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Initial characterisation of exosomes released by umbilical cord-derived mesenchymal stem cells and mature dendritic cells, under 'Good Manufacturing Practice' conditions

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Abstract:

Exosomes represent an important mode of intercellular communication and play key roles in many physiological and pathological processes. Exosomes have hitherto exhibited their capacity to modulate biological activities through their carrying of functional molecules such as proteins, lipids, and genetic materials. In the current study, we investigated exosomes released by mature dendritic cells (mDCs) and umbilical cord-derived mesenchymal stem cells (UCMSCs) under Good Manufacturing Practice (GMP) conditions. Ultracentrifugation was used to isolate and purify the exosomes. Additionally, a transmission electron microscope (TEM) and immunoblotting were used to characterise exosomal morphology and markers. The preliminary results showed that both mDCs and UCMSCs secreted exosomes into GMP culture media. Exosomes exhibited a cup-shaped morphology and showed positive for CD63. Additionally, no difference was observed between mDC-derived exosomes and UCMSC-derived exosomes regarding marker expression or morphology. This data indicates the potential for further development of GMP exosomes for clinical application.

Keywords: **dendritic cells, exosomes, mesenchymal stem cells.**

Classification number: **3.2**

Introduction

Research suggests that both normal and tumour cells secrete extracellular vesicles into the extracellular space [1-3]. Extracellular vesicles (EVs) are enclosed by a phospholipid bilayer similar to the cellular membrane, and are known to vary in size. Based on their biogenesis, EVs can be classified into three main classes: (1) apoptotic bodies (1,000-5,000 nm), which are a product of apoptosis upon programmed cell death; (2) microvesicles (100-1,000 nm), formed by direct shedding from the plasma membrane; and (3) exosomes (30-150 nm), which are formed through endocytosis and released into the extracellular milieu through the exocytosis mechanism [1, 3, 4]. Of these, exosomes are the most interesting and best studied in terms of functioning. Exosomes exhibit a cup-shaped morphology under inspection with a transmission electron microscope and are floated at a density of 1.10-1.21 g/ml in a sucrose gradient [5, 6]. During formation, exosomes are enveloped within proteins and genetic materials such as mRNAs, microRNAs, and other non-coding RNAs (ncRNAs) [7]. Such exosomal components can be delivered to both neighbouring and distant cells and modulate the behaviour of recipient cells. The mechanism of component delivery means that EVs, including exosomes, are bioactive and carry a potential for therapeutic use.

Dendritic cells (DCs) arise from progenitor cells in the bone marrow and reside in peripheral tissues in an immature state [8]. Under appropriate stimulation, immature DCs undergo maturation and express stimulatory molecules, secreting cytokines to activate tumour-specific cytotoxic T lymphocytes and B cells [9]. The capacity of DCs to present antigens makes them a candidate of interest in the field of

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cancer immunotherapy, in their potential to stimulate the immune system to eradicate tumours [10]. However, DCs which are generated *in vitro* can be affected by immune-inhibiting factors in the tumour environment [10]. It has been reported that the production of soluble factors by tumour cells might lead to a down-regulation of the entire metabolism of DCs [11].

Moreover, tumour cells secrete cytokines to inhibit tumour antigen presentation by DCs [12]. This can lead to immune tolerance and/or activate regulatory activity, or suppressor T cells, meaning that the immune response then fails to be induced [12]. Thus, the use of DC-derived exosomes in cancer immunotherapy is one way to avoid the inhibition of DC metabolism and DC antigen presentation [13]. Additionally, it has been illustrated that DC-derived exosomes have a longer half-life after injection than *in vivo* DCs [14]. Moreover, DC-derived exosomes are stable for storage for six months at -80^{0}C without being affected either structurally or functionally. This enables them to have a considerable potential for therapeutic use such as DC-derived exosome-based cancer immunotherapy [13].

Mesenchymal stem cells (MSCs) were first discovered in bone marrow in the early 1970s [15]. Currently, MSCs are isolated from a variety of tissues, but the majority are isolated from bone marrow (BM) [16], adipose tissue (AD) [17], dental pulp [18], and the umbilical cord (UC) [19]. MSCs have been shown to differentiate into a number of cell types, thus enabling MSC therapeutics [20]. In fact, the differentiation capacity of MSCs formed the original rationale for their clinical application, with the expectation that they would differentiate and replace damaged cells. However, it is difficult, perhaps impossible, to verify the actual presence of these cells at the site of injury. Thus, the correlation between functional improvement and cell engraftment or differentiation at the site of injury cannot always be recognised. It has been proposed that MSCs express their effects not through their differentiation potential, but rather, through secreted factors, notably enveloped EVs/exosomes [21-23]. Therefore, MSCs could potentially be replaced with MSC-derived EVs/exosomes in order to effect disease treatment.

In the current research, we isolate mDC-derived exosomes and UCMCS-derived exosomes from a GMP cell culture medium, comparing their characteristics in terms of morphology and marker expression.

Materials and methods

Cell lines and cell culture

A tumour cell line A549 (ATCC) was cultured in DMEM (Dulbecco's Modified Eagle's Medium with 1 g/l glucose, L-glutamine, and sodium pyruvate - Corning) with 10% foetal bovine serum and 1% penicillin-streptomycin 100X (Thermo Fisher Scientific, USA), at 37^{0}C, in a humidified atmosphere containing 5% CO_2. The cells were for use as a tumour antigen preparation for loading to DCs.

Tumour antigen extraction from A549 cell lysis and protein quantification

A number of $1x10^7$ - $2x10^7$ cells were re-suspended in 1 ml phosphate buffered saline (PBS). The cells were then processed in 10 rapid freeze-thaw cycles (in liquid nitrogen) to generate total protein lysate. Trypan blue staining was used to examine live cells. The rate of cell survival was required to reach 0% after the final cycle. Total protein lysate was centrifuged at 2,000xg/10 minutes and the pellets discarded. The collected supernatant was filtered using a 0.2 µm pore filter (Corning, USA). The concentration of purified total protein was determined using a Bradford (Sigma Aldrich) assay, following the manufacturer's instructions. The total protein lysate was stored at -80^{0}C for further use.

Dendritic cell culture and differentiation

Ethical approval for umbilical cord blood collection and cell isolation was obtained from the ethics committee of the Dinh Tien Hoang Institute of Medicine. The pregnant donor was required to be negative for HIV, CMV, HBV, HCV, and TPHA to be selected for umbilical cord blood collection for the research. Umbilical cord blood (UCB) was obtained by venipuncture from the umbilical cord veins immediately after normal full-term delivery, following informed consent. Mononuclear cells (MNCs) were isolated using density gradient separation by Ficoll-Paque/Lymphoprep™ (Stem Cell Technologies, Canada). Monocytes were then isolated by incubating MNCs ($2x10^6$ cells/ml) with Lymphocyte Serum-Free Medium KBM 551 (Corning, USA) at 37^{0}C for 1.5 hours. Adherent cells ($CD14^+$ monocytes) were cultured continuously and floating cells removed. After 24 hours (Day One), 500 U/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech, USA) and 500 U/ml of interleukin 4 (IL-4) (Peprotech, USA) were added to the medium to induce monocyte differentiation into DCs. On Day Three, the culture medium was refreshed with the addition of a half volume of fresh medium with

supplementary cytokines. On Day Five, the tumour antigen extracted from the A549 cell lines was added into the cell culture medium (50 µg tumour antigen/ml medium) in order to load the DCs with the antigen. On Day Six, tumour necrosis alpha (TNFα) 1,000 U/ml was added to the culture medium to induce DC maturation for one day. On Day Seven, the culture medium was harvested for exosome isolation and mDCs were harvested for marker analysis.

Monocyte and dendritic cell marker analysis

Adherent cells ($CD14^+$ monocytes) at Day One of the cell culture process and mDCs from Days Six and Seven were collected for cell marker analysis by flow cytometry. Typical surface markers of monocytes (CD14) and DCs (CD40, CD80, CD86, major histocompatility complex molecule - HLADR, and CD123) were used to examine cell states and phenotypes. Briefly, cells were suspended in phosphate-buffered saline (PBS) and labelled with CD14-PC7, CD40-PE, CD80-PE, CD86-PE, HLADR-FITC (Beckman Coulter, USA), and CD123-APC Vio (Miltenyi Biotech, Germany). Appropriate isotype controls, including IgG1-PE (Beckman Coulter, USA), IgG1-FITC (Beckman Coulter, USA), IgG1-PC7 (Beckman Coulter, USA), IgG1-APC (Beckman Coulter, USA), and IgG1-APC Vio (Miltenyi Biotech, Germany) were included in each experiment. Cell marker analysis was performed using the Flow Cytometry System (Beckman Coulter), equipped with Navios Software.

Umbilical cord-derived MSC culture

Ethical approval for using umbilical cord-derived MSCs was obtained from the ethics committee of Vinmec International General Hospital Joint Stock Company. The umbilical cord (UC) was collected from consenting patients. UCs were cut into 2-3 cm long segments and washed with PBS to discard traces of blood. The cleaned UCs were then minced into small pieces with Collagenase Type I (Gibco, USA) in a gentleMACS™ Dissociator (Miltenyi Biotech, Germany) for 1.5 hours, following which they were washed three times in PBS to remove collagenase and centrifuged to collect pellets. The pellets were transferred into a 25 cm^2 culture flask (Nunc™ Cell Culture Treated EasYFlasks™, Thermo Fisher Scientific, USA), which had already been coated with a xeno-free substrate (CTS™ CELLstart™ Substrate, Gibco, USA) and which contained a GMP Power Stem medium (Pan Biotech, Germany). The cells were then incubated at 37^0C with 5% CO_2 to allow cellular migration from the explants. The culture medium was replaced every three days. When the cells reached 80% confluence, they were passaged using CTS™ TrypLe™ Select Enzyme (Thermo Fisher Scientific, USA). The passage 1 cells were subsequently seeded in T75 flasks (375,000 cells/flask). The passage 1 UCMSCs were required to reach 80% confluence, at which point the supernatant was harvested for exosome isolation.

Umbilical cord-derived MSC marker analysis

The cells (UCMSCs at passage 1) were harvested following supernatant collection using CTS™ TrypLe™ Select Enzyme (Thermo Fisher Scientific, USA). The UCMSCs then were analysed for markers using the Human MSC Analysis Kit (BD Biosciences, USA), which includes positive markers for human MSCs (CD73, CD90, and CD105) as well as negative markers (CD11b, CD19, CD34, CD45, and HLA-DR). Staining was performed according to the manufacturer's instructions. Markers were detected and analysed using Navios Software equipped with a flow cytometry instrument (Beckman Coulter, USA).

Exosome isolation

The cell culture supernatant (three T75 cell culture flasks for the mDCs and three for the UCMSCs) was centrifuged at 300×g/10 minutes at 4^0C to remove cell debris, then at 2,000×g/10 minutes at 4^0C to remove apoptotic bodies. The supernatant was then continuously centrifuged at 10,000×g/30 minutes at 4^0C to remove microvesicles and at 100,000×g/70 minutes at 4^0C (Optima XPN-100 Ultracentrifuge, Beckman Coulter) to obtain exosome pellets. The exosome pellets were washed in PBS and centrifuged again at 100,000×g/70 minutes at 4^0C to obtain clean exosomes, which were re-suspended in 50 µl PBS and either used immediately or stored at -80^0C for further use.

Analysis of exosome morphology using transmission electron microscopy

Purified exosome pellets were fixed with 4% paraformaldehyde. Following this, a 5 µl drop of the suspension was loaded onto Formvar-carbon coated grids (TED PELLA Inc., CA, USA) and left to dry at room temperature for up to 20 minutes. The grids were subsequently washed with PBS and incubated with 1% glutaraldehyde for five minutes, then washed for 8×2 minutes with distilled water. The sample was then stained with uranyl-oxalate pH 7 for five minutes at room temperature, prior to being incubated with methyl cellulose-uranyl acetate for 10 minutes on ice. Finally, the grids were left

to dry at room temperature and observed with transmission electron microscopy (TEM) at 80 KV (JEM1010-JEOL).

Western blotting analysis

For each sample, exosomal proteins equivalent to 5×10^6 secreted cells were loaded per lane and separated by 4-12% polyacrylamide SDS-PAGE gel (Invitrogen, USA) at 200 V/35 minutes/4^0C. Proteins were then transferred to a PVDF membrane (Amersham™) for 200 mA/2 hours at 4^0C. The membrane was probed with antibodies to CD63 (Mouse monoclonal IgG, Santa Cruz Biotechnology), followed by incubation with anti-mouse-HRP (Amersham™). Proteins were detected through use of a chemiluminesence substrate (Amersham™) and images captured using ImageQuant LAS 500 (GE Healthcare Life Sciences).

Statistical analysis

A Student's t-test was performed to analyse significance of difference between the mean of the two groups. Error bars indicated ± SD (Standard Deviation).

Results

Characteristics of morphology and immunophenotype of monocyte-derived DCs

In order to generate mature DCs (mDCs), monocytes were cultured and induced by GM-CSF and IL-4, with observation of immature DCs after four days of monocyte culture. These immature DCs were both adherent and floating cells, expressing some short dendrites (Figs. 1A, 1A1). Mature DCs, which exhibited the specific dendritic cell morphology of long and thin dendrites, were observed at Day Seven (Figs. 1B, 1B1). Analysis of typical monocyte and DC surface markers revealed a significant difference in cell surface marker expression between Day 0 and Day Seven: expression of mDC markers such as CD40, CD80, CD86, and HLA-DR increased markedly at Day Seven of culture. Specifically, CD40 increased from 21.4 to 82.5%, CD80 from 0.42 to 58.7%, CD86 from 16.57 to 74.1%, and HLA-DR from 20.35 to 73.25%. However, expression of CD14, which is the specific marker for monocytes, showed no change between Day 0 and Day Seven. Furthermore, CD123, which is a marker of plasmacytoid DCs, showed very slight expression and did not change between Days 0 and Seven (Fig. 1C). The above information regarding markers and morphology indicates that successful differentiation of monocytes into mDCs occurs after seven days in the acculturation process.

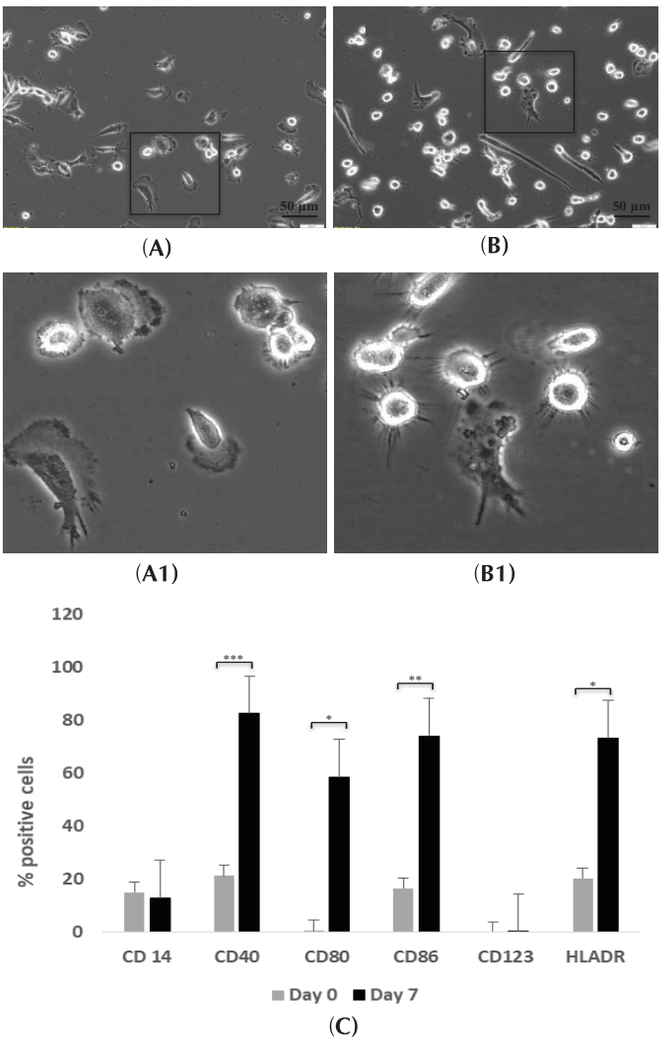

Fig. 1. Morphology and marker expression of immature and mature dendritic cells. (**A**, and **A1**) Immature DCs express short dendrites; (**B**, and **B1**) mDCs express long and thin dendrites; (**C**) Immuno-phenotype of monocytes and mDCs: different expression of CD14, CD40, CD86, CD123, and HLADR at Day 0 (monocytes) and Day Seven (mDCs) in cell culture (n=3). *: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001.

Characteristics of morphology and markers of UCMSCs

Cells were captured for morphological analysis at the time point of conditioned media collection. Typical fibroblast morphology of MSCs was observed (Fig. 2A).

After the collection of the conditioned medium for exosome isolation, UCMSCs were harvested for marker analysis. Results showed that UCMSCs under GMP-culture conditions expressed positive markers for MSCs, including CD73, CD90, and CD105, and were negative for CD11b, CD19, CD34, CD45 and HLA-DR at the time point of exosome harvest (Fig. 2B).

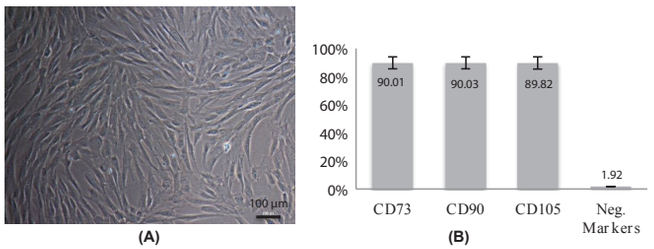

Fig. 2. Morphology and marker expression of UCMSC passage 1 at the time point of conditioned media collection for exosome isolation. (**A**) UCMSCs at passage 1 adhere to plastic culture flasks and show a fibroblast-like shape; (**B**) UCMSCs express positive markers of CD73, CD90, and CD105 and do not express MSC negative markers, including CD11b, CD19, CD34, CD45, and HLA-DR.

Exosome characteristics

Exosomes were isolated using conventional differential ultracentrifugation. The isolated exosomes exhibited a cup-shaped morphology under examination with a transmission electron microscope (Figs. 3A, 3B, 3C). It was also noted that exosomes have a size range of 50 nm to 200 nm. There were no differences in morphology or size between UCMSC-derived exosomes and mDC-derived exosomes. The biomolecular analysis of the exosomes revealed that they were expressed with CD63 (Fig. 3D). However, CD 63 was only detected in exosome samples from UCMSCs and mDCs, and not in the parental UCMSCs or mDC lysates. Additionally, there were no differences in CD63 expression between UCMSC-derived and mDC-derived exosomes.

Discussion

Exosomes, which were first observed more than 30 years ago [24, 25], are formed through endocytosis and released into the extracellular environment through the exocytosis mechanism [4]. With recent advances in science and technology, a variety of different techniques and commercial kits have become available for exosome isolation [26]. However, the choice of EV isolation technique depends on sample sources and the downstream application of the EVs [27]. The most common method used to isolate exosomes from cell culture media is differential ultracentrifugation [28]. In the current research, exosomes released by mDCs and UCMSCs were isolated from cell culture supernatant using differential centrifugation [28], allowing for the removal of dead cells, cell debris, aggregated proteins, and other extracellular vesicles (apoptotic bodies and microvesicles) [29]. Data generated from transmission electron microscopy indicated that only the exosome population had been harvested. The exosomes were of typical cup-shaped morphology and were not contaminated with other vesicle populations, which lack this form (Figs. 3A, 3B, and 3C). The cup-shaped morphology is generated by the collapse of the exosomal membrane, due to dehydration during staining [30]. Additionally, there was no contamination by protein aggregation found in the exosome fraction (Figs. 3A, 3B, and 3C). Immunohistochemistry data revealed exosomes expressed CD63 (Fig. 3D), and it is interesting that CD63, which is enriched in exosomes and considered their marker, was detectable only in the exosome fraction and not in

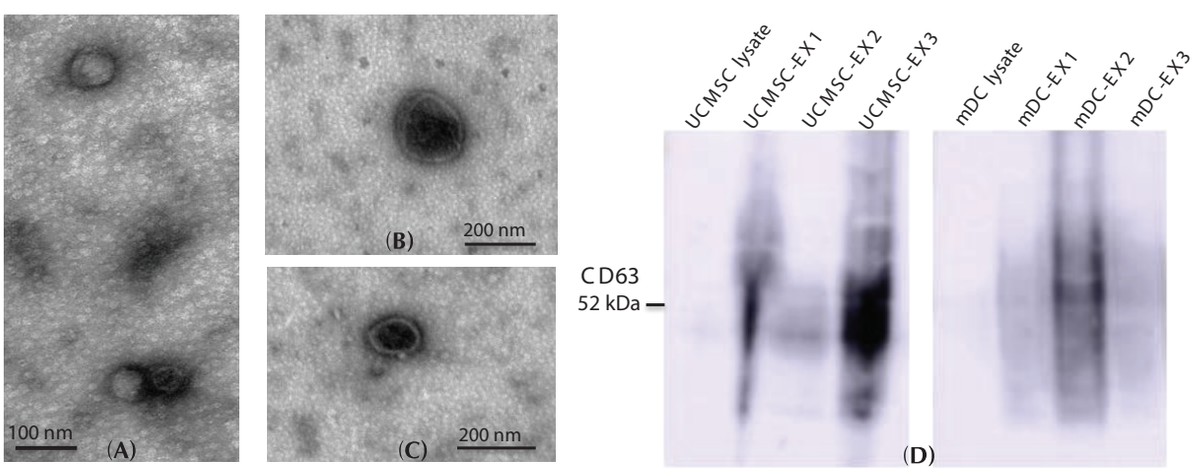

Fig. 3. Exosome morphology and marker expression. (**A**, **B**, and **C**) Exosomes exhibit a cup-shaped morphology and are sized 50 nm to 200 nm under examination with transmission electron microscopy. (**A**) Exosome representative from mDCs; (**B** and **C**) Exosome representatives from UCMSCs; (**D**) Expression of CD63 exosomal marker. Exosomal proteins were loaded per lane to the equivalent of 5×10^6 parental cells. The same number of cells were lysated and loaded as in the control. Exosomal samples were positive for CD63 but parental cells (UCMSCs and mDCs) were not. UCMSC lysate: parental UCMSC lysate, UCMSC-EX1: UCMSC-derived exosome sample 1, UCMSC-EX2: UCMSC-derived exosome sample 2, UCMSC-EX3: UCMSC-derived exosome sample 3, mDC lysate: parental mature DC lysate, mDC-EX1: mature DC-derived exosome sample 1, mDC-EX2: mature DC-derived exosome sample 2, mDC-EX3: mature DC-derived exosome sample 3.

the cell lysate. This result gives further confirmation that CD63 is highly enriched in exosomes only. These data indicate that differential centrifugation, with the final step of washing exosomes with PBS, is appropriate in harvesting clean exosomes. The washing of exosomes with PBS ensures freedom from contamination with chemicals and other contaminants such as extracellular protein aggregates, which can destroy or alter the functioning of exosomes. This is important information for the future clinical application of exosomes.

The literature suggests that exosomes are released by most cell types both *in vitro* and *in vivo*, including immune cells and stem cells. Importantly, exosomes can deliver their contents to target cells through the mechanisms of ligand/receptor interaction, direct membrane fusion, or internalisation [31]. By such mechanisms of information transmission, exosomes can alter the behaviours of recipient cells. Regarding exosomes released by DCs, Zitvogel, et al. (1998) [32] have reported the novel finding that DCs secrete exosomes bearing functional major histocompatibility complex (MHC) classes I and II, which turned exosomes becoming an antigen presenting agent. Since then, further reports have indicated that DC-derived exosomes might be novel candidates for cell-free vaccines and anti-tumour therapy [13, 33-35]. As examples, DC-derived exosomes could directly activate T cells by presenting a T cell antigen or activate natural killer (NK) cells by presenting the NKG2DL ligand to the NK cell's NKG2D receptor [35]. Additionally, DC-derived exosomes indirectly activate T cells by transferring antigens to DCs or tumour cells, thus turning DCs and tumour cells into antigen-presenters for T cells [35]. Furthermore, human UCMSC-derived exosomes have expressed a capacity for wound-healing via the Wnt-4 pathway [22], as well as for promoting cell proliferation and protection against oxidative stress-induced cell apoptosis, by activation of ERK1/2 and p38 [21]. These exosomes carry a high dose of cytokines such as IL6 and IL8 [21]. Interestingly, human UCMSC-derived exosomes are known to reduce anti-inflammatory factors such as TNFα and IL1-β but to increase the concentration of anti-inflammatory factor transforming growth factor beta (TGF-β) in the injured brains of Wistar rats [23]. These data indicate that immune regulation may also be an important mechanism in UCMSC-derived exosome tissue regeneration.

Conclusions

Increasing knowledge of exosomes and their capacities provides evidence for their application in disease treatment. This study provides preliminary data on exosomes released by UCMSCs and mDCs under GMP-culture conditions, with the ultimate aim of assessing their clinical application. Exosomes were isolated successfully from a cell culture supernatant and characterised according to their typical cup-shaped morphology and CD63 marker. Despite the limitations of this study, such as the lack of exosome quantification, exosomes were successfully generated and isolated under serum-free conditions. The exosomes will be further investigated for their role in cellular biological processes in order to further reveal their capacities.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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