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Characterization of Fibroblast Secretome: Extracelular Vesicles (EVs) Particle Count, Molecular Size and Total Protein Levels

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ABSTRACT

Introduction: The secretome of human dermal fibroblasts consists of diverse bioactive molecules including proteins and extracellular vesicles (EVs), playing a key role in skin regeneration and homeostasis. Molecular weight-based fractionation provides insights into the functional heterogeneity of the conditioned medium (CM). This study aimed to characterize size-fractionated CM from fibroblasts to reveal its molecular properties and potential bioactivity.
Methods: A descriptive laboratory study was conducted using conditioned medium from passage 5 human dermal fibroblasts cultured in serum-free conditions. Tangential flow filtration (TFF) was applied to fractionate the CM into low (LMW), medium (MMW), and high molecular weight (HMW) components. Nanoparticle tracking analysis (NTA) quantified EV particle concentration and size, while total protein content was assessed by BCA assay. Experiments were performed in triplicate (n=3).
Results: Particle concentrations increased with molecular weight, with HMW fractions showing the highest counts but slightly smaller average particle sizes compared to LMW and MMW fractions. Total protein concentration also rose with molecular weight, peaking in HMW fractions, indicating enrichment of larger protein complexes and vesicles. Fractionation maintained consistent particle size distribution across fractions, supporting effective separation.
Conclusion: Fibroblast secretome exhibits heterogeneous EV and protein populations distinguishable by molecular weight fractionation. The enriched higher molecular weight fractions contain more EVs and proteins, suggesting distinct biological roles. Combining TFF with further purification steps may enhance separation purity, facilitating therapeutic applications of secretome components.

Keywords: fractionation; molecular size; particle count; protein; secretome

INTRODUCTION

The secretome of human dermal fibroblasts, comprising a complex mixture of bioactive molecules including proteins, peptides, and Extracellular Vesicles (EVs), play a pivotal role in skin homeostasis and regenerative processes. Conditioned medium (CM) derived from fibroblasts has attracted increasing attention for its potential applications in wound healing, anti-aging therapies, and tissue engineering[1,2]. This medium contains various factors such as growth factors, cytokines, and extracellular matrix (ECM) components that influence cellular behavior and tissue remodeling.

Molecular weight-based fractionation of secretome provides a refined understanding of the functional heterogeneity within the CM, allowing the isolation of low molecular weight (LMW, 5-100

kDa), medium molecular weight (MMW, 100-300 kDa), and high molecular weight (HMW, >300 kDa) components, each associated with distinct biological activities[3].

Tangential flow filtration (TFF) is a preferred method for efficient fractionation due to its gentle processing and scalability, which preserves the bioactivity of proteins and EVs[4]. The size range of extracellular vesicles (EVs) is heterogeneous and depends on their origin and type. Generally, EVs range from about 20-30 nanometers up to 1,000 nanometers (1 micron) or even larger in some subtypes. Small EVs, including exosomes, typically range from 20 to 100 nm in diameter. Microvesicles range from approximately 100 nm up to 1,000 nm. Larger EV types, such as apoptotic bodies, can be greater than 1,000 nm[5].

Quantification of EV particle count alongside molecular size distribution and total protein content is essential to comprehensively characterize the CM's therapeutic potential[6]. Despite advancements, a detailed profile of the EV fractions from early-passage human dermal fibroblasts remains limited. Understanding the properties of each fraction—including EV abundance and protein composition—could optimize the application of CM in regenerative medicine and skin biology[7]. This study aims to characterize the fractionated CM from passage 5 human dermal fibroblasts to elucidate its molecular characteristics and potential bioactivity.

METHODS

Study Design and Subject

Human dermal fibroblasts were obtained as primary cells from discarded human preputial skin tissue collected during elective circumcision procedures after written informed consent and approval from the Research Ethics Committee of YARSI University (Approval No: 277/KEP-UY/EA.10/X/2023). Immediately after surgery, the preputial skin specimens were rinsed several times with phosphate-buffered saline (PBS) containing antibiotic–antimycotic solution, and the subcutaneous fat was carefully removed. The tissue was cut into small pieces (approximately 1–3 mm), and explant cultures were established in fibroblast growth medium in standard tissue culture flasks. Spindle-shaped adherent cells migrating from the dermal explants were allowed to expand until they reached 70–80% confluence and were then subcultured. Fibroblasts were identified based on their typical spindle-shaped morphology, plastic adherence, and homogeneous growth pattern, and passage 5 cells were used to produce conditioned medium for this study. This descriptive laboratory study used conditioned medium derived from passage 5 human dermal fibroblasts isolated from preputial skin to characterize the size-fractionated components of Human Dermal Fibroblast Conditioned Medium (HDF-CM).

The HDF-CM was prepared from 34.500.000 Human Dermal Fibroblast (HDF) cells at Passage 5, cultured for one week in a serum-free medium (DMEM/KOSR) to eliminate protein interference. Prior to processing, the medium was clarified using a 0.22 μm filter to remove cellular debris. The initial sample volume was subsequently processed using a Tangential Flow Filtration (TFF) System under controlled conditions to concentrate the final retentate. Sequential ultrafiltration was performed to isolate three distinct size fractions: High Molecular Weight (HMW), Medium Molecular Weight (MMW), and Low Molecular Weight (LMW), utilizing specific Molecular Weight Cut-Off (MWCO) membranes. Following fractionation, all collected samples, including the initial conditioned medium, were subjected to downstream characterization, which included Nanoparticle Tracking Analysis (NTA) for quantifying Extracellular Vesicle (EV) particle count and molecular size distribution, and a Bicinchoninic Acid (BCA) Assay for total protein quantification.

Serum-Free Human Dermal Fibroblast Conditioned Medium (HDF-CM) Preparation

1. Human Dermal Fibroblast (HDF) cells at Passage 5 (P5) were seeded at a density of 5,700,000 cells per T-870 flask and expanded in their standard growth medium for 7 days. The cells were incubated at 37^0 C with 5% CO2, and the medium was replaced every two days.
2. On Day 6, the medium was replaced entirely with a serum-free conditioned medium (CM) production medium (DMEM supplemented only with KOSR).
3. On Day 7, the cell count was determined to be 34,500,000 cells. The cell monolayers were first rinsed rigorously three times with warm Phosphate-Buffered Saline (PBS) to ensure the complete removal of residual serum components. Subsequently, a total volume of 200 mL of conditioned medium was collected from all T-870 flasks, yielding an approximate initial volume of 200 mL, which was then pooled into sterile collection containers.
4. The pooled CM was transferred to 50 mL conical tubes and centrifuged at 300×g for 10 minutes at 4^0 C to pellet and remove the cells.
5. The resulting supernatant was collected and subjected to a second centrifugation step at 2,000×g for 20 minutes at 4^0 C to eliminate large cellular debris and aggregates.
6. The final supernatant (clarified HDF-CM) was then sterilized by passing the entire volume through a 0.22 µm low-protein binding filter (PES).
7. The sterile, filtered HDF-CM was immediately aliquoted and stored at -80^0 C until the subsequent Tangential Flow Filtration (TFF) process.

Molecular Weight Fractionation of Human Dermal Fibroblast Conditioned Medium using Tangential Flow Filtration (TFF)

1. Prepare the conditioned medium sample from human dermal fibroblasts, ensuring its clarification by microfiltration through a 0.22 µm membrane filter to remove cells and particulate debris.
2. Assemble the Pall Minimate EVO TFF System, which comprises a feed reservoir, a peristaltic pump, fluidic conduits, in-line pressure transducers, and a TFF membrane module equipped with membranes of appropriate molecular weight cut-off. The initial sample volume for processing is 170 ml.
3. Initiate the peristaltic pump to circulate the sample tangentially across the membrane surface at a controlled crossflow velocity of 40 rpm.
4. Perform sequential fractionation of the sample utilizing membranes with descending molecular weight cut-offs to achieve the following fractions; Low Molecular Weight (LMW) Fraction: 5–100 kDa, Medium Molecular Weight (MMW) Fraction: 100–300 kDa, High Molecular Weight (HMW) Fraction: >300 kDa.
5. Collect the permeate (filtrate) and retentate fractions separately during each filtration step.
6. Recirculate the retentate stream back to the feed reservoir throughout the diafiltration or concentration process to maintain continuous tangential flow and facilitate the progressive concentration of retained macromolecules until the final retentate volume reaches 20 ml.
7. Maintain and precisely control the transmembrane pressure (TMP) at 10 psi and the crossflow velocity at 40 rpm to optimize filtration flux, maximize separation efficiency, and mitigate membrane clogging.
8. Continue the filtration process until the requisite volume reduction or target fractionation is attained for each designated molecular weight range.
9. Collect and archive the resulting fractionated samples under appropriate storage conditions for subsequent analytical assays.

Nanoparticle Tracking Analysis (NTA) Protocol for HDF-CM Fractions Using The NanoSight system

1. Retrieve the -80^0 C HDF-CM fractions (HMW, MMW, LMW, and the initial CM control) and thaw them rapidly in a 37^0 C water bath. Bring the samples to room temperature before analysis.
2. Determine Optimal Dilution, due to the high concentration of TFF-processed samples, the fractions must be diluted so that the particle concentration is within the NTA system's recommended range. Prepare three technical replicates of the optimized dilution for each of the four sample types (HMW, MMW, LMW, and initial CM).
3. Load the first diluted sample replicate into the clean syringe pump attached to the NTA flow cell.
4. Inject the sample slowly and smoothly into the chamber until a stable flow is achieved and air bubbles are cleared. Adjust the Camera Focus and the Camera Shutter Level until the particles appear bright against a dark background, optimizing for the brightest particles without saturation. Record three 60-second videos per sample replicate using the same Camera Level and Detection Threshold settings established for the calibration standard. Between each sample type, thoroughly clean the fluid path by flushing it multiple times with the particle-free buffer to prevent cross-contamination. Repeat steps for sample replicates (n=3).
5. Use the NTA software to analyze the recorded videos. Apply the same Detection Threshold across all videos for consistent analysis.
6. The software will track the Brownian motion of individual particles to calculate the hydrodynamic diameter (nm) using the Stokes-Einstein equation.
7. The software will report: Particle Concentration: Particles per milliliter (particles/mL). Mean and Mode Size: The average and most frequently observed particle sizes (nm).

Total Protein Examination Procedure

1. Total protein concentration was determined using the Coomassie (Bradford) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.
2. Briefly, 5 µL of each protein standard and sample were pipetted into individual wells of a 96-well microplate. Then, 250 µL of Coomassie Brilliant Blue reagent was added to each well, and the plate was incubated at room temperature for 10 minutes to allow the dye to bind to the proteins.
3. The absorbance was measured at 595 nm using a microplate reader. All samples and standards were assayed in triplicate (n=3) to ensure accuracy and reproducibility.
4. Protein concentrations were calculated based on a standard curve generated from known concentrations of bovine serum albumin (BSA). Data are presented as mean ± standard deviation (SD).

RESULTS

Characterization of Extracellular Vesicles (EVs) Particle Count and Molecular Sise

Nanoparticle Tracking Analysis (NTA) measurements revealed both particle concentration and average size across different molecular weight fractions. The data indicate substantial variation in particle count between fractions, while the average particle size remains relatively consistent (as shown in Table 1).

The particle counts increase with higher molecular weight categories, with HMW (>300 kDa) having the highest concentration at 16 x 10^9 particles/ml. Despite variation in concentration among the fractions, the average particle size remains relatively consistent, with the HMW fraction being slightly smaller (157 nm) compared to the LMW and MMW fractions. These data suggest that higher molecular

weight fractions contain more particles per volume but with a marginally smaller average size, which may reflect differences in particle composition or aggregation state among the fractions.

These findings suggest not only a clear relationship between molecular weight fraction and particle concentration, but also highlight the robustness of the fractionation method in maintaining a consistent particle size distribution across different fractions. This pattern is typical in nanoparticle isolation studies, indicating that as molecular weight increases, the number of particles per volume tends to rise, while their size distribution remains within a narrow range. Such consistency in size, despite concentration shifts, supports the effectiveness of the separation technique and reflects the homogeneity often desired in nanoparticle preparations. It indicates a correlation between molecular weight category and particle concentration, while maintaining similar size ranges, consistent with typical nanoparticle fractionation profiles[8].

Table 1. Particle Concentration and Average Size in Different Molecular Weight Fractions

Fractination	Particle count (particles/ml)	Molecular size (nm)
LMW (5-100 kDa)	1,3 x 10^9 particles/ml	161 nm
MMW (100-300 kDa)	2,7 x 10^9 particles/ml	170 nm
HMW (>300 kDa)	16 x 10^9 particles/ml	157 nm

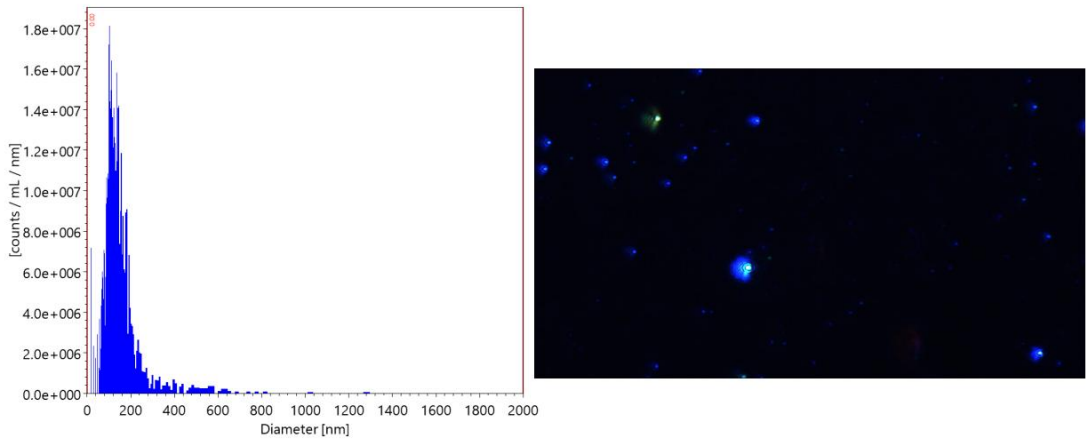

Figure 1. Nanoparticle Tracking Analysis results of LMW 5–100 kDa secretome, illustrating the distribution of particle sizes (left) and visually confirming the presence of individual nanoparticles in suspension (right)

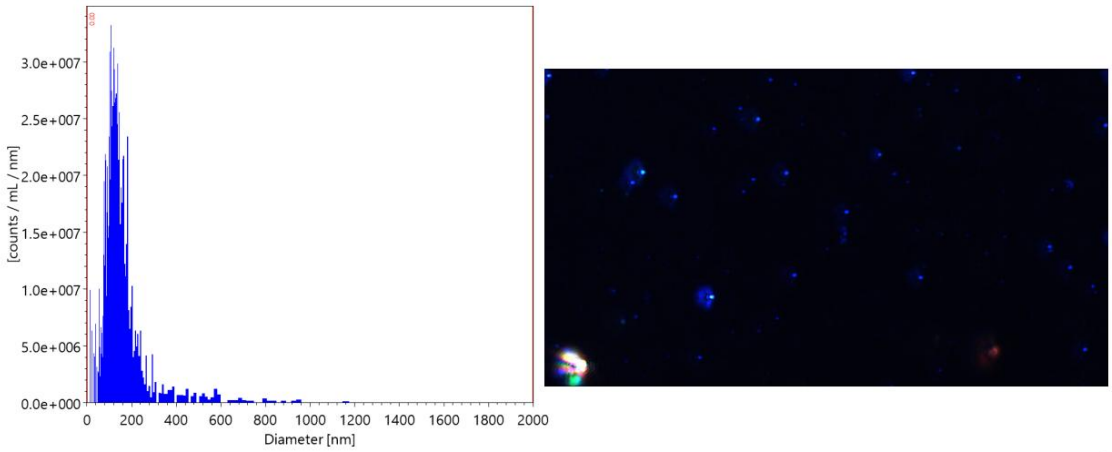

Figure 2. Nanoparticle Tracking Analysis results of MMW 100–300 kDa secretome, illustrating the distribution of particle sizes (left) and visually confirming the presence of individual nanoparticles in suspension (right)

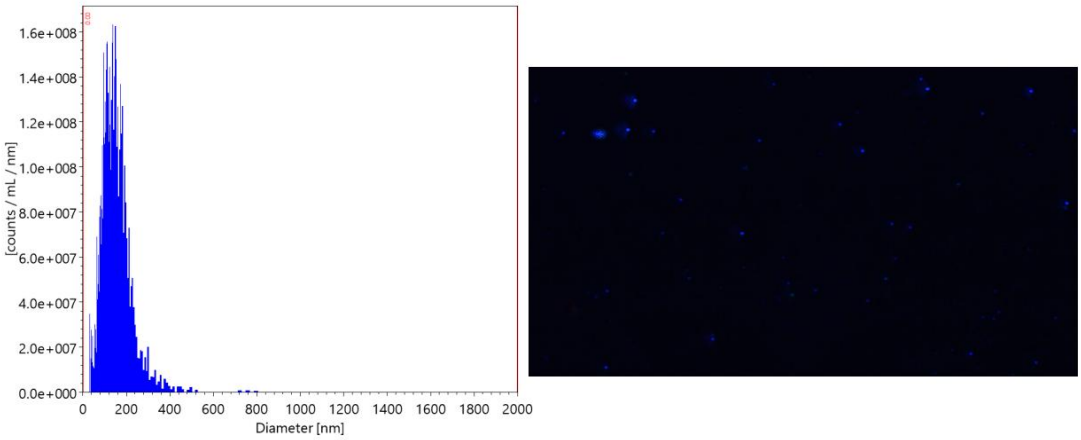

Figure 3. Nanoparticle Tracking Analysis results of MMW >300 kDa secretome, illustrating the distribution of particle sizes (left) and visually confirming the presence of individual nanoparticles in suspension (right)

Distribution of Protein Concentration in Secretome

The total protein concentration was measured in molecular weight fractions obtained from 10^4 cells over 24 hours. The results demonstrate a clear increase in protein concentration with rising molecular weight, highlighting the differences in protein distribution across size fractions (as shown in Table 2 and Figure 2).

Table 2. Total Protein Concentration in Molecular Weight Fractions

Fractination	Total protein of 10^4 cells/24 hour (µg/ml)
Raw (0,22 µm)	3227,91 µg/ml
LMW (5-100 kDa)	5673,72 µg/ml
MMW (100-300 kDa)	14438,45 µg/ml
HMW (>300 kDa)	24195,46 µg/ml

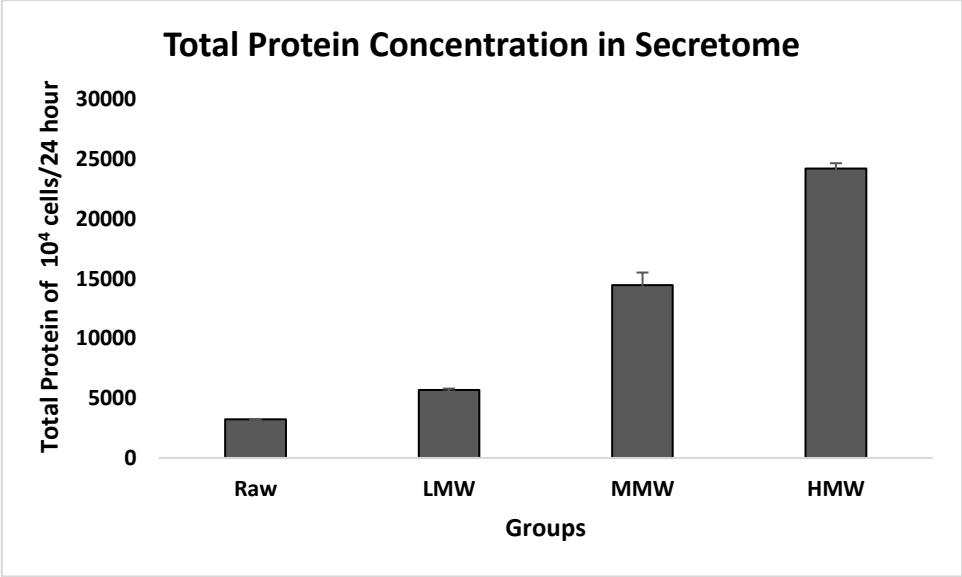

Figure 2. Total protein concentration in secretome samples measured from 10^4 cells over 24 hours across different groups. The data represent the mean values from three independent replicates (n=3). Higher molecular weight fractions (MMW and HMW) show progressively increased protein concentrations compared to the raw and LMW groups, indicating that higher molecular weight fractions contain more total protein.

Protein concentration increases significantly with molecular weight, with the HMW fraction exhibiting the highest concentration. This trend suggests that higher molecular weight fractions contain substantially more protein, which may be due to the presence of larger protein complexes or aggregates. Such fractionation data are consistent with typical protein characterization studies where protein content correlates positively with molecular weight size[9].

DISCUSSION

Characterization of Extracellular Vesicles (EVs) Particle Count and Molecular Sise

The characterization of fibroblast secretome fractions revealed mean sizes of low molecular weight (LMW), medium molecular weight (MMW), and high molecular weight (HMW) fractions were 161 nm, 170 nm, and 157 nm, respectively. The characterization of fibroblast secretome fractions revealed mean particle concentrations for Groups LMW, MMW, and HMW as 1.3×10^9, 2.7×10^9, and 16×10^9 particles/ml, respectively.

These size distributions are consistent with the heterogeneous nature of EV populations, which include exosomes and microvesicles[10,11]. The size of exosomes derived from fibroblast secretome typically ranges between 100 and 150 nm, while microvesicles derived from fibroblast secretome generally range from 30 to 300 nm[12,13].

The observed particle sizes for these groups suggest a mix of small to medium-sized EVs, potentially comprising typical exosomes along with larger vesicles such as microvesicles or vesicles formed through different biogenetic pathways. This heterogeneity is a well-documented characteristic of EV populations isolated from biological fluids and cell secretomes, where overlapping size distributions are common due to the shared biophysical properties and overlapping functions of EV subtypes[10].

These differences in concentration and molecular weight fractions likely reflect distinct populations of extracellular vesicles (EVs) with varying sizes and cargo compositions. The LMW fraction typically corresponds to small EVs such as exosomes, which range around 30-150 nm and carry specific proteins and nucleic acids. The MMW fraction likely represents a mix of exosomes and larger EVs, such as microvesicles, which are formed by budding from the plasma membrane and are generally larger (100-1000 nm). The HMW fraction, with the highest particle concentration, probably contains a heterogeneous collection of larger microvesicles, apoptotic bodies, protein aggregates, and other macromolecular complexes, which contribute to the greater molecular weight and particle numbers observed[11,14].

Using tangential flow filtration (TFF) is an efficient and scalable method for isolating extracellular vesicles (EVs), including exosomes, from biological samples such as fibroblast secretome. However, TFF alone cannot fully separate exosomes from microvesicles because these particles often overlap in size and share similar physical properties. Both exosomes (30-150 nm) and microvesicles (100-1000 nm) may coexist after TFF fractionation since the method is based primarily on size and molecular weight cut-offs rather than specific vesicle markers[15,16].

To overcome this limitation and enhance the purity between exosomes and microvesicles, following tangential flow filtration (TFF) with size exclusion chromatography (SEC) can further refine the isolation by separating vesicles based on more precise size differences. Additionally, immunoaffinity capture targeting specific surface markers, such as CD63 and CD81 for exosomes, can be employed after size-based separation to selectively enrich exosomes[17].

Distribution of Protein Concentration in Secretome

The total protein concentrations measured in the raw secretome and molecular weight fractions (LMW, MMW, and HMW) showed a clear increasing trend: raw secretome at 3227,91 µg/ml, LMW at

5673,72 µg/ml, MMW at 14438,45 µg/ml, and HMW at 24195,46 µg/ml. This suggests that proteins are more concentrated in the higher molecular weight fractions as separated by tangential flow filtration (TFF).

This pattern may be explained by the presence of large extracellular vesicles such as microvesicles and protein aggregates and macromolecular complexes predominantly in MMW and HMW fractions, while smaller soluble proteins and peptides contribute less to total protein in the LMW fraction[14,18]. The distribution might also reflect differential retention and possibly partial aggregation of proteins during TFF isolation.

The higher protein concentration in HMW can include large vesicles, protein complexes, or extracellular matrix components that are retained in these fractions, contributing significantly to the total protein mass[19]. This has implications for interpreting functional bioactivity since different molecular weight fractions may contain distinct bioactive proteins and vesicle populations.

Initially, the raw secretome contains a complex mixture of soluble proteins, extracellular vesicles (EVs), protein aggregates, and other macromolecules, resulting in an overall moderate protein concentration measured in bulk. When the secretome is processed through molecular weight fractionation methods such as tangential flow filtration (TFF), proteins and complexes are separated based on their size and MW. The increase in protein concentration in higher MW fractions is due to the selective enrichment and concentration of large protein complexes, vesicles (microvesicles, apoptotic bodies), and aggregated proteins in the MMW and especially HMW fractions. These large complexes contribute disproportionally to total protein by mass. In contrast, smaller soluble proteins and peptides enrich the LMW fraction but tend to have lower mass concentration overall[20,21].

The fractionation step effectively concentrates proteins in each molecular weight range, which elevates their measured concentration compared to unfractionated raw secretome. This enrichment enables better functional characterization of proteins according to their molecular weight, clarifying the biological roles of different secretome components[22].

CONCLUSION

In conclusion, the analysis of fibroblast secretome fractions reveals a complex and heterogeneous population of extracellular vesicles and proteins. Particle size and concentration data indicate distinct populations of vesicles, with smaller exosomes predominating in low molecular weight (LMW) fractions and larger microvesicles and aggregates found in medium (MMW) and high molecular weight (HMW) fractions. Total protein concentrations increase with molecular weight fractionation, reflecting the concentration of larger protein complexes, extracellular vesicles, and aggregates in higher molecular weight fractions. This highlights the importance of optimizing isolation techniques such as combining TFF with size exclusion chromatography and immunoaffinity capture to improve purity and specificity in secretome analysis.

However, this study is limited by the lack of proteomic profiling or functional assays to identify specific bioactive components, and its descriptive design without validation in biological models such as wound healing assays. Fibroblasts from preputial skin at passage 5 may also limit generalizability to other sources or passages. Overall, these findings stress the need for careful methodological considerations, including future functional studies, to accurately characterize and utilize fibroblast secretome components in research and therapeutic applications.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The experimental protocol for this study was reviewed and approved by the Research Ethics Committee of YARSI University (Approval No: 277/KEP-UY/EA.10/X/2023) under the project title "The Role of the Concentrated Fibroblast Secretome in Improving the Function of Fibroblast Senescent Cells" on October 16, 2023. All procedures involving cell cultures were conducted in accordance with the ethical standards of the Institutional Research Committee.

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