Original Article

Isolation of exosomes from serum of patients with lung cancer: a comparison of the ultra-high speed centrifugation and precipitation methods

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Background: Extracellular vesicles (EVs) is a group of heterogeneous cell-derived membrane structures, which is composed of a large number of exosomes released by cells, microbubbles (MVs) and apoptotic bodies. The formation of exocrine body is a process of fine regulation, which includes four stages: initiation, endocytosis, polycystic body formation and exocrine body secretion. Ultracentrifugation is currently the gold standard for external body separation; it includes a series of centrifugation steps at a rotation speed of 100,000 rpm or more to purify exocrine bodies from protein contaminants. Thus far, some *in vitro* separation methods, such as ultracentrifugation, polymer-based exosome separation kits and immune affinity-based isolation using antibodies against exosome surface proteins, have been used for tumor exosome isolation. It is not very clear which method is more suitable for the separation of serum exosomes from lung cancer patients.

Methods: Two methods for the extraction of exosomes from serum samples of lung cancer patients, namely, ultra-high speed centrifugation (Ultra-Exo) and precipitation (Prekit-Exo), were analyzed and compared. The biological morphologies of the extracted exosomes were studied by negative staining matter with transmission electron microscopy and cryo-electron microscopy. The particle size and the distribution were detected using nanoparticle tracking analysis (NTA).

Results: Bio-transmission electron microscopy revealed that the morphologies of exosomes extracted by ultracentrifugation were superior to exosomes extracted with the Prekit-Exo kit. Ultracentrifugation was able to extract more exosomes compared to the Prekit-Exo kit. NTA showed that the exosomes obtained by ultra-high speed centrifugation had a smaller particle size compared to exosomes obtained by precipitation (30.4±26.8 *vs.* 150.3±6.8 nm, respectively). It is possible that the precipitant used in the precipitation kit was extracted with the exosomes, thereby causing the particle size to increase. Notably, the particle size of the exosomes extracted by the precipitation kit method showed a relatively narrow range in size. This could be due to the coating effect of the precipitation reagent, reducing the difference in the particle size of the exosomes.

Conclusions: Exosomes collected from the serum of lung cancer patients using the two extraction methods differed in morphology and numbers, with the ultracentrifugation method being superior to the precipitation method.

Keywords: Exosome; ultracentrifugation; kit; precipitation; cryo-electron microscopy

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Introduction

Exosomes are nano capsules secreted by cells through endogenous pathways. They have a cup-shaped or concave hemispherical structure, with a particle size distribution of 30–200 nm (1). Exosomes can be found in a range of bodily fluids including blood, urine, ascites, saliva, amniotic fluid, milk, semen, and cerebrospinal fluid. These extracellular vesicles (EVs) contain a variety of lipids, proteins, and nuclei acids, which act as intracellular biological signals to adjacent or distal cells for uptake by target cells (2). Therefore, exosomes are widely involved in physiological and pathological processes and play an important role in cell communication. Furthermore, exosomes have been recognized as the cause of many diseases and as such, may act as potential biomarkers or therapeutic targets (3).

The classical classification of EVs includes apoptotic bodies, microvesicles, and exosomes (4,5), all of which act via different mechanisms, have different particle sizes, and varying content. Apoptotic bodies and microvesicles are formed by direct budding of cells, but apoptotic bodies can only bud from apoptotic cells. However, complicated endogenous pathways are involved in the formation of exosomes (6-8). First, the cell membrane invaginates to form early endosomes. The membrane of the early endosome, forming intracavitary vesicles in the early endosome, and then matured into the late endosome. In the late stage, the internal body membrane continues to invaginate and the vesicles in the cavity continue to accumulate, thus forming multi-vesicular bodies (MVBs). The multi-vesicular membrane fuses with the cell membrane, and the inner vesicle, that is, the exosome, is then expelled. Despite the different properties of the exosomes at each stage of formation, they cannot be effectively separated due to technical limitations. In fact, the lack of an efficient isolation method is currently a major obstacles in the study of exosomes. This present investigation compared two methods of extracting exosomes from the serum of lung cancer patients, namely, the precipitation kit method and the classical ultra-high speed centrifugation method. Identification and optimization of an efficient extraction method is crucial for future research regarding exosomes.

We present the following article in accordance with the MADR reporting checklist (available at http://dx.doi.org/10.21037/atm-21-2075).

Methods

Sample preparation

Serum samples were collected from a patient with primary lung cancer who received surgical treatment and was confirmed by pathological examination in Renji Hospital in December 2018. The patient voluntarily participated in this research project and signed an informed consent form (approved by Renji Hospital Ethics Committees: 2018-160). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Peripheral venous blood (15 mL) was collected from the patient in a serum separation test tube (without heparin). The sample was kept at room temperature for 30 minutes prior to centrifugation at 2,000 g for 15 minutes at 4 ℃. The upper pale-yellow layer of serum was collected and divided into 2 centrifuge tubes each with 3 to 5 mL serum. Samples were kept on ice as much as possible and stored at –80 ℃. The above process should not exceed 6 hours.

Instruments and reagents

The Avanti JXN-30/26 Intelligent High-Efficiency Centrifuge and the associated ultracentrifuge tube, Prescot-Exokit, were purchased from a company (Umibio Science and Technology Group, Shanghai, China). The following equipment was used: a biological transmission electron microscope (Tecnai G2 Spirit Biotin/*Tecnai G2 Spirit Biotin), a FEI Talos F200C 200 kV electron microscope, a carbon support membrane for the Mesoscope, and a Malvern NanoSight NS300.

Extraction of exosomes from serum by ultra-high speed centrifugation

Serum samples were centrifuged at 2,000 g for 5 minutes

at 4 ℃. The supernatant was transferred to a new tube and centrifuged at 3,500 g for 15–20 minutes at 4 ℃. The supernatant was again transferred to a new tube and centrifuged at 10,000 g for 30–40 minutes at 4 ℃. The supernatant was then transferred to an ultra-high speed centrifuge tube and centrifuged at 120,000 g for 140 minutes at 4 ℃ in an ultra-high speed centrifuge. The supernatant was discarded and the precipitate was resuspended in 1× phosphate buffered saline (PBS) and passed through a 0.22 μm sterile filter. The supernatant was again collected and centrifuged at 120,000 g for 70 minutes at 4 ℃. The supernatant was then carefully discarded. The small amount of liquid at the bottom of the centrifuge tube containing the serum exosomes was resuspended in 100 mL 1× PBS solution and stored at 4 or –80 ℃.

Extraction of exosomes from serum by the precipitation method

For the extraction of serum exosomes via precipitation, the Prekit-Exo kit ((Umibio Science and Technology Group, Shanghai, China) was used according to the manufacturer's instructions. Serum samples were centrifuged at 3,000 g for 15–20 minutes at 4 ℃. The supernatant was collected and filtered through a 0.45 μm filter. The filtered supernatant was then incubated with the reagent in the PreKit-Exo kit ($V_{sample\ liquid}$: V_{kit} =1/4) at 4 ℃ overnight. Samples were then centrifuged at 1,500 g for 30–40 minutes at 4 ℃. The resultant supernatant was discarded and the precipitate was centrifuged again at 1,500 g for 5–10 minutes at 4 ℃ to fully remove the supernatant. The remaining precipitate containing the exosomes was washed with 100 mL 1× PBS and centrifuged for at 1,500 g for 5 minutes. The exosome pellet was then resuspended in PBS and stored at 4 or –80 ℃.

Biological transmission electron microscopy of serum exosomes

The exosome suspension (10 mL) was transferred as a droplet into the copper mesh grid of the electron microscope. The drop was left on the copper mesh for more than 3 minutes. After negative staining with 2% uranium diacetate solution for 1 minute, the excess solution was dabbed with a piece of filter paper and the samples were air dried at room temperature. The copper mesh samples were observed and photographed under a 120 kv biological transmission electron microscope.

Cryo-electron microscopy and image processing

The cryo-electron microscopy was performed in the Center of Cryo-Electron Microscopy Zhejiang University. For each glow discharge porous carbon grid (Quantifile CuR 1.2/1.3), a 3.5 μL sample with a particle concentration of about 1 μM was used. The mesh was then sucked dry with Whatman 55 mm filter paper for 1.5 seconds, quickly frozen in liquid nitrogen, and cooled in FEI Vitrobot Mark IV. The grid was transferred to an FEI Talos electron microscope running at an accelerating voltage of 200 kV and equipped with a Gatan K2 Summit direct electronic counting camera. The micrograph was recorded in super-resolution mode using the semi-automatic low-dose acquisition program UCSF-image 4, with a nominal magnification of 22,500×, which is equivalent to the pixel size of 1.31 Å at the sample level. The total exposure time of each image is 8 seconds, which is divided into 32 sub-frames.

Analysis of particle size distribution and concentration of exosomes using the nanolayers particle size tracking meter

Serum exosome samples (10 mL) were dilute to 1,000 mL with deionized water. Samples were loaded with a 1 mL syringe and the concentration and size distribution of exosomes were measure using the nanolaser particle size tracking meter.

Statistical analysis

SPSS 24.0 software was used for statistical analysis. The experimental data were expressed as mean ± standard deviation ($\bar{x}$ ± s). The independent sample *t*-test and one-way analysis of variance (ANOVA) were applied, and the homogeneity of variance test was performed at the same time. When the variance was homogeneous, the LSD statistical method was used for comparisons between groups. $P<0.05$ indicated a significant difference.

Results

Transmission electron microscopy and cryo-electron microscopy

Biotransmission electron microscopy was used to detect the morphology of exosomes extracted from the serum of lung cancer patients. Exosomes obtained by ultra-

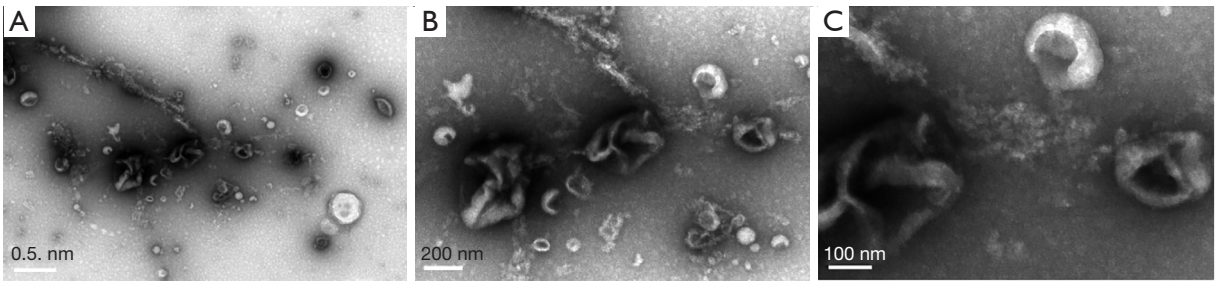

Figure 1 Transmission electron micrographs of serum exosomes extracted by ultra-high speed centrifugation. Scale bars: (A) 0.5 μm, (B) 200 nm, and (C) 100 nm.

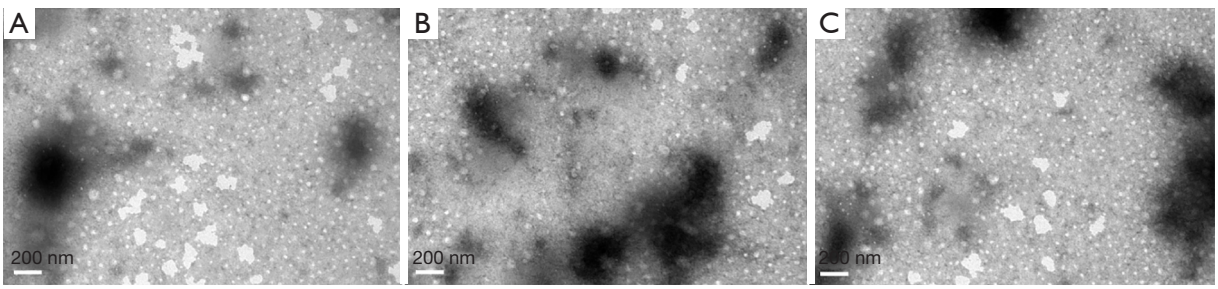

Figure 2 Transmission electron micrographs of serum exosomes extracted using the precipitation kit method under 3 different fields of vision. (A), (B), and (C), (scale bar =200 nm).

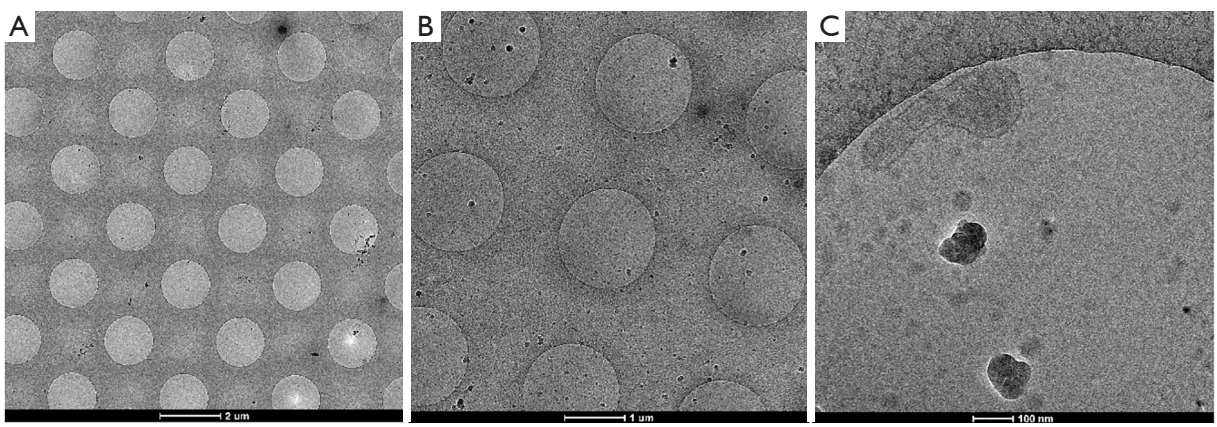

Figure 3 Cryo-electron microscopy of serum exosomes extracted by ultra-high speed centrifugation. Scale bars: (A) 2 μm, (B) 1 μm, and (C) 100 nm.

high speed centrifugation showed obvious cup-shaped or concave hemispherical structure, and some were clustered together (*Figure 1*). However, the exosomes extracted by the precipitation kit method did not show an obvious structure and impurities could be observed (*Figure 2*).

Cryo-electron microscopy showed that exosomes obtained by ultra-high speed centrifugation had obvious circular morphology or Whistle shape structure, as showed in *Figure 3*.

Nanoparticle tracking analysis (NTA)

The particle size distribution and concentration of exosomes were analyzed by using a nanolaser particle size

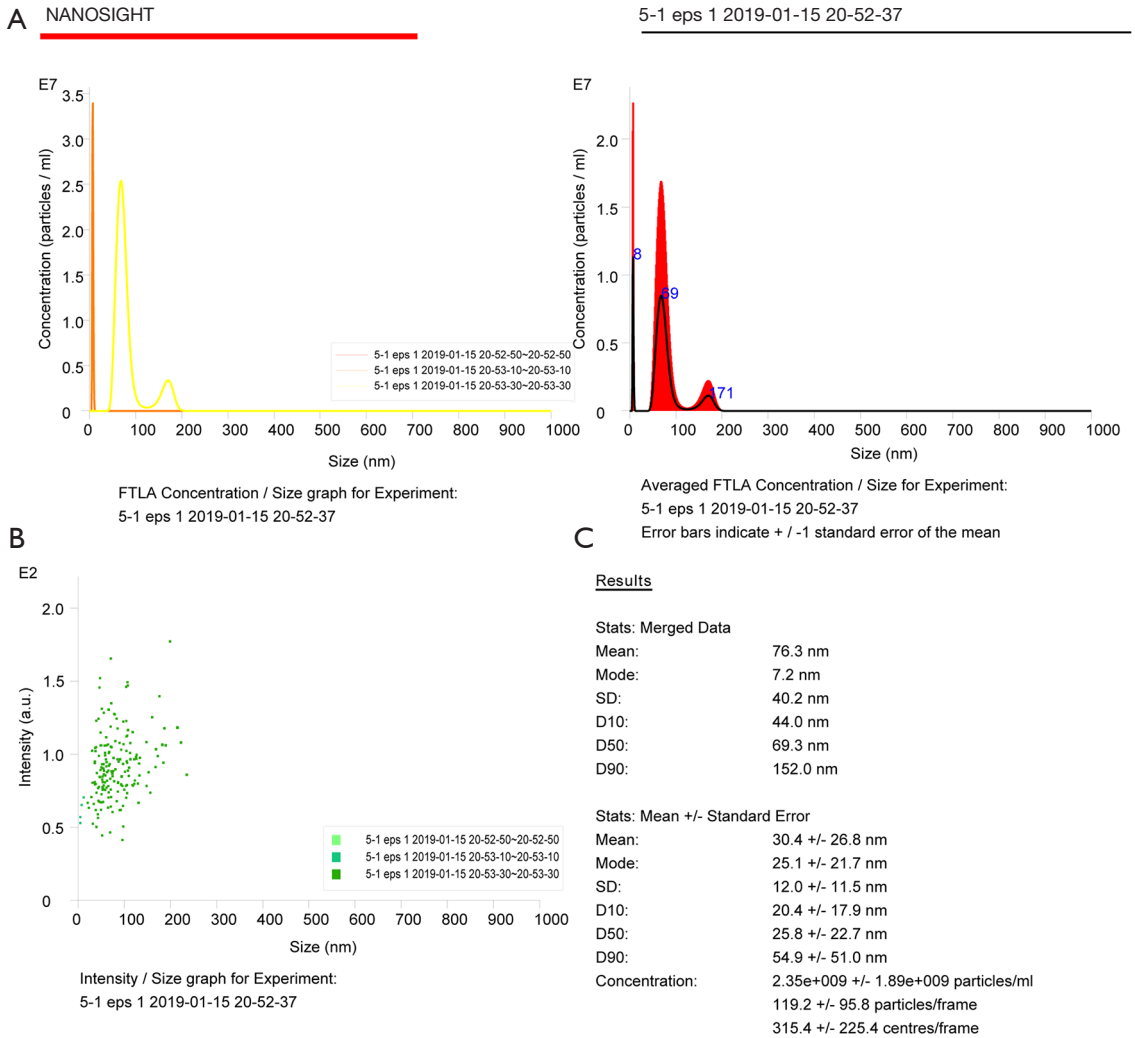

Figure 4 Nanoparticle tracking analysis (NTA) of the particle size of serum-derived exosomes extracted by ultra-high speed centrifugation. (A) FTLA (left) or averaged FTLA (right) concentration/Size graph. (B) Intensity/size graph. (C) The results of merged data and mean ± standard error. FTLA (particles/frame against size distribution), Finite Track Length Analysis (FTLA) was used for size determination.

tracking meter. Take the sample 3 times at a time.

The average diameter of exosomes obtained by ultra-high speed centrifugation was smaller (30.4±26.8 nm) (*Figure 4*) than that of exosomes extracted using the precipitation kit method (150.3±6.8 nm) (*Figure 5*). It is possible that the precipitant in the precipitation kit was extracted together with the exosomes to cause the increased particle size. Furthermore, the particle size of exosomes extracted by the precipitation kit method was within a relatively narrow range (*Figure 5*), which may be due to the coating effect of the precipitation reagent covering up the differences in the particle sizes of the exosomes themselves.

Discussion

First reported in 1981, exosomes are vesicles produced from cells that can be found in body fluids or cell supernatants (9-12). In recent years, there has been much interest in exosomes due to its role in cell protection, intercellular communication, and disease diagnosis and treatment (13). However, the efficient isolation and purification of exosomes from various sources, especially from the serum,

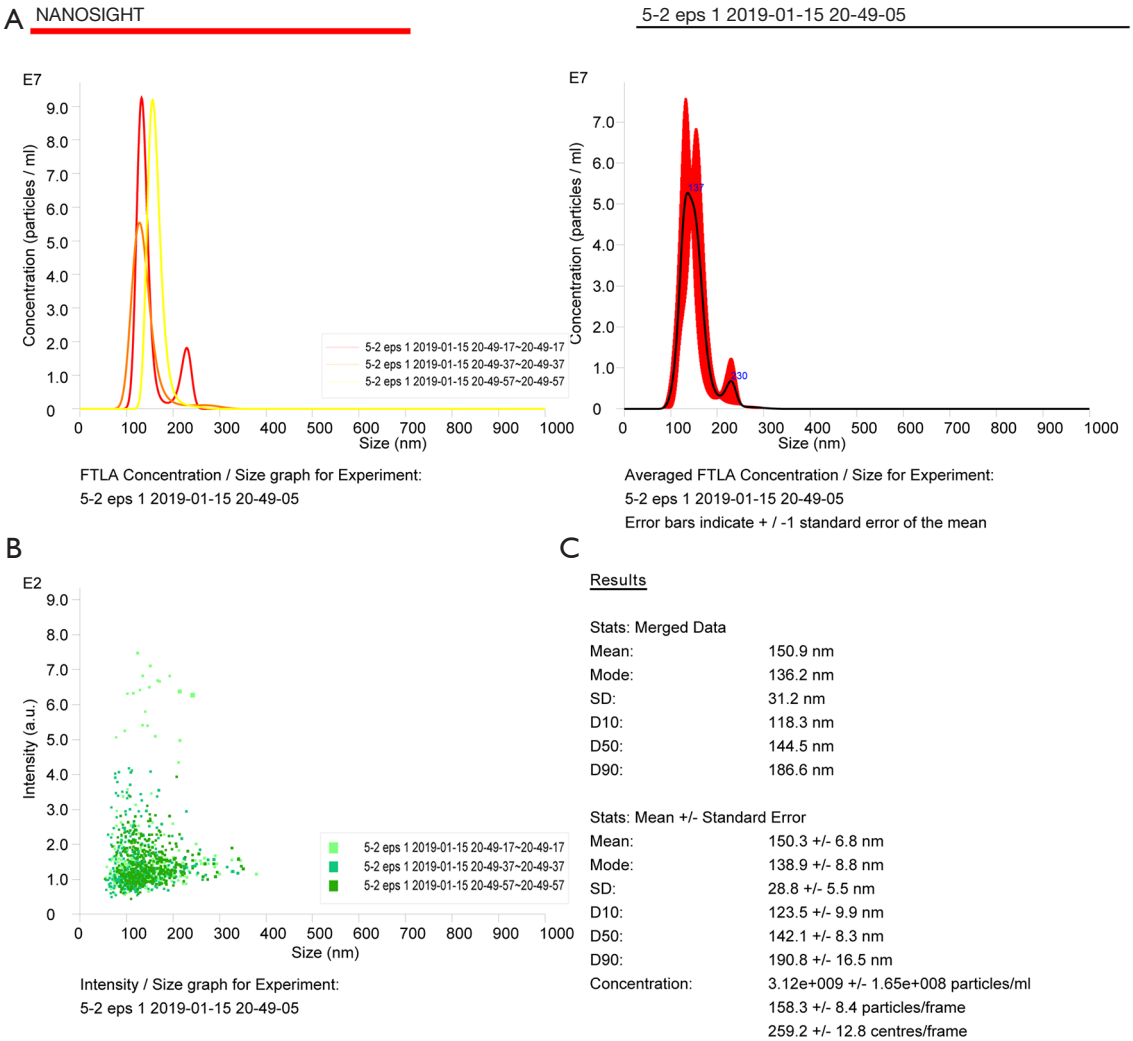

Figure 5 Nanoparticle tracking analysis (NTA) of the particle size of serum-derived exosomes extracted by the precipitation kit method. (A) FTLA (left) or averaged FTLA (right) concentration/size graph. (B) Intensity/size graph. (C) The results of merged data and mean ± standard error. FTLA (particles/frame against size distribution), Finite Track Length Analysis (FTLA) was used for size determination.

are preliminary problems that need to be resolved (14-16). At present, methods for the extraction and purification of exosomes include ultracentrifugation, membrane separation based on particle size, magnetic bead immunocapture, and precipitation kits. However, each method has its advantages and disadvantages. Many exosome isolation products have come onto the market, however, these products generally have not been standardized and thus it is important to examine the quality of the resultant isolated exosomes (17-22). In this investigation, exosomes isolated and purified from the serum of lung cancer patients by ultracentrifugation and a precipitation kit were compared. The exosomes extracted by ultra-high speed centrifugation were abundant and showed good shape, with cup-shaped or concave hemispherical structure typical of exosomes. The background was clean and exosomes were observed to gather together. Moreover, the exosomes showed a relatively uniform particle size distribution with a small particle size of 30.4±26.8 nm and a NTA concentration of 2.35×10^9 cells/mL. In contrast, the particle size of the exosomes extracted by the precipitation kit method was larger (150.3±6.8 nm) and within a relatively narrow range. This was likely due to the coating effect of the precipitation reagent masking the differences in particle sizes of the

exosomes themselves. Although the classical ultra-high speed centrifugation method requires the use of an ultra-high speed centrifuge, the quality of exosomes extracted by this method was superior to that obtained with the precipitation kit method. Furthermore, the precipitation kit was expensive and the separation purity was of concern. It is crucial that researchers pay attention to the quality and efficacy of the extraction method so as to produce good quality exosomes for further research regarding their properties and functions.

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Footnote

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Data Sharing Statement: Available at http://dx.doi.org/10.21037/atm-21-2075

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-21-2075). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics board of Renji Hospital (No.: 2018-160) and informed consent was taken from all individual participants.

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