# Interaction of mesenchymal stem/stromal cell-derived exosomes with human cell lines for different biomedical applications

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## Abstract

Mesenchymal stem/stromal cells (MSCs) are multipotent cells characterized by their unique biological properties, already explored for the treatment of several conditions. However, accumulating evidence indicates that most MSC therapeutic properties are related to their secretome, amongst which are exosomes, small membrane-vesicles that have been shown to play an important role in intercellular communication and numerous biological processes. The mechanisms underlying exosome function are still mostly unknown, as is also their potential in the treatment of pathologies. The main objective of this work was to perform a preliminary characterization of the biological function of exosomes obtained from MSCs of different tissues and healthy donors expanded in bioreactors. In a first approach, the effects of these vesicles on the proliferation of two adenocarcinoma cell lines (MCF-7 and A549) and one representative of the blood-brain barrier (hBMEC) were assessed and a response pattern was noted for both MCF-7 and A549. In addition, A549 invasion through Matrigel was also evaluated and the presence of the MSC-exosomes was seen to be stimulating. Furthermore, control exosomes were isolated, for comparison with the functional activity of MSC-derived exosomes, and were characterized using BCA, NTA and Western Blot analyses. For the tested samples, size distribution and markers characteristic of exosomes were seen. Finally, the labelling of the vesicles was attempted with an anti-CD63 antibody conjugated with FITC and three protocols were tested and compared. These results highlight the possibility for the application of MSC-exosomes for therapeutic purposes, however further assessment of their mechanisms of action is still necessary.

## 1. Introduction

Extracellular vesicles (EVs) are secreted membrane vesicles that originate from most cell types and can be found in the majority of bodily fluids. Depending on biogenesis, size and specific membrane markers, EVs can be divided into different categories, of which the three main types are exosomes (40 nm-100 nm), apoptotic bodies (50 nm-5 µm) and microvesicles (50 nm-1µm). Exosomes are nano-sized vesicles lined by a lipid bilayer and secreted upon fusion of multivesicular bodies (MVBs) with the plasma membrane (PM). In fact, exosomes correspond to the intraluminal vesicles (ILVs), which are accumulated within the MVBs due to inward budding of the endosomal membrane. As a consequence of their origin, the released exosomes will contain proteins, lipids and RNA molecules, entrapped inside the ILVs at the time of their formation, that may be representative of the parent cell. Nevertheless, a conserved set of proteins has been identified in exosomes, regardless of cellular origin, that could be used as markers of these specific vesicles. These include the MVB biogenesis molecules Alix and TSG101, the tetraspanins CD63, CD81 and CD9 and the heat shock protein HSP70.[1]

Exosomes were first described in the 1980's and were thought to be a mechanism characteristic of maturating reticulocytes for the shedding of specific PM components [2]. However, over the years more biological functions have been attributed to exosomes and these vesicles have been demonstrated to play an important role in intercellular communication. In fact, exosomes are now known to take part in the maintenance of normal body physiology and also to have an important role in pathogenicity. The functions of the released exosomes depend on their cellular origin and are believed to be mostly mediated by the transfer of proteins and RNA molecules, that will prompt the alteration of the target cell's phenotype [3]. Given this, the application of these vesicles towards therapeutic purposes has attracted considerable interest, as they can be isolated from several different sources and have multiple possible clinical applications.

Over the past years, many efforts have been made towards the development of stem cell-based therapies and, with this, interest on the use of mesenchymal stem/stromal cells (MSCs) has risen. MSCs are non-hematopoietic, fibroblast-like cells, that have the ability to be expanded *in vitro* while maintaining their capacity to differentiate into multiple mesenchymal lineages such as the osteogenic, chondrogenic and adipogenic cell lines. These cells can be found and extracted from most tissues, including adipose tissue (AT), peripheral blood and several birth-associated tissues, like amniotic fluid and umbilical cord matrix (UCM). Nevertheless, bone marrow MSCs, the first to be identified, are considered the gold standard for MSC

based therapies and can be used for the comparison of MSCs from other sources [4].

Their wide availability, relatively ease of harvest and lack of ethical concerns, are some of the major advantages of MSCs over other stem cells. Furthermore, they have been shown to have the ability to home to injured sites, a high plasticity which allows for their differentiation into cells of all germ layers and a broad spectrum of immuno-modulatory capacities. These properties have been explored in a series of studies and results show that MSCs do in fact exhibit elevated efficacy in a variety of disease models [5]. Initially, it was thought that MCSs' healing properties were mediated by their engraftment and direct interaction with the injured site. However, new hypotheses suggest these may actually be a result of alternative modes of action that modulate the surrounding microenvironment, as is the secretion of regulatory and trophic factors (i.e. growth factors, cytokines and chemokines) as well as extracellular vesicles [6].

For these reasons, MSCs are an attractive source of exosomes, as it is thought that these vesicles will retain the parent cells' healing abilities, allowing for their application in the conditions where MSC-based therapy has already been employed and thus overcoming some of the issues associated with cell therapy. In this context, many techniques have been developed towards the isolation of exosomes from conditioned cell culture media, which can be separated into five main types: Ultracentrifugation-based, Size-based, immunoaffinity-based, precipitation and microfluidics-based. Currently, ultracentrifugation-based methods are the most commonly used for EV isolation. However, this method can be quite expensive, as it requires specific equipments, and entails considerable amounts of time for a successful isolation. Given this, the development of other methods has been advantageous, as is the example of the commercially available exosome precipitation kits. These are mostly comprised of polymers which promote the alteration of the vesicle's solubility by tying up water molecules and consequently forcing their precipitation. Despite the fact that these methods are quite convenient and straightforward, which is a major benefit, the resulting yields are variant and there is a risk of co-precipitation of other components, possibly compromising the purity of the isolated sample [7].

Several studies have demonstrated the extent of the potential of MSC-exosomes, from their successful use in the treatment of certain conditions to their application as drug delivery agents. However, the mechanisms of exosome action and uptake by the recipient cells, as well as their full therapeutic effects, are still unknown and their determination remains a challenge.

The main goal of this work was to perform an initial characterization of the functional activity of MSC-derived exosomes, in a way to understand what effects they may have on cells from different disease and healthy cell lines and determine the mechanisms underlying those effects, thus possibly establishing a baseline for future assays. This was attempted for exosomes obtained from MSCs, expanded in vertical-wheel bioreactors, of different tissue sources and healthy donors, thus conceivably also allowing for a comparison between these parameters and the determination of their contribution towards the effects of their secreted vesicles.

In a first approach, these vesicles were used for treatment of two human cancer cell lines and a human brain microvascular endothelial cell line, a major component of the blood-brain barrier, with the aim of monitoring their proliferation and, for one of the tumour cell lines, the capacity of *in vitro* invasion through Matrigel in response to the treatment with MSC-derived exosomes. Furthermore, exosomes were also isolated from other sources, following the same isolation protocol, and characterized in order to be used as controls in parallel experiments.

Finally, the determination of the molecular mechanisms by which these vesicles are taken into the cells is also of interest, and so a confocal microscopy approach is intended. Consequently, in this work, the labelling of these vesicles was attempted with an immune-fluorescence approach, in opposition to a lipid membrane staining method, and further characterization of stained exosomes was also performed.

## 2. Materials and Methods

### 2.1. Cell Lines and Cell Culture

Human breast cancer (MCF-7), human non-small cell lung cancer (A549), human embryonic kidney 293 (HEK293) and human brain microvascular endothelial (hBMEC) cell lines were obtained from ECACC (European Collection of Authenticated Cell Cultures).

MCF-7, A549 and HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO™), supplemented with 10% heat-inactivated (HI) fetal bovine serum (FBS, GIBCO™), 100 IU/mL penicillin and 100 mg/mL streptomycin (1% PenStrep, GIBCO™).

hBMEC cells were cultured in Roswell Park Memorial Institute Medium (RPMI-1640) supplemented with 10% HI FBS, 10% Nu-serum, 1% MEM-vitamins, 1% PenStrep, 1% MEM non-essential aminoacids and 1% Sodium pyruvate.

The cells were maintained in culture in T-Flasks, at 37ºC in a humidified atmosphere with 5% $CO_2$ and passaged by chemical detaching with Trypsin 0.05%, when ~90% confluence was reached.

### 2.2. Serum Inactivation

Exosome-depleted FBS (GIBCO™), was inactivated by heat (Heat-inactivation, HI). Briefly, the serum was thawed at room temperature and swirled every 10-15 min. After completely thawed, it was submerged in a temperature-controlled water bath at 56ºC for 30 min and gently swirled every 5-10 min. The temperature of the water bath was monitored using a control bottle with water and a suspended calibrated thermometer. After the 30 min, the serum was removed from the bath, gently swirled again and allowed to cool at RT. The heat inactivated serum was then stored at -20ºC, until use.

### 2.3. Exosome Samples

Exosomes derived from MCF-7, A549 and HEK293 cell lines were isolated from cells' conditioned media using total exosome isolation reagent (Invitrogen). Briefly, the cells were cultured in DMEM supplemented with 10% FBS and 1% PenStrep until 80-90% confluence was reached. Afterwards, cells were washed two times with PBS and medium was exchanged to DMEM supplemented with 10% HI Exosome-Depleted FBS, for 48 h. The conditioned media was then collected and centrifuged at 2,000x*g* at room temperature (RT) for 30 min. The supernatant was transferred to a centrifuge tube and 1/2 of the volume of total exosome isolation reagent was added and well homogenized. This solution was incubated at 4ºC overnight (12h - 18h) and subsequently centrifuged at 10,000x*g* at 4ºC for 1 h. The resulting supernatant was discarded, and the exosome-pellet was resuspended in PBS (high-grade) in order to achieve a concentration factor of 40. The total protein concentration of the samples was determined using a Pierce™ BCA Protein Assay Kit (Thermo Scientific™), following manufacturer's instructions, and the exosomes were stored at -80ºC until further use.

As a control, DMEM medium supplemented with 10% HI Exosome-Depleted FBS, not conditioned by cells was also processed by the same method.

MSC-derived exosome samples isolated from umbilical cord matrix (donors UCM#40 and UCM#45, UCM-MSCs(1) and UCM-MSCs(2), respectively), bone marrow (donors M79A15 and F99A18, BM-MSCs(1) and BM-MSCs(2), respectively) and adipose tissue (donor L140326, AT-MSCs) were provided by PhD student Miguel Fuzeta, as they were obtained in the scope of his work. Briefly, the cells were initially expanded in T-flasks and cultured in DMEM supplemented with 5% human platelet lysate (hPL). Subsequently, they were inoculated in vertical wheel bioreactors and grown attached to microcarriers, in the same medium. For conditioning, the medium was exchanged to basal DMEM for 48h. Exosomes were then isolated from the conditioned medium using total exosome isolation reagent, as described.

### 2.4. Protein extraction and Western blotting analysis

Cells conditioned for exosome isolation were washed twice with PBS and resuspended and lysed in catenin lysis buffer (1% Triton X-100, 1% Nonidet-P40 in deionized PBS) supplemented with 1:7 proteases inhibitor (Roche Diagnostics GmbH, Germany), for 10 min at 4ºC. The lysates were collected, vortexed three times, for 10 seconds each time, and centrifuged at 14,000 rpm at 4ºC for 10 min. The supernatant was kept and the total protein concentration was determined by Bradford method.

30 µg and 20 µg of total protein of exosome samples and of whole cell lysates, respectively, were prepared with Laemmli sample buffers (1x and 4x), denaturated at 100ºC for 5 min and separated by electrophoresis in 12% polyacrylamide gels.

Gels were transferred onto nitrocellulose membranes (RTA Transfer Kit, BioRad), using Trans-Blot Turbo Transfer System (BioRad), following the manufacturer's instructions. Transfer was confirmed with Ponceau S staining solution and non-specific binding sites were blocked for 1 h with 5% (w/v) non-fat milk in PBS-Tween-20 (0.5% v/v). Subsequently, the membranes were incubated in an agitator overnight at 4ºC with primary antibody [anti CD63, 556019, BD Pharmingen™, diluted 1:100 in 5% (w/v) non-fat milk in PBS-Tween-20 (0.5% v/v)].

The membranes were then washed three times with PBS-Tween-20 (0.5% v/v), for 5 min, and incubated for 1 h, at room temperature, with the proper secondary antibody, conjugated with horseradish peroxidase [ m-IgGκ BP-HRP: sc-516102, Santa Cruz Biotechnology, diluted 1:2000 in 0.5% PBS-Tween-20 (0.5% v/v)]. Afterwards, they were washed five times with PBS-Tween-20 (0.5% v/v), for 5 min and developed by adding ECL substrates (Pierce) and chemiluminescence was captured by Fusion Solo equipment (Vilber Lourmat).

### 2.5. Nanoparticle Tracking Analysis

Particle concentration and size distribution of the isolated exosome samples were determined using a NanoSight LM10 instrument (Malvern, Worcestershire, UK) and NTA 3.1 software. Samples were diluted in PBS (high-grade), to a final volume of 1.5 mL, to achieve a particle concentration ranging from $5\times10^8$ to $2\times10^9$ particles/mL. Ten, runs of 30 seconds, were recorded for each sample, at 20ºC, with a camera level of 13, and, analysed with a detection threshold of 13. All parameters were optimized by pre-testing with 100 nm silica microspheres (Polysciences).

### 2.6. Cell Viability assays

For the establishment of the appropriate cell density for the viability experiments, MCF-7 and A549 cells were seeded in 96-well plates at different cell densities ($1\times10^5$, $5\times10^4$, $2.5\times10^4$, $1.25\times10^4$, 3125 and 780 cells/well), and as a control, wells containing fresh medium were used. After 24 h, a PrestoBlue™ (PB™, Life Technologies) viability assay was performed by washing the cells twice with PBS and adding 100 µL of DMEM with 10% PB™ reagent to each well. Fluorescence intensity was measured using a microplate reader (FilterMax F5,

Molecular Devices) with 535 nm excitation and 595 nm emission, for 6 h, every 1 h, meanwhile the cells were incubated at 37ºC, 5% $CO_2$.

To determine the influence of media exchange on the viability of MCF-7 and A549 cells,$1\times10^4$ cells were seeded in 96-well plates and cultured for 24 h. After this time, cells were treated by exchanging the medium to DMEM+10% HI exosome-depleted FBS, or, as control, the medium was exchanged for fresh culture medium. Viability was assessed at 24, 48 and 72 h time points through PB™ Viability and MTT [3-(4,5 dimethylthiazol-2-yl-2,5 tetrazolium bromide)] assays. PB™ viability assay was performed as described and the fluorescence intensity measured for 4 h. For the MTT assays, after incubation in the different media, cells were washed with PBS and 100 µL of DMEM and 20 µL of MMT (5 mg/mL) were added to each well, followed by incubation at 37ºC for 3.5 h. The reaction was stopped with the addition of 150 µL of 40 mM HCL in isopropanol and MTT formazan formed was spectrophotometrically read at 590 nm in a microplate reader. The viability values were calculated from the fluorescence intensity (at 3.5 h time point)/absorbance values as a percentage of the control cells.

For the assessment of the effect of media changes for hBMEC cells, a PB™ viability assay was conducted. $5\times10^4$ and $1\times10^4$ cells were seeded in a 96-well plate and cultured in fully supplemented RPMI-1640 (control) or DMEM+10% HI exosome-depleted FBS for 24 h. The media were then exchanged for fresh corresponding media and the cells were left to incubate for 48 h. Viability was then determined as previously described. In addition, hBMEC cells were also seeded in rat-tail collagen type I coated 96-well plates at different cell densities ($5\times10^4$, $2.5\times10^4$, $1.25\times10^4$, 3125 and 780 cells/well), as a control wells containing fresh medium were used. After 48 h, a PB™ viability assay was performed and fluorescence intensity was measured as described, for 4 h, every 30 min.

For the assessment of the effect of exosome and conditioned media samples in MCF-7, A549 and hBMEC cells, $1\times10^4$ cells were seeded in a 96-well plate and cultured in exosome-depleted medium for 24h. The cells were washed twice with PBS and treated with 100 µL of exosome solutions at different total protein concentrations (200, 100, 50, 25, 10 or 5 µg/mL) or 100 µL of conditioned medium solutions. Dilutions were performed using PBS and medium supplemented with HI exosome-depleted serum, for the conditioned medium solutions the volumes used for the dilutions were the same as those used for the 100 µg/mL solution of the correspondent exosome samples. As a control, cells were treated with a solution of medium and PBS, using the same volumes as the prepared exosome solutions (0 µg/mL). After 48 h of incubation, PB™ viability assays were performed as described and the viability values were calculated.

### 2.7. Transwell Invasion Assays

The invasion capacity of A549 cells through Matrigel was assessed using a 24-well-transwell migration assay (Corning® BioCoat™ Matrigel® Invasion Chambers) with cell culture inserts containing an 8 µm pore size PET membrane treated with a thin layer of Matrigel (Falcon®). The inserts were rehydrated with basal DMEM for 3h, at 37ºC, according to manufacturer's instructions.

After rehydration, the medium was removed and 750 µL of exosome-depleted medium were added to the bottom chamber. Cell suspensions (250 µL) were prepared in DMEM+10% HI exosome-depleted FBS, containing $5\times10^4$ cells as well as different dilutions of exosome samples (5, 10, 25 and 50 µg/mL), in PBS, and added to the upper chamber. As a control, cell suspensions were prepared with either a solution of medium and PBS, using the same volumes as the prepared exosome solutions (0 µg/mL), or with processed basal DMEM diluted in medium, in the same volumes as the prepared exosome solutions. An initial experiment where cells were resuspended in either normal culture medium or exosome-depleted medium, was also conducted to assess the influence of the different media in the invasion capacity of the cells.

Following a 48h incubation period, at 37ºC, the inserts were washed with PBS and non-migrated cells were removed from the upper side of the chamber with cotton swabs dipped in PBS. Migrated cells were fixed in cold methanol (4ºC) for 10 minutes. After complete drying, the membranes were removed using a scalpel, placed in a microscope glass slide and stained with VECTASHIELD® Hardset™ mounting medium with DAPI (Vector Laboratories). The slides were stored at 4ºC overnight or -20ºC for longer periods. Cells of ten independent fields were counted under a fluorescence microscope (Zeiss), using the ImageJ software setting "Analyse Particles" [Size ($pixel^2$): 800-1000; Circularity: 0.1-1.0] and the mean number of migrated cells per condition was determined by the average of all fields. Results are presented as the fold change in migration of the cells when compared to the control (0 µg/mL).

### 2.8. Fluorescent Labelling of Exosomes

Exosomes from HEK293 cells were labelled with an anti-CD63 antibody coupled with FITC (0.04 mg/mL mouse anti-Human CD63 FITC, EXBIO) in three different experiments. Initially, a volume equivalent to $6.5\times10^8$ particles of exosome specimens was diluted in 500 µL of PBS+0.2% BSA and then mixed with 20 µL of the labelled antibody and incubated for 2h, at RT, in the dark. For the following experiments, volumes equivalent to $9.6\times10^9$ and $1.1\times10^{10}$ particles of exosome specimens were mixed with 50 µL of the labelled antibody, and incubated at different conditions (2h, at RT and overnight, at 4ºC, respectively) in the dark. Any necessary dilutions were made in PBS+0.2% BSA. For a blank control, the antibody was replaced by PBS+0.2% BSA.

To wash out unbound antibodies, the samples were transferred to an ultrafiltration unit (Amicon®, Merk) with a 100 kDa cut-off and three centrifugations at 5000 rpm for 1 min were performed, with the addition of PBS+0.2% BSA for each to wash the sample. The membrane was thoroughly washed with PBS+0.2% BSA and the labelled exosomes solutions were stored at 4ºC, in the dark.

To determine the effectiveness of the washout steps, particle concentration and size distribution of the samples were determined using NTA, and to confirm the effectiveness of the labelling strategy, fluorescence emission spectra were obtained from 480 nm to 600 nm, in a spectrofluorometer (Fluorolog®-3-21, HORIBA Scientific, USA) for an excitation wavelength of 460 nm.

## 3. Results

### 3.1 Establishment of conditions for MCF-7, A549 and hBMEC cells viability assays

Initially, the establishment of the conditions for the viability experiments was necessary. For this several cell densities, of all three cell lines, were tested for 4 to 6 hours, depending on the assayed cell line, using a PrestoBlue™ viability assay. Analysing the results (data not shown) It was determined that all viability assays on these cells should be performed using cell densities in the $1x10^4$ cells per well range with fluorescence intensity being measured for 4 hours. In the case of hBMEC cells, it was also determined that these cells should be cultured in 96-well plates coated with rat-tail collagen type I, as a considerable amount of cell detachment was observed in some wells, when these cells were cultured directly in non-coated plates.

Subsequently, it was necessary to determine whether exchanging the medium of cells during the assay had some influence on their viability, seeing as, for the assays using exosome samples, medium supplemented with HI exosome-depleted serum must be used, in order to ensure that there is no interference of other vesicles (present in FBS) in the obtained results [8]. Also, for hBMEC cells there was the need of determining whether the cells would remain viable when cultured, and subsequently assayed, in DMEM medium, as they are usually maintained in RPMI-1640. Results showed that the alteration of the culture medium after seeding did in fact affect the viability of the cells, hence the assay should be established directly in the medium containing exosome-depleted FBS, in order to allow for the cells to adapt more effortlessly to the new medium and consequently avoid the interference of this effect on the obtained values for cell viability. Additionally, hBMEC cells were not seen to be significantly affected when cultured and assayed in this medium, and so it was concluded that all testing on these cells could be performed in DMEM culture medium.

### 3.2 Influence of MSC-derived exosomes on MCF-7, A549 and hBMEC cell viability

Once the experiments were established the effects of treatment with MSC-derived exosomes from different sources on the viability of the cells, were assessed. For this, four different exosome samples, obtained from Umbilical Cord Matrix (UCM), Adipose Tissue (AT) and Bone-Marrow (BM) mesenchymal stem/stromal cells cultured in vertical-wheel bioreactors, were diluted, in order to achieve six solutions with concentrations up to 200 µg/mL of total protein, for the UCM-MSC derived samples, and 100 µg/mL, for the remaining. MCF-7, A549 and hBMEC cells were then seeded in the conditions previously determined and exposed to these solutions, after 48 hours of incubation a PB™ viability assay was performed. The graph in **Figure 1** was created using the values of viability calculated for the cells exposed to exosome samples, relatively to the cells to which no exosomes were added (control – 0 µg/mL).

Results obtained for both tumour cell lines were comparable, demonstrating a similar response pattern for all three sources of MSCs. In general, it was seen that lower concentrations of exosome solutions had a stimulating effect and promoted the proliferation of the cells, while, on the contrary, this effect was attenuated for the cells exposed to higher concentrations of exosomes, which were shown to have lower viability values in comparison. Nevertheless, and despite the fact that these results were overall comparable, it was noted that A549 cells had much less significant variations in cell viability when compared to what was seen for the MCF-7 cells, demonstrating that these vesicles have a smaller impact in their proliferation.

In contrast, when it came to the response of hBMEC cells to this treatment no cohesive pattern was observed for the different samples tested, however these cells seemed to be more affected by the MSC-exosomes than the previous ones, with the exception of the UCM-MSCs derived sample, which led to a slight increase in cell viability.

Overall, these results demonstrated that some effects are exerted over the cells by the experimented exosomes, which can be different depending on the assayed cell line and, in some cases, on the source of the tested exosome samples. Therefore, this should be further assessed in a way to understand the mechanisms underlying these effects.

### 3.3 Biophysical and biochemical characterization of isolated control exosomes

As suggested in MISEV2018 [9], when the functional activity of specific exosome samples is being studied, certain controls should be employed and produce minimal effects over the tested cells. Considering this, several negative or background

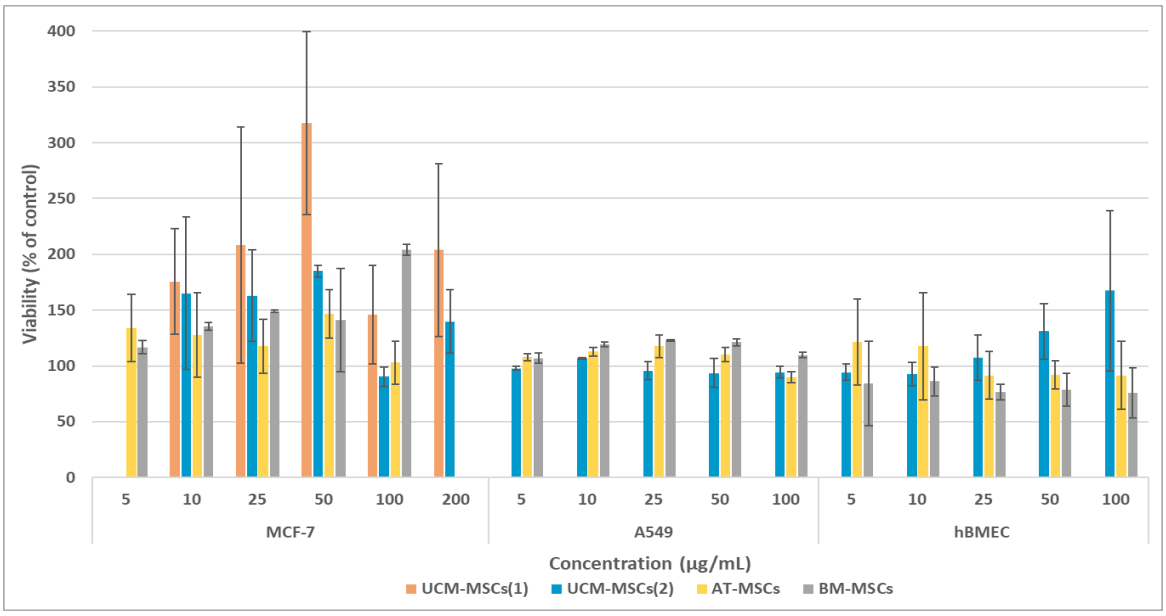
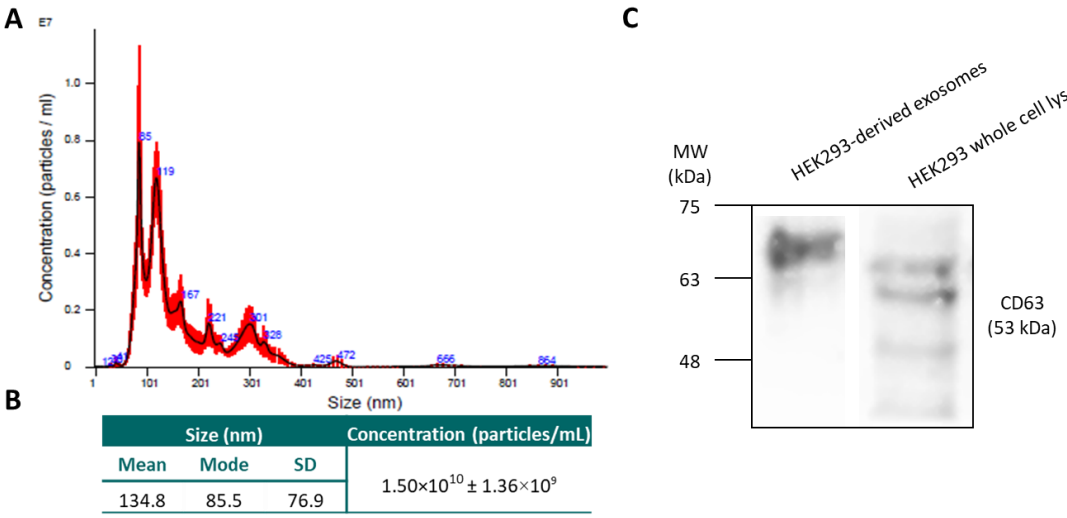



**Figure 1 –** Cell viability assessed with PrestoBlue™ viability assay for MCF-7, A549 and hBMEC cells after 48 hours of treatment with increasing concentrations of exosome samples from three different MSC sources (Umbilical Cord Matrix – UCM-MSCs(1) and UCM-MSCs(2) – Adipose Tissue – AT-MSC – and Bone Marrow – BM-MSC(1)) cultured in bioreactors. Untreated cells received medium with PBS and no exosome samples and their viability was admitted as 100%. Results are represented by the mean of 3 replicates ± SD.

control situations were established and for some cases an isolation of exosomes was performed using a total exosome isolation reagent, similar to what had been done for the MSC-derived samples.

On the one hand, "mock" exosomes from DMEM culture medium were isolated and, on the other hand, vesicles were extracted from HEK293 cell conditioned medium, as these cells have not been described to have intrinsic therapeutic properties and, thus, can be used as an exogenous negative control in this situation. Furthermore, specifically for the cancer cell lines, exosomes were extracted from medium conditioned by the cells to be assayed, in this case MCF-7 and A549 cells.

Following their isolation, certain characterizations were proceeded, beginning with the quantification of their total protein concentration, using a BCA assay. This was performed in a way to standardize the following experiments relatively to those performed with the MSC-derived exosome samples, as it represented a comparable straightforward parameter to quantify the dose of the samples for the treatment of the cells. Also, NTA and Western Blot analysis were performed for some of the isolated controls, and in general the obtained results were representative of the presence of vesicles with exosome-like characteristics. As can be seen in **Figure 2**, the tetraspanin CD63 was seen to be




| Size (nm) | | | Concentration (particles/mL) |
|---|---|---|---|
| Mean | Mode | SD | $1.50\times10^{10} \pm 1.36\times10^{9}$ |
| 134.8 | 85.5 | 76.9 | |

**Figure 2 –** Characterization of an isolated HEK293-derived exosome sample. A) Particle concentration and size distribution assessed by Nanoparticle Tracking Analysis (NTA). (B) Mean and mode size (nm), and standard deviation (SD) of results, of the vesicles present in the HEK293-exosome sample, assessed by NTA. C) Confirmation of the presence of a specific exosomal marker in the tested sample through Western Blot. The tetraspanin CD63 (53 kDa) was detected both in the cell lysates (positive control) and the tested exosome sample, in different forms.

enriched in the tested sample and the size distribution of the particles fell inside the range described for these vesicles (from 60 to 200 nm).

Although it was not possible to perform these analyses for all of the samples, these results allow for a general idea of the properties of the isolated controls, and therefore support the suitability of these samples for the experiments.

### 3.4 Influence of vesicles isolated from same cells and other background controls on MCF-7, A549 and hBMEC cell viability

Once isolated and characterized these controls were then diluted in order to achieve six solutions, as was described for the MSC-derived samples, to which all three cell lines were exposed and after 48 hours a PB™ viability assay was performed. In addition, the influence of culture medium conditioned by UCM-MSCs and HEK293 cells, in the viability of these cells was also assayed, in a way to better understand whether the effects previously seen were in fact related to the presence of exosomes or solely to other soluble factors secreted by the cells that may have been present in the samples. The graph depicted in **Figure 3** was then created using the values of viability calculated for the cells exposed to these controls, relatively to the cells that received no treatment (control – 0 µg/mL).

Generally speaking, when cells were treated with the background controls (exosomes from self-conditioned culture medium and non-conditioned culture medium processed with the isolation protocol), as well as the chosen exogenous negative control (exosomes and conditioned medium from HEK293 cells), there was no response in cell proliferation, meaning that the viability of the cells was relatively close to that of the control cells, and the same was observed for A549 cells upon treatment with medium conditioned by UCM-MSCs. On the other hand, an exception to what was described was registered for MCF-7 cells when treated with exosomes derived from A549 cells, as a significant proliferative effect was seen, nevertheless this could be explained by the fact that extracellular vesicles derived from cancer cells have been demonstrated to have a role in tumorigenicity and tumour-related pathologies, and thus could be stimulating the growth of these cells [10]. Also, hBMEC cells were shown to be significantly affected by the isolated "mock" exosomes from processed DMEM medium supplemented with exosome depleted serum. Finally, medium conditioned by umbilical cord matrix (UCM)-MSCs was seen to slightly induce cell proliferation in these cells, which was not observed for A549 cells as their viability was maintained relatively close to the control after treatment with this sample.

All in all, these results were significantly different from those obtained for the MSC-derived samples and thus help support the specificity of their effect over the assayed cells.

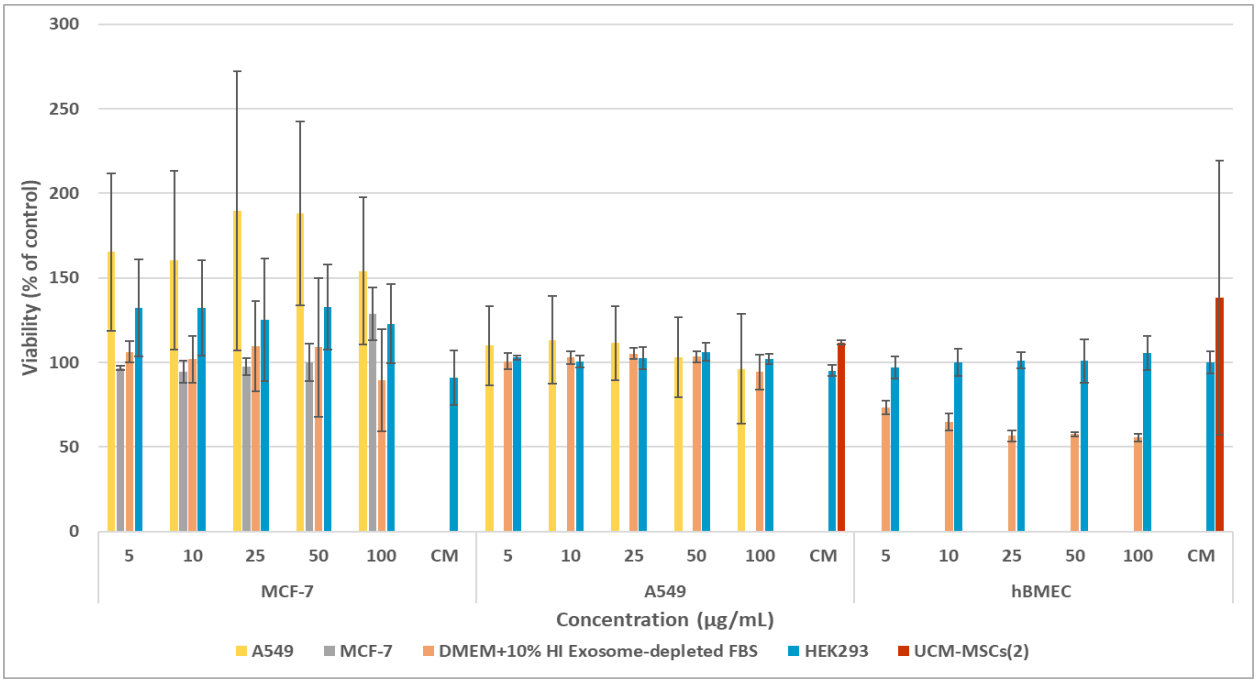



**Figure 3 –** Cell viability assessed with PrestoBlue™ viability assay for MCF-7, A549 and hBMEC cells after 48 hours of treatment with increasing concentrations of exosome or conditioned medium (CM) samples from four different sources (HEK293, A549 and MCF-7 cells, and Umbilical Cord Matrix MSCs – UCM-MSCs(2)), and increasing concentrations of processed culture medium supplemented with HI exosome-depleted serum. Untreated cells received only medium with PBS and their viability was admitted as 100%. Results are represented by the mean of 3 replicates ± SD.

### 3.5 Influence of MSC-derived exosomes on A549 cells invasion capacity in vitro

For the A549 cells, the viability assay results were complemented with the assessment of their invasive capacity *in vitro* when exposed to the MSC-derived exomes, achieved using a transwell migration assay with Matrigel™ coated cell culture inserts which allowed for an approximation to the *in vivo* environment.

In general, the results obtained from this experiment (**Figure 4**) demonstrated that the presence of these exosomes stimulated the invasive capacity of this cell line, with results, once again, varying between samples from different sources. For this assay, HEK293-derived exosomes were also used as exogenous controls, however results were not in agreement with the viability assays, as in this case there was unexpectedly a response similar to what was seen after treatment with BM-MSCs exosomes. However, results were not consistent between the different assays performed using this sample which may indicate that the sample itself has great variability, consequently resulting in varying responses. Also, isolates of non-conditioned basal DMEM also produced unexpected results, by decreasing the invasive potential of the cells by more than 50%, nevertheless this sample seemed to be degraded and this was in agreement with a viability assay performed using the same sample in which there was a great loss in cell viability (data not shown).

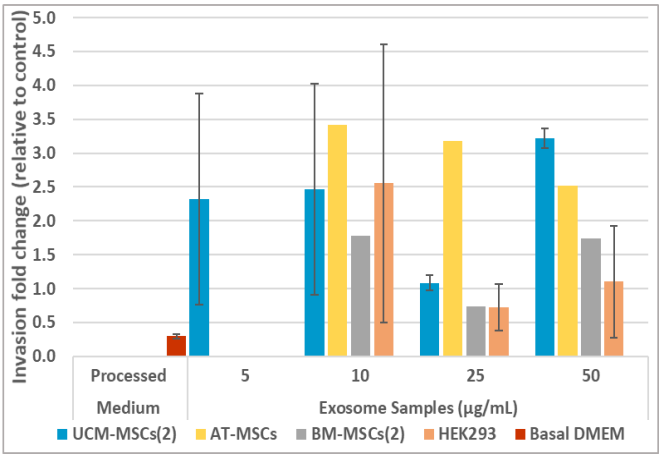



**Figure 4 –** Invasion potential of A549 cells after a 48-hour incubation period with increasing concentrations of exosome samples from three different MSC sources (Umbilical Cord Matrix –UCM-MSCs(2) – Adipose Tissue – AT-MSC – and Bone Marrow – BM-MSC(2)) cultured in bioreactors and HEK293 cells, and with processed basal DMEM culture medium. Results are represented as the fold change in invasion relatively to control cells, to which no exosomes were added (cells were resuspended in DMEM medium supplemented with exosome-depleted FBS and PBS). UCM-MSCs(2) results are represented by the mean of 2 independent experiments ± SD. HEK293 and Basal DMEM results are represented by the mean of 3 independent experiments ± SD.

Combined with the viability assays, these results demonstrate the impact that MSC-derived exosomes have over these cells, further indicating that these vesicles retain some of the biological properties of their parent cells.

### 3.6 Establishment of a protocol for fluorescent labelling of exosomes

As aforementioned, one of the main goals of this work was to assess the mechanisms involved in the uptake of MSC-derived exosomes into the different cell lines tested. For this, it was first necessary to determine a method to fluorescently label these vesicles. The most common labelling technique for extracellular vesicles is through lipophilic dyes, nevertheless these will often lead to false positive signals as they'll non-specifically label the vesicles along with other cellular components and unbound dyes may even stain the recipient cells [11]. Given this, it was thought that staining of exosome membrane markers using an immune-fluorescence strategy would be ideal for the indented assays, as it would allow for the specific targeting of these vesicles (avoiding the described false positives) and possibly for a better insight on the mechanisms involved in their internalization.

With this in mind, exosomes were once again isolated from HEK293 cells and subsequently characterized with an immunoblot analysis, in a way to confirm the presence of the tetraspanin CD63, as an antibody for this exosome marker conjugated with fluorescein isothiocyanate (FITC) was chosen for the labelling of the vesicles. Subsequently, three staining experiments were performed with varying conditions: Amount of antibody, total number of labelled particles, temperature and incubation time. As a blank control, exosomes were processed in the same conditions used in the first experiment and the antibody was replaced by PBS+0.2% BSA. To wash out unbound antibodies, for all experiments, an ultrafiltration was performed.

After labelling, a fluorescence emission spectrum was obtained from 480 nm to 600 nm using an excitation wavelength of 460 nm, for each of the samples (data not shown) and the concentration of the labelled samples was determined, as well as the ratio of antibody or FITC molecules per particle for each of the samples (**Table 3**).

By analysis of the spectra and concentration values obtained, it was possible to conclude that the conditions in which the exosomes are labelled do in fact have an impact on the effectiveness of the

**Table 1 –** Anti-CD63 FITC concentration, determined using the fluorescence intensity values obtained by the integration of the emission spectra obtained from 480 nm to 600 nm, for an excitation wavelength of 460 nm, for exosomes labelled in three different experiments. The ratio of antibody molecules per particle was calculated using the total number of particles in the sample, obtained through NTA, and the FITC molecules per particle ration was calculated assuming an FITC to antibody stoichiometry of 4-7:1.

| | Concentration (μM) | Anti-CD63 molecules/Particle | FITC molecules/Particle | |
|---|---|---|---|---|
| | | | Min | Max |
| 1st Experiment | $2.1\times10^{-3}$ | 98 | 392 | 686 |
| 2nd Experiment | $4.9\times10^{-3}$ | 162 | 650 | 1137 |
| 3rd Experiment | $7.9\times10^{-3}$ | 142 | 569 | 996 |

staining and their detection through fluorescence spectroscopy. Overall, the results from these experiments indicated that the amount of labelled antibody co-incubated with the exosomes and the number of vesicles to be labelled were the parameters that mostly influenced the effectiveness of the labelling strategy, as the second and third experiments (where higher amounts of both were tested) were seen to result in better labelling yields.

Finally, to assess whether the purification of the samples using an Amicon® ultrafiltration unit was successful in the retrieval of the stained vesicles and whether there was any aggregation of the exosomes caused by either the labelling or the purification steps, an NTA assay was performed for the samples stained in the second and third experiments. The results from this analysis were directly compared with those obtained for the exosome samples in question before labelling (data not shown) and it was concluded that this protocol did not affect the distribution in size of the vesicles or the particle concentration of the samples, indicating that it doesn't cause aggregation of the particles and there isn't a significant loss of vesicles during this process.

In conclusion, the results obtained from this experiment indicated to the effectiveness of these labelling strategies. Nevertheless, further assays should be performed in order to assure the specificity of the chosen technique.

## 4. Discussion

This study was developed with the intent of elucidating the biological functionality of the exosomes isolated from human mesenchymal stem/stromal cells (MSCs), obtained in the scope of the framework of the PhD studies of Miguel Fuzeta who is aiming at the scalable production of these vesicles expanding the cells in bioreactors and, in future works, their application for drug delivery in cancer therapy.

For this, the first approach was to determine the effects of these MSC-derived exosomes on the proliferation of two adenocarcinoma cell lines (MCF-7 and A549) and one representative of the blood-brain barrier (hBMEC): Proliferation was assessed using a fluorescence-based PrestoBlue™ (PB™) viability assay and, for lung adenocarcinoma (A549) invasion through Matrigel was also evaluated in transwell platforms.

Overall, the results from these experiments indicated that MSC-derived exosomes do in fact have a functional effect over the cells tested, which was not observed for exosomes from other sources (HEK293). Nevertheless, these effects were seen to change with the cell line in test and with the source of the MSC-exosomes. Despite being more obvious for hBMEC cells, the results obtained for MCF-7 and A549 cells also demonstrated notable differences in the variation of cell viability from source to source and, for the UCM-derived samples, even between both donors, regardless of the fact that there was a similar response pattern after treatment with all samples. This was not unexpected as it has been previously reported that the properties of these cells vary with their source and donor [12], and thus, the same would be true for their secreted vesicles.

Nonetheless, it is worth emphasizing that these are only preliminary results and despite the fact that these vesicles stimulate and promote the proliferation of cancer cells it might not be indicative of their effect *in vivo,* as the conditions in which they were assayed do not truly resemble the tumour microenvironment and thus it might be possible that in *in vivo* conditions there would be more favourable results. Therefore, at this point these results shouldn't be seen as discouraging when it comes to their application for cancer therapy. In fact, in their work, Mendt *et al.*[13] reported comparable observations when assaying exosomes isolated from BM-MSCs cultured in conditions similar to those used to obtain these vesicles. When applied for *in vitro* treatment of pancreatic cancer cells, these MSC-derived exosomes were overall not seen to cause an increase in apoptosis, in some cases even demonstrating a slight decrease in the percentage of apoptotic cells, relatively to the control. Nonetheless, when tumour-bearing mice were treated with these vesicles their survival rate was seen to be slightly improved comparatively to that of the mice treated solely with their vector (control). These results could then be promising when it comes to the application of MSC-exosomes for cancer therapy, as, all in all, they show the possibility of a large-scale production of GMP compliant exosomes with positive *in vivo*

outcomes, despite of what was observed *in vitro*. In addition, as mentioned, the main goal for these vesicles is their use for drug delivery, an application which was also shown to be possible by these authors, who were able to effectively load siRNA into the vesicles and consequently demonstrated significantly increased treatment efficacies in both testing conditions.

In a similar way, the same should also be noted for the results obtained for the hBMEC cells, as the observation that these cells seem to be affected by treatment with the tested vesicles might not be truly indicative of their possible effects *in vivo*. As a matter of fact, these cells were assayed since there is interest in their application as an *in vitro* model of the blood-brain barrier (BBB), in a way to possibly elucidate the capacity of these vesicles to cross this barrier and, hopefully, allow for their future application for treatment of neurodegenerative and other brain related disorders. Therefore, it is relevant to understand that the conditions to which these cells would be subjected when mimicking the BBB [14,15] are substantially different to those used in these assays, and consequently the impact caused by these vesicles might also be changed. Also, several studies have shown that systemic administration of MSC-derived exosomes allows for the delivery of drugs into target regions of the brain [16] and also for the recovery of neurological function and neurovascular plasticity [17,18] in mouse models of stroke, indicating that these vesicles are indeed capable of crossing the BBB without causing adverse effects on the tested subjects, and consequently providing a promising outlook on the application of the produced MSC-derived exosomes.

The establishment and isolation of controls for the mentioned experiments was performed taking into consideration MISEV2018 [9], where the authors suggest the importance of using certain negative controls which should result in minimal functional effects, consequently allowing for the substantiation that the impact caused by the MSC-derived samples was in fact related to the presence of purified exosomes and not associated with other factors.

Following their isolation, certain characterizations were proceeded, beginning with the quantification of their total protein concentration, using a BCA assay. This parameter was used mostly for the preparation of the exosome solutions later utilized in the viability and invasion assays, for both the controls and the MSC-derived samples. It might be suggested that other parameters would be more appropriate for the quantification of these samples, e.g. the total number of particles which can be obtained by Nanoparticle Tracking Analysis (NTA), as medium components might have co-precipitated with the vesicles, consequently resulting in an overestimation of the concentration of the samples. Nevertheless, it was not possible to perform a NTA quantification for all of the isolated samples, due to low resulting yields after isolation and lack of time. Furthermore, for the MSC-derived samples, a comparison between calculated ratios of total protein per vesicle was performed (data not shown) and it was possible to conclude that there is a consistent correlation between these two parameters for all of the samples, which was expected as all exosomes were isolated using the same methodology, consequently resulting in similar yields. This comparison then supports the use of the total protein concentration for the quantification of the dose of the samples for the treatment of the cells.

Besides this, NTA and western blot analysis were performed and in general the obtained results were representative of the presence of vesicles with exosome-like characteristics and were in agreement with what has been obtained for MSC-derived vesicles isolated in static conditions. Although it was not possible to perform these analyses for all of the samples, these results allow for a general idea of the properties of the isolated controls, and therefore support the suitability of these samples for the experiments.

Finally, as there was the intention of using a confocal microscopy approach for the determination of the mechanisms involved in the uptake of MSC-derived exosomes, the labelling of HEK293-derived exosomes was attempted using an immune-fluorescence strategy. This was thought to allow for a more specific targeting of these vesicles when compared to the more commonly used lipophilic dyes, possibly circumventing issues regarding their use. Results from three separate experiments, although only preliminary, indicated that certain parameters influence effectiveness of the labelling strategy. Also it was seen that the protocol applied for the removal of unbound antibodies was adequate for the intended purposes, as it did not affect the distribution in size of the vesicles or the particle concentration of the samples, indicating that it doesn't cause aggregation of the particles and there isn't a significant loss of vesicles during this process.

Once again, these results are only preliminary since, due to time constraints, it was not possible to proceed to further testing. In fact, fluorescence fluctuation spectroscopy (FFS) analysis is intended and expected to allow for a more in-depth

characterization of these vesicles and of the overall labelling strategy [19].

## 5. Conclusions and Future Perspectives

All in all, this work provides an outlook on the biological properties of exosomes derived from three different sources of MSCs (AT, UCM and BM), demonstrating that the vesicles isolated from the conditioned medium obtained from the expansion of cells in bioreactors are not inert when co-incubated with different cell lines and, consequently, highlighting the possibility for their future application for therapeutic purposes. The main objective of this experiment was to determine an overall behaviour pattern of cells in response to the treatment with these vesicles which was achieved for the two tumour cell lines (MCF-7 and A549) assayed. However, further experiments are still needed in order to confirm these results and to achieve a better understanding of how these effects are produced.

Given this, for future works there is the intent of performing more specific assays, in order to better elucidate on the actual mechanisms underlying MSC-exosomes' effects, such as the assessment of exosome uptake and intracellular routes. As mentioned, permeability studies using an *in vitro* blood-brain barrier model (achieved with hBMEC cells) are also intended, in order to clarify the potential of their application for treatment of neurodegenerative disorders. Moreover, and considering that the main goal for the production of these vesicles is their use for drug delivery in cancer therapy, their loading with therapeutic molecules will be attempted and thus different encapsulation techniques will be tested.

Finally, it is worth mentioning that the production of these vesicles from medium conditioned by MSCs cultured in vertical-wheel bioreactors seems to be yielding vesicle concentrations in the same order of magnitude as those obtained by other authors who reported a large-scale production exosomes from bone marrow–derived MSCs [13]. The method used for the isolation of these vesicles was a precipitation based one, using a total exosome isolation reagent, which allows for a fast recovery of the intended vesicles with good yields, nevertheless, this technique has been reported as having increased chances of contamination with medium components or even polymeric materials. With this in mind, it would be of interest to experiment with other isolation techniques, such as a sucrose cushion ultracentrifugation which has been shown to allow for better yields with high purity, by Chopra *et al.*[20], when compared with other methods.

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