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OPTIMIZATION OF PROTEIN QUANTIFICATION IN WHARTON JELLY-DERIVED MESENCHYMAL STEM CELL EXOSOMES

WHARTON JELİ MEZENKİMAL KÖK HÜCRE EKZOZOMLARINDA PROTEİN MİKTARININ OPTİMİZASYONU

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ABSTRACT

Objective: Exosomes are small intracellular membrane-based vesicles of different compositions that are involved in various biological and pathological processes. They are secreted by all cell types and can be found in most body fluids, including the blood, saliva, and urine. Exosomes are nanometer-sized microvesicles, approximately 30-200 nm in diameter, containing DNA, mRNAs, non-coding RNAs, membrane proteins, and cytosolic proteins. stem cells exert paracrine effects through exosomes. Recent studies have shown that exosomes have important potential as new alternatives to cellular therapies. In this study, we isolated and characterized exosomes from Wharton-Jelly derived mesenchymal stem cells (WJ-MSCs). It was aimed to determine the most effective exosome lysis solution using different lysis chemicals over a certain number of nanoparticles and to optimize protein quantification in these exosomes.

Methods: WJ-MSC exosomes were isolated and characterized, and changes in protein levels were determined after treatment with 1 billion/ml particle exosomes with commonly used chemicals such as RIPA buffer, Mammalian Protein Extraction Reagent (M-PER), Tris-TritonX, and Tris-SDS.

Results: As a result, approximately 2.5 µg/ml of 1 billion/ml particulate exosomes were detected with the bicinchoninic acid (BCA) kit. The protein concentration in these exosomes increased 3-4 times as a result of disintegration of the bilayer membranes in the cell membrane structure of these exosomes with RIPA buffer.

Conclusion: These data can be used in future studies, particularly for the quantification of mesenchymal stem cell exosomes.

Keywords: Wharton-Jelly, mesenchymal stem cells, exosomes, lysis buffer, protein quantification

ÖZ

Amaç: Eksozomlar, çeşitli biyolojik ve patolojik süreçlerde yer alan farklı bileşimlere sahip küçük hücre içi membran bazlı veziküllerdir. Tüm hücre tipleri tarafından salgılanırlar ve kan, tükürük ve idrar dahil olmak üzere çoğu vücut sıvısında bulunabilirler. Eksozomlar, yaklaşık 30-200 nm çapında, DNA, mRNA'lar, kodlamayan RNA'lar, membran proteinleri ve sitozolik proteinler içeren nanometre boyutunda mikroveziküllerdir. Kök hücreler eksozomlar aracılığıyla parakrin etkiler gösterir. Son çalışmalar, eksozomların hücresel tedavilere yeni alternatifler olarak önemli bir potansiyele sahip olduğunu göstermiştir. Bu çalışmada, Wharton-Jeli kaynaklı mezenkimal kök hücrelerden (WJ-MSCs) eksozom izolasyonu ve karakterizasyonu yapılmıştır. Belirli bir nanopartikül sayısı üzerinden farklı lizis kimyasalları kullanılarak en etkin eksozom lizis solüsyonunun tespiti ve bu eksozomlarda protein miktar tayini optimizasyonu amaçlanmıştır.

Yöntem: WJ-MKH ekzozomları izole edilip karakterize edilmiş ve 1 milyar/ml partikküllü ekzozomların RIPA tamponu, Mammalian Protein Ekstraksiyon Reaktifi (M-PER), Tris-TritonX ve Tris-SDS gibi yaygın olarak kullanılan kimyasallarla muamelesinden sonra protein seviyelerindeki değişiklikler belirlenmiştir.

Bulgular: Bicinchoninic asit (BCA) kiti ile yaklaşık 2,5 µg/ml 1 milyar/ml partikül ekzozom tespit edilmiştir. Bu eksozomların hücre zarı yapısındaki çift tabakalı membranların RIPA tamponu ile parçalanması sonucunda bu eksozomlardaki protein konsantrasyonu 3-4 kat artmıştır.

Sonuç: Bu veriler gelecek çalışmalarda, özellikle mezenkimal kök hücre ekzozomlarının kantifikasyonu için kullanılabilir.

Anahtar Kelimeler: Wharton-Jeli, mezenkimal kök hücre, eksozom, lizis tamponu, protein miktar tayini

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Introduction

Mesenchymal stromal/stem cells (MSCs) can be isolated from different tissues or organs such as bone marrow, adipose tissue, placenta, umbilical cord, amniotic fluid, liver, teeth, and peripheral blood. The most commonly used sources of MSCs are bone marrow, adipose tissue, and, more recently, MSCs derived from the umbilical cord Wharton's Jelly region with high proliferation capacity. Isolations from adult tissues such as perinatal organs, bone marrow, and adipose tissue have several risks such as invasiveness, high risk of infectious diseases, and limited proliferation potential due to the advanced age of the donor.[1,2]

The umbilical cord (UC) is a perinatal organ that connects the placenta and fetus to facilitate nutrition and the exchange of gases (oxygen and carbon dioxide). The UC is composed of two layers: the umbilical cord membrane, which is epithelial and mesenchymal; the perivascular region (PVB), which surrounds and protects the blood vessels; and the central part of the UC, the Wharton's Jelly (WJ) region, which is rich in glycosaminoglycans such as hyaluronic acid and chondroitin sulfate and has a gelatinous structure that provides elasticity to the cord.[3,4] The collection of UC is noninvasive, ethically treated as waste material, and does not pose ethical issues. Furthermore, UC is a good source for obtaining a significant number of MSCs. Recently, it has become the preferred source of MSCs for therapeutic purposes compared to bone marrow and adipose tissue.

Similar to other multipotent stromal cells, these cells were positive for CD73, CD90, and CD105 and negative for CD45, CD14, CD34, CD19, and HLA-DR surface antigens. They are characterized by their ability to adhere to plastic surfaces and differentiate into other cell types such as adipocytes, chondrocytes and osteoblasts.[5] Due to the low expression of HLA class II proteins, immunogenicity is almost non-existent; therefore, allogeneic use in cellular therapies is not considered a problem.[6,7] Another reason why these cells are preferred in the clinic is that they secrete various growth factors and cytokines, such as G-CSF, HGF, PDGFAA, TGF-β, IL-6, and IL-8, which play important roles not only in immunomodulation, but also in cell proliferation, differentiation, growth, and tissue repair.[8,9]

Distant intercellular communication is facilitated by molecules, such as hormones, that signal to other parts of the body through the circulatory system. Another type of remote intracellular communication occurs via extracellular vesicles (EVs), which are membrane-based structures. These EVs act as vehicles to transport different types of cellular cargo such as lipids, proteins, receptors, and effector molecules to recipient cells.[10] These include apoptotic bodies, microvesicles and exosomes.[11] Apoptotic bodies range in size from 50 to 5000 nm and contain cellular contents such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and histone proteins. During apoptosis, apoptotic bodies present these contents to macrophages, resulting in cell engulfment.[12] Microvesicles are formed by outward budding and fission from plasma membranes, with sizes ranging from 50 nm to 1000 nm. Once microvesicles are formed, they carry specific proteins and lipids and deliver their cargo to the designated recipient cell.[11] The final category of EVs is exosomes, which differ from microvesicles mainly in their intracellular origin and size. Exosomes are small intracellular membrane-based vesicles with different compositions that are involved in various biological and pathological processes. The use of exosomes as drug delivery vehicles offers significant advantages over drug delivery systems such as liposomes and polymeric nanoparticles. Exosomes are non-immunogenic in nature because of their similar composition and size as their own cells.[13]

Exosomes are small endosome-derived vesicles ranging in size from approximately 30 to 200 nm.[10,14,15] They are secreted by all cell types and can be found in most body fluids, including the blood, saliva, and urine. The exosome is a "nanosphere" with a bilayer membrane containing various types of lipids and proteins derived from the host cell. Some of these include transport proteins, heat shock proteins, and multivesicular body-associated proteins. In addition to proteins, exosomes are composed of different types of lipids, such as cholesterol, sphingolipids, phosphoglycerides, ceramides, and saturated fatty acid chains.[11] The composition of exosomes is important, as they serve as biomarkers and provide an indication of their function in biological processes. Recent studies have also shown that exosomes have significant potential as novel alternatives to cellular therapies.[16]

After exosome isolation, various characterization procedures are required.[15,17] In addition, protein quantification was performed for standardization. This is a critical step because it forms the basis of subsequent in vitro and in vivo studies. In the original articles, it is stated that in-vitro and in-vivo experimental studies were performed at concentrations of "10-50 µg", but there is no mention of the use of any chemicals related to the amount of protein contained in the exosomes in bicinchoninic acid BCA[18-20] and in recent studies and treatment applications, quantification is made based on particle number. Nanoparticle measuring devices are used for this purpose, but this process is available in very few laboratories. After prolonged culture and multi-stage centrifugation, the amount of protein obtained from the exosomes was small. In our study, we aimed to establish a protocol for the isolation and characterization of exosomes from mesenchymal stem cell culture by ultracentrifugation, and to determine the amount of protein contained in the exosomes obtained in the most accurate way. For this purpose, after nanoparticle tracking analysis (NTA) of Wharton Jelly Derived Mesenchymal Stem Cell (WJ-MSC) exosomes obtained by ultracentrifugation, the protein quantification efficiency of one billion particles diluted in one milliliter (ml) isotonic solution was investigated after treatment with four different lysis solutions.

Methods

Exosome Isolation from WJ-MSCs

The cells used in the experimental phase were WJ-MSCs donated with patient consent and cultured and propagated in the laboratories of "STEMBIO Cord Blood, Cell and Tissue Center-MARTEK/Gebze" approved by the Ministry of Health of the Republic of Turkey under Good Manufacturing and Practice (GMP) conditions. Exosome isolation was also performed in this center.

In this study, differential ultracentrifugation was used for exosome isolation, which provided exosome isolation with high efficiency and purity. It is based on the separation of exosomes from non-vesicular particles, such as large bioparticles, proteins, and protein/RNA aggregates, by centrifugation at high speed and centrifugation of the medium taken from the MSC culture with different densities, shapes, and sizes.

After the WJ-MSC culture dishes reached 80-90% confluency, the media were poured and washed twice with PBS, and exosomes and microvesicles from Fetal Bovine Serum (FBS) were removed. After MSCs were cultured for 48 h in DMEM/F12 medium with 1% Pen/Strep antibiotic supplement without FBS for 48 h, the media were collected into 50 ml falcon tubes[21]. In the first stage of exosome isolation, WJ-MSC culture media was centrifuged at 300 × g for 10 min at 4°C, and dead cells were removed. In the 2nd stage, the medium was centrifuged at 15,000 g for 20 min at 4°C and the supernatant was transferred to new tubes. The supernatant was passed through a 0.22 µm pore size filter (BIOFIL, China) to remove microvesicles larger than 220 nm. The pre-purified cell media were centrifuged 2 times at 110,000 g for 70 min at 4°C for 70 min using a CS150FNX (Hitachi Himac, Japan) ultracentrifuge and S50A model rotor, and the pelleted exosomes remaining after supernatant removal were homogenized with a small amount (~100-600 µl) of PBS or saline. After centrifugation in the isolation phase, washing procedures were carried out in a cold environment on ice. All the centrifugation steps were performed at 4 ^{0}C.

Exosomes maintained their protein structure for one week at 4°C during the experimental stages. Exosomes were stored at -20°C and/or -80°C if the experiment was planned for a later period for long-term storage.

Characterization of WJ-MSC Exosomes

For exosome characterization, exosome surface markers were determined by flow cytometry, size and shape analysis by electron microscopy, and size and quantity determination by NTA.

Immunophenotypic Characterization by Flow Cytometry

For exosome characterization, exosome surface markers were labeled with the appropriate antibodies. The ExoStepTM Kit (Immunostep, Salamanca, Spain) was used for this purpose. Exosomes labeled on a flow cytometer (Beckman Coulter, California, US) were analyzed for marker positivity. CD63 capture antibodies, consisting of 6 µm diameter magnetic beads coated with a specific antibody that can be detected by the device, are used as secondary antibodies with CD9 secondary/detector antibodies labeled in red fluorescence, allowing them to be detected by the flow cytometry fluorescence detector (Figure 1).

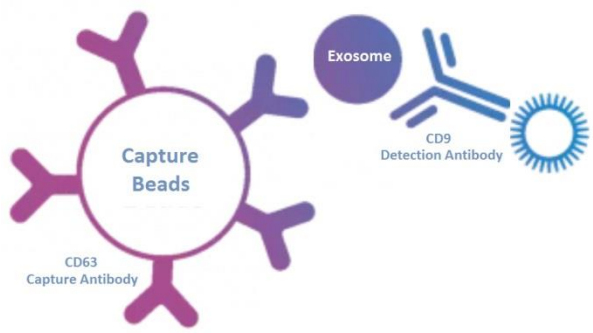

Figure 1. Working principle of exosome characterization by immunophenotyping[21]

Morphological images of exosomes obtained by electron microscopy (Quadro, Thermo Scientific, USA) were captured at the Boğaziçi University, Center for Life Science. A 5µl sample was dropped onto the grid, and the instrument was switched to the environmental SEM (ESEM) mode. The instrument mode was visualized at 30 kV, and a spot size of 1. The device was set to 160,000 × magnification under high vacuum to visualize the sample in aqueous form.

Nanoparticle Tracking Analysis (NTA)

Size and quantity analyses of exosomes were performed using a NanoSight NTA 3.4 instrument (Malvern Panalytical, UK) at Yeditepe University.

Exosomes were lysed with radioimmunoprecipitation assay (RIPA) buffer, mammalian protein extraction reagent (M-PER), Tris-Triton, and Tris-SDS, and protein quantification was performed using bicinchoninic acid (BCA) chemical. The protein content of each lysis solution was evaluated as a blank, without any treatment.

Lysis with RIPA Buffer

RIPA buffer (Thermo Fisher Scientific, Illinois, USA) is a lysis solution containing 25 mM Tris.HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS. After incubation for 30 minutes, equal amount of RIPA buffer was added to the exosomes and placed in a cold sonication bath for 30 seconds. After this step, the samples were gently mixed on ice for 15 minutes.[22]

Lysis with M-PER Buffer

Equal amount of M-PER Buffer (Thermo Fisher Scientific, Illinois, USA) was added to the exosomes and gently mixed for 1-2 min for protein quantification.

Lysis with Tris-SDS

EVs were added to equal amount Tris-SDS buffer [2% (w/w) sodium dodecyl sulfate-SDS, 20 mM, Tris-HCl pH 8] and kept at 95°C for 5 min. EVs were placed in a cold sonication bath and vortexed six times for 30 s at 30-second intervals on ice to reduce heating.[23]

Lysis with Tris-Triton

Exosomes were treated with equal amount of Tris-Triton buffer [120 mM NaCl pH 7.5, 1% Triton-X 100 to lysate with 25 mM Tris-HCl], mixed, incubated on ice for 3 h, and vortexed every hour.[24]

Exosome Quantification by Micro-BCA Assay

Protein quantification was performed using a micro-BCA protein assay kit (Booster, Pleasanton, CA, USA). The bicinchoninic acid (BCA) method is a calorimetric assay based on the reduction of proteins in alkaline solution from Cu^{+2} to Cu^{+1} with 'Biuret' reagent and spectral measurement of the color change from green to purple in proportion to the amount of protein. According to the kit protocol, standard solutions prepared with bovine serum albumin (BSA) and exosome samples were read at 562 nm wavelength using a microplate reader (Versamax, USA), and the chromogenic reaction was measured. Protein concentrations of the samples were evaluated by comparing the measured absorbance values with the standard curve. All experiments were repeated thrice.

Results

Characterization of WJ-MSC Exosomes

CD63 and CD9 surface markers were positive over 96,64± 1,20% (Figure 2).

Electron microscopy images showed that the exosome size was approximately 163,34±10,72 nm (Figure 3).

NTA results also showed the size of the exosomes was 116.4±46.6 and the number of particles per ml of exosomes was also determined by this analysis (Figure 4).

The experiments carried out for characterization all confirm that the EVs obtained are exosomes.

Protein Quantification with Micro-BCA Kit

For BCA quantification, the absorbance values of the isotonic solution (saline) in which the isolated exosomes were reconstituted and stored and the absorbance values of each lysis chemical after treatment with BCA solution alone were evaluated according to the standards during the treatment with exosomes. Each chemical contained a blank. When we subtracted the blank values of all lysis chemicals from their mixtures with exosomes, the highest amount of protein was observed in the RIPA buffer. The results showed that the RIPA buffer was approximately 3.3 times more effective than the other lysis solutions (Figure 5).

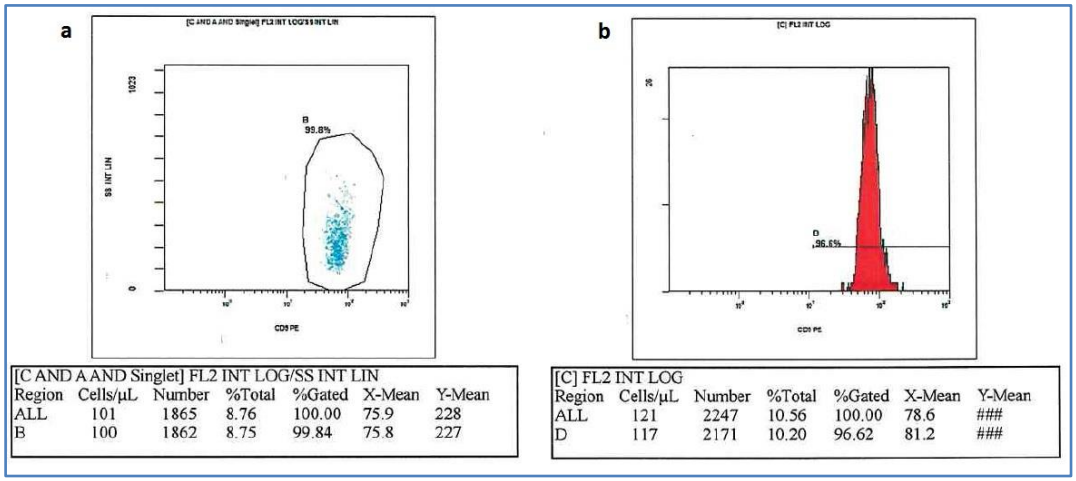

Figure 2. Flow cytometry analysis of exosomes. a. Graph showing the gating of the target cell cluster. b. Graphical display of positive cells within the cell cluster.

Discussion

Mesenchymal stem cells (MSCs) are a cell population with a remarkable ability to differentiate in multiple ways. MSCs can differentiate into several lineages and are important for physiological systems. In addition to their capacity for differentiation and self-renewal, mesenchymal stem cells (MSCs) emit a variety of substances that affect the immune system. It is thought that EV secretion may have many physiological effects on MSCs. The fact that EVs are involved in important processes and events, such as cancer, fibrosis, and inflammation, suggests that they may contribute to the onset of disease[25]. Additionally, it has been demonstrated that EVs produced from stem cells are helpful in a variety of therapeutic procedures. Exosomes derived from MSCs are small vesicles that play important roles in extracellular communication. The therapeutic use of exosomes has important advantages over that of MSCs: [25] (i) they can be stored at low temperatures (e.g. -80^0C) until required for cellular therapy; (ii) their contents such as cytokines, growth factors, transcription factors, and RNA are encapsulated, that is, covered by a lipid double membrane, which avoids some of the problems associated with rapidly degrading small soluble

molecules and provides protection against degradation in vivo; (iii) they are highly stable and long-lasting; (iv) they can be injected intravenously, reaching remote locations because the vesicles are small and circulate easily, whereas MSC are very large and therefore may have difficulty circulating through thin capillaries; (v) they can cross the blood-brain barrier; and (vi) they are hypoimmunogenic, with no risk of unwanted side effects such as rejection by the immune system.[26] Several studies have shown that the immunosuppressive, immunoregulatory and regenerative effects of exosomes produced by MSCs preconditioned with cytokines, hypoxia and chemicals are enhanced.[27,28] In addition, it has been reported that the amount of exosomes and the lipid and protein compositions of exosomes obtained from mesenchymal stem cells cultured in serum-deprived media change.[29]

Exosomes contain a lipid bilayer and protein content within the membrane. They transfer the proteins they contain to the host cell by fusion and demonstrate their effects.[25] Therefore, protein quantification should be performed by bursting the membranes. This is the reason why NTA analysis is often preferred for exosome characterization and quantification, but this analysis is expensive and not always accessible. Subedi et al. evaluated the effectiveness of different chemical treatments for detecting protein content using spectrometric (MS) analysis of a head-and-neck cancer cell line and concluded that RIPA was the best lysis buffer. The study noted that RIPA contains a combination of both ionic and non-ionic detergents, resulting in the highest number of EV peptides and proteins identified by MS-based identification.[22]

In this study, hWJ-MSC exosomes were isolated and characterized, and protein amounts were determined from these exosomes after treatment with different chemicals. The results showed that the RIPA buffer was approximately 3.3 times more effective than the other lysis solutions.

In conclusion, accurate and reliable protein quantification from exosomes derived from mesenchymal stem cells is important for understanding the biological properties of these nanovesicles and developing their potential therapeutic applications. Further studies should evaluate the efficacy of different chemicals in exosome analysis, and comparatively investigate the accuracy and sensitivity of the detection methods used.

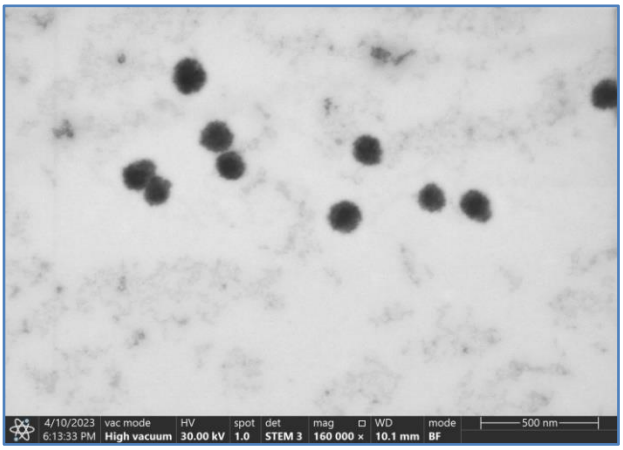

Figure 3. Electron microscopy analysis of exosomes. Exosome clusters are similar in size and spherical in shape.

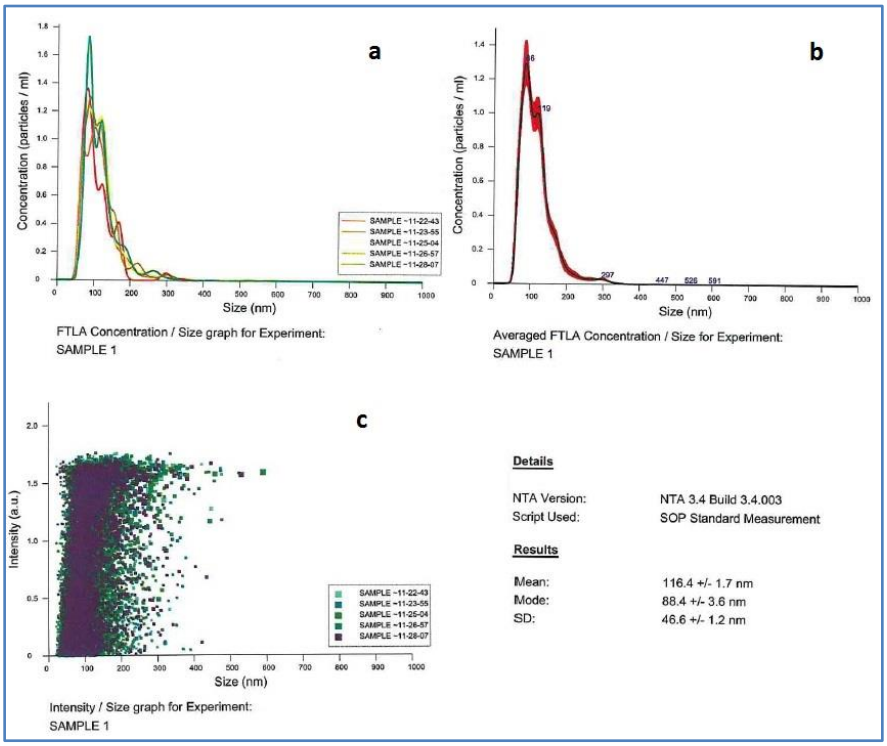

Figure 4. NTA analysis result. a. Graph showing the size and concentration per ml in five different readings of the exosome sample by the instrument. b. graph showing the average concentration. c. Dot plot of density and size of exosome particles.

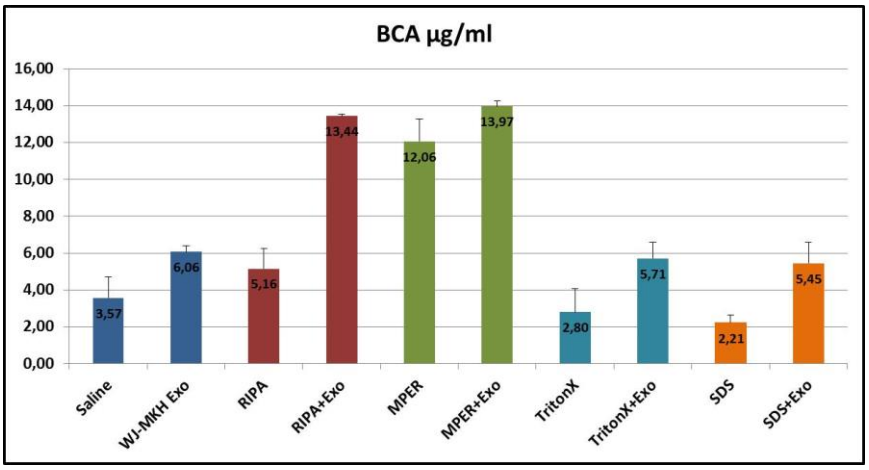

Figure 5. Protein quantification with micro BCA kit. Each lysis buffer was grouped into different colors.

Compliance with Ethical Standards

Exosomes obtained from STEMBIO Cord Blood, Cell, and Tissue Center were produced after obtaining production and release authorization for cells and cell-derived products issued by the Ministry of Health and patient consent. Ethical approval was not required for the experimental procedures.

Conflict of Interest

The author declares no conflicts of interest.

Author Contribution

ZSH: Conceived the ideas; ZSH, ZEUK, CDS, KCK, BA: Designed the experiments; ZSH, ZEUK, CDS, KCK, BA, YY: Performed the experiments, analyzed the data; ZSH, ZEUK, KCK: Wrote the manuscript. All authors have read and approved the final version for publication.

Financial Disclosure

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