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ORIGINAL RESEARCH

Quality Control of Fetal Wharton's Jelly Mesenchymal Stem Cells-Derived Small Extracellular Vesicles

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Background: Quality control (QC) is an important element in ensuring drug substances' safety, efficacy, and quality. The dosing regimen for sEVs can be in the form of protein concentration or the number of particles based on the results of a series of quality controls applied as in-process control.

Methods: Wharton's Jelly Mesenchymal Stem Cells (WJMSCs) were isolated from four independent umbilical cord samples and were characterized following the International Society for Cellular Therapy (ISCT) guidelines. Small extracellular vesicles (sEVs) were isolated separately from these four WJMSCs samples using the Tangential Flow Filtration (TFF) method and were characterized per Minimal Information for Studies of Extracellular Vesicles (MISEV2018) guidelines. Each isolated and concentrated sEV preparation was standardized and its purity was determined by the ratio of the number of particles to protein concentration.

Results: All the WJMSCs samples passed the Mesenchymal Stem Cells (MSCs) characterization QC tests. Qualitatively, EVs-positive markers (CD63 and TSG101) and intact bilipid membrane vesicles were detected in all the sEV preparations. Quantitatively, the protein and particle concentrations revealed that all the sEV preparations were "impure" with $< 1.5 \times 10^9$ particles/µg protein. Albumin was co-isolated in all the sEV preparations.

Conclusion: In short, all characterized and standardized individual and pooled sEV preparations were deemed "impure" due to albumin co-isolation using the TFF method. For therapeutic development, it is essential to report protein and particle concentrations in EV preparations based on these QC results.

Keywords: quality control, umbilical cord, Wharton's jelly mesenchymal stem cells, tangential flow filtration, small extracellular vesicles, purity

Introduction

Extracellular vesicles (EVs) as natural nanovesicles have become an imperative, intensifying potential biotherapeutics, and this can be seen clearly with the increasing number of registered clinical trials involving EVs worldwide together with the published clinical studies.[1] Pharmaceutical industries are competing too intensely to translate this promising field of EVs into clinical settings. EV cargos are highly enigmatic due to the isolated EVs subpopulation's complexity and heterogeneity. A smooth translation depends on one of the crucial components of regulatory compliance: the quality of the product, both the drug substance (DS) and the finished products.[2] The final therapeutic outcome of EVs is highly influenced by the "raw material", including the cells, production processes, and in-process quality control (QC) with the acceptance criteria. For potential biotherapeutics agents like EVs, where the potency assay and the mechanism of action

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(MOA) are not fully elucidated, the best approach to adopt is the "process is the product" approach.[3] The manufacturing process defines the product's quality and consistency in production in biological products, especially new products such as EVs, which are complex. Currently, all manufacturing processes and QC shall comply with Common Technical Document Module 3, as described in the International Council of Harmonisation (ICH) Topic M4Q guideline.[4] Since EVs are produced from living cells, any changes and disturbances in the production environment (both macro and microenvironments to the living cells) may significantly impact the DS produced.

On the other hand, the absence of standardized EVs isolation and enrichment methods adds more burden for researchers to validate their research and inadequate recording and reporting of experimental parameters. A meta-analysis was published revealing extensive EVs isolation methods with 190 distinctive isolation methods and 1038 unique procedures.[5] EVs researchers face manufacturing obstacles, especially isolation, quantification, and characterization. The International Society for Cell and Gene Therapy (ISCT) has published guidelines[6] for the characterization of MSCs, and the International Society for Extracellular Vesicles (ISEV) has published the Minimal Information for Studies of Extracellular Vesicles (MISEV2018) guideline[7] for researchers interested in EVs' work. These two guidelines may help design minimum QC parameters to produce quality EVs products, achieve prudent in-process QC during production, and not rely entirely on the final product release specification alone.

Based on ICH Q6B Specifications: test procedures and acceptance criteria for biotechnological/biological products,[8] impurities may be related to manufacturing or the product, and it is important to identify and quantify potential impurities as part of the QC process. A good-quality product is not based solely on the active ingredients alone; controlling the unwanted substances is equally important to ensure the product's quality and safety aspects based on the nature of the impurities.

This study aims to isolate, concentrate, and characterize human fetal Wharton's jelly MSCs (WJMSCs)-derived small EVs (sEVs) in vitro. In addition, QC parameters to standardize the sEV preparations were evaluated based on the MISEV2018 guidelines.[7] The consistency of sEVs production using the tangential flow filtration (TFF) method with different fetal WJMSCs sources was also determined in this study.

Materials and Methods

Ethics Approval and Study Design

This research protocol has been reviewed and approved by the National University of Malaysia (UKM) Research Ethics Committee (JEP-2022-065), Medical Research and Ethics Committee, Ministry of Health (NMRR ID-22-00306-ZN0), and Research Ethics Committee, Military Health Services, Ministry of Defence (PKA/JKE/28-08). The experimental design is illustrated in Figure 1. In this study, Wharton's jelly-derived mesenchymal stem cells (WJMSCs) were isolated and characterized. The conditioned medium was collected at passage 3, and small extracellular vesicles (sEVs) were isolated and concentrated using a tangential flow filtration (TFF) system. Subsequently, the isolated sEVs were characterized individually using several techniques: BCA assay for protein quantification, nanoparticle tracking analysis (NTA) for size distribution, Western blot for specific protein markers, and transmission electron microscopy (TEM) for morphological assessment.

Fetal Umbilical Cord Collection

Informed consents were obtained from the umbilical cord (UC) donors for the collection, storage, and use of biological samples prior to study commencement. The fetal part of the human UC, as shown in Supplementary Figure 1, was used to isolate fetal WJMSCs. These fetal UCs were collected from healthy-term pregnant mothers with informed consent from the Department of Obstetrics & Gynaecology at Hospital Angkatan Tentera Tuanku Mizan, Wangsa Maju, Ministry of Defence, Kuala Lumpur, and the Department of Obstetrics & Gynaecology at Hospital Canselor Tuanku Muhriz (HCTM), Kuala Lumpur via either spontaneous vaginal delivery or caesarean section. Inclusion criteria for UC donation involve the donor being over 18 years old, having a gestational age of 37–40 weeks, having no medical complications, and not being under any medications. Pregnant mothers who tested positive for any transmissible infectious diseases like human immunodeficiency virus (HIV), hepatitis virus types B and C, and syphilis were excluded from this study.[9,10]

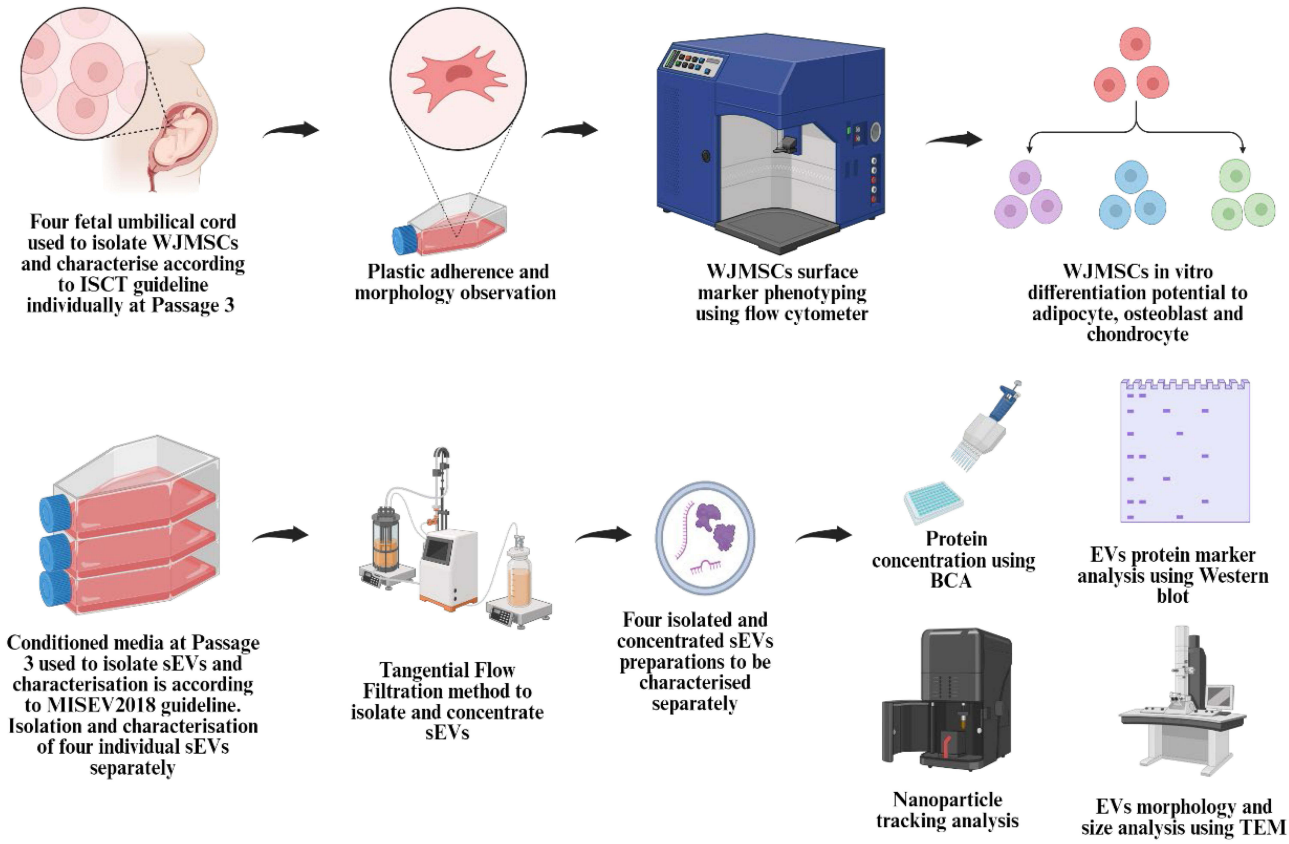

Figure 1 Overview of quality study design. WJMSCs were isolated and characterized. Conditioned medium at passage 3 was used to isolate and concentrate sEVs via TFF. The isolated sEVs were then characterized using BCA, NTA, Western blot, and TEM.

Isolation and Culture of Fetal WJMSCs

The four independent UCs were transferred in sterile conditions to the laboratory within 24 hours for processing. The sample processing and isolation of fetal WJMSCs were performed as previously reported.[11] The local arteries and veins were carefully excised, and the remaining white parenchyma was shredded into thin strips (0.5–1.0 mm^2). The tissue was digested in 0.6% Collagenase Type 1 (Worthington, United States) and incubated in a 37°C shaker incubator for one hour. A complete culture medium of α-MEM was prepared by mixing 1% Glutamax (Gibco, Germany), 1% HEPES (Gibco, Germany), 1% antibiotic-antimycotic (Gibco, Germany), and 10% in-house human platelet lysate (hPL). The hPL was prepared as previously reported.[12] A complete medium was added in equal volume with the digested fetal WJMSCs suspension to neutralize the collagenase activity. Following centrifugation at 5000 rpm for 5 minutes, the supernatant was discarded, and the pellet was resuspended in a complete medium. The cells were seeded at an initial 3000 cells/cm^2 density and maintained in a 37°C incubator with 5% CO_2. The culture medium change is performed after the first 24 hours and thereafter, every three days. At 90% confluency, the cells were harvested using TrypLe Express Enzyme (Gibco, Germany) and subcultured. By passage 1, the cells were enumerated and cryopreserved at 1 million cells per cryovial.

Characterisation of Fetal WJMSCs

Fetal WJMSCs were trypsinized from the flask and the cell suspension was transferred to a new centrifuge tube and centrifuged for 5 minutes at 5000 rpm at room temperature. The pellet was collected and resuspended in a complete medium. An equal volume of this cell suspension was added with trypan blue solution and transferred to the haemocytometer. Cells were viewed under the inverted light microscope. The number of blue-stained cells (dead) and non-stained (live) cells were counted.

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$$\text{Cell counting} = \text{Average number of live cells} \times \text{dilution factor} \times 10^4(\text{cells/mL}) \times \text{total volume of cell suspension}$$

$$\text{Cell viability (\%)} = \text{Live cell counted/Total cell counted} \times 100\%$$

Cell Count and Viability Test

WJMSCs were trypsinized from the flask and the cell suspension was transferred to a new centrifuge tube and centrifuged for 5 minutes at 5000 rpm at room temperature. The pellet was collected and resuspended in a complete medium. An equal volume of this cell suspension was added with trypan blue solution and transferred to the haemocytometer. Cells were viewed under the inverted light microscope. The number of blue-stained cells (dead) and non-stained (live) cells were counted.

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Isolation of Fetal WJMSCs-Derived sEVs

Isolation, purification and concentration of the WJMSCs-derived sEV preparations were adopted from the TFF manufacturer's protocol and as previously reported.[13] WJMSCs at passage 3 were used for the EV harvesting. Four independent fetal WJMSCs were initially seeded at a seeding density of 3000/cm^2 in a culture flask. The culture medium change was performed every three days. When the fetal WJMSCs reached 70–80% confluency, the culture medium was discarded, and the cells were gently washed twice with PBS before being replaced with a phenol red-free DMEM-LG basal medium. After 24 hours, the conditioned medium (CM) was collected and transferred into 50 mL conical tubes. The CM was centrifuged at 2000 × g for 15 minutes at 4°C to remove cell and cell debris. Then, the supernatant of the CM was collected immediately and frozen at −80°C until further use. Approximately 200 mL of CM was collected for each sample. Thawed CM was used to isolate sEVs using the Minimate™ TFF system and Minimate™ TFF capsule with Omega™ polyethersulfone (PES) ultrafiltration 100 kDa membrane (Pall Corporation, US). The enriched sEVs were sterile-filtered through a 0.22 μm filter and stored at −80°C until further use. The final amount of sEV preparation collected was 6 mL for each sample.

Characterization of Fetal WJMSCs-Derived sEVs

Protein Concentration Determination

All four independent fetal WJMSCs-derived sEV preparations were characterized separately based on the MISEV2018 guidelines.[7] Pierce BCA Protein Assay Kit (Thermo Fisher Scientific™, United States) was used following the manufacturer's instructions to determine the protein concentration. Briefly, 25 μL of each standard and sample were added to a 96-well plate. Then, each well was added 200 μL of working reagent (mixed 50 parts of BCA Reagent A with 1 part of BCA Reagent B). The 96-well plate was covered and incubated at 37°C for 30 min. The absorbance was measured at 562 nm on a spectrophotometric multi-well plate reader.

Particle Size and Distribution Analysis

Particle size and distribution analysis were performed using nanoparticle tracking analysis (NTA) with NanoSight NS300 (Malvern Panalytical, UK). Fetal WJMSCs-derived sEV preparations were diluted in filtered PBS to fit in 20–100 particles/frame. The diluted sample at ambient temperature between 25.5°C and 26.8°C was injected using disposable 1 mL syringes into the NTA chamber. The measurement settings were adjusted as follows: the camera level was set to level 14, the detection threshold was set to include as many particles as possible while only counting 10–100 red crosses, and the number of blue crosses was set to five (red crosses are valid tracks, while blue crosses are near the threshold).

Protein Marker Analysis

Fetal WJMSCs-derived sEV preparation's protein markers were analyzed for positive markers (CD63 and TSG101), negative markers (Grp94), and purity control (albumin) using a standard Western blot procedure. The selection of protein markers was according to the MISEV2018 guidelines.[7] Cell lysates of fetal WJMSCs were collected after 5 minutes of radioimmunoprecipitation assay (RIPA) buffer (Pierce™ BCA Protein Assay Kits, United States) and protease and phosphatase inhibitor (Halt™ Protease and Phosphatase Inhibitor Cocktail (100×) Thermo Scientific™, United States) incubation with WJMSCs at 4°C. The lysed samples were centrifuged at 14,000 × g at 4°C for 15 minutes. The collected supernatant is designated as cell lysates. The standard electrophoresis method was used to separate the EV marker proteins. A nitrocellulose membrane, 0.45 µm (Thermo Fisher, United States), was used to transfer the protein from the gel. The primary and secondary antibodies (Cell Signalling Technology, United States) were used as listed in Table 1. The protein markers were detected using ECL detection reagents (Pierce^{TM} ECL Western Blotting Substrate, Thermo Fischer, United States) and viewed under the gel documentation viewer (Amersham Imager 600, GE Healthcare Life Sciences, United Kingdom).

Morphology and Size Analysis

The membrane structure of the sEVs and their size were viewed and confirmed with transmission electron microscopy (TEM) analysis. Briefly, diluted sEVs with PBS were dropped on a Copper grid (300 Mesh) and left to dry for 5 minutes. The excess was wiped with a tissue. One drop of phosphotungstic acid (Sigma-Aldrich, United States) as a negative staining agent was dropped on the same grid and left to dry again for 5 minutes. Again, the excess was wiped with tissue paper. This grid was dried for 72 hours (at room temperature in a drying cabinet with silica) before viewing under the transmission electron microscope (Leo Libra 120, Germany).

Quality Control Monitoring Parameters

Fetal WJMSCs Quality Control Parameter

The four independent fetal WJMSCs were evaluated on qualitative parameters, including cell morphology and trilineage differentiation capabilities. The quantitative parameters were cell count, cell viability, and WJMSCs' surface marker phenotyping assessment.

sEVs Quality Control Parameters

The qualitative parameters for sEV preparations assessment include protein marker analysis (Western blot), with membrane structure and size (TEM) analysis. Quantitative parameters were protein concentration, number of particles, particle size (mode and mean), total protein yield per sample, total particles per sample, total protein in one million cells, and total particles in one million cells.

$$\text{Total protein yield per sample } (\mu g) = \text{Protein concentration } (\mu g/\text{mL}) \times \text{volume of sEVs (mL)}$$

$$\text{Total particles per sample} = \text{Number of particles per mL} \times \text{volume of sEVs (mL)}$$

$$\text{Total protein in one million cells } (\mu g/\text{million cells}) = \text{Total protein yield per sample } (\mu g)/10^6 \text{cells}$$

$$\text{Total number of particles in one million cells (number of particles/million cells} = \text{Total number of particles per sample}/10^6 \text{cells}$$

Table 1 Primary and Secondary Antibodies Used in Western Blot

Antibodies	Catalogue No.	Clonality	Dilution Factor
CD63 (Primary)	13917	Monoclonal	1:1000
TSG101 (Primary)	72312	Monoclonal	1:1000
Albumin (Primary)	4929	Monoclonal	1:1000
Grp94 (Primary)	2104	Monoclonal	1:1000
Anti-rabbit IgG, HRP-linked (Secondary)	7074	Polyclonal	1:1000

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sEVs Standardization Quality Control Parameter

Each individual characterized sEV preparation was standardized by the ratio of the total number of particles to total protein concentration before being pooled together. This ratio is again determined after pooling all four sEV samples. This ratio is also used to evaluate the purity of each isolated sEV preparation and the pooled sEV preparation.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 9.0.0 (GraphPad Software, California, United States). All quantitative variables were presented as mean ± standard error mean (SEM). Comparisons between independent fetal WJMSCs and their sEV preparations were conducted through one-way Analysis of Variance (ANOVA) with Geisser-Greenhouse correction. Multiple comparisons analysis was performed using Tukey's post-hoc test. A difference at $p \leq 0.05$ was considered statistically significant.

Result

Fetal WJMSCs Quality Control

As shown in Figure 2(A), all four independent fetal WJMSCs at passage 3 fulfilled the criteria of ISCT for the characterization of MSCs where all the cells were found to be attached to plastic (culture flask) and their fibroblastic-like morphology with spindle-shaped cells with large nuclei. WJMSCs surface marker phenotype result is shown in Table 2, where all the samples passed the QC acceptance criteria by ISCT, where ≥95% of CD73, CD90, and CD105 and ≤2% of CD11b, CD19, CD34, CD45, and HLA-DR. All the fetal WJMSCs samples passed the QC for in vitro differentiation to adipocytes (Figure 2(B)), osteoblasts (Figure 2(C)), and chondroblasts (Figure 2(D)). Overall, all four independent samples passed the minimal criteria for defining MSCs by ISCT regarding qualitative and quantitative assessment. Other quantitative QC parameters are shown in Figure 3, where there are no statistically significant differences between the independent samples regarding cell count and cell viability. All these four samples were used to produce sEVs as they were confirmed as MSCs.

Fetal WJMSCs-Derived sEVS Quality Control

As for the qualitative QC parameters for the fetal WJMSCs-derived sEVs, the results were illustrated in both Figure 4 for the Western blot analysis and Figure 5 for the TEM analysis. Positive EV markers, ie CD63 and TSG101, were present in all the preparations for EVs, including cell lysates. However, both sEVs samples and cell lysates detected albumin as purity control. Grp94, as an EVs negative marker, was only present in the cell lysate and was absent in all the sEVs samples. TEM analysis showed the presence of membrane vesicles with a size of less than 200 nm isolated from all the sEV preparations, with bigger microscopic images shown in Supplementary Figure 2.

The assessment of quantitative QC testing performed on all sEV preparations is shown in Figure 6(A–H). The protein concentration per mL showed a statistically significant difference between EV2 and EV3 ($p \leq 0.05$). EV3 preparation demonstrated the highest protein concentration and particle number (1112.80 ± 117.87 µg and $1.296 \times 10^{11} \pm 4.358 \times 10^{10}$ particles per mL, respectively) compared to other sEV preparations. Rationally, EV3 has the highest protein yield and particles per sample. The protein concentration and particles in a million cells also showed a similar trend. The average mean and mode of the particle size were 98.9 ± 7.97 nm and 75.12 ± 7.15 nm, respectively.

sEVs Standardization Quality Control

Table 3 summarises the average number of particles and total protein per sample for each six mL of sEVs pooled together. The pooled result was 1.00×10^{8} particles per µg of protein.

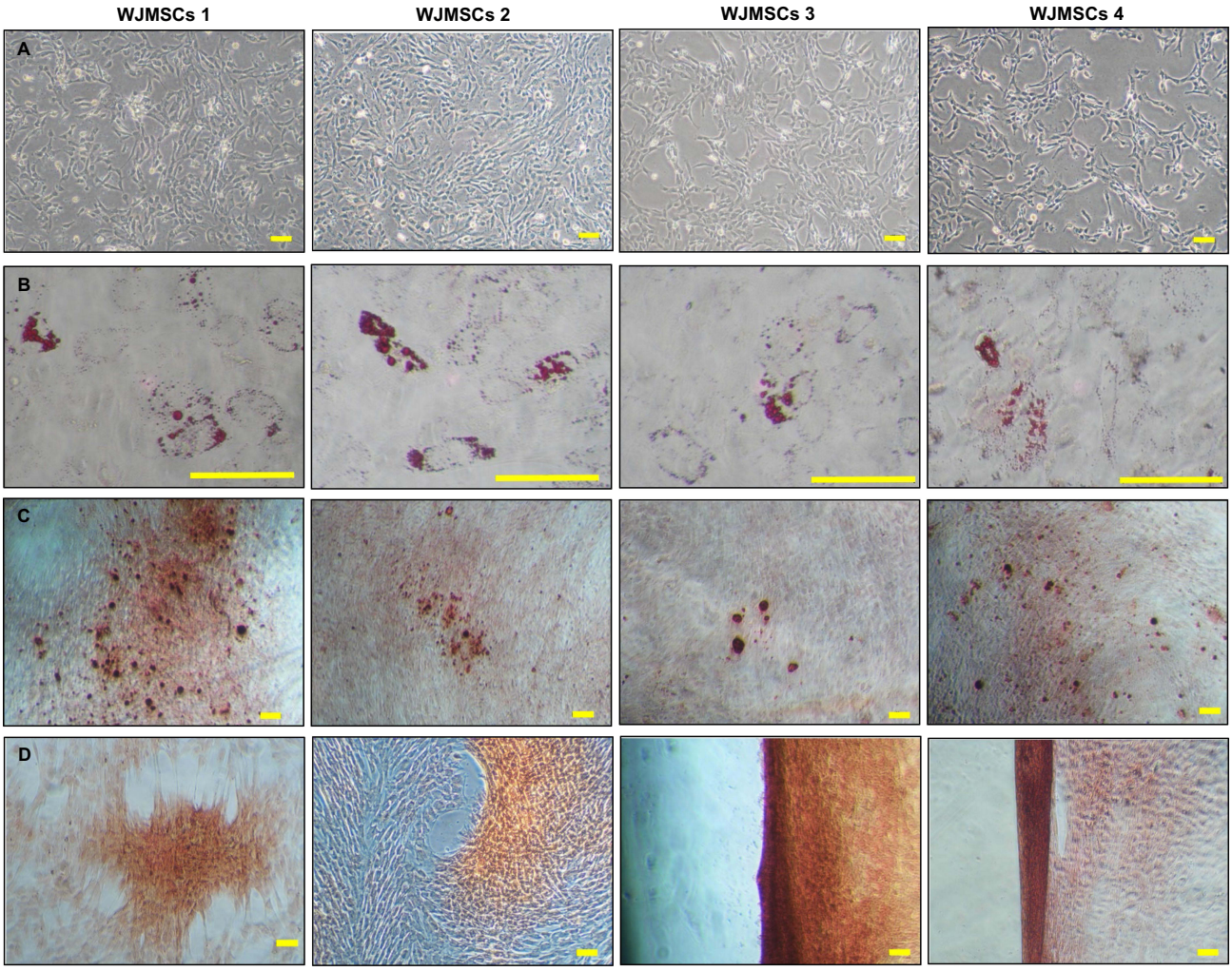

Figure 2 Morphology and trilineage capabilities of fetal WJMSCs at passage 3. (**A**) Morphology of fetal WJMSCs with fibroblast-like shape cells with large nuclei (scale bar 100 μm, magnification 40×). (**B**) Oil red O-stained intracellular lipid droplets in adipogenic-induced fetal WJMSCs (scale bar 100 μm, magnification 200×). (**C**) Alizarin red-stained calcium deposits in osteogenic-induced fetal WJMSCs (scale bar 100 μm, magnification 100×). (**D**) Safranin O-stained glycosaminoglycans in chondrogenic-induced fetal WJMSCs (scale bar 100 μm, magnification 100×).

Discussion

Researchers worldwide are fascinated with stem cell therapy, especially dealing with MSCs, due to the many outstanding potentials of these cells in treating a broad spectrum of diseases. However, this uplifted curiosity has also generated many uncertainties and anomalies in this field due to the unstandardized laboratory practices among researchers. In 2006, the

Table 2 Fetal WJMSCs Surface Marker Phenotype Analysis Results

Sample	Positive Surface Marker (%)			Negative Surface Marker (%) *Cocktail	Overall results
	CD105	CD73	CD90		
WJMSCs 1	99.30	98.73	99.65	0.33	Pass
WJMSCs 2	99.50	99.47	99.99	0.61	Pass
WJMSCs 3	99.70	98.18	100.00	0.69	Pass
WJMSCs 4	99.73	99.83	99.98	0.24	Pass

Notes: *Cocktail: CD34, CD45, CD11b, CD19, and HLA-DR.

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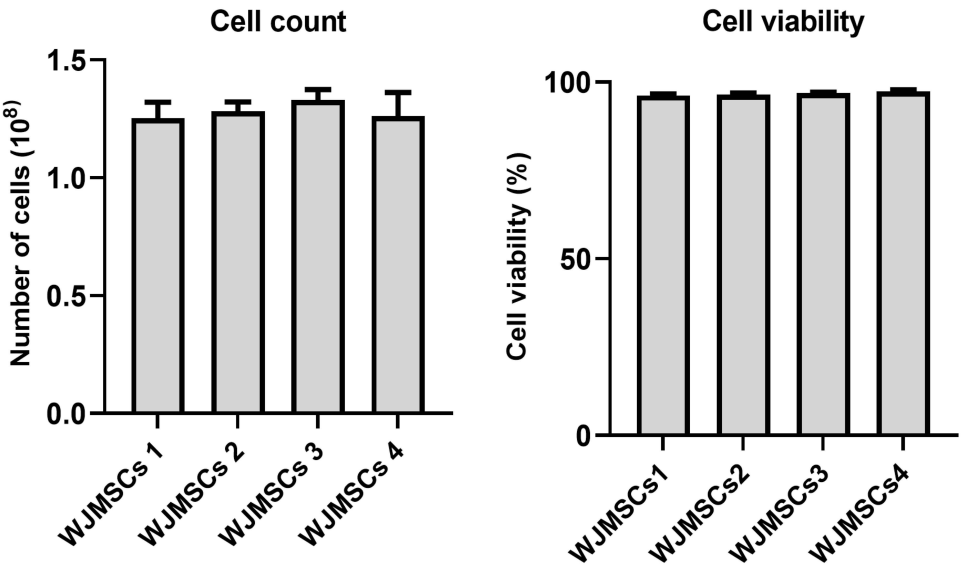

Figure 3 Cell count and viability for fetal WJMSCs. Fetal WJMSCs at passage 3 were used. Data are presented as mean ± SEM (n=4). A difference at * $p \leq 0.05$ was considered statistically significant.

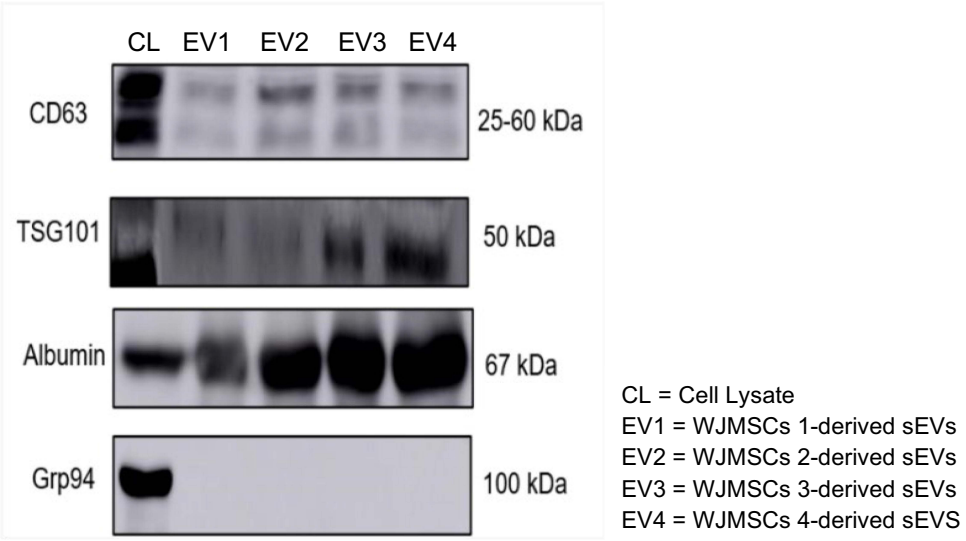

Figure 4 Characterisation of sEV preparation protein markers by Western blot. CD63 and TSG101 as positive markers, albumin as a purity control, and Grp94 as a negative marker.

ISCT published a position paper[6] to help MSC researchers with globally accepted criteria to standardize and define their MSCs used in research. The proposed criteria were plastic adherence, surface marker phenotyping, and multipotent differentiation potential based on the human MSCs. This begins a journey to standardize the MSCs used in research, industry, and regulatory fields. MSCs minimum criteria ensure the reproducibility of results and serve as QC criteria for the "raw materials", which are the MSCs themselves, to guarantee good quality products are manufactured. This study used these minimum criteria for the QC of fetal WJMSCs. All four independent UC samples from different healthy donors were harvested, and the fetal WJMSCs were cultivated separately and characterized individually. Rigor QC shall be applied to the starting material and, in this case, the fetal WJMSCs as part of in-process QC. Therefore, no pooling of the fetal WJMSCs was conducted as per the guidelines.[14] Consistency in production was observed in all four independent WJMSCs regarding the number of cells and viability, where no statistical significance was observed.

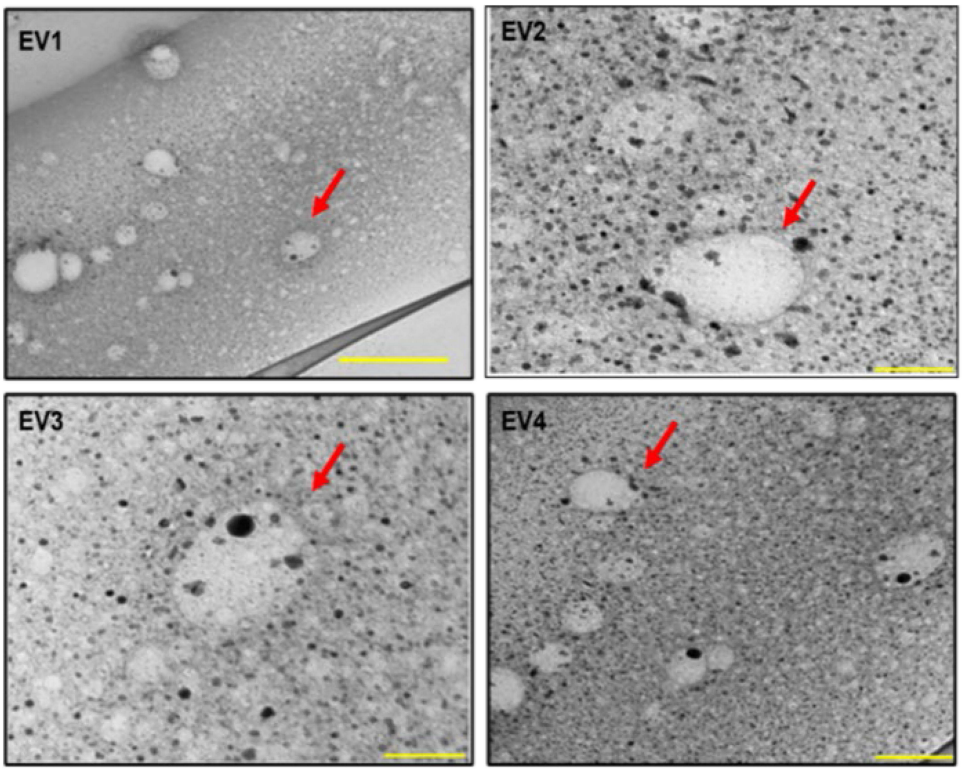

Figure 5 TEM images of sEV preparations. The red arrows indicate the sEVs. Scale bar for EV1 (500 nm), EV2 and EV3 (100 nm), and EV4 (200 nm). Magnification for EV1 (10,000×), EV2 and EV3 (50,000×) and EV4 (20,000×).

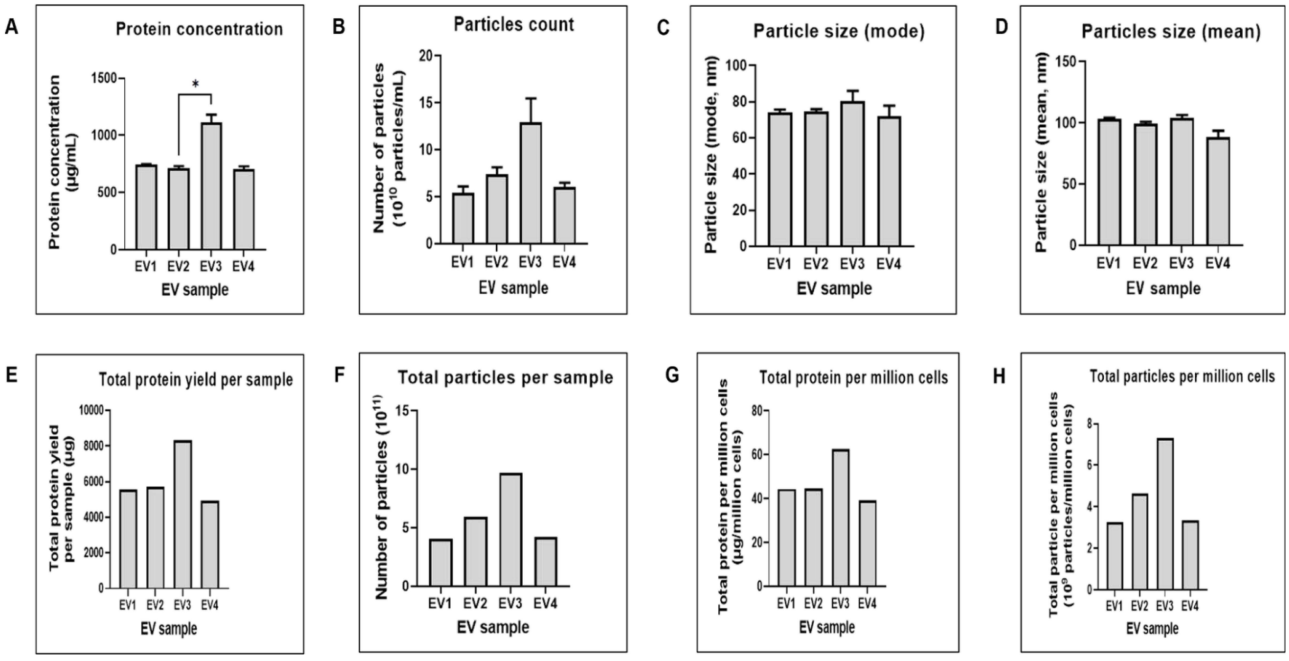

Figure 6 Quantitative quality control parameters of fetal WJMSC-derived sEV preparations. (**A**) Protein concentration, (**B**) Particle count. (**C**) Particle size (mode). (**D**) Particle count (mean). (**E**) Total protein per sample. (**F**) Total particles per sample. (**G**) Total protein per million cells. (**H**) Total particles per million cells. Data in panels (**A**) to (**D**) are presented as mean ± SEM (n=4). A difference at * $p \leq 0.05$ was considered statistically significant.

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Table 3 Characteristics of the Individual and Pooled Fetal WJMSC Derived sEV Preparations

Sample	Average Number of Particles Per mL	Average Protein Concentration (µg/mL)	Ratio Number of Particles/µg Protein	Volume of sEVs (mL)	Total Number of Particles Per Sample	Total Protein Per Sample (µg)
EV1	5.437×10^{10}	741.79	7.28×10^{7}	6	3.262×10^{11}	4450.74
EV2	7.407×10^{10}	714.40	1.04×10^{8}	6	4.444×10^{11}	4286.40
EV3	1.296×10^{11}	1112.80	1.17×10^{8}	6	7.776×10^{11}	6676.80
EV4	6.003×10^{10}	723.52	8.30×10^{7}	6	3.602×10^{11}	4341.12
Pooled	Total number of particles				1.908×10^{12}	
	Total amount of protein (µg)					19,755.06
	Total number of particles/µg protein				1.0×10^{8}	

Note: The colored cells in the table indicate the absence of results in those specific areas.

The same goes for EVs where the ISEV published the first guideline for EVs-based research in the year 2014 with the latest version MISEV2023[15] where this document was produced from the contributions of more than 1000 EVs scientists. These guidelines provide the latest nomenclature, collection and pre-processing, separation and concentration, reporting, characterization, release, and uptake, and in vivo studies involving EVs. All EVs stakeholders can refer to this position paper as the basic guidance for EVs QC and standardization. In this study, the isolated sEVs-enriched preparations were characterized independently to evaluate the in-process QC according to MISEV2018[7] and, as reviewed.[2,3]

The presence of EVs protein markers, ie CD63 and TSG101 with the absence of negative markers, ie Grp94 indicated the EVs were harvested in the preparation. TEM displayed EV morphology and its lipid membrane. The size of EVs can be estimated via NTA and size visualization in TEM analysis. However, due to the differences in instrumental principles, limits of detection, and algorithms, the size of EVs measured by NTA and TEM varied in this study. Yet, the TFF method managed to isolate EVs with a size of less than 200 nm, and there is no significant size difference among the four independent sEV preparation. All these characterizations and QC enable us to confirm the presence of EVs in the preparations and give assurance to be classified as small EVs. Apparently, characterization and QC of EV preparations distinguished between EVs and non-EVs components and assessed the consistency of production.

EVs preparations are high in heterogeneity, evasive, and hard to explore due to the diverse populations, physical properties, and complexity of their cargo that depend on many internal and external factors.[16] This heterogeneity hinders the reproducibility of the protocols and experiments, making it challenging to standardize the purification process and the acceptance of in-process QC and release criteria.[17] Concretely, the properties of the isolated EVs in the EVs preparations rely upon the isolation protocol chosen for the separation and enrichment of the CM or biofluid.[18] The same parent cells used for EVs production with different EVs isolation and enrichment protocols resulted in different properties of EVs preparations. Compared to the conventional ultracentrifugation method, which was claimed as the "gold standard method" by some researchers, TFF is more beneficial for therapeutic development with its scalability in EV production.[19] Using the ultrafiltration 100 kDa membrane with the TFF, we managed to isolate and enrich sEVs with a particle size of less than 200 nm with average mode and mean particle size of 75.12 ± 7.15 nm and mean 98.9 ± 7.97 nm, respectively. Consistency in production was observed in EVs productions in all the sEV preparations for the number of particles per mL, particle size (mode), and particle size (median), where no statistical significance was observed. Here, we concluded that different fetal WJMSCs samples produced a consistent number of particles and size of EVs with the same tissue harvesting method, culture condition, and sEVs isolation, purification, and enrichment methods.[20,21]

EVs isolation and enrichment using MSCs CM permit a more manageable environment than biofluid collection.[22] Serum-free medium or EV-depleted serum medium are commonly used for EVs harvest. Both approaches may influence the EVs yield and properties. Sudden changes by adding a serum-free medium may cause stress to the EVs-producing

cells, which may affect the production and secretion of EVs.[23] Meanwhile, EV-depleted serum is less preferred due to the time-consuming method with multiple stages,[24] and it was also reported that this method does not exclude contamination from RNAs found in bovine serum albumin.[25] It is recommended to use a xeno-free, chemically defined medium to minimize the low serum-induced stress and eliminate the contamination from fetal bovine serum.[26,27] In this study, a serum-free medium was used during the preparation of EVs-containing CM to reduce the co-isolation of serum-derived EVs. Albumin is recognized as the predominant protein contaminant in EV preparations. Unfortunately, we found the presence of albumin in all the sEV preparations using Western blot, which was found mainly in serum. This co-isolated albumin contributed to the final protein concentration of the sEV preparations. Therefore, the protein concentration results were not solely for EVs but may include other "protein contaminants" as co-isolates. Stolk & Seifert reported that albumin may complicate the process because it may still contain natural vesicles and cause inaccurate protein concentration.[28] Although study[29] reported that lipoproteins and non-EVs proteins were isolated from a serum-containing medium, the presence of albumin in our sEV preparations from serum-free sEVs-containing CM suggests that the transition step from serum-containing medium to serum-free medium is worth discussing to better manage the unwanted "protein contaminants". Our study also emphasized the importance of determining soluble protein and lipoprotein with regard to EVs isolation protocol. On the other hand, recent studies[30–33] demonstrated that these co-isolated "protein contaminants" or soluble proteins in the EVs preparations provide positive functional and stability effects on the isolated EVs. In short, in addition to focusing on the quality of EVs, much attention should be given to the QC of the EVs production "contaminants" as these co-isolates contribute a certain level to the functional efficacy, safety, and dosing of EVs therapy.[34] Yet, the production of these co-isolates in EVs preparation is unique to the methods used for EVs isolation, purification, and enrichment.[35]

The ratio of particles of EVs to the protein concentration is recommended for purity determination in EVs preparation.[15] The purity determination in the EVs preparations can be divided into three categories: higher vesicular purity ($>3 \times 10^{10}$ particles/μg), low purity (2×10^{9} to 2×10^{10} particles/μg), and unpure ($<1.5 \times 10^{9}$ particles/μg).[36] In our study, all the sEV preparations were "unpure" based on these criteria, consistent with the findings from Cheng & Kalluri,[17] who harvested EVs using the TFF method. However, this purity category by Webber & Clayton was not included in the MISEV 2018[7] and MISEV2023[15] due to the different LOD of each assay or instrument. Hence, it is recommended to report the absolute protein and particle concentrations separately.[15] There are differing opinions on whether the purity of EVs preparations contributes to their attributes and functionalities, with some agreeing and others disagreeing on its impact.[19,33,37] Therefore, the potency assay and consistency assay of the EVs preparation are the crucial aspects of QC to ensure the EVs functionality and reproducibility of EVs production, especially for therapeutic use.[38] A limitation of this study is the absence of functional assays on both isolated fetal WJMSCs and the sEV preparation after characterization. On the other hand, high-throughput analytical approaches such as mass spectrometry, Raman spectroscopy, and next-generation sequencing should be utilized to determine the components of EV preparations, providing a more comprehensive analysis.

The "no pooling" of the fetal WJMSCs was emphasized in the previous paragraphs to reduce the risk of consistency from living cells.[14] However, biological DS has high variation due to the complexity of the product, especially EVs (heterogeneity, cargo, cell source); therefore, pooling of the individual DS is encouraged to ensure the consistency of the production and to reduce the batch-to-batch variations. This approach also ensures sufficient EVs are produced for further use when the EVs are required on a larger scale.[39,40] The idea is to pool only the sEV preparations that passed the QC, and this is to avoid wastage of characterized sEV preparations when pooled with the sEV preparations that failed the QC.

There are no specific guidelines for dosing of EVs, and most of the dosing regimen was determined from the literature review. A meta-analysis review[41] provides some EVs dosing information based on 64 preclinical studies. A significant obstacle in the EVs field is the disparities in EVs production and characterization, and therefore, it is difficult to create clear dosing guidelines. EVs dose can be in the form of protein concentration or particle number. Most of the studies used protein concentration as EVs dosing. However, we need to consider protein concentration overestimation due to EVs co-isolates, especially when a less specific method is used in EVs isolation and enrichment. In our study, we concluded that the presence of albumin in sEV preparations led to overestimated sEVs protein concentration values. Additionally, the lack of specificity of EVs detection for NTA can result in inaccurate sEVs concentration measurements. Hence, it is

important to note that these values do not represent actual EVs concentrations. The estimation of the abundance of sEVs in the preparations is recommended by testing protein concentration, particle number, and/or lipid content separately.[15]

EVs are rapidly cleared from the systemic system.[42,43] Consequently, determining the optimal administration route and frequency of EVs administration is critical to ensure their therapeutic efficacy. Determination of the dose was based on the consideration of EVs purification, in vivo dosing, and disease type of meta-analysis data of the EV dosing review by D. Gupta and also the ongoing clinical trial of "The Use of Exosomes for the Treatment of Acute Respiratory Distress Syndrome or Novel Coronavirus Pneumonia Caused by COVID-19" (ClinicalTrials.gov ID: NCT04798716).

Conclusion

In this study, all the WJMSC samples passed the minimum criteria of ISCT guidelines and the good QC "raw materials" were used for EV productions. By utilizing the TFF method to harvest and concentrate sEVs from different human fetal WJMSCs samples, we observed the variability of protein concentration among the four sEV preparations, while the number and the size of sEVs particles for all the preparations showed no statistical differences. Quantification of albumin or any other co-isolates contributed to QC data, such as in-process release criteria for the albumin as impurities or co-isolates.[44] In future, the detection and quantification of other lipoproteins are also important since they might affect the NTA results as well.

Abbreviations

sEVs, small extracellular vesicles; QC, quality control; WJMSCs, Wharton's jelly mesenchymal stem cells; ISCT, International Society for Cell and Gene Therapy; TFF, tangential flow filtration; MISEV, minimal information for studies of extracellular vesicles; DS, drug substance; MOA, mechanism of action; ICH, International Council of Harmonisation; UC, umbilical cord; hPL, human platelet lysate; CM, conditioned medium; NTA, nanoparticle tracking analysis; TEM, transmission electron microscopy; ISEV, International Society for Extracellular Vesicles.

Ethical Statement

The studies were reviewed and approved by the National University of Malaysia (UKM) Research Ethics Committee (JEP-2022-065), the Medical Research and Ethics Committee of the Ministry of Health (NMRR ID-22-00306-ZN0), and the Research Ethics Committee of the Military Health Services, Ministry of Defence (PKA/JKE/28-08). This research adheres to the principles outlined in the Declaration of Helsinki. All participants provided written informed consent prior to their involvement in the study.

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Disclosure

Dr. Min Hwei Ng reports a patent approval is pending to Medixcell Sdn. Bhd. for WJMSCs-sEVs for metabolic syndrome. Mrs. Shathiya Rajamanickam reports a patent is pending for Medixcell Sdn. Bhd. Dr. Yogeswaran Lokanathan reports grants and research collaboration from Medixcell Sdn. Bhd. during the conduct of the study. In addition, Dr. Yogeswaran Lokanathan has also reported a patent approval is pending for WJMSCs-sEVs to Medixcell Sdn. Bhd. and Universiti Kebangsaan Malaysia. This paper was presented at the SHIFT-MTERMS-EWA'S BRPM 2024 Conference as an oral presentation with interim findings. The abstract proceeding was published in *Med & Health Apr 2024; 19(2) (Suppl): 01*. https://doi.org/10.17576/MH.2024.s1902. All authors report no other conflicts of interest in this work.

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