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COMPARATIVE ANALYSIS OF METHODS FOR THE PURIFICATION OF EXTRACELLULAR VESICLE SUSPENSIONS FROM HUMAN MESENCHYMAL STEM CELL CULTURE

Aim. *To evaluate the impact of isolation methods and culture conditions on the purity, yield, and phenotype of extracellular vesicles (EVs) derived from human mesenchymal stromal cells (MSCs).* ***Methods.*** *EVs were isolated using tangential flow filtration (TFF) with membranes of different molecular weight cut-offs (100–300 kDa), combined with ultracentrifugation (UC), under serum-containing and serum-free conditions. The particle concentration and size distribution (40–1000 nm) were analyzed by flow cytometry, total protein content was measured fluorometrically, morphology was assessed by transmission electron microscopy, and expression of tetraspanins (CD9, CD63, CD81) was determined by immunophenotyping.* ***Results.*** *The serum-containing media yielded a higher number of EVs but resulted in substantial protein contamination. The purest isolates (0.09–0.2 mg/mL total protein) were obtained under the serum-free conditions using TFF with <100 kDa membranes combined with ultracentrifugation. Under these conditions, CD63$^+$ exosomes predominated (up to 98%). Transmission electron microscopy confirmed typical EV morphology with diameters of 30–200 nm.* ***Conclusions.*** *TFF combined with UC enables modulation of EV purity and yield. The serum-free culture significantly improves the biochemical purity and phenotypic specificity of EV isolates, making them more suitable for downstream applications. Optimization of filtration parameters remains critical to balance vesicle concentration and quality.*

Keywords: *extracellular vesicles, mesenchymal stem cells, serum-free cultivation, characteristics of extracellular vesicles, tangential flow filtration, exosome purity.*

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Introduction

Extracellular vesicles (EVs) are membrane-bound nanostructures secreted by cells into the extracellular space, which participate in the transport of biomolecules, intercellular communication, and the regulation of physiological and pathological processes [1, 2].

EVs contain proteins, lipids, microRNAs, mRNAs, and DNA, that can be transferred from donor cells to recipient cells. Due to these properties, vesicles are considered promising biomarkers and delivery systems for targeted therapeutic agents.

For analytical and therapeutic applications, EVs must be efficiently isolated with minimal contamination. Among the most common methods is ultracentrifugation (UC), which enables the precipitation of small EVs (30—150 nm) but is often accompanied by co-precipitation of protein aggregates [3, 4].

An alternative method is tangential flow filtration (TFF), which allows preliminary size-based fractionation and reduces mechanical damage to EVs. The use of membranes with different cut-off thresholds allows for control of protein contaminant levels [5, 6].

Other methods, such as size-exclusion chromatography (SEC), provide a high degree of purification but are labor-intensive and require specialized equipment [7, 8]. Microfluidic systems, which integrate separation, concentration, and EV analysis within a single device, are also gaining popularity [9—12].

Protein contamination is one of the main challenges in obtaining EV isolates. Contamination may arise from the culture medium, particularly fetal bovine serum (FBS), as well as due to specific isolation protocols [13]. The methods utilizing density gradients (e.g., OptiPrep) can reduce protein contamination through selective density-based separation [14, 15].

Current research focuses on developing hybrid approaches that combine the advantages of different isolation methods. Combining UC with SEC or TFF allows for high purity without significant losses in EV yield [16, 17].

The aim of this study was to compare the protein removal efficiency and EV quality obtained via UC and tangential flow filtration for the isolation of EVs from Human mesenchymal stem cells (MSCs), depending on the culture conditions and membrane separation parameters.

Materials and Methods

Cell Culture, Immunophenotyping, and Extracellular Vesicle Collection

Human mesenchymal stem cells (MSCs) were isolated from adipose tissue following enzymatic digestion with 0.2% type II collagenase (Sigma Aldrich). Donors provided written informed consent. Cells were cultured in DMEM/F12 (Sigma Aldrich) supplemented with 5% fetal bovine serum (FBS) (Sigma Aldrich), 10 ng/mL FGF2 (Biotech), and 1 × antibiotic/antimycotic (Sigma Aldrich) at 37 °C in 5% CO_2. Cells were passaged using 0.05% trypsin/EDTA, and MSCs at passage 3 were used for experiments with an initial cell seeding density of 1 million cells per T175cm^2 flask. The volume of medium in each flask was 25ml. For serum-free conditions, only FGF2 (10 ng/mL (Biotech)) and antibiotic/antimicotic were added to DMEM/F12. Cultures were maintained until reaching 85—90% confluency on 4-th day. Immunophenotyping of MSCs was performed by flow cytometry (CytoFLEX S, Beckman Coulter). The expression of CD73, CD90, and CD105, as well as the absence of CD45, CD34, and HLA-DR, was evaluated. Cells were detached with 0.05% trypsin/EDTA, centrifuged at 450 g for 10 min, counted, and incubated with antibodies (CD73 APC, CD90 FITC, CD105 V450; BD Pharmingen). Unbound antibodies were removed by centrifugation in 1 × PBS. A total of 5,000 events were recorded, and data were analyzed in CytExpert (Beckman Coulter) based on fluorescence intensity in FITC, APC, and V450 channels.

For extracellular vesicle (EV) collection, cells were washed twice with PBS (Biowest) and incubated for 7 days in either serum-containing or serum-free medium. The conditioned medium

was filtered through a 0.70 μm filter (Stainer) prior to EV isolation.

Methods for Extracellular Vesicle Isolation and Concentration

A total of 600 mL of both serum-containing (+FBS) and serum-free (−FBS) conditioned medium was collected. Three EV isolation methods were applied:

Ultracentrifugation (OPTIMA XPN80, Beckman Coulter) was performed as follows: 2,000 g for 10 min (supernatant collected), followed by 10,000 g for 10 min (supernatant collected). The supernatant was then ultracentrifuged at 100,000 g for 70 min. The resulting pellet was resuspended in 3 mL PBS for further analysis.

Tangential Flow Filtration (Millipore Labscale TFF System) was carried out via sequential filtration of the medium through 0.22 μm and 0.1 μm filter cassettes (Millipore Pellicon XL Cassette), separating fractions >0.22 μm, 0.1—0.22 μm, and <0.1 μm. The <0.1 μm fraction was subsequently passed through 300 kDa and 100 kDa membranes (Millipore Pellicon XL Cassette), yielding two fractions: >100 kDa and <100 kDa. This allowed EV separation based on both size and molecular weight.

Combined Method (TFF + UC): Conditioned medium was filtered through four cartridge combinations:

- 0.22 μm → 300 kDa
- 0.22 μm → 100 kDa
- 0.1 μm → 300 kDa
- 0.1 μm → 100 kDa

Each filtered fraction was further concentrated by ultracentrifugation at 100,000 g for 70 min. This approach enabled EV isolation with different degrees of purification and fractionation.

Quantification of Total Protein in EV Preparations

The protein content in EV isolates obtained by the three methods was determined using a Qubit™ fluorometer (ThermoFisher) with the Qubit™ Protein Assay Kit. Working solution was prepared at a 1 : 200 ratio; 190 μL of reagent and 10 μL of standard or sample (1—20 μL) were added to each tube, adjusting the final volume to 200 μL. After 2 min incubation at room temperature, fluorescence was measured and protein concentration was calculated based on a standard curve. Each analysis was performed in duplicate.

Determination of EV Concentration and Size by Flow Cytometry

EV analysis was performed with CytoFLEX Nano flow cytometer (Beckman Coulter). Samples were diluted 1 : 100 in sterile 10× PBS. Calibration was performed with Nanosphere™ particles (40—400 nm) and QC kits. Particles of 40—150 nm were analyzed in the VSSC1 channel, and those of 80—1000 nm in the VSSC2 channel. A 3 μL sample volume (1 min acquisition) was analyzed, and concentrations were normalized per 1 mL. EV subpopulations were identified with modal sizes of 40—50 nm, 60—150 nm, and 200—300 nm.

EV Surface Marker Profiling by Flow Cytometry

Expression of CD9, CD63, and CD81 was evaluated by flow cytometry on a CytoFLEX S (Beckman Coulter). EVs (1×10^9 particles) were incubated with latex beads (4 μm, 0.01%) in PBS in the presence of monoclonal antibodies: CD9-APC-A750, CD63-FITC, CD81-V450 (Beckman Coulter). Incubation was performed for 20 min at room temperature in the dark, in a total volume of 200 μL. The data were collected in three channels (FITC, V450, APC-A750), analyzing 10,000 events. Negative controls included beads without EVs and beads without antibodies. No nonspecific antibody binding was detected.

Morphological Assessment of EVs by Transmission Electron Microscopy (TEM)

The EV morphology and size were examined using TEM. Copper grids (0.1 mm) with Formvar

coating (SPI-Chem, UK) were used for sample preparation. A 30 μL aliquot of EV suspension was applied to the grids, incubated for 45 min in a humid chamber, and rinsed with cacodylate buffer containing glutaraldehyde. After vacuum drying, samples were fixed with 0.1% OsO_4 for 5 min, contrasted with 2% uranyl acetate for 40 min under humid conditions, and rinsed in phosphate-buffered saline (PBS) (5 × 10 min). Following final vacuum desiccation over NaOH, grids were examined according to standard TEM protocols.

Statistical test

Results are presented as mean ± SD from three independent experiments. Group comparisons were made using the two-tailed Student's t-test, with significance set at $P \leq 0.05$.

Results and Discussion

Fractional Composition of Extracellular Vesicles in Suspension

EVs were isolated by UC and by a combination of TFF with UC. For TFF, the membranes with molecular weight cut-offs of 100 kDa and 300 kDa were used.

In serum-free medium, the small vesicles measuring 40—60 nm, typical of exosomes, predominated. Using a 300 kDa cartridge, 40 nm particles accounted for 66%, whereas for the 100 kDa cartridge, they accounted for 55%.The particles larger than 200 nm represented a minimal fraction, indicating effective removal of aggregated structures and microvesicles.

In the presence of FBS, the total number of small EVs increased; however, the protein content in the isolates was higher, indicating the presence of exogenous vesicles. For instance, with UC in 5% FBS, 40 nm particles accounted for 55% and 60 nm particles for 33.9%. Cartridges with a 300 kDa cut-off provided better selection of small EVs due to reduced protein contamination. Membranes with a 100 kDa cut-off retained a broader spectrum of particles, including EVs > 150 nm. These results are consistent with literature reports [18, 19].

Comparison of methods showed that UC effectively concentrates small EVs but results in co-precipitation of proteins, particularly in serum-containing media. The combined TFF+UC approach, consistent with findings in [20], produces isolates with lower protein content, especially under serum-free conditions.

Thus, TFF with 100—300 kDa membranes optimizes the isolation of small EVs with a high degree of purity. Culture conditions, particularly the presence of FBS, affect the qualitative composition of fractions, which should be considered when selecting an isolation method.

Concentration of Extracellular Vesicles in Suspension

Two EV isolation methods — ultracentrifugation and tangential flow filtration with 100 kDa and 300 kDa membranes — were evaluated under three conditions: with fetal bovine serum (+FBS), without serum (–FBS), and in 5% FBS in saline (control) (Table 1).

Table 1. **Concentration of extracellular vesicles (EVs) under different isolation methods and condition**

Method	+FBS (EV/mL)	–FBS (EV/mL)	5% FBS in saline (EV/mL)
UC	7.5×10^9	5.4×10^9	2.9×10^{10}
TFF > 300 kDa	2.9×10^{10}	4.4×10^9	1.6×10^{10}
TFF < 300 kDa	6.8×10^7	7.0×10^7	2.0×10^7
TFF > 100 kDa	1.7×10^8	1.6×10^8	6.2×10^{10}
TFF < 100 kDa	8.0×10^7	5.4×10^7	1.8×10^8

With UC, EV concentrations were 7.5 × × 10^9 EV/mL (+FBS) and 5.4 × 10^9 EV/mL (−FBS). In the control sample (5% FBS), the concentration reached 2.9 × 10^{10} EV/mL, indicating the presence of exogenous vesicles in FBS.

With TFF using >300 kDa membranes, EV concentrations increased to 2.9 × 10^{10} EV/mL in +FBS, 4.4 × 10^9 EV/mL in −FBS, and 1.6 × 10^{10} EV/mL in the control, suggesting efficient removal of protein contaminants and accumulation of target vesicles.

In contrast, with TFF using <300 kDa membranes, concentrations were significantly lower: 6.8 × 10^7 EV/mL (+FBS), 7 × 10^7 EV/mL (−FBS), and 2 × 10^7 EV/mL (control). A similar trend was observed with > 100 kDa membranes-1.7 × × 10^8 EV/mL (+FBS), 1.6 × 10^8 EV/mL (−FBS) — but with a sharp increase to 6.2 × 10^{10} EV/mL in the control. For <100 kDa membranes, results were 8 × 10^7, 5.4 × 10^7, and 1.8 × 10^8 EV/mL, respectively.

These data demonstrate that the choice of membrane cut-off substantially affects the EV concentration and purity. The membranes >300 kDa facilitate the removal of large protein complexes, improving the purity and yield of small vesicles. The membranes <300 kDa, on the other hand, retain protein components, which may hinder efficient EV recovery.

These findings align with those of Nordin *et al.* (2015), who reported that 100 kDa cartridges effectively reduced protein contamination in EV isolates [22]. At the same time, ultracentrifugation ensures a high yield but does not eliminate exogenous EVs from FBS.

Therefore, optimization of TFF with consideration of membrane molecular cut-off is critical for obtaining highly purified and concentrated EV fractions, which is essential for the reliable biochemical and functional analyses.

Protein Concentration in EV Suspensions

The protein concentration in EV isolates varied significantly depending on the culture conditions and isolation method (Table 2). At ultracentrifugation in FBS-containing medium, the protein content was 4.2 mg/mL; in contrast, it was 0.2 mg/mL in serum-free medium. In the control sample (5% FBS in saline), the concentration was 1.7 mg/mL, indicating a substantial contribution of exogenous protein contaminants from the serum.

In serum-free media, UC yielded a minimal protein level (0.2 mg/mL), suggesting a purer isolate predominantly consisting of vesicles of cellular origin. Such isolates are potentially suitable for therapeutic applications, including intravenous administration.

Using TFF with >300 kDa membranes, protein concentrations in +FBS, −FBS, and 5% FBS samples reached 15.68 mg/mL, 0.44 mg/mL, and 18.8 mg/mL, respectively. Membranes with <300 kDa cut-off reduced protein levels to 0.25 mg/mL (+FBS), 0.18 mg/mL (−FBS), and 1.31 mg/mL (control). A similar trend was observed with >100 kDa membranes (up to 14.8 mg/mL in +FBS), whereas <100 kDa membranes resulted in only 0.09—0.54 mg/mL.

Table 2. **Protein concentration in EV isolates obtained under different conditions and isolation methods**

Method	+FBS (mg/mL)	−FBS (mg/mL)	5% FBS in saline (mg/mL)
UC	4.20	0.20	1.70
TFF > 300 kDa	15.68	0.44	18.80
TFF < 300 kDa	0.25	0.18	1.31
TFF > 100 kDa	14.80	0.43	23.20
TFF < 100 kDa	0.09	0.18	0.54

Thus, larger cut-off membranes (>300 kDa) produce higher protein concentrations in serum-containing media due to efficient removal of large protein aggregates (including albumin), thereby facilitating the accumulation of small vesicles. In serum-free media, they also provide acceptable isolate purity.

Smaller cut-off membranes (<300 kDa) promote additional removal of protein components, reducing contamination levels, but may also retain some target vesicles, thus lowering the overall yield.

These findings are consistent with reports [22, 19] demonstrating that 100 kDa membranes reduce protein contamination, while serum-free conditions yield isolates with a protein profile characteristic of intracellularly derived EVs [20].

Therefore, selecting the appropriate membrane cut-off in TFF is critical for obtaining highly concentrated and pure EV isolates. The membranes >300 kDa are particularly effective in serum-containing conditions, whereas in serum-free environments, the membranes <300 kDa are more appropriate. This approach ensures high-quality biomaterial suitable for further biochemical and functional analyses.

Transmission Electron Microscopy of Isolated EVs

Morphological analysis of EVs was performed using transmission electron microscopy (TEM). TEM micrographs of EVs obtained from mesenchymal stem cell (MSC) culture medium by ultracentrifugation (Fig. 1*a*) revealed a heterogeneous population of spherical vesicles ranging from 30 to 200 nm in diameter, corresponding to the size range of exosomes and microvesicles [23].

Most vesicles exhibited round or polygonal shapes with well-defined membranes. Some showed electron-dense shells, indicative of protein or lipid components, while their internal content was predominantly homogeneous. In several vesicles, electron-lucent areas or dense internal structures were observed, likely associated with ribonucleoprotein complexes [24—26].

TEM images of EVs isolated by tangential flow filtration (Fig. 1*b*) revealed structures with electron-lucent centers and varying membrane densities. Certain vesicles showed signs of partial degradation or hollow morphology. The absence of bilayered structures allowed these EVs to be distinguished from apoptotic bodies.

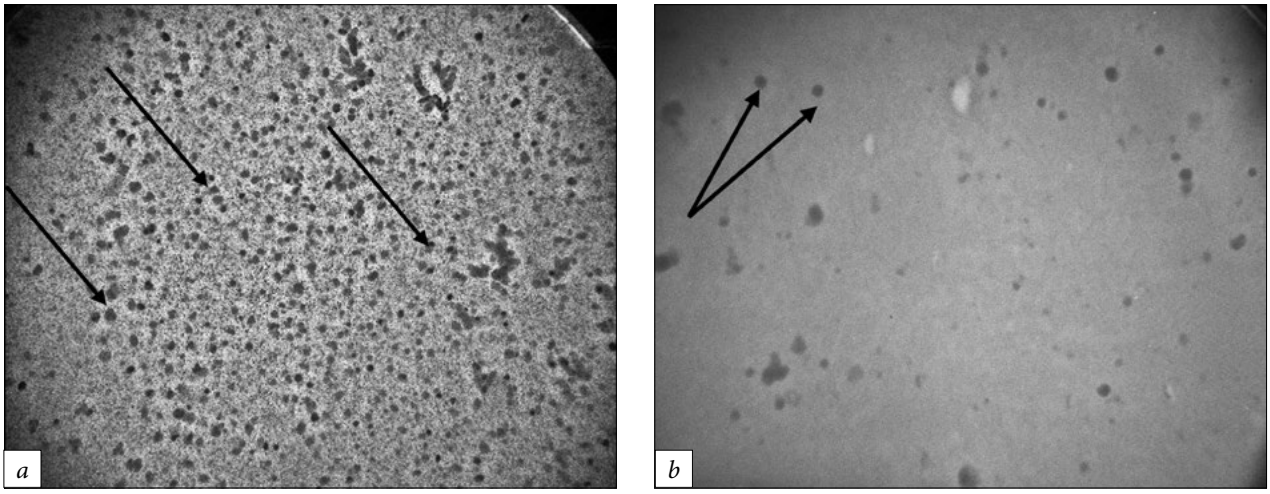

Fig. 1. Electron micrograph of Evs. *a* — EVs isolated from culture medium by ultracentrifugation. *b* — EVs isolated from culture medium by tangential flow filtration followed by ultracentrifugation

The morphological characteristics of isolates obtained by different methods are consistent with previous reports [14, 15] and confirm TEM as an effective tool for visualizing EV size, shape, and ultrastructure.

Expression of Tetraspanin Markers (CD9, CD63, CD81) and Their Ratios on Extracellular Vesicles

Tetraspanins CD9, CD63, and CD81 are key markers of EVs, particularly exosomes, and are involved in the vesicular transport and intercellular communication processes [28, 29].

In this study (Tabl. 3—5), CD9 expression ranged from 42.2% to 67.2%, reaching its maximum under serum-free conditions with TFF using <100 kDa membranes. The lowest values were observed with UC in the presence of FBS (45.1%), which likely reflects the influence of exogenous vesicles.

CD63 expression remained consistently high (81.1—98.4%) under all conditions, particularly in the presence of serum, where levels of 97.3—98.4% were recorded. This confirms the role of CD63 as a universal exosome marker and a reliable indicator of EV isolation efficiency [23].

Table 3. **Tetraspanin Expression in Extracellular Vesicles under Serum-Supplemented Conditions (+FBS)**

Method	CD9, %	CD63, %	CD81, %	CD9/CD63, %	CD9/CD81, %	CD63/CD81, %
UC + FBS	51,3	94,2	8,9	49,8	2,6	5,8
TFF > 300 kDa + FBS	47,8	87,6	7,9	43,0	2,0	4,9
TFF < 300 kDa + FBS	46,8	93,8	3,2	44,1	1,1	2,1
TFF > 100 kDa + FBS	45,3	81,1	3,9	43,6	1,4	2,4
TFF < 100 kDa + FBS	56,9	97,3	3,9	55,5	1,5	2,5

Table 4. **Tetraspanin Expression in Extracellular Vesicles under Serum-Free Conditions (–FBS)**

Method	CD9, %	CD63, %	CD81, %	CD9/CD63, %	CD9/CD81, %	CD63/CD81, %
UC –FBS	58,7	82,5	19,2	52,9	8,4	14,1
TFF > 300 kDa –FBS	52,6	90,9	6,7	48,9	1,5	4,0
TFF < 300 kDa –FBS	66,3	98,4	6,1	65,6	2,2	3,9
TFF >100 kDa –FBS	58,4	83,4	5,9	56,1	1,8	3,3
TFF < 100 kDa –FBS	67,2	98,1	5,6	66,6	1,9	3,3

Table 5. **Tetraspanin Expression in Extracellular Vesicles in 5% FBS Control Medium**

Method	CD9, %	CD63, %	CD81, %	CD9/CD63, %	CD9/CD81, %	CD63/CD81, %
UC 5% FBS	45,1	88,6	3,8	40,3	1,5	2,6
TFF > 300 kDa 5% FBS	42,2	83,2	3,5	35,6	1,2	2,4
TFF < 300 kDa 5% FBS	44,2	94,0	3,2	42,1	1,1	2,2
TFF > 100 kDa 5% FBS	43,9	81,4	3,8	35,3	1,8	2,9
TFF < 100 kDa 5% FBS	49,3	94,2	3,4	46,5	1,2	2,4

CD81 expression was lower, ranging from 3.2% to 19.2%, with the highest values found in UC isolates obtained under serum-free conditions. This may indicate the presence of EVs with an altered phenotype or the contribution of protein contaminants from serum.

These findings indicate that both the culture conditions and isolation method significantly influence tetraspanin expression. The samples obtained under serum-free conditions using TFF with low-cut-off membranes demonstrated the highest CD9 levels and CD9/CD63 ratios, suggesting a predominance of cell-specific exosomes.

Conclusions

The fractional distribution and quality of extracellular vesicles are strongly influenced by both the isolation method and the composition of culture medium. The serum-containing culture conditions led to a marked increase in EV concentration (up to 2.9×10^{10} particles/mL in 5% FBS) but were accompanied by substantial contamination with exogenous vesicles and protein impurities, complicating the recovery of specific cellular secretome.

In contrast, serum-free culture combined with TFF and UC yielded highly purified isolates with a low protein background (0.09—0.2 mg/mL for TFF <100 kDa) and a predominance of small exosomes (up to 66% of 40 nm particles with TFF 300 kDa). While TFF with >300 kDa membranes concentrated larger numbers of EVs, it also retained a higher protein content (up to 15.68 mg/mL in +FBS), necessitating additional purification steps.

Ultracentrifugation confirmed its effectiveness in concentrating small EVs but showed lower selectivity under the serum-containing conditions, where broad size distribution and high contamination levels were observed.

Morphological analysis by transmission electron microscopy confirmed the presence of characteristic spherical and polygonal vesicles with diameters ranging from 30 to 200 nm, consistent with exosomes and microvesicles. Immunophenotyping revealed high expression of the tetraspanins CD9, CD63, and CD81, supporting the exosomal nature of the isolates. The highest fluorescence intensity was detected for $CD63^+$ (81—98%), while the CD9/CD63 ratio (35—66%) provided a detailed assessment of vesicle phenotype.

Thus, the combination of serum-free culture, TFF with an optimal membrane cut-off (<300 kDa), and ultracentrifugation is the most effective approach for obtaining pure, high-quality, and phenotypically homogeneous exosome isolates suitable for further research and potential clinical applications.

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ПОРІВНЯЛЬНИЙ АНАЛІЗ МЕТОДІВ ОЧИЩЕННЯ СУСПЕНЗІЙ ПОЗАКЛІТИННИХ ВЕЗИКУЛ З КУЛЬТУРИ МЕЗЕНХІМАЛЬНИХ СТОВБУРОВИХ КЛІТИН ЛЮДИНИ

Мета. Оцінити вплив методів ізоляції та умов культивування на чистоту, вихід і фенотип позаклітинних везикул (ПВ), отриманих з мезенхімальних стромальних клітин людини. ***Методи.*** ПВ ізолювали методом тангенціальної фільтрації (ТФ) із мембранами молекулярно-масового порогу 100—300 kDa у поєднанні з ультрацентрифугуванням (УЦФ), за умов з додаванням сироватки та без неї. Концентрацію частинок і розподіл за розмірами (40—1000 нм) визначали методом проточної цитометрії, вміст білка — флуорометрично. Морфологію оцінювали за допомогою трансмісійної електронної мікроскопії, а експресію маркерів CD9, CD63 і CD81 — методом імунофенотипування. ***Результати.*** Сироваткові середовища сприяли вищому виходу ПВ, але супроводжувались значним білковим забрудненням. Найчистіші ізоляти (0,09—0,2 мг/мл білка) отримано у безсироваткових умовах із застосуванням ТФ < 100 kDa та УЦФ. За цих умов переважали $CD63^+$ екзосоми (до 98%). Електронна мікроскопія підтвердила характерну морфологію ПВ діаметром 30—200 нм. ***Висновки.*** Поєднання ТФ та УЦФ дозволяє регулювати чистоту та вихід ПВ. Безсироваткові умови культивування значно покращують біохімічну чистоту та фенотипову специфічність ізолятів, що робить їх більш придатними для подальших досліджень. Оптимізація параметрів фільтрації залишається ключовою для досягнення балансу між концентрацією та якістю ПВ.

***Ключові слова*:** позаклітинні везикули, мезенхімальні стовбурові клітини, безсироваткове культивування, характеристика позаклітинних везикул, тангенціальна фільтрація потоку, чистота позаклітинних везикул.