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Comparison of Two Tangential Flow Filtration Methods in Isolating CD63+/CD9+ Mesenchymal Stem Cell Exosome

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ABSTRACT

Background: Extracellular vesicles, particularly CD63+/CD9+ Mesenchymal Stem Cell Exosome (MSC-Exo), have emerged as crucial mediators of intercellular communication and potential therapeutic agents, including regenerative medicine and immunomodulation. However, the precise isolation and purification of MSC exosomes pose critical challenges. Tangential Flow Filtration (TFF) has gained recognition as an efficient exosome isolation method, offering scalability and versatility. In this study, we address the pressing need for standardized exosome isolation methods by comparing two distinct TFF-based protocols for isolating CD63+/CD9+ MSC exosomes based on filter size pore order. **Methods**: MSC-Exo were conducted from the Stem Cell and Cancer Research Laboratory (SCCR Indonesia), which were then processed through TFF using different filter sizes and orders. There are two filtration methods compared, first, MSC-Exo was filtered with 1000-5-500-300-100-50-10-5 filter order. Second procedure, MSC-Exo was filtered using 1000-500-300-100-50-10-5 filter order. **Result**: Flow cytometry analysis revealed variations in the percentage of CD63+/CD9+ in the MSC-Exo based on filter order. The results indicate that the choice of filter order significantly influences the size range with the highest concentration of CD63+/CD9+ MSC-Exo. **Conclusion**: This research underscores the importance of optimizing TFF-based isolation methods for CD63+/CD9+ MSC exosomes, especially in the order of filter pore size.

Keywords : MSCs, Exosome, TFF, CD63, CD9.

INTRODUCTION

Extracellular vesicles secreted by various stem cell types or commonly are referred as the exosome, have emerged as vital mediators of intercellular communication and as potential therapeutic agents in regenerative medicine, cancer therapy, and immunomodulation[2,14,23]. Among the diverse exosomal cargo, Mesenchymal Stem Cell-Exosomes (MSC-Exo) have gained significant attention due to their regenerative and immunomodulatory properties, making them promising candidates for cell-free therapy[2,24,25]. However, the effective isolation and purification of MSC-Exo pose critical challenges, as their isolation is essential for the precise characterization and harnessing of their therapeutic potential[2,22,26]. Tangential Flow Filtration (TFF), also known as Cross-Flow Filtration, is a filtration technique commonly used in various scientific and industrial applications, including biotechnology, pharmaceuticals, and the isolation of nanoparticles such as exosomes[3,8,18]. It is a versatile and efficient method for separating particles from a liquid based on their size, shape, and

Mesenchymal Stem Cell Exosomes (MSC-Exo) CD63+/CD9+, have emerged as important mediators in the field of intercellular communication and have tremendous potential as therapeutic agents, especially in the domain of regenerative medicine and immunomodulation[1–3]. CD63 is a commonly used marker for the identification and isolation of exosomes. CD63 is one of the proteins that can be found on the surface of exosomes, and its presence is used to characterize and isolate these vesicles[3,4]. CD9 is found on the cell surface, where it interacts with other proteins, including integrins and other tetraspanins, to modulate cell adhesion and signaling[4,5]. Both CD63 and CD9 are important molecules in cell biology, with diverse functions that extend beyond their roles as cell surface markers[3,4]. They are widely studied in various research fields, including immunology, cell biology, and cancer biology, due to their involvement in critical cellular processes and their potential as therapeutic targets.

TFF has been recognized as an efficient method for exosome isolation, offering some advantages including scalability and versatility[6,7]. The CD63 and CD9 proteins are abundantly distributed on the surface of exosomes, thus commonly used as markers for exosome characterization and isolation[3,5]. Despite the growing recognition of TFF as a valuable exosome isolation technique, the field still grapples with the challenge of standardizing isolation methods, as different TFF protocols, particularly those differing in filter size pore order, may yield divergent results[6,8]. In this research study, we aim to address the pressing need for standardized exosome isolation methods by comparing two distinct TFF-based protocols for isolating CD63+/CD9+ MSC exosomes based on filter size pore order. This investigation seeks to evaluate the efficiency, purity, and yield of the two TFF methods and to provide insights into their respective suitability for downstream applications.

METHODS

Research Design

This study was conducted in the Stem Cell and Cancer Research Laboratory (SCCR Indonesia) from November to December 2022.

MSC-Exo Preparation

MSCs cultured in serum-free complete medium were incubated under hypoxia conditions in the hypoxic chamber maintaining a gas mixture composed of 5% O_2 and balanced N2 at 37 °C for 12 h[9,10]. MSCs conditioned medium was then collected after 12-hour incubation. The collected MSCs conditioned medium was centrifuged at 2000 rpm for 5 minutes to remove cell debris and passed through a 0.22-µm filter membrane (Corning, NY, USA) to remove the remaining cell debris. The conditioned medium was collected then filtered using Tangential Flow Filtration (TFF) with different filters; 5-10, 10-5-, 50-100, 100-300, 300-500 kDa and different procedures. First procedure, MSCs conditioned medium was filtered with 1000-5-500-300-100-50-10-5 filter order. Second procedure, MSCs conditioned medium was filtered with 1000-500-300-100-50-10-5 filter order. The S-HMSCs were kept at 2-8°C temperature until the next analysis.

Exosomes Detection on MSC-Exo

Exosomes detection on MSC-Exo was conducted using Exosome Isolation and Analysis Kit CD63/CD9 (Abcam). MSC-Exo pretreatment by centrifugation at 200 x g, 5 minutes, 4˚C, then subsequently centrifugation at 14.000 x g, 5 minutes, 4˚C. 100 µL MSC-Exo mixed with 50 µL capture beads CD63+ and incubated overnight, in the dark, at room temperature, then washed with assay buffer 1X and centrifuged at 2.500 x g, 5 minutes. 5 µL anti-CD9 antibody PE was added to bead-bound exosome (pellet) and incubated 1 h, in the dark, at 2-8 ˚C, then washed with assay buffer 1X and centrifuged at 2.500 x g, 5 minutes. The pellet was resuspended in 350 µL assay buffer 1X and then was analysed using flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

Statistical analysis was accomplished with the software GraphPad Prism 9 software. All data are presented as mean ± standard deviation (SD). Data analysis used one-way ANOVA and continued with the Least Significant Difference (LSD) post hoc test using a *p*-value <0.05.

RESULT

MSC Characterization

The morphological characteristic of isolated cells under microscopical observation was demonstrated by their plastic adherent, homogenous shape, typical MSC fibroblast-like phenotype, and round nucleus. We proved, using flow cytometry, that the separated cells had the MSC-specific markers CD90.1, CD29, but lacked CD45 and CD31. While the results of the validation of osteogenic differentiation demonstrated that MSCs could differentiate into osteocytes as indicated by the red calcium deposits in the MSCs population using Alizarin Red staining, the results of the validation of the morphology of the MSC culture were obtained as an image of cells attached to the bottom of the flask with spindle-like cell morphology under microscopic observation (Figure 1A and B). The results of isolated MSC cells were verified using flow cytometry to demonstrate that MSCs were able to express multiple MSC surface markers, which is consistent with their osteogenic capacity. According to the validation findings, MSCs could express CD90.1 (99.80%) and CD29 (94.20%), CD45 (1.60%) and CD31 (6.60%) (Figure 1C).

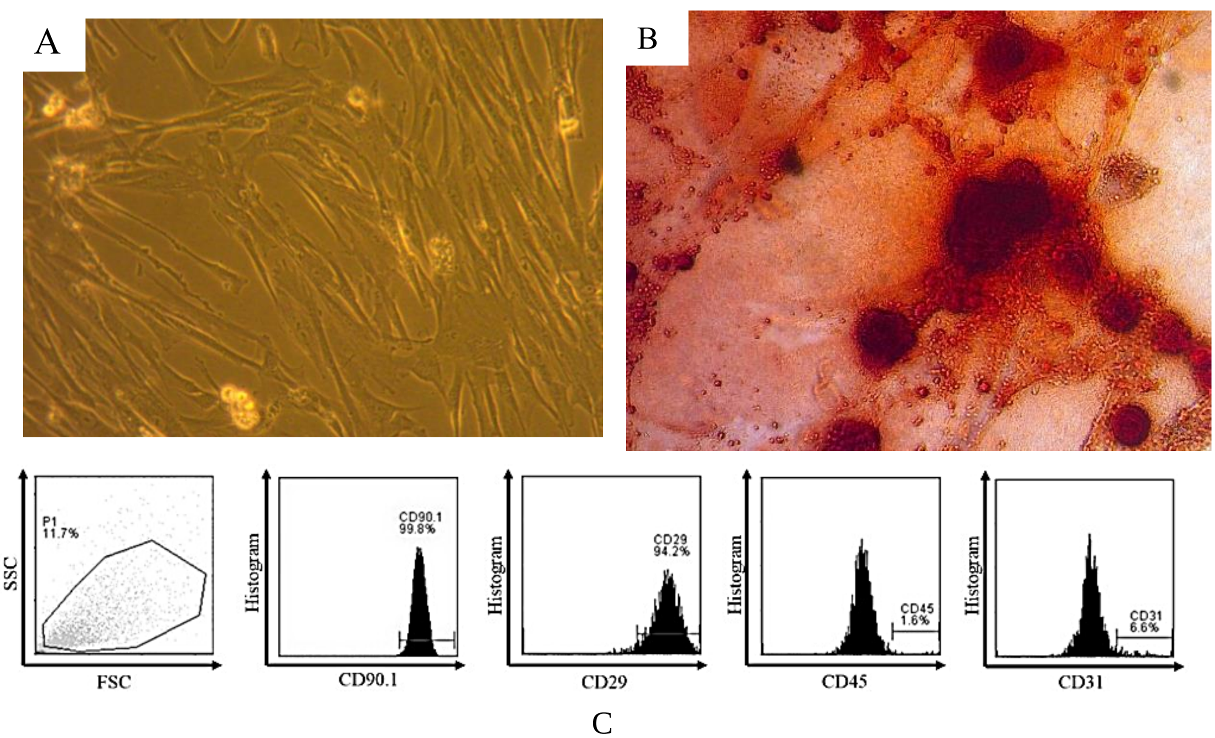

Figure 1. MSC Validation. (A) Isolated MSCs with 80% confluent showed spindle-like cells (pointed by arrows) at 100x magnification. (B) Osteogenic differentiation using Alizarin Red staining appears in the MSC population at 100x magnification. (C) Flow cytometry analysis of the expression of CD90.1, CD29, CD45, and CD31.

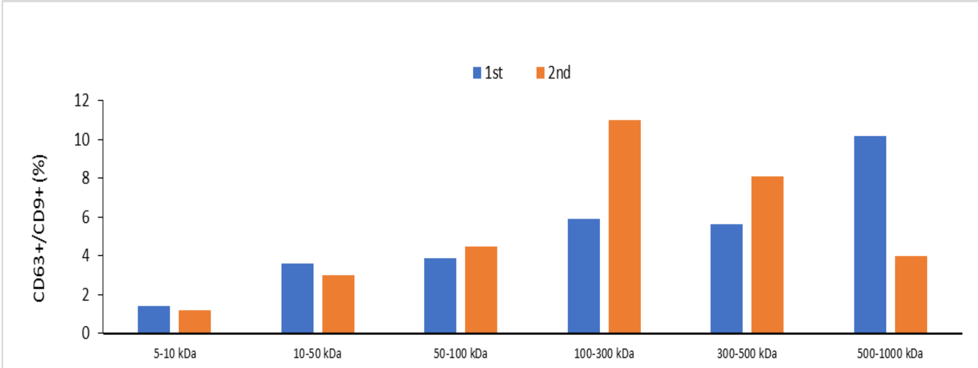

Figure 1. Percentage of $CD63^{+}/CD9^{+}$ detected in MSC-Exo filtered with different filter sizes.

DISCUSSION

Isolation of Extracellular Vesicles (EVs) from MSC is a crucial task in biomedical research, as these tiny membrane-bound vesicles carry several signalling molecules critical for intercellular communication and various physiological processes including suppressing inflammation and wound healing[10–15]. Various methods to effectively and selectively isolate CD63+/CD9+ EVs have been proposed including TFF, Size Exclusion Chromatography (SEC), and ultracentrifugation[5,16,17]. However, each method has its own advantages and limitations, making the choice of technique dependent on the specific research goals and available resources. TFF method presents notable advantages over other common EV isolation methods such as ultracentrifugation and SEC[3,18–20]. TFF is efficient, high-purity and preserves their structural and functional integrity, which is a significant advantage over ultracentrifugation and SEC[7,16,21].

In this study, we compare two procedures of TFF to isolate CD63+/CD9+ EVs from MSC based on size pore filter order. In the first procedure we used 1000-5-500-300-100-50-10-5 filter order while for the second procedure it is 1000-500-300-100-50-10-5 filter order. This research reveals that differences in filter order will affect the size range that has the highest number of CD63+/CD9+ EVs. The study shows the greatest accumulation of CD63+/CD9+ EVs in the 500-1000 kDa range was found using the first procedure, whereas the second procedure in the 100-300 kDa range. The size of CD63+/CD9+ exosomes derived from MSCs can vary, within a range of approximately 60 kDa to 150 kDa[2,22].

Based on the results, the 5 kDa filter that is used after 1000 kDa in the first procedure will reduce the volume of MSC conditioned medium in exchange for increased concentration of protein and EVs. We suggest that the increased concentration will increase the blockage of the 500 kda filter thus retaining CD63+/CD9+ EVs in the 500-1000 kDa range. Clogging in the TFF system can potentially hinder the filtration process and affect the purity and yield of isolated EVs.

CONCLUSION

This research underscores the importance of optimizing TFF-based isolation methods for CD63+/CD9+ MSC exosomes especially in the order of filter pore size.

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None

AUTHORS' CONTRIBUTION

AP concept and design the study and wrote the manuscript, IA prepared and analyzed the data, APO and SP contributed to revisions of the manuscript and approved it for publication.

COMPETING INTERESTS

The authors declare no competing interests.

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