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**Y.M. Khmelnytska^{1, 2}, D.M. Pykhtieiev¹, A.S. Melnyk¹,
B.V. Yuriev¹, V.A. Kordium², M.I. Gumeniuk¹**

¹ Biotechnology Laboratory of the Bank of Umbilical Cord Blood,
Tissues and Other Human Cells BU «Reocell»
10, Amosova Str., Kyiv, Ukraine 03141

² Institute of Molecular Biology and Genetics, NAS of Ukraine
150, Akademika Zabolotnoho Str., Kyiv, Ukraine, 03143
yuliia.khmelnytska@reocell.com

A COMPARATIVE ANALYSIS OF EXTRACELLULAR VESICLE PRODUCTION BY MESENCHYMAL STEM CELLS OF DIFFERENT TISSUE ORIGINS UNDER SERUM-FREE CULTURE CONDITIONS

Introduction. Mesenchymal stem cells (MSCs) are a source of biologically active secretions containing extracellular vesicles (EVs) that possess regenerative and immunomodulatory potential, but their characteristics depend on MSC origin and culture conditions. **Methods.** MSCs derived from adipose tissue (AD-MSCs), umbilical cord (UC-MSCs), menstrual blood (EN-MSCs), and neural crest-derived tissues (NC-MSCs) were cultured in serum-free medium for 8 days. EV concentration and size were assessed by flow cytometry. Total protein, IL-6, TGF- β 1, VEGF, and M-CSF were measured in EV-depleted conditioned medium. CD9, CD63, and CD81 expression was assessed immunocytochemically. **Results.** All MSC cultures maintained high viability (>97%). UC-MSCs showed the highest EV production ($89,7 \times 10^9$ vesicles/mL; $4,62 \times 10^5$ vesicles/cell), followed by EN-MSCs, AD-MSCs, and NC-MSCs. EVs of 40–80 nm predominated in all samples. UC-MSCs showed elevated IL-6 and TGF- β 1 levels, whereas AD-MSCs showed higher VEGF and M-CSF levels. Total protein concentration did not correlate with the number of EVs. **Conclusions.** Growth factors produced by MSCs mediate paracrine effects, but their characteristics depend on the cell source. UC-MSCs are the most effective source for large-scale production of EVs, whereas AD-MSCs offer advantages for angiogenic and immunomodulatory applications. These results support rational selection of MSC sources depending on the intended EV application.

Keywords: mesenchymal stem cells, extracellular vesicles, serum-free culture, secretome

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Introduction

Mesenchymal stem cells (MSCs) are a promising tool in regenerative medicine due to their immunomodulatory, angiogenic, and reparative properties [1]. It has been established that the therapeutic effects of MSCs are primarily mediated through paracrine mechanisms, a key component of which is a secretory fraction containing cytokines, growth factors, and extracellular vesicles (EVs) [2, 3].

Particular attention is paid to EVs, which are considered a promising tool for cell-free therapy due to their ability to transport biologically active molecules that modulate the processes of angiogenesis, immune response, and tissue regeneration [4]. Unlike cell therapy, EVs are characterized by a higher safety profile and the possibility of standardizing their production [5–7].

It has been shown that the efficacy of EVs is determined by their molecular composition and the origin of the producer cells, which accounts for their functional heterogeneity even under similar culture conditions [8–10]. It has been shown that MSCs from adipose tissue, the umbilical cord, the endometrium, and neural crest-derived tissues differ in their secretory profile, including the production of growth factors, cytokines, and EVs [11, 12]. This determines their angiogenic, immunomodulatory, and regenerative potential [13–16].

Despite this, there is no clear consensus on which specific source of MSCs is optimal for obtaining stem cells with specific functional properties. An additional complicating factor is the influence of culture conditions, particularly the use of serum-free media, which helps prevent contamination but can induce cellular stress and alter the quantitative and qualitative characteristics of EVs as well as the secretory profile of cells [17–21].

The study aimed to conduct a comparative analysis of MSCs of different tissue origins based on their ability to produce EVs, to assess their size distribution, and to determine the characteristics of the secretory profile under serum-free culture conditions to justify the rational selection of an

MSC source for obtaining EVs with specified functional properties.

Materials and Methods

Isolation and culture of mesenchymal stem cells from adipose tissue, umbilical cord, menstrual blood, and neural crest-derived tissues

MSCs from adipose tissue (AD-MSCs), umbilical cord (UC-MSCs), menstrual blood (EN-MSCs), and neural crest-derived gingival tissue (NC-MSCs) were obtained from healthy donors aged 22–35 years with informed consent and tested for HIV 1/2, HBV, HCV, EBV, and syphilis. AD-MSCs and NC-MSCs were isolated by enzymatic disaggregation, UC-MSCs were isolated by explantation, and EN-MSCs were isolated using a modified protocol by Dalirfardouei *et al.* [22].

Cultivation of human mesenchymal stem cells

EN-MSCs were cultured in DMEM high glucose (Gibco), while AD-MSCs, UC-MSCs, and NC-MSCs were cultured in DMEM/F12 (Sigma-Aldrich) supplemented with 5% FBS (Sigma-Aldrich), 10 ng/mL FGF2 (BioTechne), and 1% Antibiotic/Antimycotic (Gibco) under standard conditions (37 °C, 5% CO₂). Passaging was performed at 80–90% confluence using 0.05% trypsin/EDTA.

At 3–4 passages, the cells were characterized by the phenotype CD73+, CD90+, CD105+ (Becton Dickinson); CD45-, CD34-, HLA-DR- (Beckman Coulter) using flow cytometry.

For the experiments, the cells from 5th passage were seeded into T175 flasks (5×10^6 cells/flask). After 24 hours of adhesion, the cells were washed twice with DPBS (Biowest) and cultured in serum-free IMDM (Sigma Aldrich) containing 14 ng/mL FGF2 for 8 days.

Cell viability was assessed by 7-AAD staining (Beckman Coulter) followed by flow cytometric analysis.

Isolation of extracellular vesicles

After 8 days of serum-free culture, the conditioned media were collected and centrifuged at $2,000\times g$ for 20 min at $+4\text{ }^{\circ}\text{C}$ to remove residual cells, cellular debris, and large cellular structures. The resulting supernatants were subsequently ultracentrifuged at $100,000\times g$ for 1 hour at $+4\text{ }^{\circ}\text{C}$. The EV-enriched pellets were collected and resuspended in DPBS for further analysis. The post-ultracentrifugation supernatants were retained as EV-depleted conditioned media (EV-depleted CM) and used for the analysis of soluble secretome components.

Quantification of total protein, growth factors, and IL-6 in the culture medium

The concentration of total protein in the EV-depleted conditioned medium was determined using the Lowry method according to the standard protocol [23]. The concentrations of TGF- β 1, VEGF, and M-CSF in the EV-depleted CM were determined by ELISA using commercial kits (Abcam) according to the manufacturer's instructions. The concentration of IL-6 was determined by chemiluminescent immunoassay (CLIA), which was performed in triplicate.

Immunocytochemical detection of membrane markers of EVs by MSCs

The expression of CD9, CD63, and CD81 (Invitrogen by Thermo Fisher Scientific) was assessed by immunocytochemistry using the corresponding primary antibodies and the PolyVue HRP/DAB Detection System (Diagnostic BioSystems) in accordance with the manufacturer's instructions. The proportion of positively stained cells was quantified by morphometric analysis using the Avtandilov point-counting method. Five independent biological samples were analyzed for each MSC culture. Microphotographs were obtained using a Leica DM2000 LED light microscope at $\times 400$ total magnification ($\times 40$ objective, $\times 10$ eyepiece).

Quantification and analysis of EVs concentration in the isolated suspension using a nano flow cytometer

EVs concentration and size were determined on a CytoFLEX Nano (Beckman Coulter) after calibration with Nanosphere™ standard particles (ThermoFisher brand) in the range of 40–600 nm. Samples were diluted in sterile PBS (1 $\times$) at a 1 : 100 ratio. EVs detection was performed in the 40–150 nm and 80–1000 nm ranges. EV size distribution was analyzed within the 40–400 nm range following calibration with standard nanoparticles. For comparative analysis, the predominant size classes (40–80 nm, 100–150 nm, and 200–400 nm) are presented in this study.

Determination of the receptor profile of EV using flow cytometry

Immunophenotyping of EVs for CD9, CD63, and CD81 was performed by flow cytometry DxFlex after immobilizing 1×10^9 EVs on 4- μm latex particles (0.01% in 1 $\times$ PBS). The samples were incubated with fluorophore-conjugated antibodies for 20 minutes at room temperature in the dark. A total of 10,000 events were recorded. The particles without EV and EV-immobilized particles without antibodies served as controls.

Phenotyping of EV by Western blot

EVs samples were denatured in Lemli buffer at $95\text{ }^{\circ}\text{C}$ for 5 min and separated on a 10% polyacrylamide gel. Proteins were transferred to a PVDF membrane (0.45 μm), blocked with 5% BSA, and incubated with primary antibodies against CD9, CD63, and CD81 (1 : 1,000) overnight at $4\text{ }^{\circ}\text{C}$. After incubation with the corresponding HRP-conjugated secondary antibodies (1 : 5,000), the signal was visualized using the ECL method on the ChemiPRO Imaging System.

Statistical Analysis

The results are presented as the mean $\pm$ standard deviation (mean $\pm$ SD) from at least three independent experiments. Statistical analysis was performed using one-way ANOVA and the t-test. Differences were considered statistically significant at $p \leq 0.05$.

Results

Characterization of MSCs

AD-MSCs, UC-MSCs, NC-MSCs, and EN-MSCs were adherent and, by the fifth passage, exhibited a typical fibroblast-like morphology with elongated cytoplasmic processes characteristic of MSCs. The MSC phenotype, regardless of the source from which the cells were isolated, was at least 96% for the markers CD73, CD90, and CD105, and no more than 3% for the markers CD45, CD34, and HLA-DR.

Immunocytochemical analysis of MSCs

Immunocytochemical analysis of tetraspanin marker expression revealed source-specific changes in their membrane expression after 8 days of serum-free culture (Table 1).

Serum-free culture and source of MSCs influence the expression of tetraspanins in MSCs. The most pronounced changes were observed in AD-MSCs and EN-MSCs, mainly due to a decrease in CD63 expression, while NC-MSCs maintained a stable CD9/CD63/CD81 profile. Changes in cellular tetraspanin expression may reflect adaptive remodeling of endosomal trafficking and EV biogenesis associated with cellular adaptation to serum-free culture [20, 24–28]. However, the underlying mechanisms were not investigated in this study. The pronounced decrease in CD63 expression observed in AD-MSCs after serum-free culture may reflect remodeling of the endosomal compartment during adaptation to nutrient deprivation rather than a reduction in EV production. Since EV yield increased despite lower cellular

CD63 expression, cellular tetraspanin levels should not be interpreted as direct indicators of vesicle secretion. Thus, serum-free culture serves not only as a technical approach for purifying the secretome from serum impurities but also as a functional stimulus for the biogenesis of EVs. Serum-free culture induced source-specific changes in MSC tetraspanin expression, reflecting cellular adaptation rather than EV-producing activity.

Phenotypic characterization of isolated extracellular vesicles

Immunophenotyping by flow cytometry using latex particles revealed the presence of the characteristic tetraspanin markers CD9, CD63, and CD81 in the EVs of all cultures studied (Fig. 1).

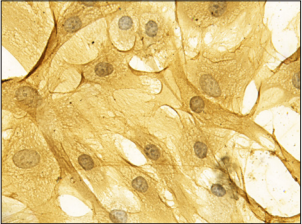
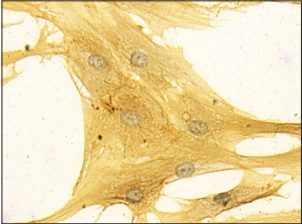
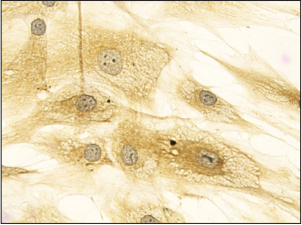
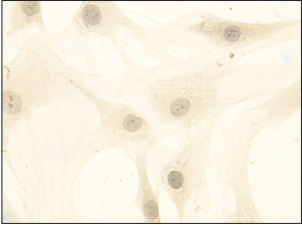
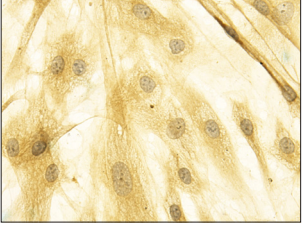
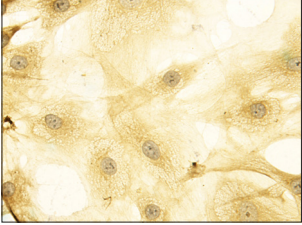
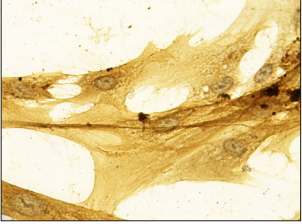
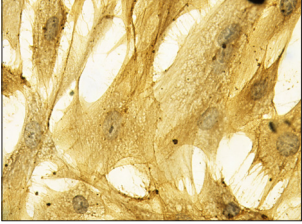
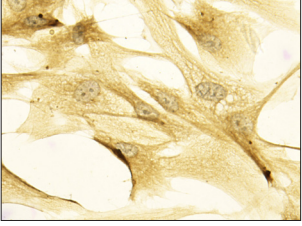
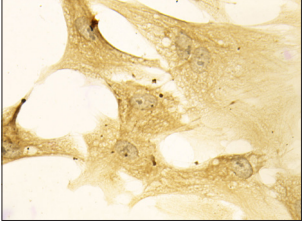
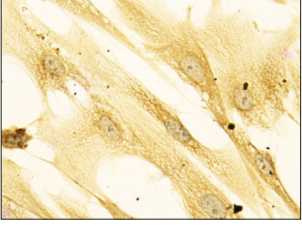
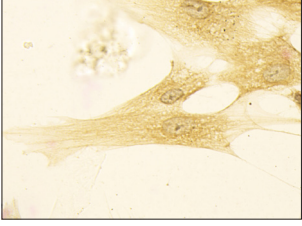
The presence of these markers in the isolated fractions was further confirmed by Western blot analysis (Fig. 2). Taken together with the determined size distribution, the results confirm that the isolated particles correspond to the characteristics of extracellular vesicles.

Concentration and fractional distribution of EVs in the EV-depleted conditioned medium

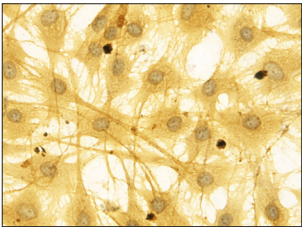
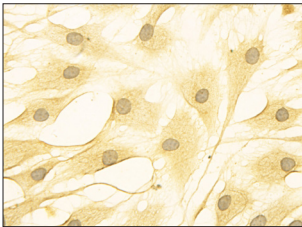
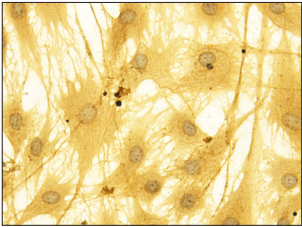
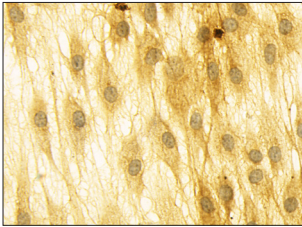
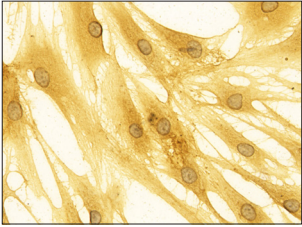
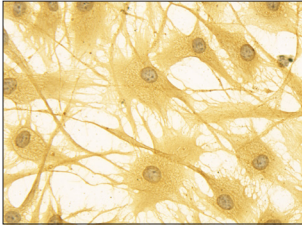
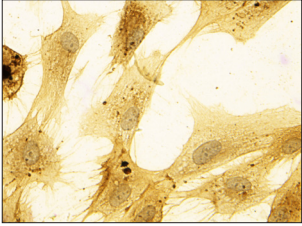
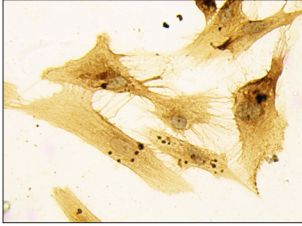
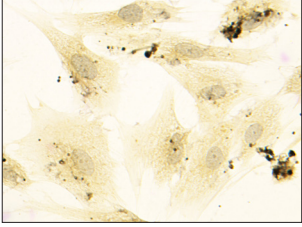
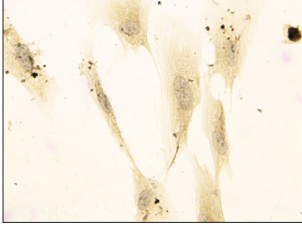
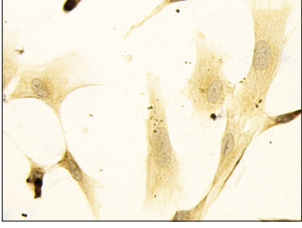
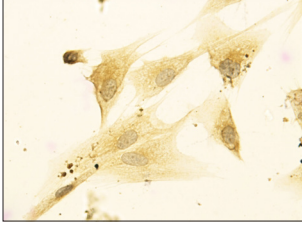
During serum-free culture, a difference in the secretory activity of extracellular vesicles in the EV-depleted CM was observed on day 8, depending on the source of MSC. The highest concentration of EV in the culture supernatant was observed for UC-MSCs — $89,7 \pm 4,2 \times 10^9$ EVs—which was nearly twice the EV production compared to EN-MSCs — $42,6 \pm 1,4 \times 10^9$ EVs. The lowest EV concentration was observed in AD-MSCs and NC-MSCs, at $20,5 \pm 2,0 \times 10^9$ EVs and $14,4 \pm 1,1 \times 10^9$ EVs, respectively, which may indicate lower EV secretory activity by these cells under serum-free culture conditions.

UC-MSCs were characterized by the highest EVs production both per unit volume of EV-depleted CM and per cell, which is consistent with the data on increased secretory activity of

Table 1. Immunocytochemical analysis of CD9, CD63, and CD81 expression in MSCs of different tissue origins before and after 8 days of serum-free culture

Cell culture	CD Marker	Day 0, %		Day 8, %	
AD-MSC	CD9		100%		99%
	CD63		88%		25%
	CD81		62%		64%
UC-MSC	CD9		98%		72%
	CD63		79%		78%
	CD81		86%		55%

End of Table 1

Cell culture	CD Marker	Day 0, %		Day 8, %	
NC-MSC	CD9		98%		94%
	CD63		92%		88%
	CD81		96%		95%
	CD9		95%		92%
	CD63		68%		44%
	CD81		60%		50%
EN-MSC					

Notes: Representative immunocytochemical images of CD9, CD63, and CD81 expression. HRP/DAB staining, original magnification ×400.

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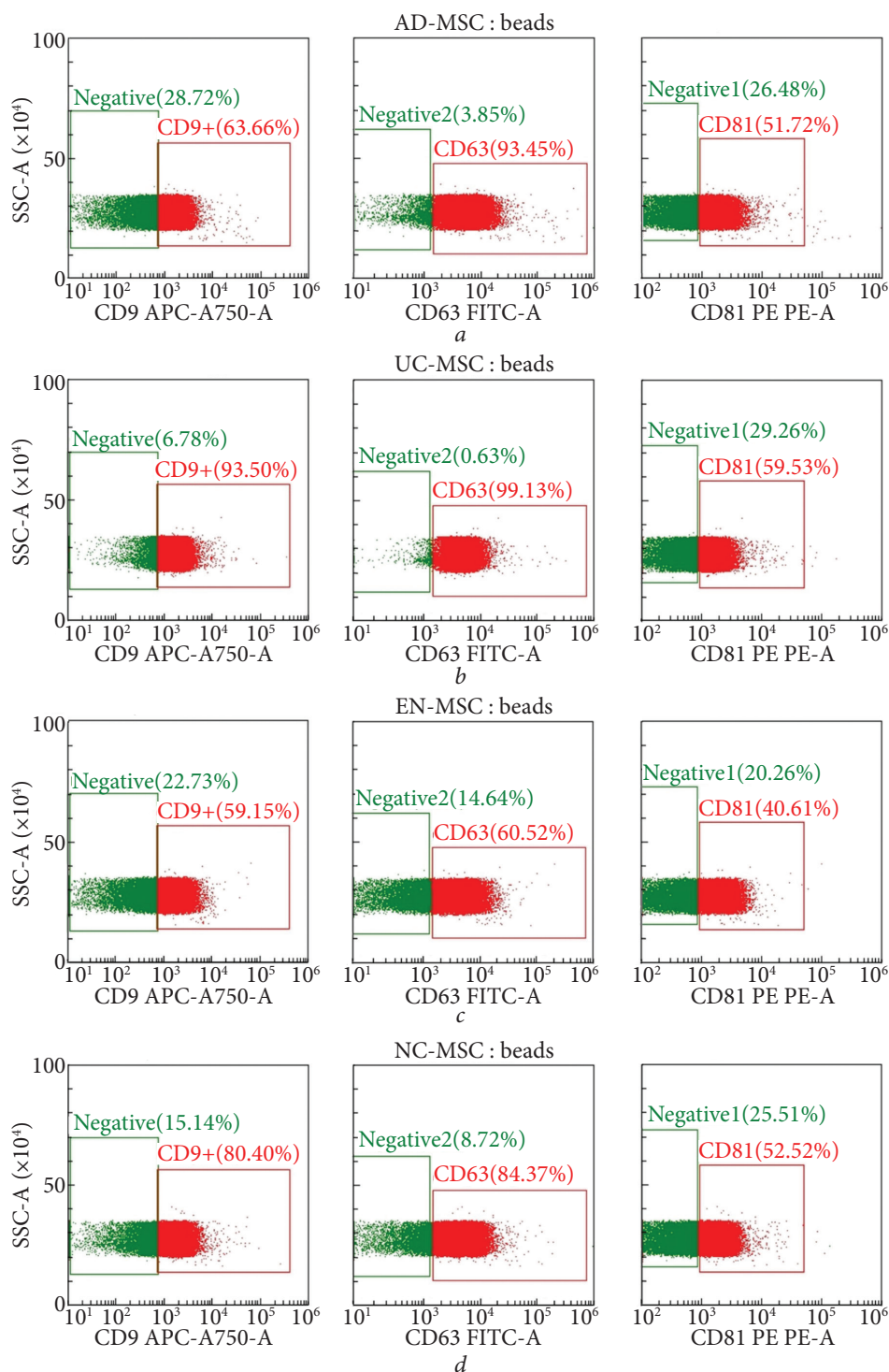


Fig. 1. CD9, CD63, and CD81 expression in MSC-derived extracellular vesicles assessed by bead-based flow cytometry. *a* — AD-MSCs; *b* — UC-MSCs; *c* — EN-MSCs; *d* — NC-MSCs. The population of positive events is marked in red, and the negative control is marked in green

perinatal MSCs [29—31]. All cultures maintained high viability (>97%), indicating that differences in EV production were associated with source-specific secretory activity rather than cell death [29—31].

On day 8 of culture, the vesicle yield per cell was $4,6 \times 10^5$ vesicles/cell for UC-MSCs, $2,9 \times 10^5$ EV/cell for EN-MSCs, $1,5 \times 10^5$ EV/cell for AD-MSCs, and $1,0 \times 10^5$ EV/cell, confirming the highest vesicle-producing activity of UC-MSCs regardless of the cell concentration in the culture.

Analysis of EV size (Table 2) showed that the fraction of small EVs (40—80 nm) was predominant in all MSC cultures, while the fraction of larger vesicles was minimal. UC-MSCs showed a higher selection of medium-sized EVs, which may reflect source-dependent differences in EV secretion [30, 32].

Protein concentration in EV-depleted conditioned medium from MSCs of various sources

Total protein concentration in EV-depleted CM remained stable among all MSC sources (0.75—0.80 $\mu\text{g/mL}$), indicating that increased EV production was not directly related to total protein secretion, consistent with previous studies of the MSC secretome [33, 35, 36]. This observation is consistent with our previous data showing that EV purification methods and serum-derived protein components can markedly influence the apparent protein content of EV preparations [34].

Concentrations of pro-inflammatory cytokines and growth factors

To assess the pro-inflammatory activity of EV-depleted CM under serum-free culture conditions, the concentration of the pro-inflammatory cytokine IL-6 was measured. In the control sample, which was a freshly prepared serum-free medium, the IL-6 level was <2.5 pg/ml, confirming the absence of exogenous sources of the cytokine (Fig. 3, Table 3).

IL-6 secretion was dependent on MSC origin, with the highest level in UC-MSC conditioned medium [37]. The marked difference in IL-6 secretion between UC-MSCs and NC-MSCs may reflect tissue-specific characteristics of the cells and their different responses to prolonged serum-free culture. UC-MSCs are characterized by high paracrine activity, whereas gingiva-derived NC-MSCs may exhibit a less pronounced inflammatory secretory response under the same conditions. However, donor-specific variability

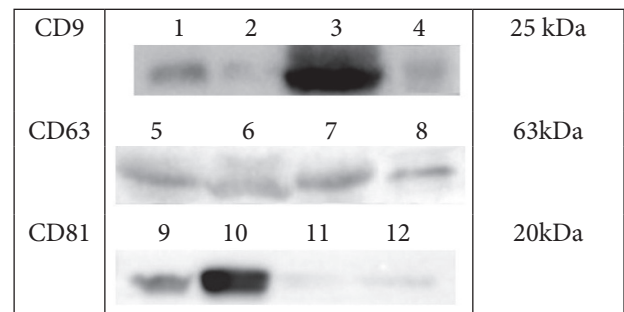


Fig. 2. Western blot analysis of CD9, CD63, and CD81 in MSC-derived EVs. Lanes 1, 5, 9 — AD-MSC; 2, 6, 10 — UC-MSC; 3, 7, 11 — EN-MSC; 4, 8, 12 — NC-MSC

Table 2. Distribution of size classes of extracellular vesicles secreted by MSCs of different origins

Modal classes	AD-MSC, %	UC-MSC, %	NC-MSC, %	EN-MSC, %
40—80 nm (Mean $\pm$ SD)	$85 \pm 0,5$	$75,8 \pm 0,8$	$84,9 \pm 0,9$	$87,6 \pm 0,6$
100—150 nm (Mean $\pm$ SD)	$1,56 \pm 0,3$	$2,7 \pm 0,5$	$2,1 \pm 0,4$	$0,7 \pm 0,2$
200—400 nm (Mean $\pm$ SD)	$0,02 \pm 0,01$	$0,05 \pm 0,02$	$0,05 \pm 0,02$	$0,01 \pm 0,005$

Notes: Only the predominant EV size classes are presented. Intermediate size ranges (80—100 nm and 150—200 nm) are not shown; therefore, the percentages do not sum to 100%.

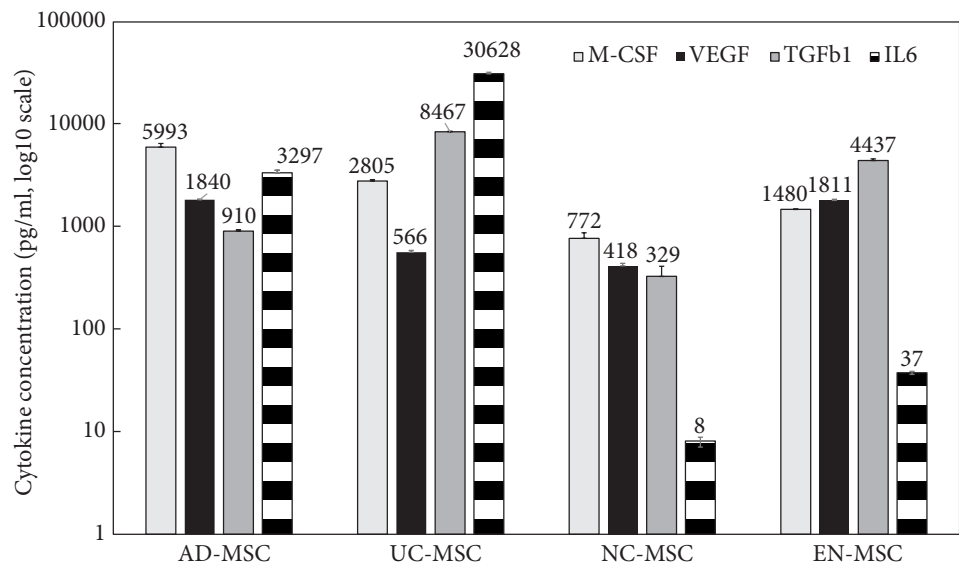


Fig. 3. Concentrations of cytokines and growth factors secreted by MSCs from different tissue sources after 8 days of serum-free culture. IL-6, TGF-β1, VEGF, and M-CSF concentrations were determined in EV-depleted conditioned medium from AD-MSCs, UC-MSCs, NC-MSCs, and EN-MSCs. TGF-β1, VEGF, and M-CSF concentrations were determined by ELISA, whereas IL-6 concentration was determined by CLIA. Data are presented as mean ± SD (n = 3 independent experiments). The y-axis is shown on a logarithmic (log10) scale

Table 3. Concentrations of cytokines and growth factors in EV-depleted conditioned medium from MSCs of different tissue origins after 8 days of serum-free culture

Cell culture	IL-6, pg/mL (Mean ± SD)	TGF-β1, pg/mL (Mean ± SD)	VEGF, pg/mL (Mean ± SD)	M-CSF, pg/mL (Mean ± SD)
AD-MSC	3297 ± 220	910 ± 28,38	1840 ± 26,92	5993 ± 512,76
UC-MSC	30628 ± 1027	8467 ± 110,70	566,3 ± 24,11	2805 ± 82,12
NC-MSC	8,04 ± 0,61	329 ± 83,06	418 ± 45,04	772 ± 17,73
EN-MSC	36,81 ± 1,42	4437 ± 178,88	1811 ± 22,31	1480 ± 102,04

cannot be excluded and should be considered when interpreting these results.

The concentrations of the growth factors TGF-β1, VEGF, and M-CSF in MSC culture supernatants were also analyzed on day 8 of serum-free culture (Table 3).

Growth factor analysis showed source-dependent differences in the secretory profiles of MSCs. UC-MSCs exhibited increased TGF-β1 secretion, whereas AD-MSCs showed higher VEGF and M-CSF levels. These findings suggest

a potentially pro-angiogenic and macrophage-modulating secretory profile; however, functional assays are required to confirm these biological activities [39—42].

Conclusions

MSCs from different tissue sources retained a typical phenotype and high viability under serum-free conditions, confirming their suitability for standardized EV production.

UC-MSCs demonstrated the highest EV production among all investigated MSC sources. Their conditioned medium also contained elevated levels of IL-6 and TGF- β 1, indicating a distinct and highly active secretory profile under serum-free conditions.

AD-MSCs demonstrated moderate EVs production but were characterized by the most pronounced angiogenic and macrophage-modulating profile, as evidenced by high concentrations of VEGF and M-CSF.

EN-MSCs produced a moderate amount of EVs with a balanced growth factor profile, whereas

NC-MSCs were characterized by the lowest secretory activity.

Regardless of the MSC source, the main population of EVs consisted of vesicles 40–80 nm in size.

The results obtained confirm the advisability of making a rational choice of MSC source depending on the intended application of the vesicles. UC-MSCs can be considered a highly productive source of EVs for further regenerative applications, whereas the elevated VEGF and M-CSF levels observed in AD-MSC conditioned medium warrant further functional evaluation of their angiogenic potential.

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Ю.М. Хмельницька^{1,2}, Д.М. Пихтєєв¹, А.С. Мельник¹,
Б.В. Юр'єв¹, В.А. Кордюм², М.І. Гуменюк¹

¹ Біотехнологічна лабораторія Банку пуповинної крові,
тканин та інших клітин людини БЮ «Реоцелл»
вул. Амосова, 10, Київ, Україна, 03141

² Інститут молекулярної біології і генетики НАН України
150, вул. Академіка Заболотного, Київ, Україна, 03143
yuliia.khmelnyska@reocell.com

ПОРІВНЯЛЬНИЙ АНАЛІЗ ПРОДУКЦІЇ ПОЗАКЛІТИННИХ ВЕЗИКУЛ МЕЗЕНХІМАЛЬНИМИ СТОВБУРОВИМИ КЛІТИНАМИ РІЗНОГО ТКАНИННОГО ПОХОДЖЕННЯ В УМОВАХ БЕЗСИРОВАТКОВОГО КУЛЬТИВУВАННЯ

Вступ. Мезенхімальні стовбурові клітини (МСК) є джерелом біологічно активного секретому з позаклітинними везикулами (ПВ), які мають регенеративний та імуномодуючий потенціал. Проте їх характеристики залежать від походження МСК та умов культивування. **Методи.** МСК, отримані з жирової тканини (AD-MSC), пуповинного канатика (UC-MSC), менструальної крові (EN-MSC) та тканин похідних нервового гребня (NC-MSC) культивували в безсироваткових умовах протягом 8 діб. Концентрацію та розмір ПВ визначали методом проточної цитометрії. Загальний білок, IL-6, TGF- β 1, VEGF та M-CSF визначали в кондиціонованому середовищі, збідненому на ПВ. Експресію CD9, CD63 та CD81 визначали імуноцитохімічно. **Результати.** Усі культури МСК зберігали високу життєздатність (>97%). Найвищу продукцію ПВ продемонстрували UC-MSC ($89,7 \times 10^9$ ПВ/мл; $4,62 \times 10^5$ ПВ/клітину), далі EN-MSC, AD-MSC та NC-MSC. У всіх зразках переважали ПВ розміром 40–80 нм. UC-MSC характеризувалися підвищеними рівнями IL-6 та TGF- β 1, тоді як AD-MSC — вищими рівнями VEGF та M-CSF. Концентрація загального білка не корелювала з кількістю ПВ. **Висновки.** ПВ, що продукуються МСК, опосередковують їх паракринні ефекти, однак їх характеристики залежать від джерела клітин. UC-MSC є найбільш ефективним джерелом для масштабного отримання ПВ, тоді як AD-MSC мають переваги для ангіогенних та імуномодуючих застосувань. Отримані результати обґрунтовують раціональний вибір джерела МСК залежно від цільового застосування ПВ.

Ключові слова: мезенхімальні стовбурові клітини, позаклітинні везикули, безсироваткове культивування, секретом.