

Development of a rinsing separation method for exosome isolation and comparison to conventional methods

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Abstract. – **OBJECTIVE:** Exosomes contain valuable biomarkers for many diseases. Tragically, standardized isolation methods and subsequent characterization criteria for exosomes remain limited. Therefore, we developed a new exosome isolation method, termed rinsing separation, and compared its advantages and weaknesses relative to the existing ultracentrifugation and ExoQuick precipitation methods.

MATERIALS AND METHODS: Rinsing separation utilizes heparin and glutaraldehyde as a fixative to isolate exosomes, and was developed using the culture supernatant from mesenchymal stem cells (MSCs). The isolated exosomes were characterized and compared by nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), and Western blot.

RESULTS: Consistent with known exosome parameters, exosomes isolated using each method ranged in size from 30 to 150 nm and demonstrated the characteristic cup-shaped morphology. Moreover, the exosome markers CD63 and TSG101 were observed in the lysate of all exosome samples that were isolated using each method. Several advantages and drawbacks were noted for each exosome isolation method. Most notably, ultracentrifugation resulted in fewer, but highly pure, exosomes, and samples generated using the ExoQuick precipitation method contained the most contaminating debris. Samples obtained using pour rinsing separation method represented an amalgam of these two fractions, but were isolated in significantly less time.

CONCLUSIONS: In this study, we propose rinsing separation as a new method of isolating exosomes. This method is convenient, and the resulting exosomes are highly pure. Moreover, rinsing separation offers time- and cost-efficiency advantages, making it a promising approach for exosome isolation for clinical applications.

Key Words

Mesenchymal stem cell, Exosome, Rinsing separation

Abbreviations

EV, extracellular vesicles; MSC, mesenchymal stem cell; NTA, nanoparticle tracking analysis; TEM, transmission electron microscopy; T2DM, type 2 diabetes mellitus.

Introduction

As a source of biomarkers, extracellular vesicles (EVs) play an important role in the diagnosis and prognosis of many diseases, including many cancers and cardiovascular diseases¹. EVs can be classified into exosomes and microvesicles based on several different properties. Exosomes range in size from 30 to 150 nm and are released extracellularly from a variety of cells, as well as from the fusion of multi-vesicular bodies²⁻⁴. Exosomes carry a wide variety of proteins and nucleic acids that circulate in bio-fluids or are transferred to cells and tissues to impart functions⁵. As such, exosomes can facilitate intercellular drug transfer, which contributes to the pharmacodynamics in neighboring cells. Therefore, the contents of exosomes are often regarded as ideal candidates for cancer biomarkers. For example, TRIM3 serves as a biomarker for gastric cancer diagnosis and the use of TRIM3 for diagnosis provides a new avenue for gastric cancer therapy⁶. Additionally, tumor-derived exosomes can carry lncRNAs that have been shown to affect tumor cell resistance⁷. Thus, many researchers have be-

gun to think of exosomes as a promising tool for studying cancer and other diseases. Beyond their role as a source of cancer biomarkers, exosomes from mesenchymal stem cells (MSCs) play an important role in maintaining cell homeostasis and responding to external stimuli⁸. This function is especially important when a tissue microenvironment is destroyed by disease or injury⁹. For instance, exosomes from MSCs have been shown to alleviate type 2 diabetes mellitus (T2DM) by reversing peripheral insulin resistance and relieving beta-cell destruction¹⁰. Importantly, compared with MSCs, secreted exosomes are smaller, less complex, easier to produce and store, and avoid some of the regulatory problems that arise from using MSCs for therapeutic interventions¹¹. Moreover, exosomes derived from MSCs have no cellular activity and are less immunogenic than MSCs due to a decreased membrane-bound protein content¹¹. Therefore, we selected MSCs as a source of exosomes in this study.

At present, no standardized purification method for the evaluation of the usability and purity of exosomes derived from cell culture conditioned media and biological fluids such as plasma exists¹². Many methods have been used to purify a subset of exosomes, such as differential ultracentrifugation, immunoaffinity isolation, size exclusion chromatography, gradient density centrifugation, different micro-fluidic techniques, polymeric precipitation, gel exclusion, membrane affinity, etc¹³⁻¹⁶. An estimated 56% of exosome researchers utilize ultracentrifugation, making differential ultracentrifugation the most common approach for exosome isolation¹⁷. Due to the heterogeneity and considerable overlap of EV size, two common drawbacks of ultracentrifugation include the damage caused to vesicles by repeated ultracentrifugation, which may reduce the quality of the exosomes, and the aggregation of soluble proteins, which may result in sample contamination¹⁸. Of note, exosomes derived using the ExoQuick precipitation technique contain a large amount of contaminating protein, including many macromolecular proteins that substantially impact subsequent proteomic analysis¹⁹.

To circumvent these issues, we have developed an approach to isolate exosomes, termed rinsing separation that accounts for protein aggregation during sample preparation and does not require ultracentrifugation. Here, we present our comprehensive comparison of the rinsing separation, differential ultracentrifugation, and ExoQuick precipitation methods using three

currently recognized approaches to characterize the exosomes isolated using these techniques, namely, nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), and Western blotting.

Materials and Methods

Materials

Glutaric dialdehyde solution (Pentane-1,5-dial) was manufactured by Sigma-Aldrich Inc. (St. Louis, MO, USA). Antibodies raised against human TSG101 and CD63 were obtained from Abcam (Cambridge, MA, USA). ExoQuick was provided by System Biosciences (Palo Alto, CA, USA). Sprague-Dawley (SD) Rat MSC Osteogenic Differentiation Basal Medium and SD Rat Bone Marrow MSC Adipogenic Differentiation Basal Medium were manufactured by Cyagen (Guangzhou, China).

Animals

All animals were raised and treated in accordance with the Shanghai Animal Center for the Ethical treatment of animals. The animal experiments in this study were approved by the Ethical Committee Guidelines (No. 20140266-095) from Institutional Animal Care and Use Committee of Shanghai Medical College Fudan University (Shanghai, China). Adult male SD rats, which weighted 150-200 g, were obtained from the Shanghai Laboratory Animal Monitoring Institute (Shanghai, China). During this study, the animals had access to standard laboratory diet and drinking water *ad libitum*.

Purification and Identification of MSCs

MSCs used in this study were isolated from SD rats. After rats were anesthetized with pentobarbital (40 mg/kg, intravenous), femurs and tibiae were taken, and bone marrow cells were flushed out into culture flasks with MSC culture medium (Stem Cell Technologies Inc., Vancouver, BC, Canada). The bone marrow cells were then cultured in MSC medium supplemented with 10% fetal bovine serum (FBS) (Stem Cell Technologies Inc., Vancouver, BC, Canada) in an incubator with an atmosphere of 5% CO₂ at 37°C. The culture medium was replaced every 3 days, and nonadherent hematopoietic cells were removed as needed. SD Rat MSC Osteogenic Differentiation Basal Medium and SD Rat Bone Marrow MSC Adipogenic Differentiation Basal Medium were used to promote the differentiation

of MSCs. Osteogenic and adipogenic induction was carried out and confirmed by Alizarin Red and Oil Red O staining, respectively, in accordance with previous protocols^{20,21}. MSCs at passage three were used for further experiments.

Flow Cytometry

Isolated single-cell suspensions were incubated with anti-rat CD45, CD80, CD73 or CD90 antibodies for 30 min at 4°C. After washing, the cells were then processed with a fixation/permeabilization kit (eBiosciences, San Diego, CA, USA) and stained with Foxp3-antibody. The cells were then observed using a Flow cytometer (Thermo Fisher, Waltham, MA, USA). The results were analyzed using Flowjo software.

Exosome Isolation

Cell culture supernatant was collected from MSCs by gently rocking culture dishes to suspend exosome microcapsules in media and the culture solution was then transferred to a centrifuge tube for subsequent exosome isolation.

Rinsing separation

The collected cell culture supernatant was rinsed 1:1 with PBS containing 2/10 sodium heparin (12500 U). The mixture was vortexed for 10–20 s in a 50 Hz vortex mixer. Samples were then centrifuged at 800 rcf (\times g) for 5 min at 4°C or room temperature. After centrifugation, the supernatant was transferred to a new centrifuge tube, and 100 μ l of fixative (2.5% glutaraldehyde) was added, and the samples were then vortexed for 20 s with a 50 Hz vortex mixer. Samples were then centrifuged at 8,000 rcf (g) for 10 min. If no white precipitate was evident, the samples were centrifuged again at 12,000 rcf (g) for 10 min. After centrifugation, the supernatant was discarded and 1 ml of PBS and 200 μ l of fixative (2.5% glutaraldehyde) were added. Samples were then mixed by shaking for 10 s, ensuring that the final concentration of the fixative solution was not greater than 0.5%. Samples were, then, fixed at 4°C for 30 min. Once fixation was complete, the samples were centrifuged at 2,000 or 2,500 rcf (g) for 10 min at room temperature. After centrifugation, 1 ml PBS was added and the samples were mixed by shaking for 10 s. The samples were, then, ready to be send for analysis. Samples were centrifuged again at 2,000 or 2,500 rcf (\times g) for 10 min. The supernatant was discarded and 200 μ l of water were, then, added. The samples in centrifuge tubes were then placed in a tube rack with an appropriate amount of ice in

an icebox. The centrifuge tube wall never directly touched the ice.

Ultracentrifugation

The collected cell culture supernatant was split into centrifuge tubes and subjected to gradient centrifugation at 4°C. Samples were centrifuged for 15 min at 300 g, and the supernatant was collected and then additional samples were centrifuged at 2,000 g for another 15 min. Next, the supernatant was collected again and the remaining samples were centrifuged at 10,000 g for 40 min. The supernatant from each sample was filtered through a 0.22- μ m filter to remove cells and cell debris. The supernatant from each sample was then centrifuged at 110,000 g for 75 min. 1.5 ml of the original solution remained. PBS was added to re-suspend and equilibrated the tubes. These samples were, then, centrifuged again at 110,000 g for another 75 min. Finally, the supernatant was discarded completely, and the pellet was re-suspended in 50 μ l of PBS.

ExoQuick Precipitation

The collected cell culture supernatant was centrifuged at 3,000 g for 15 min to remove cells and cell debris. Next, the supernatant was transferred to a sterile tube, and an appropriate volume of ExoQuick was added to the bio-fluid. For the best recovery of both RNA and protein, 1 ml of ExoQuick was added to 5 ml of tissue culture media, as recommended by the manufacturer. The ExoQuick and cell culture supernatant were mixed well by inverting or flicking the tubes. The samples were then refrigerated upright overnight (at least 12 hours) at 4°C without rotation. The next day, the mixture was centrifuged at 1,500 g for 30 min. Centrifugation was performed at either room temperature or 4°C with similar results. After centrifugation, exosomes appeared as a beige or white pellet at the bottom of the tube. The supernatant was aspirated, and the residual ExoQuick solution was spun down by centrifugation at 1,500 g for 5 min. All traces of fluid were removed by aspiration, and the precipitated exosomes in the pellet were not disturbed. The exosome pellet was finally re-suspended in 50 μ l of sterile PBS.

Characterization of Exosomes Transmission Electron Microscopy

For TEM morphologic observations, 5 μ l of an exosome suspension was absorbed onto formvar carbon-coated electron microscopy

grids. Two or three grids were prepared for each exosome sample. The membranes were allowed to absorb for 5 min at room temperature. An absorbent paper was used to gently remove excess liquid. The grids were then positioned on a paper with the coated side up and allowed to air dry for an additional minute. Then, the exosome suspension was subjected to 2.5% uranyl acetate staining for 8 min. The grids were washed one to three times with PBS and were maintained in a semi-dry state or stored in grid boxes for future examination by TEM (JEOL USA, Peabody, MA, USA).

Nanoparticle Tracking Analysis

The exosomes were diluted (1:10) in PBS for NTA using a NanoSight LM20 instrument (NanoSight, Malvern Panalytical Ltd, Malvern, UK). The Brownian motion of each particle was tracked between frames, and the size was calculated with the Stokes-Einstein equation.

Western Blot

Exosome samples were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors. The protein concentration was measured using a bicinchoninic acid (BCA) assay (Pierce, Waltham, MA, USA). Equal volumes of lysates (50 μ l) were subjected to SDS-PAGE using 12% sodium dodecyl sulfate-polyacrylamide gels and then transferred to a polyvinylidene difluoride (PVDF) membranes (MDI Membrane Technologies, Harrisburg, PA, USA) using a wet transfer system (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked in 5% bovine serum albumin (BSA) in TBST and immunoblotted by incubation in CD63 primary antibody (1:5,000; Abcam, Cambridge, MA, USA) or TSG101 primary antibody (1:500; Abcam, Cambridge, MA, USA) overnight at 4°C. The blots were then washed several times in TBST, incubated with secondary antibody, and developed with an ECL imager (Invitrogen, Carlsbad, CA, USA).

Statistical Analysis

Data are expressed as the mean \pm standard error of mean (SEM). Differences were compared by one-way analysis of variance (ANOVA) or Student's *t*-test, as appropriate. A value of $p < 0.05$ was considered statistically significant. SPSS 13.0 (SPSS Inc. Chicago, IL, USA) was used to analyze the data.

Results

To assess if our new method of isolating exosomes was superior to ultracentrifugation or the ExoQuick isolation method, the profiles of exosomes derived from MSCs were assessed using three different methods. The identification of exosomes focused on commonly accepted criteria, including size ranging between 30 and 150 nm, spherical morphology observed by TEM, and exosome markers as revealed by Western blot analysis.

Characterization and Differentiation Potential of MSCs

Prior to isolating exosomes, MSCs were isolated from the bone marrow tissue of adult rats. These MSCs were characterized by flow cytometry for surface markers. Specifically, the isolated bone marrow MSCs were positive for CD73 and CD90 and negative for CD80 and CD45, as expected (Figure 1A). The differentiation potential of MSCs was also evaluated by Alizarin Red and Oil Red O staining, markers of osteogenic and adipogenic differentiation, respectively (Figure 1B and 1C).

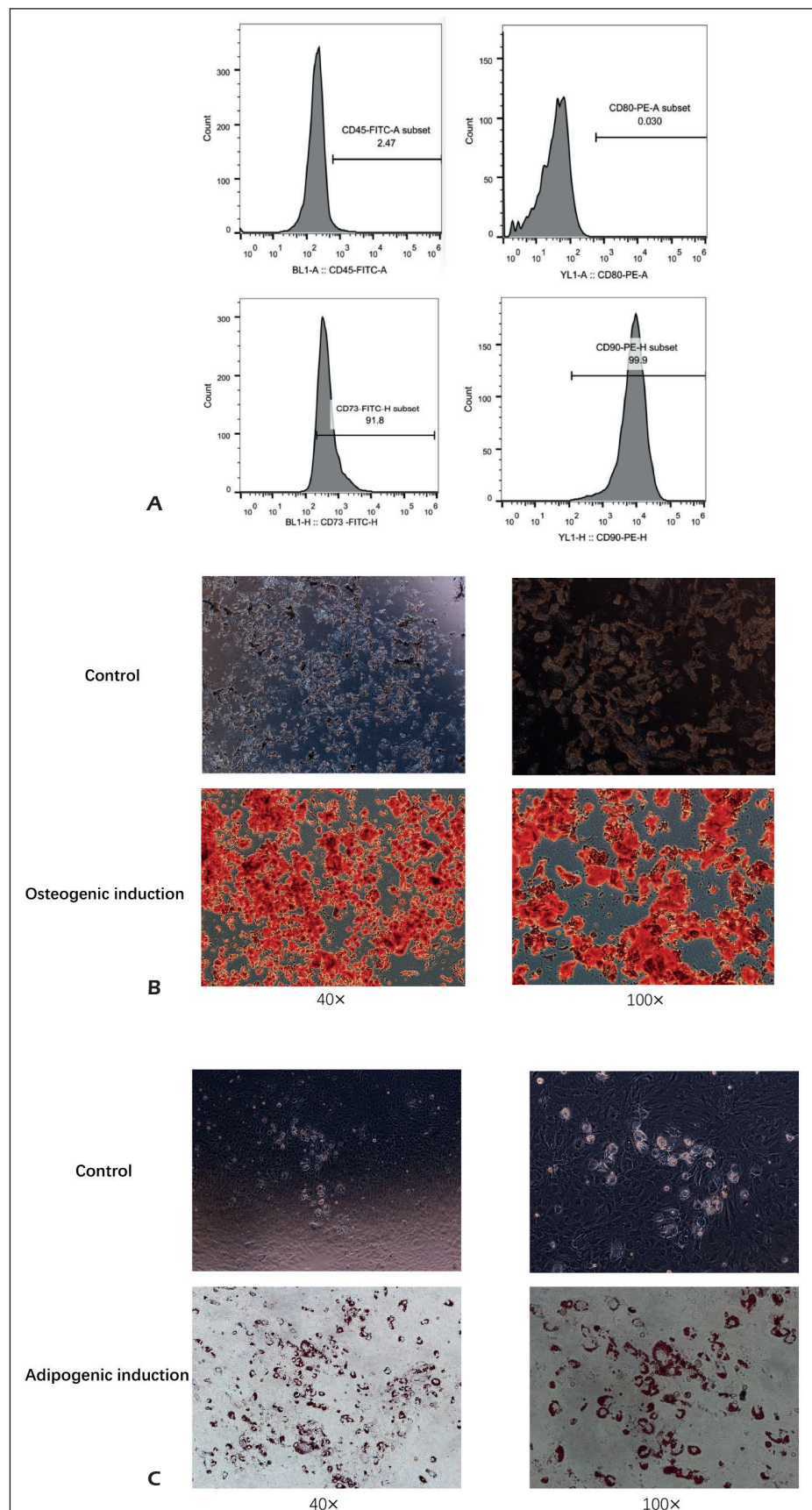
Morphometric Analyses of Exosomes by TEM

TEM analysis confirmed that all of the three methods used in this study successfully isolated exosomes within the expected size range and with general exosome-like morphology, consistent with the previous studies²². Cup-shaped vesicles were observed, along with smaller micro-vesicles and aggregated structures in all cases (Figure 2). Interestingly, the number of exosomes present in the rinsing separation samples was higher than the other methods. As shown in Figure 2, the ExoQuick method appeared to have higher levels of contaminants compared to other methods.

Comparative Evaluation of Exosome Size and Number by NTA

Next, the particle size of the exosomes isolated using the three methods was assessed by NTA. Consistent with our TEM observations, the size of all isolated exosomes was within the appropriate size range of 30 to 150 nm (Figure 3A-3D). A statistically significant increase in the concentration of exosomes was measured using the rinsing separation method in comparison with other methods. Specifically, the concentrations of exosomes isolated by ultracentrifugation, rinsing separation,

Figure 1. Characterization of MSCs. **A**, Flow cytometry for the MSC positive markers CD73 and CD90, and MSC negative markers CD80 and CD45 relative to Fox3p. **B**, Osteogenic differentiation potential was determined by Alizarin S staining of the extracellular mineralized matrix. **C**, Adipogenic differentiation potential was determined by Oil Red O staining of lipid droplets. (Left) Images taken at 40× magnification. (Right) Images taken at 100× magnification. Abbreviation: MSC, mesenchymal stem cell.



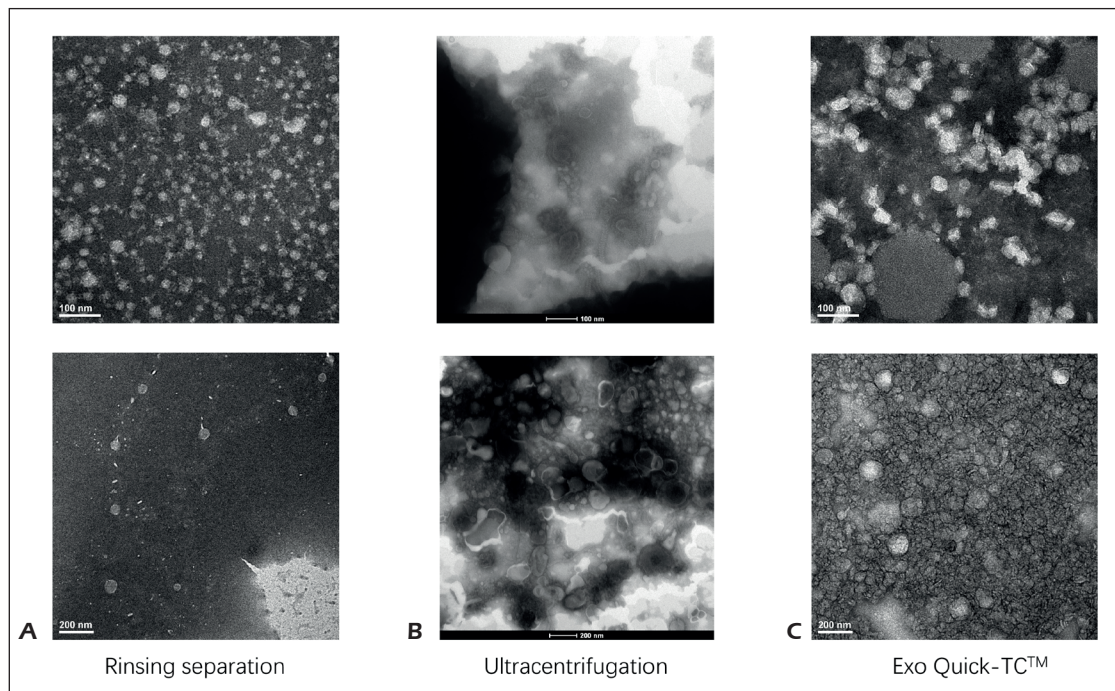


Figure 2. TEM analysis of exosome samples. Representative TEM images of exosomes isolated by (A) rinsing separation, (B) ultracentrifugation, and (C) ExoQuick precipitation.

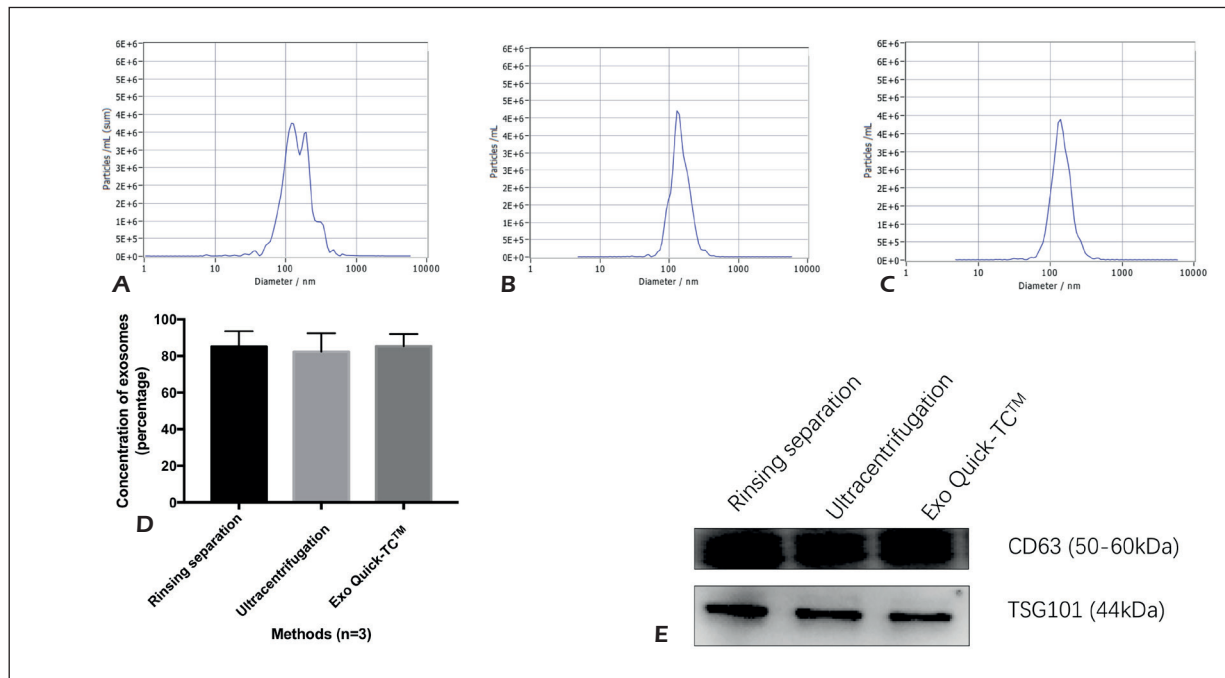


Figure 3. Size distribution and concentration of MSC-derived exosomes and exosome marker profiling by Western blotting. As measured by NTA, the particle sizes of exosomes isolated by (A) rinsing separation, (B) ultracentrifugation, and (C) ExoQuick precipitation were within the 30 to 150 nm size range. (D) Concentrations of exosomes isolated with the three methods. (E) Protein expression of CD63 and TSG101 in exosomes isolated with each method (50 μ l of total protein per sample). Results are depicted as the mean \pm standard error of the mean and represent three independent experiments. * = significant with p-value of less than 0.05.

and ExoQuick precipitation were 97.6%, 98.6%, and 97.7%, respectively. Additionally, the three isolation methods resulted in differing size distribution profiles. In comparative terms, the size distribution curves obtained with ultracentrifugation and ExoQuick were similar. In all three cases, the size distribution curves confirmed that vesicles could reach 150 nm in size. Rinsing separation exhibited nearly the same high size range compared to ultracentrifugation and ExoQuick precipitation, demonstrating that this method performs similarly to these two other common exosome isolation protocols.

Confirmation of Exosomes by Western Blot

The exosomes derived from MSCs were then assessed by Western blot using antibodies specific to exosome surface markers. The protein concentration of exosomes isolated using rinsing separation was higher than those isolated using ultracentrifugation or ExoQuick precipitation. Equal volumes of lysate from eluted exosomes were resolved by Western blotting to observe the variation in total yield. CD63, a commonly used specific protein marker, and TSG101, were observed in the lysate of all exosomes (Figure 3E). Finally, densitometric ratios were calculated and the ratio was higher for the rinsing separation method than for ultracentrifugation and ExoQuick precipitation.

Discussion

In this study, we compared rinsing separation, our newly developed method for isolating exosomes, with the common ultracentrifugation and ExoQuick precipitation methods for exosome isolation (Figure 4). As a result of these comparisons, we determined that rinsing separation had several advantages over these two conventional methods. We found that rinsing separation was particularly useful for protein analyses due to the reduced amount of non-exosome protein contamination. Additionally, rinsing separation was more time-efficient and cost-efficient compared to ultracentrifugation of ExoQuick precipitation, respectively.

In general, three different approaches, NTA, TEM, and Western blot analysis, were used to characterize the presence of exosomes. From our Western blot results, the tetraspanins CD9 (trans-

membrane protein) and CD63 (transmembrane protein), as well as TSG101 (cytosolic protein), were substantially enriched in exosomes isolated by rinsing separation. Tang et al²³ showed that CD9 was only found in exosomes isolated by ultracentrifugation, but CD63 and TSG101 were present in all exosome samples. The molecular signatures of exosomes remain unclear, and the reduced sensitivity of Western blotting relative to mass spec may be contribute to the differences in our results relative to Tauro et al²⁴.

NTA can be used to calculate the size and total concentration of vesicles, but it cannot adequately differentiate between synthetic nanoparticles and biological vesicles²⁵. Our NTA results revealed that the particle numbers obtained from ultracentrifugation-derived exosomes were lower than the number obtained using either of the two other methods. Two possible explanations for this discrepancy could be: a) biological vesicles were damaged by ultracentrifugation, resulting in lower particle recovery, or b) the higher particle recovery of the ExoQuick method was due to polymeric precipitation. As mentioned above, the higher particle recovery of the ExoQuick method could also be due to the presence of protein aggregate particles. Neither the ultracentrifugation nor the ExoQuick precipitation methods include a step to separate exosomes and high-density protein aggregates. In the rinsing separation protocol, the purpose of the initial centrifugation step (800 rpm, 5 min) was to remove impurities in the shortest possible time and to preserve the integrity of any exosomes present. Extended centrifugation times should affect the autolysis of exosomes. After the initial brief centrifugation, the purpose of transferring the supernatant to a new centrifuge tube is to remove any precipitated impurities, which should also reduce the contamination of exosomes during the TEM preparation process. Rinsing separation also accounts for any potential additional damage to the isolated exosomes by using PBS for resuspension following fixation. Another benefit of this step is that exosome samples can then be prepared in pure water for TEM observation.

Our TEM observations revealed that each of the three-exosome isolation methods successfully resulted in cup-shaped exosomes. However, the exosome samples isolated by the ExoQuick precipitation method contained more contamination than the other two methods. The contaminants consisted of differing degrees of protein aggregates. This adherent protein debris was



Figure 4. Comparison of exosome isolation methods. Exosome isolation using the newly developed rinsing separation (left) and other methods: ultracentrifugation (center) and ExoQuick (right).

derived from cell secretions during the culture process and from the original serum proteins in the culture medium. To allow exosomes to form independent units, exosomes must be rinsed. If exosomes are rinsed with PBS alone, it is difficult to dissolve proteins in a short time, resulting in the observed adhesion of exosomes and other proteins in TEM images. Due to the consistency of the pulp, most proteins can be combined with heparin sodium²⁶. Therefore, we diluted heparin sodium in PBS to prevent coagulation of proteins during the rinsing process. Additionally, we used a 50 Hz vortex mixer to vortex rinse samples and successfully separate exosomes into independent units. As a result, exosomes obtained by our rinsing separation methodology appeared to be clearer, with fewer adherent proteins being co-isolated.

Rinsing separation includes an additional innovative step that does not exist in the ultracentrifugation or ExoQuick precipitation protocols. Exosome samples are pre-fixed with 2.5% glutaraldehyde to preserve the tissue structure and cell morphology by coagulating proteins and other components. The benefits of fixation include precipitating or immobilizing proteins and antigens in cells, maintaining the original position of proteins in the cell, terminating or reducing the reaction of exogenous and endogenous degrading enzymes, preventing tissue autolysis during prolonged *in vitro* time, and reducing cell solubility, dispersion, destruction, and loss of proteins, fats, and carbohydrates. At the same time, the tissue can be hardened for subsequent embedding, sectioning, and dyeing. Due to tissue shrinkage as a result of fixation, we determined the optimal final concentration of a glutaraldehyde fixative solution to be 0.5% of the total sample volume for a 30 min incubation time. In terms of time-efficiency, rinsing separation took approximately 90 minutes, while the ultracentrifugation method required at least 150 minutes, and ExoQuick precipitation was completed in approximately 30 minutes only after an overnight incubation. Therefore, at an operational level, ultracentrifugation not only took the most time but also was more complicated compared to the other methods. Although ultracentrifugation provides the highest purity protein, it is not usually applicable in clinical samples due to the amount of material and time required. ExoQuick precipitation was the most expensive method evaluated, due to specialized reagents for processing.

Conclusions

Although exosomes possess diagnostic and therapeutic potential, a common challenge in their use is the method used for exosome isolation. In this study, we proposed rinsing separation to isolate exosomes and compared this strategy with two other isolation strategies, ultracentrifugation and ExoQuick precipitation. The repeated centrifugation steps in ultracentrifugation damaged exosomes, and exosomes from ExoQuick precipitation contained large amounts of protein, which may impact subsequent proteomic analyses. In contrast, rinsing separation was more convenient than the other methods and resulted in highly pure exosome samples. Each exosome isolation method had its own advantages, thus, it is important to select the isolation approach that is the most suitable for each type of subsequent analysis. A major limitation of this study is that the exosomes used were isolated from only one cell type. Although each step was optimized, we cannot rule out the possibility that other cell types may require modified parameters to successfully isolate highly pure exosomes. Therefore, rinsing separation should be optimized for use in a variety of cells types and states in future studies.

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Conflict of Interests

The authors declare that they have no conflict of interests for disclosure.

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