profile for the three sources throughout the 8 days was analyzed (**Figure 5A**). To be used as control, a fresh sample of the medium employed in MSC culture was incubated in a T-Flask for 2 days (corresponding to the time of medium conditioning) under the same conditions of MSC culture and handled equally to quantify the particles present.

Since EVs are intercellular communication mediators, cells in culture capture and internalize both the particles and EVs fed within the culture medium supplied, as well as the EVs produced and secreted by other cells in culture. In this manner, the optimization of EV production must be based on the maximization of EV production relatively to EV uptake. The constant production and uptake of EVs throughout cell culture was considered in this work.

Considering that the amount of particles present in the medium used (7.39×10^{11} particles/mL) is added to the culture in the beginning of the experiment, and that it is the initial amount of particles in MSC culture, it seemed that the uptake of particles by cells was higher than the EV production until day 3 for the three sources (**Figure 5A**). UCM MSCs yielded the highest average EV concentration in the CM at this day ($2.59 \pm 0.69 \times 10^{11}$ particles/mL). The average EV concentration at day 3 was lower and similar for BM and AT MSC (0.87×10^{11} and $1.08 \pm 0.26 \times 10^{11}$ particles/mL, respectively; EV concentration of BM MSC at day 3 from one donor only). The higher uptake of particles and lower EV secretion in the beginning of cell culture is expected as cells are in the lag phase and adapting to the culture conditions, consuming more to initiate the growth. At day 3, the medium was renewed completely and therefore, it is plausible to consider that the EVs existent in the CM at that time were removed and that culture medium-particles were introduced with the culture medium added. From day 3 until day 6, the uptake of particles and vesicles by the cells seemed more balanced with the EV production (**Figure 5A**). The EV concentration in the CM was higher at day 6 comparing to day 3, and more similar to the particle concentration present in the culture medium, but this differed between cell sources. BM MSCs yielded the highest average EV concentration in the CM at day 6 ($8.56 \pm 3.89 \times 10^{11}$ particles/mL) but also the most heterogeneous among donors (n = 3), followed by UCM MSCs ($5.77 \pm 1.83 \times 10^{11}$ particles/mL) and then AT MSC ($2.90 \pm 0.56 \times 10^{11}$ particles/mL). At day 6, three different percentages of MC were applied to investigate the influence in MSC-EV production. Therefore, there are three different possible analysis for the EV concentration in the CM at day 8. In the case of 0% MC, the EVs present in CM at day 6 were not removed and neither particles from the culture medium were added. At this stage, the cells were in exponential growth phase leading to high confluence at day 6 (around 80%), which means that there were increasingly more cells to uptake particles and EVs but also to produce EVs. Therefore, it seems that for this condition, in the case of BM and UCM MSC, the uptake of EVs was higher than the production between day 6 and day 8, since the EV concentrations at day 8 ($3.56 \pm 0.65 \times 10^{11}$ particles/mL and $2.68 \pm 0.33 \times 10^{11}$ particles/mL, respectively) were lower than at day 6 (**Figure 5A**). For AT MSCs, EV concentration at day 8 ($2.19 \pm 0.52 \times 10^{11}$ particles/mL) was similar to day 6, which possibly indicates that the EV uptake and production balanced each other between these days. The EV concentration varied similarly to the condition of 0% MC. In the case of 25% MC, some of the EVs present in the CM at day 6 were removed and particles from the culture medium were added. The EV concentration varied similarly to the condition of 0% MC (**Figure 5A**). For BM and UCM MSC the uptake of EVs seemed also higher than the production between day 6 and day 8 (EVs concentrations at

day 8, 3.97 ± 0.51 × 10^{11} particles/mL and 3.57 ± 0.06 × 10^{11} particles/mL, respectively, were lower than at day 6, and for AT MSC seemed that balanced each other (EVs concentration of 2.59 ± 0.55 × 10^{11} particles/mL at day 8). It is important to remark that the discrepancy on EV concentration at day 6 was relatively high between the different donors from BM and UCM, and thus, the differences in the averages of EV concentration between day 6 and 8 observed for the conditions of 0% and 25% of MC for these sources could not be statistically significant. These observed heterogeneities emphasize the importance of testing MSC from multiple donors of each cell source to assess for intrinsic biological variability.

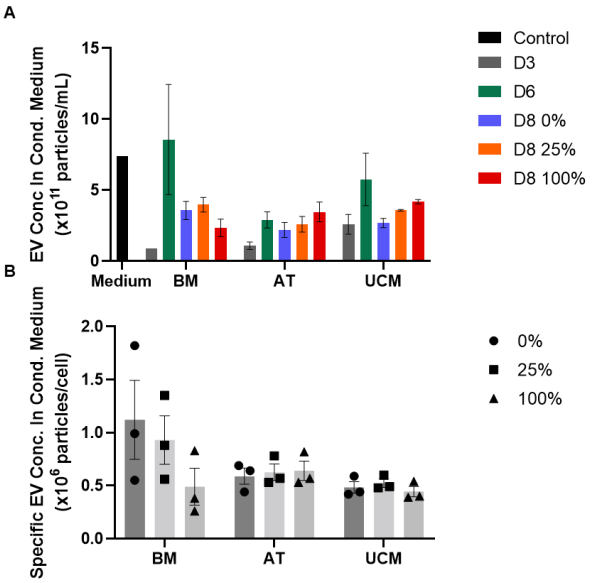

Figure 5. (A) EV concentration (x10^{11} particles/mL) in the cell culture conditioned medium from BM, AT, and UCM MSC cultures in static system. Samples of conditioned medium were harvested at day 3 (grey), 6 (green) and 8, and EVs were isolated with PEG solution. A fresh sample of the medium used as control (black). Three different percentages of medium change were applied at day 6 (before medium conditioning stage), 0 % (blue), 25% (orange) and 100 % (red). MSC from three different donors were used for each tissue source (i.e., n = 3 biological replicates). Results are presented as mean ± SEM (n = 3; except BM sample from day 3 and fresh medium sample, n=1). **(B)** Specific EV concentration (x10^{6} particles/cell) in the cell culture conditioned medium from BM, AT, and UCM MSC cultures in static system. MSC from three different donors were used for each tissue source and for each donor three different percentages of medium change were applied at day 6, 0 % (circle), 25% (square) and 100 % (triangle). Results are presented as mean ± SEM (n = 3). To reveal the variation across the measurements, these data are plotted as individual points, and the average of these measurements is also shown. BM, bone marrow; AT, adipose tissue; UCM, umbilical cord matrix.

Finally, when 100% of the medium was changed at day 6, it is expected that the EVs present in CM at day 6 were totally removed and particles from the culture medium were added. Here, it is necessary to compare again the EV concentration at day 8 with particles concentration from the medium added. In this manner, the uptake of EVs seemed also to surpass the production between day 6 and day 8 for the three sources, more sharply for BM MSC (**Figure 5A**). The EV concentration at day 8 (2.34 ± 0.61 × 10^{11} particles/mL, 3.46 ± 0.69 × 10^{11} particles/mL and 4.18 ± 0.15 × 10^{11} particles/mL, for BM, AT and UCM MSC respectively) were lower than the particle concentration in the culture medium added.

These three conditions of MC were also compared in terms of EV productivity (**Figure 5B**), that in the end is the factor that matters for the optimization of EV production per cell, the specific EV concentration. For that, EV concentration in the CM at the end of experiment (day 8) was divided by the cell concentration at that time. BM MSC donors showed more heterogenous EV specific concentration between the three MC conditions (between 0.49 ± 0.17 × 10^{4} particles/cell and 1.12 ± 0.37 × 10^{4} particles/cell) which is indicative that donor variability could be reflected in the EV productivity. AT and UCM MSC EV specific concentration showed similar values between MC conditions (between 0.59 ± 0.07 × 10^{4} particles/cell and 0.64 ± 0.09 × 10^{4} particles/cell; and between 0.44 ± 0.04 × 10^{4} particles/cell and 0.52 ± 0.04 × 10^{4} particles/cell, respectively). Generally, the different percentages of MC seemed not to impose statistically significant variations in EV productivity.

Purity assessment

The EVs were isolated from MSC CM using a method based on precipitation with a PEG solution. However, this approach is considered to result in low purity as the precipitation agent co-isolates contaminants and the molecules of biopolymers can also integrate the EV fraction and possibly interfere with further analysis of the sample (Konoshenko et al. 2018).

Therefore, to investigate the purity of the EV samples obtained, the protein to particle ratio (PPR) was determined by dividing the total protein concentration (determined by microBCA protein assay) (data not) by the EV concentration of the same sample (determined by NTA, **Figure 5A**) (Webber and Clayton 2013). The lower the PPR values, the lower is the amount of co-isolated protein contaminants after EV isolation and higher the sample purity. The PPR values from the EV samples obtained (**Figure 6**) were heterogeneous between days of sample harvesting and conditions of MC. BM MSC-EV sample from day 3 presented the higher PPR (9 fg protein/ EV particle), and in other words, presented the lower purity. While UCM MSC EV sample from day 6 presented the lower PPR (2.20 ± 0.73 fg protein/EV particle), which means the higher purity. Comparing these values with the PPR from the unpurified CM that were much higher (data not shown), it is possible to observe that the purity was largely increased after the EV isolation and that the majority of non-vesicular materials were removed from the CM samples. The values of concentration factor, calculated dividing the EV concentration (determined by NTA) from EV samples (**Figure 5A**) by the EV concentration from CM samples (data not shown), are also in agreement with this, ranging between 7.89 and 40.86 ± 17.17 and showing that besides the variability in the concentration efficiency and in the EV isolation, the EV samples were concentrated to a high extent after the isolation with PEG solution.

However, although it was observed a significant increase in purity, the EV samples presented considerable contaminants. Attending the purity classification proposed by Webber and Clayton in one of the few studies with a detailed purity analysis of EV samples using the PPR values (Webber and Clayton 2013), the EV samples obtained in this experiment were still not pure. According to this purity

classification, PPR lower than 0.03 fg protein/EV particle is high purity, PPR between 0.05 and 0.5 is low purity and PPR higher than 0.67 is impure. Nevertheless, Webber and Clayton evaluated the purity of EVs isolated by other method (ultracentrifugation), from different cell type (several cancer cell lines) and using different culture medium, which could influence these purity classification levels.

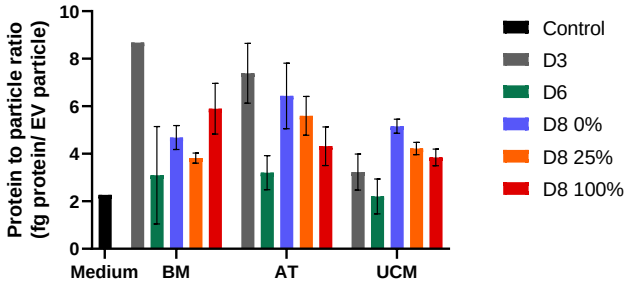

Figure 6. Purity assessment through the protein to particle ratios (PPR) (fg protein/ EV particle) of EV samples obtained throughout BM, AT and UCM MSC culture in static system, at day 3 (grey), 6 (green) and 8 (blue-0%; orange-25%; and red-100% medium change). MSCs from three different donors were used for each tissue source. Results are presented as mean ± SEM (n = 3; except BM sample from day 3 and fresh medium sample, n=1).

If the EV samples would be used as therapeutic product, a second method of EV purification should be applied in order to increase the purity of the EV samples and to remove the PEG precipitation agent.

Size distribution

NTA was also used to determine the size distribution of MSC-EVs. MSC-EV samples from the three sources showed no visible variation in size distribution profile between each other and throughout the experiment (**Figure 7A**), except in the case of EV samples from day 3 of the experiment, that showed a more heterogenous size distribution profile and a more notorious presence of vesicles with higher sizes, for the three different sources. Furthermore, MSC-EV samples were mostly enriched in small EVs (<200 nm) (**Figure 7B**). Curiously, the sample of fresh medium used as control showed a more heterogenous size distribution profile and higher mode size of particles (**Figure 7A** and **Figure 7B**). The presence of particles derived from the culture medium in the EV samples from day 3 could explain the more heterogenous size distribution profile and the presence of particles with higher sizes.

To produce MSC-EVs for therapeutic applications, the ideal would be the use of a culture medium that was not a source of particles that will be co-isolated with the MSC-EVs. For this work, SP medium was chosen for being well-defined and xeno/serum-free and thus considered a better option for therapeutic applications than, for example, FBS or hPL supplemented media that have a large amount of protein and vesicle contents prone to be co-isolated with the EV fraction, thus contaminating the end product. However, as shown by NTA analysis (**Figure 5A**- black bar), this medium also presented particles that could be interfering in MSC-EV quantification. To avoid this interference and the contamination of MSC-EV fractions, the StemPro® supplement could have been removed at the end of the MSC expansion period and StemPro® supplement-free medium used for the medium conditioning period. As seen when the medium was not change (0% MC), that the number of cells was lower at the end of experiment comparing to the other conditions of MC (**Figure 1**), the elimination of the supplement can be considered a possible stress factor for cell culture and could induce a different molecular profile to the MSC CM and EVs. Nevertheless, in the future, a comparison between supplemented and non-supplemented culture medium, as well as comparing with supplemented filtered medium (to ensure the removal of the culture medium particles) could be considered.

The fact the particles present in the SP medium used for MSC expansion and medium conditioning could have interfered in the quantification and characterization of EVs produced by MSCs in the time point experiment, led to the suggestion of using filtered medium to try to remove those contaminant particles. Since the NTA analysis of a fresh sample of SP medium identified that most of the particles present had size above 100 nm (**Figure 7**), it was decided to filter the medium with 0.1 µm filter to try to remove those particles and study MSC-EV production.

StemPro *vs* filtered StemPro medium experiment

Attempt of removing particles from the medium by filtration

In the following experiment, the MSC-EV production was studied for cells from one selected donor of BM cell source cultured using the normal SP medium in comparison with 0.1 µm filter SPf medium. In this case, a period of 5 days was selected for MSC expansion, considered the necessary time to achieve around 80% cell confluence and do not impose stress on cells due to the lack of space to attach and grow during medium conditioning. The next 2 days were considered for medium conditioning. The medium was totally changed at day 3 to support MSC expansion and two percentages of medium change at day 5 (0 and 100% MC) were applied. Here, CM samples were harvested only at day 7 and MSCs detached from the flasks for cell counting.

It was observed that the total cell number was slightly lower for the conditions using filtered medium, 1.48×10^6 cells for 0% MC of SPf compared with 1.79×10^6 cells for 0% MC of SP, and 2.26×10^6 cells for 100% MC of SPf compared with 3.19×10^6 cells for 100% MC of SP. Even so, the use of filtered medium seemed to be feasible for MSC expansion.

In terms of EV concentration in the CM (determined using NTA), the preliminary results showed that the variation between conditions was similar (**Figure 8A**). When the SPf was used, EV concentrations were lower comparing to SP, more sharply in the case of 0% MC (from $4.85 \pm 0.11 \times 10^{10}$ particles/mL to $2.86 \pm 0.09 \times 10^{10}$ particles/mL) than 100% MC (from $3.08 \pm 0.09 \times 10^{10}$ particles/mL to $2.42 \pm 0.08 \times 10^{10}$ particles/mL), although it did not seem significant. These differences in EV concentration could indicate that the filtered medium added less vesicles to the culture than the not filtered medium. Comparing the conditions of 0% and 100% MC (with or without medium filtration), EV concentration was higher when the medium was not changed at day 5. This could indicate that when totally changed at day 5, the medium did not add many more vesicles to the culture than the EVs produced by MSCs.

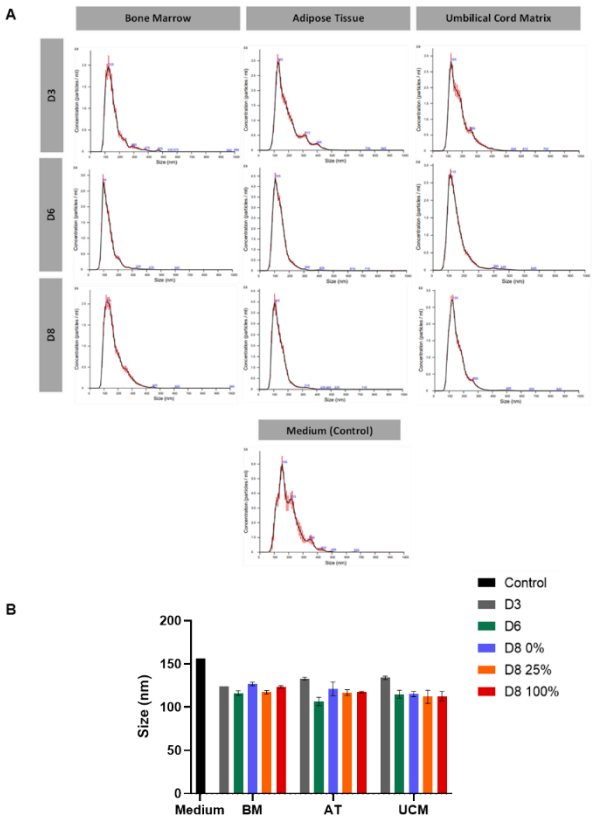

Figure 7. Size distribution of MSC-EVs. **(A)** Representative size (in nm) distribution curves of EV samples obtained at day 3, 6 and 8 of BM, AT, and UCM MSC cultures in static system, and from fresh SP medium used in the experiment. **(B)** Mode sizes (nm) of EV samples obtained at day 3 (grey), 6 (green) and 8 (blue-0%; orange-25%; and red-100% medium change) from BM, AT, and UCM MSC cultures in static systems, and from fresh SP medium used in the experiment (black). Results are presented as mean ± SEM. MSC from three different donors were used for each tissue source (i.e., n = 3 biological replicates). BM, bone marrow; AT, adipose tissue; UCM, umbilical cord matrix.

In the case of EV productivity (**Figure 8B**), it seemed to be higher when the medium was not changed at day 5 (filtered medium or not), as seen before for BM MSC in the time point experiment (**Figure 5B**). Although it seemed that cells grow healthier when the medium was completely changed at day 5, leading to a higher cell number at the end of the experiment, it had not positively influence in EV productivity.

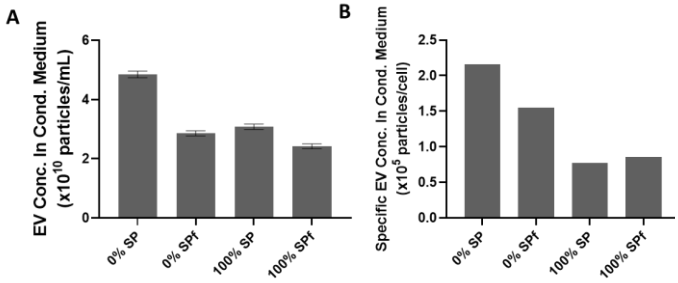

Figure 8. (A)- EV concentration (x10^{10} particles/mL) in the cell culture conditioned medium from BM MSC in the end of the 7 days of culture. Results are presented as mean ± SD. **(B)**- Specific EV concentration (x10^{5} particles/cell) in the cell culture conditioned medium from BM MSC culture in static system. (n=1) SP- StemPro® MSC SFM XenoFree medium; SPf- filtered StemPro® MSC SFM XenoFree medium.

Generally, the size of the EVs were similar between the different conditions, slightly higher than 100 nm, and also similar to the size of EVs present in the unpurified samples of cell culture CM.

In relation to purity of EV samples, it was observed a significant increase in purity after isolation with PEG solution, since the PPR values from EV samples decreased significantly comparing to unpurified CM samples. However, the PPR values from EVs samples were still elevated (between 11.79 fg protein/EV particle and 22.7 fg protein/EV particle) and higher than in case of samples from time point experiment (**Figure 6**), indicating that samples were still impure. The higher protein contamination could be due to lower yield of EV isolation method used, possibly a consequence of incomplete removal of supernatant after the centrifugation step (and therefore lower removal of protein) and of higher dilution of the EVs in the final resuspension with DNase/RNase-Free PBS 1x.

Without data from samples throughout MSC expansion and before medium conditioning, it is difficult to understand the profile of uptake/production of EVs and to be sure that the filtration of the medium in fact removed enough particles from the medium to avoid the contamination of MSC-EV samples and the interference in MSC-EV quantification and characterization. These doubts led to another experiment using again SP *vs* SPf.

Improved analysis of medium filtration and EV uptake/production profile

wasp and SPf were used again to study MSC-EV production when no medium change was performed throughout the 7 days of culture. The goal was to try to better understand the profile of EV uptake/production by MSCs without having the influence of particles added once the medium is changed. Another donor of BM was selected and MSC-EV production was studied for 7 days. Samples of CM were harvested at day 1, 3, 5 and 7 for EV isolation and further characterization. Samples of fresh SP and SPf were also processed and considered as day 0.

After filtration, the mean size of particles present in the medium decreased from 134 ± 1.2 nm to 116.2 ± 1.2 nm, and the mode size from 104.2 ± 3.1 nm to 92.7 ± 2.3 nm, showing the impact of the filtration with 0.1 µm filter. However, particles with size higher than 100 nm were still observed in the SPf medium and, although the particle concentration decreases in relation to the SP medium (1.91 ± 0.04 × 10^{10} particles/mL), it is also observed a high particle concentration in the SPf medium (1.62 ± 0.03 × 10^{10} particles/mL) (**Figure 9** - Day 0). Therefore, it seems that the SPf medium could still add particles to the culture and possibly contaminate the MSC-EV samples.

In relation to the understanding of EV uptake/production profile throughout the experiment, the preliminary (n=1) results seem to indicate that the uptake of EVs was increasing in relation to production during MSC expansion (from the beginning until day 5), since the EV concentration seemed to decrease for both CM (**Figure 9**). This agrees with the necessities of MSC growth. After cells reach 80% confluence (around day 5), EV concentration seemed to maintain identical values which could mean that EV uptake and production balanced each other. After day 5, there is still cell growth although to a lower extent compared to day 3 to 5 of the exponential phase. Nevertheless, it is expected the uptake of EVs by cells. Furthermore, during this period there

are more cells to uptake EVs. However, there is also more cells to produce EVs. Therefore, it seems that the increased EV uptake is balanced by the increased EV secretion.

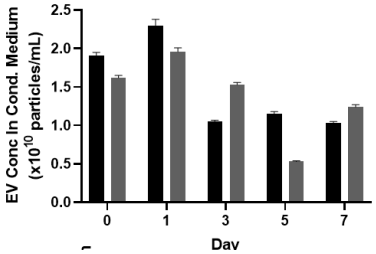

Figure 9. - EV concentration (x10^{10} particles/mL) in BM MSC-CM at days 1, 3, 5 and 7 of culture, when using SP medium (black bars) and SPf medium (grey bars). Fresh samples of the media were considered as day 0. Results are presented as mean ± SD. **(B)-** Specific EV concentration (x10^6 particles/cell) in MSC-CM. SP- StemPro® MSC SFM XenoFree medium; SPf-filtered StemPro® MSC SFM XenoFree medium.

Without medium change throughout 7 days, it is possible that particles added from the medium in the beginning of the experiment were totally consumed during the first 5 days of culture, not causing a significative contamination of EV samples. However, it is difficult to be sure of that and thus, the use of a medium without particles seems to be the best option. On other hand, proving that particles from the medium used are not toxic and perhaps additionally beneficial for therapeutic applications, as reported before for example for hPL supplement (Witwer et al. 2019), the total removal of the particles from the medium could not be extremely necessary.

Influence of hypoxia and temperature in MSC-EV production

To further explore the impact of culture conditions on MSC-EV production, the influence of oxygen tension (normoxia *vs* hypoxia) and temperature (37ºC *vs* 33ºC) in MSC-EV production was also studied using SP and SPf. MSC expansion and medium conditioning were studied under normoxia (21% O_2) or hypoxia (5% O_2), and a temperature of 37ºC or 33ºC was studied during medium conditioning.

Samples of CM were also harvested at day 1, 3, 5 and 7 for EV isolation and characterization. In the case of the hypoxia condition, this allowed to have an overview of EV uptake/production profile throughout the MSC expansion and medium conditioning. For the temperature condition, since the 33ºC were just applied during medium conditioning to not affect MSC expansion, during the first 5 days MSCs were under the same conditions as for the study of filtered *vs* unfiltered medium. Therefore, it is only possible to analyze the EV uptake/production during medium conditioning.

The analyses of the preliminary results of EV concentration in the CM throughout the 7 days when the SP medium was used (**Figure 10A**), indicate that hypoxia seemed to have influence in EV uptake/production during MSC expansion and medium conditioning (blue bars). Comparing with normoxia at 37ºC during MSC expansion (grey and green bars), the condition of hypoxia at 37ºC shows a lower amount of EVs per volume of CM at day 1, and at day 3 it shows a considerable higher amount. The EV uptake seemed to be higher in the beginning of MSC expansion and followed by a higher EV production possibly conjugated with a decrease in EV uptake. After day 3, the EV production seemed to decrease, or the uptake to increase, or both. During the medium conditioning (between day 5 and 7), the EV production seemed to increase again or be balanced with the uptake. When the SPf medium was used, this possible influence of hypoxia in EV uptake/production was not notorious (**Figure 10C**). The EV concentration in CM from this condition (blue bars) seemed to vary the same way than from the condition of normoxia at 37ºC (grey bars), no considerable variations were observed in EV concentration from samples of each day of the experiment.

In the case of the culture at 33ºC in normoxia during medium conditioning, comparing the EV concentration from days 5 and 7, it seems that the EV uptake by cells was higher than the production during that period when SP medium was used (**Figure 10A**- green bars) and that EV uptake and production were more balanced when SPf medium was used (**Figure 10C**- green bars). However, these variations were not much different from normoxia at 37ºC. Overall, it seemed that EV uptake was higher than the production throughout MSC expansion and that balanced each other during medium conditioning.

In relation to EV productivity, the change of temperature to 33ºC during conditioning seemed to have some positive influence (**Figure 10B** and **Figure 10D**- green bar). The specific EV concentration in the CM was higher for this condition (6.12 × 10^6 particles/cell for SP medium and 5 × 10^6 particles/cell for SPf medium) relatively to normoxia 37ºC (4.96 × 10^6 particles/cell and 3.66 × 10^6 particles/cell) and hypoxia conditions (5.8 × 10^6 particles/cell and 3.27× 10^6 particles/cell), that showed similar values. However, these improvements could not be significative, and it is necessary to perform more tests with MSC from other tissue and cell donors to confirm it. Actually, the higher specific EV concentration when applied 33ºC during conditioning could be related with the observed lower number of cells, contrarily to the condition of hypoxia.

The size of EVs did not present significant differences between the conditions. The particle size seemed to decrease throughout the experiment period, more precisely from day 3 onwards. This could indicate that the particles added by the medium in the beginning and that appear to have higher sizes than EVs produced by MSCs (**Figure 7B**), could have been captured by cells during the expansion, and at least, be present in relatively lower amount in the MSC-EV samples in the end of experiment.

The EV samples processed in this final experiment seem to have similar PPR values relatively to samples from the previous experiments, in general lower than 10 and higher than 2, showing a low purity degree.

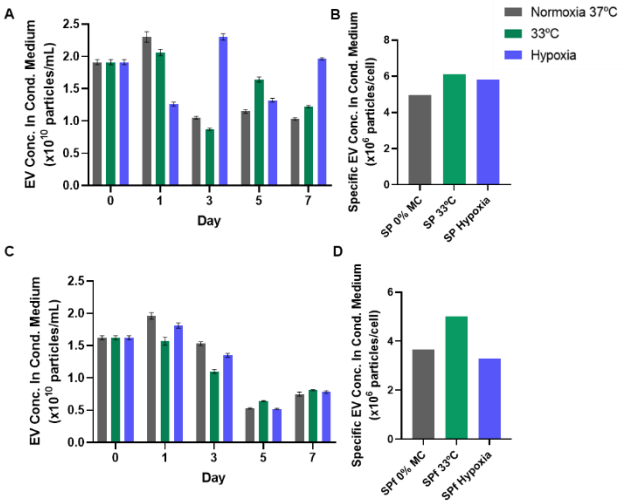

Figure 10. Comparing the influence of oxygen tension and temperature in MSC-EV production using BM MSCs (n=1). **(A) and (C)-** EV concentration (x10^{10} particles/mL) when using SP and SPf medium, respectively, for the conditions of normoxia at 37ºC (grey), normoxia at 33ºC (green), and hypoxia (5% O_2) at 37ºC (blue). Samples of MSC-CM were harvested at day 1, 3, 5 and 7. SP and SPf medium were used as controls (day 0). Results are presented as mean ± SD**. (B) and (D)-** Specific EV concentration (x10^6 particles/cell) when using SP and SPf medium, respectively, for the conditions normoxia at 37ºC (grey), normoxia at 33ºC (green), and hypoxia (5% O_2) at 37ºC (blue). SP- StemPro® MSC SFM XenoFree medium; SPf-filtered StemPro® MSC SFM XenoFree medium.

CONCLUSIONS AND FUTURE WORK

The present work provides insights regarding the influence of different cell culture parameters in MSC-EV production , including cell source, donor variability, time of EV collection, medium change, culture medium, oxygen tension and temperature, as well as about EV degradation and adhesion to plastic throughout cell culture and the MSC-EV uptake/production profile. Overall, the MSC source and medium change seemed not to imply significant variability in EV production and productivity, and the time point after MSC expansion and medium conditioning seemed the best one for EV collection. The EV uptake seemed higher than the production during MSC expansion and balanced each other during the medium conditioning. Regarding the removal of contaminating culture medium particles, a filtration with 0.1 µm filter seemed inefficient. The temperature of 33ºC applied during conditioning medium seemed to increase the EV productivity, contrarily to hypoxia, but further studies are necessary to confirm if the increase observed is statistically significant. After the isolation of EVs from the conditioned media with PEG solution, it was observed that a large part of contaminants was removed, but the values of PPR were still high, indicating that the EV samples obtained were not pure. Thus, for therapeutic applications, a second method for EV purification would be needed.

In the future, culture medium filtration using a filter with smaller pores or alternative methods (ultracentrifugation) could be considered for a more efficient particle removal and to remove particles below 0.1 µm, as well as additional testing to evaluate the influence of hypoxia and 33ºC in MSC-EV production. Other culture parameters could be studied, and the optimal culture conditions translated to dynamic systems, such as spinner flasks and fully controlled bioreactors. Different serum/xeno-free culture media and several microcarriers could be tested, as well as different oxygen concentrations, temperatures, and shear stress that in this case could be better controlled. Exposure of MSCs to inflammatory conditions, by adding pro-inflammatory cytokines to the cell culture, could also be tested. Furthermore, functional studies will be required to evaluate the applicability and potency of MSC-EVs produced and to investigate their therapeutic potential, that can differ depending on the MSC source and donor.

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