ISOLATION AND CHARACTERIZATION OF ADIPOSE-DERIVED MESENCHYMAL STEM CELL EXOSOMES: AN IN-VITRO STUDY

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ABSTRACT

Exosomes from Mesenchymal Stem Cells (MSC) are currently one of the highlighted research due to their more specific action on the target tissue. The exosome is one of the MSC Extracellular Vesicles (EVs) that mediate cellular communication. However, not so many literatures have succeeded in elaborating on its isolation and characterization process. This study aimed to explain the method used in exosome isolation from Adipose-Derived MSC (ASC) and its elaborate characteristics. It is important to fill in the information needed on this bioactive molecule of great interest due to its potential use in cellular therapies. This is an in-vitro study performed in the Stem Cell and Tissue Engineering (SCTE) Indonesia Medical Education and Research Institute (IMERI) laboratory. The ultracentrifugation method was performed to isolate exosomes from ASC. Prior to characterization, sterility tests were performed to ensure a safe environment for the exosome growth. The exosome was characterized based on their particle size, morphology, and CD63 and CD81 expression for its purity. We were able to isolate sterile exosomes from ASC by performing differential ultracentrifugation. The mean size of the exosome was 88.7 nm ± 40 nm SD and showed expression of CD63 and CD81. Exosome was successfully isolated from ASC using the ultracentrifugation method, and characterization following the MISEV standard should be implemented in order to meet the therapeutic efficacy and safety issues as a regenerative agent.

Keywords: Isolation; Characterization; Exosome; Adipose-Derived Mesenchymal Stem Cells

INTRODUCTION

Mesenchymal Stem Cells (MSC) is currently one of the most studied topics in regenerative medicine research. Various sources have been described as a source of MSC, including the umbilical cord, bone marrow, adipose tissue, placenta, and many more.[1,2,3] Adipose-derived MSC (ASC) is one of the most studied sources of MSC due to its lower risk of donor morbidity and abundant source, while its multi-lineage capacity offers the potential repair of various tissues.[4]

However, MSC transplantation possessed several drawbacks, such as production and storage limitations, cell senescence, and ethical consideration that hindered commercial usage in the clinical setting.[5] Current research proved that MSC potency is caused by the paracrine effect rendering the massive highlighted research in MSC metabolite.[6,7] Exosome is one of the MSC metabolites, which is an

extracellular vesicle (EV). While having the same effects as MSC, they have the advantage of more targeted delivery, low immunogenicity, and high repairability.

There are many different techniques that have been developed to isolate exosomes, such as ultracentrifugation, ultrafiltration, chromatography, polymer-based precipitation, and affinity capture.[8] Isolation of exosome is aimed to obtain a pure sample in order to be able to study their action and effectivity in the biomedical setting.

Exosome is one of the EVs that has a size of 30-100 nm. While the two other EVs are microvesicles (100-1000 nm) and apoptotic bodies (1000-5000 nm).[9] Exosomes contain proteins, cytokines, lipids, mRNAs, miRNAs, tRNAs, non-coding RNA, and DNA. They have certain surface markers that help mediate exosome secretion and arrange the exosomes by assembling tetraspanin-enriched microdomains (TEMs). The most common surface marker of exosomes are CD9, CD63, and CD81.[10]

The characteristics of exosome have been proposed by the International Society for Extracellular Vesicles in the Minimal Information for Studies of Extracellular Vesicles (MISEV) guideline 2018.[11] During its isolation, it is paramount to obtain pure exosome which properties comply with the MISEV guideline in order to ensure its purity.

To date, although production and clinical translation of MSCs have been quite widespread in our country, exosome from MSC have never been successfully isolated. Therefore this study aimed to isolate exosome from one of its most practical sources ASC and characterize them according to the MISEV guideline.

MATERIAL AND METHODS

This is an in-vitro study performed in the Stem Cell and Tissue Engineering (SCTE) cluster of Indonesia Medical Education and Research Institute (IMERI), Faculty of Medicine Universitas Indonesia. Written consent was obtained from all patients involved following the Helsinki Declaration of 1975.

Samples of adipose tissue were obtained from healthy donors who performed surgical procedures at the affiliated hospital. The samples were harvested using sterile techniques and stored in a nitrogen tank in Dulbecco's Modified Eagle's Medium (DMEM) with 1% (v/v) penicillin/streptomycin.

ASC Isolation and Culture

Cryotubes containing ASC were defrosted from the nitrogen tank in a beaker glass containing 37^0C normal water for a maximum of 2 minutes. Afterward, cryotubes were kept in the biosafety cabinet (BSC). A complete medium for ASC was prepared to contain 10% platelet-rich plasma (PRP), 1% heparin (1000 U/mL), 1% amphotericin B (250 ug/mL), 1% pen-strep (10.000 U/mL penicillin, and 1000 ug/mL streptomycin, and αMEM. A total of 9 ml of complete medium were added into the centrifuge tube with 1 mL of cell from the cryotube, making a total of 10 mL volume before starting the centrifugation at 1200 rotation per minute (RPM) for 10 minutes. Afterward, the supernatant was removed, the pellet was collected, and added 1-2 mL of complete medium. The cells were cultured with the density of $5x10^3$ cells per cm^2 in a T-75 flask with an addition of 9 mL of the complete medium.

Exosome Isolation by Ultracentrifugation

Culture medium from MSCs that have reached 70% of confluency was collected and stored at a temperature of -20° C before further use. Frozen CM was defrosted by immersing the frozen CM container in a room temperature water. CM solution was centrifuged with speed of 750xg at 20^0C for 15 minutes. The supernatant was collected then further centrifuged at 2000xg speed for 15 minutes. Then the supernatant was collected and spinned in 10000xg for 45 minutes. The supernatant was collected and filtered using a 0.2 µm syringe filter then ultracentrifugation with the speed of 100000xg at 4°C was performed for 90 minutes. Afterward, the supernatant was removed, and the pellet containing EVs was

moved to a 15 mL falcon tube. Cold D-PBS was added until the volume reached 5 mL then re-suspension was performed. EVs were then aliquoted into a 1 mL cryovial and collected in a cryo-box chiller with a temperature of -20°C or freezer with a temperature of -80°C for 1-year storage.

Exosome Characterization

Prior to characterization, all of the exosome results were performed with sterility tests.

Evaluation of Size and Distribution of Exosome

Exosome size and distribution were evaluated using a particle size analyzer (PSA) using a Horiba SZ 100z machine which can also measure the zeta potential and molecular weight of the suspension sample. Zeta potential was carried out at 25^0C for each experimental triplicate.

Flowcytometry Assay

In order to ensure the purity of exosome, CD63 and CD81 expressions were evaluated using the protocol from BD Sciences. The tube containing EVs suspension was centrifugated using microcentrifuge Fresco 17 at a 500 xg speed for 5 minutes. Then, the supernatant was removed, and the pellet was collected and labeled with CD63 and CD81 antibodies by adding 2.5 µL of antibody in the tube containing the EVs pellet. Afterward, the antibody was homogenized with a pellet using a 10 µL pipette using the up and down technique and then incubated for 20 minutes in a lightless room. Then, the mixture was vortexed for a few seconds and analyzed using BD FACS ARIA III using 50000-100000 cells/second event, and software FACS Diva 8.0 was used to determine the obtained gate from EVs population that were not incubated with antibody.

RESULTS

The exosome was successfully isolated from ASC using the ultracentrifugation technique. The mean size of the exosome was 88.7 nm ± 40 nm standard deviation (SD), and the distribution of the samples was non-homogenous (Figure 1).

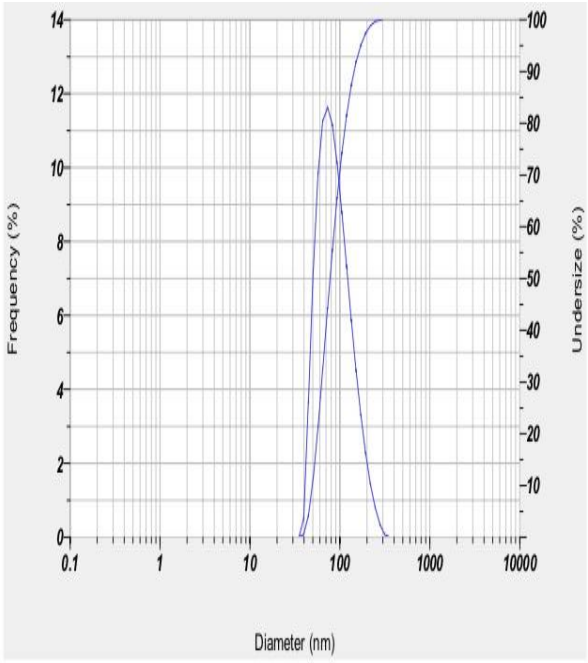

Figure 1. Exosome particle size from Particle Size Analyzer (PSA)

To evaluate the stability of exosome, the zeta potential was measured. The Zeta potential of all exosome preparations was negative and distributed within the range of -1.4 mV and -10.2 mV (Figure 2). The conductivity was between 14.945 mS/cm and 14.982 mS/cm.

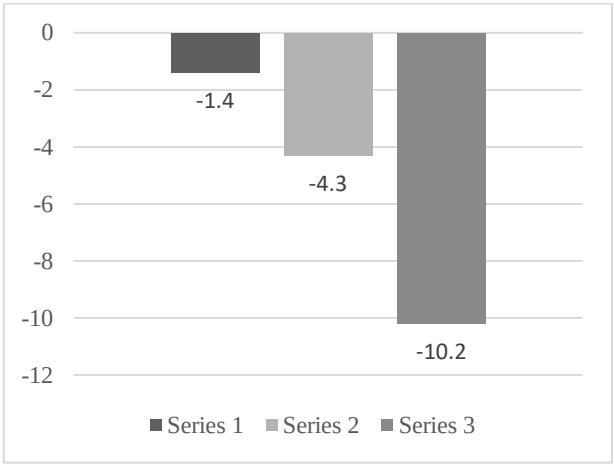

Figure 2. Zeta Potential Evaluation of Exosome Stability in 3 Sample Series. Each bar represents values of zeta potential in mS/cm

Flowcytometry Assay

The surface expression of several CD molecules was analyzed using flow cytometry to characterize the exosomes in the conditioned medium. All samples were sorted for CD63+, labeled with CD81 antibody, and showed positive results.

DISCUSSION

Recent literature showed the superiority of MSC metabolite products such as exosome and secretome compared to the original MSC. Exosome is one of the smallest extracellular vesicles (EVs) that has the ability to alter cellular communication, hence is one of the promising cell-free products that can create a lower immunogenic response with a more targeted effect.[12] While adipose is the most potent source to obtain MSC commercially, exosome-derived isolation method and characterization is still rare, especially in the Southeast Asian region.

There are several methods mentioned regarding the isolation technique of exosomes such as ultracentrifugation, ultrafiltration, chromatography, polymer-based precipitation, and affinity capture.[8] However, until now ultracentrifugation technique is still mentioned as the golden standard and the most commonly used method. The advantage of this technique is that highly enriched EV fractions can be produced while also allowing for the collection of additional vesicle fractions (larger vesicles first, then later smaller ones).[12] Although this technique requires multiple steps and a specific unit (ultracentrifuge) and needs special expertise to be performed correctly until now. This method is still the golden standard of exosome isolation.[13]

Since many methods have been used to isolate exosome, there have been questions regarding exosome purity and result in comparison. Hence, all experts in the exosome MSC gathered and determined guidelines for exosome characterization in the MISEV 2018.[11]

Characterization of the exosome, like many other EVs, is technically challenging due to a plethora of reasons, including their heterogeneity[14]. However, the MISEV 2018 guidelines pointed out several characteristics that exosome must possess, including particle size and surface protein marker[11]. Our study showed the mean diameter of EV was 88.7 nm ± 40 nm), which matches the exosome's size at 30-200 nm.

Zeta potential (ZP) is an indicator of colloidal stability influenced by the surface charge and can be measured from electrophoretic mobility in a suspension.[15] In the dispersed situation, the ZP determines the stability of particle-particle and particle-medium interactions hence the tendency of particles to aggregate. As nanoparticles (NP), nonfunctionalized exosome carry a net negative surface charge due to the nature of molecules expressed at the surfaces. The values of ZP were most often found below -10 mV, which is the minimum threshold value commonly accepted for sample stability in dispersion preventing aggregation.[16]

Our study also showed a negatively charged ZP, ranging from -10.2 until -1.4 in the conductivity between 14.945 mS/cm and 14.982 mS/cm, which indicate a stable exosome in the solution.

Another characteristic of exosome is the expression of specific protein markers. Several studies described CD63 and CD81 as the most commonly identified proteins in exosomes and considered a classic marker for exosome.[10,17] Our study also showed positive expression of CD63 and CD81 hence more emphasizing the characteristics of exosome.

CONCLUSION

Exosome was successfully isolated from ASC using the ultracentrifugation method, and characterization following the MISEV standard should be implemented in order to meet the therapeutic efficacy and safety issues as a regenerative agent.

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