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Optimization of conditions for the production of small extracellular vesicles from human umbilical cord-derived mesenchymal stem cells



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ABSTRACT

Extracellular vesicles (EVs), derived from mesenchymal stem cells (MSCs), play crucial roles in the physiological functions of MSCs and their therapeutic effects. Compared with cellular products, the composition of EVs, their biological properties, mechanisms of action, and superior stability make them attractive candidates for regenerative medicine, heralding a new era in cell therapy.

AIM – to identify optimal conditions for obtaining small EVs from cultured human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) based on quantitative indicators of EVs yield and the morpho-functional characteristics of MSC cultures depending on cultivation conditions.

MATERIALS AND METHODS. The MSCs were isolated from the human umbilical cord through enzymatic digestion. The process of optimizing the cultivation medium was conducted as follows. The first ways – culture medium was replaced with fresh medium and maintained for the subsequent 24 hours. The second ways – culture continued in the unaltered medium. Then they were changed to two media: Hank's solution and MEM alpha medium, both without devoid of xenogeneic serum and exogenous growth factors. Cultivation and extracellular vesicles production from hUC-MSCs under serum-free conditions were carried out for 24, 48, and 72 hours. All vesicles were examined to confirm their specific size distribution, determined by nanoparticle tracking analysis (NTA), and the presence of CD63 and CD81 markers, as assessed by ELISA.

RESULTS. Replacing the growth medium in the hUC-MSC culture 24 hours before switching to serum-free medium led to a significantly higher yield of extracellular vesicles. Upon transitioning the culture to serum-free medium, we identified a dependency on both the composition of the serum-free medium and the duration of MSC cultivation in these conditions. The highest yield of extracellular vesicles was observed after 48 and 72 hours of cultivation, with no significant difference observed between the serum-free medium compositions. We attribute this result to the adaptation period of the culture during the first 24 hours of serum-free cultivation. Given that no significant difference in extracellular vesicle yield was found between 48 and 72 hours, we propose 48 hours as the optimal cultivation time.

CONCLUSION. The findings of this study indicate that the optimal conditions for the cultivation of umbilical cord mesenchymal stem cells for the production of small extracellular vesicles in serum-free medium are as follows: culturing hUC-MSCs in complete growth medium with regular medium exchanges every 2-3 days until the culture achieves 70 % confluence; replacing the growth medium 24 hours prior to transitioning to serum-free medium; cultivating the hUC-MSC culture for 48 hours in Hank's solution or MEM, without the addition of xenogeneic serum.

KEY WORDS: mesenchymal stem cells; extracellular vesicles; exosomes; serum-free medium.

The therapeutic effect of cultured mesenchymal stem cells (MSCs) arises from their ability to differentiate into the appropriate tissue type and directly stimulate the regeneration of damaged tissue after transplantation [1-5]. Transplanted MSCs colonize the damaged tissue and

differentiate into the corresponding cell types capable of restoring the damage [6-10]. Although *in vitro* differentiation along various lineages is a fundamental feature of MSCs, such differentiation *in vitro* does not reflect their mechanism of action *in vivo* [11-15]. Several studies have

demonstrated the long-term presence of differentiated MSC-derived cell types *in situ* [16-19]. Therefore, another mechanism may be involved in tissue repair processes via MSCs. The paracrine effects of a large number of biologically active molecules and cytokines produced by MSCs may explain the functional benefits observed in animal models and in the treatment of patients [20]. MSCs release a variety of cytokines, growth factors, chemokines, and microvesicles, which can influence tissue healing [21-23]. These mechanisms have attracted considerable interest in recent years but remain not fully understood.

The extracellular vesicles (EVs) obtained from MSCs are of particular interest [23]. EVs retain all the therapeutic effects of MSCs, including tissue regeneration, wound healing, and immunomodulation. It has been shown that MSC-derived EVs reduce apoptosis by stimulating tissue repair, promote the reduction of fibrotic tissue, induce cell proliferation, new blood vessel formation, suppress inflammatory processes, and provide long-term neuroprotection. Furthermore, by loading EVs with target molecules, it is possible to enhance their efficacy and directed action.

Animal-derived sera are widely used in cell biology and biotechnology and are typically included in culture media, as they are a source of biologically active molecules necessary for cell metabolism and maintaining normal viability *in vitro* [24-28]. However, there is a global trend toward phasing out such additives due to the variability in composition and concentration of molecules across different manufacturers and reagent lots [29-33]. Moreover, sera are a source of xenogeneic substances of various types (especially protein molecules), which limits their use in pharmaceutical manufacturing [34-37]. Another significant issue is the considerable variability in experimental results when protocols containing xenogeneic additives are used [38-42]. Therefore, the development and implementation of alternative protocols for working with MSCs in modified media compositions is relevant and requires further study.

THE AIM of this study was to determine the optimal conditions for obtaining exosome-containing products from cultured human umbilical cord-derived MSCs (hUC-MSCs) based on quantitative indicators of exosome yield and the morpho-functional characteristics of MSC cultures depending on cultivation conditions. To this end, it was determined whether the yield of extracellular vesicles depends on changes in the nutrient growth medium, the composition of serum-free medium, and the duration of MSC cultivation in serum-free conditions.

MATERIALS AND METHODS

The experiments with the use of human cell culture *in vitro* were carried out in accordance with the human experiment issues of the Code of Ethics of the World Medical Association (Declaration of Helsinki). In all cases, voluntary informed consent was signed by MSC donors in accordance with the laws of Ukraine. The study protocol was approved by the Bioethics Committee of the Institute of Genetic and Regenerative Medicine, M. D. Strazhesko National Scientific Center of Cardiology, Clinical and Regenerative Medicine, National Academy of Medical Sciences of Ukraine (Kyiv, Ukraine). The cell culture was carried out at the biotechnological laboratory Medical Company "Good Cells" (Kyiv, Ukraine) according to License to operate the banks of human cord blood, other tissues and cells; issued by the Ministry of Health of Ukraine AE No. 2088, dated 11.09.2020, and No. 2214, dated 29.09.2020. hUC-MSCs samples (n = 6) were obtained from healthy donors (3 females and 3 males) with normal somatometric and biochemical parameters without viral or microbial infection.

Cell isolation and culture. MSCs were isolated as previously described [43-46]. hUC-MSCs were isolated from umbilical cord tissues through enzymatic digestion with 0.1 % collagenase and 0.1 % pronase in 2 % fetal bovine serum (FBS) (all – *Sigma-Aldrich*, USA) for 1 hour at 37 °C with constant agitation. All the debris and connective tissue were separated

from cells by centrifugation (600 ×g, 5 min) using a centrifuge (*Eppendorf*, Germany). hUC-MSCs were seeded with a density of 1000 cells per 1 cm². After being washed, cells were cultivated up to 3 passages in the growth medium containing basal α-MEM supplemented with 10 % FBS, 2 mM L-glutamine, 1 % antibiotic-antimycotic solution, and 1 ng/mL bFGF (all – *Sigma-Aldrich*, USA). Cells were cultured in a multi-gas incubator CB210 (*Binder*, Germany) at 37 °C in a humidified atmosphere with 5 % CO₂ and 5 % O₂. Biological properties, morphological changes, population doubling time, viability, migratory capacity, and proliferation rate of six cell lines at passages 1-3 were assessed.

Optimization of cultivation medium selection. The process of optimizing the cultivation medium was conducted as follows:

Upon reaching 70-80 % confluence of the MSC culture at passage 2:

A) The culture medium was replaced with fresh medium and maintained for the subsequent 24 hours.

B) The culture continued in the unaltered medium.

Once the MSC culture achieved 90 % confluence, the growth medium was aspirated, and the cells were washed twice with a buffered solution to remove serum residues and cellular debris.

Then, 25 mL of the following media was added:

A) Hank's solution (*BioWest*, France), devoid of xenogeneic serum and exogenous growth factors, in culture flasks.

B) MEM alpha medium (*Thermo Fisher Scientific*, USA), without phenol red, xenogeneic serum, or exogenous growth factors, in culture flasks.

Cultivation and extracellular vesicles production from hUC-MSCs under serum-free conditions were carried out for 24, 48, and 72 hours.

Colony-forming unit assay. A detailed description of the method was presented in previous studies [43, 44]. To assess clonogenic potential, cells were seeded at a cell seeding density of 100 cells per ø100 mm gelatin-coated Petri dishes (*SPL*, Korea) in growth medium (as described above) supplemented with 20 % FBS and cultured for 14 days. The cell colonies were fixed for 20 min with 96 % ethanol, washed with PBS, and stained with azure-eosin by Romanowsky-Giemsa (all: *Makrokhem*, Ukraine) for 20 min.

Colony formation efficiency (or plating efficiency, PE) was calculated according to the standard formula [47]:

$$PE, \% = (\text{no. of colonies counted} / \text{no. of cells inoculated}) \times 100$$

The cell population doubling time (PDT) were calculated according to the following standard formula [47]:

$$PDT = T / 3.31 \times \lg (X_k / X_0);$$

where X_k is the number of obtained cells; X_0 is the number of plated cells; T is the cell culture time.

Flow cytometry analysis of cell surface marker expression. A detailed description of the method was presented in previous studies [43-46]. The cell phenotype was assessed by fluorescence-activated cell sorting on BD FACSAria flow cytometer (*Becton Dickinson*, USA). Staining with monoclonal antibodies (PerCP-Cy5.5 mouse anti-human CD105, APC mouse anti-human CD73, FITC mouse anti-human CD90, PE-Cy5 mouse anti-human HLA-DR, APC mouse anti-human CD34, FITC mouse anti-human CD45) in accordance to manufacturer's instructions (*BD Pharmingen*, USA). The obtained data were analyzed using BD CellQuest software (*BD*, USA).

Directed multilineage differentiation assay. The protocol of directed multilineage differentiation assay was described earlier in details [43-46]. Briefly, cells were seeded on the 6-well plate (*SPL*, Republic of Korea) at the seeding density of 50×10³ cells per well. After 24 hours of cultivation, the quality of the culture was assessed based on adhesion to plastic and fibroblast-like morphology. The growth medium was changed every 2-3 days until the culture reached 90 % confluency. After this the medium was replaced either with growth medium or with induction medium for directed differentiation.

Osteogenic differentiation was induced by culturing in basal α -MEM medium supplemented with 10 % FBS, 100 nM dexamethasone, 10 mM β -glycerophosphate, 50 μ g/mL ascorbate-2-phosphate (all Sigma, USA) and 1 % antibiotic/antimycotic. Adipogenic differentiation was induced by culturing in high-glucose DMEM medium (4.5 g/L) (*BioWest*, France) supplemented with 10 % FBS, 1 μ M dexamethasone, 200 μ M indomethacin, 500 μ M isobutylmethylxanthine, 5 μ g/mL insulin (all from *Sigma-Aldrich*, USA), 5 % horse serum (*BioWest*, USA) and 1 % antibiotic/antimycotic solution. The medium was changed every 2 days for 21 days. Adipogenic and osteogenic differentiation assays were carried out on collagen substrate.

To confirm the osteogenic and adipogenic differentiation, the cells were fixed for 20 min in 4 % formalin (*Makrokhem*, Ukraine), washed with PBS (*Sigma-Aldrich*, USA), and stained for 20 min with 2 % solution of Alizarin Red S (pH 4.1; for detecting calcified extracellular matrix deposits) or 0.5 % solution of Oil Red O (for staining of neutral lipids). Cell cultures were photographed with phase contrast during the cultivation of cells at points of change in the culture medium. Assessment of the level of cytochemical detection of the efficiency of adipo- and osteogenic differentiation was carried out by visual control in transmitted light. Cultures were visualized and photographed using an Axio ObserverA1 microscope, an Axio Cam ERc 5s camera, and ZEN 2012 software (all – *Carl Zeiss*, Germany).

Isolation of EVs. When the cell cultures reached 70 % confluency, they were washed with phosphate-buffered saline (PBS) to eliminate residual serum and cellular debris. Subsequently, 25 mL of induction medium without phenol red (*Gibco*, UK) and devoid of xenogenic serum and exogenous growth factors were added. The MSCs were cultured for an additional 24, 48 and 72 hours. The conditioned medium, containing secreted growth factors and EVs, was carefully collected and subjected to centrifugation at 3000 \times g for 15 minutes to remove residual cell debris and apoptotic bodies. An 5810R centrifuge was used (*Eppendorf*, Germany). Following centrifugation, the supernatant was filtered through a 0.22 μ m filter (*Sarstedt*, Germany) to ensure the removal of any remaining larger particles. To further purify the EVs, the filtered medium was ultracentrifuged at 100,000 \times g for 90 minutes using ultracentrifuge Avanti JXN-30 (*Beckman Coulter*, USA). The pellet, containing the EVs, is then resuspended in Hank's solution (*Biowest*, France) for downstream applications. The isolated EVs were characterized via nanoparticle tracking analysis (NTA) and the Pierce BCA protein assay as described below to confirm their identity and purity.

Nanoparticle tracking analysis. Nanoparticle tracking analysis (NTA) was applied to determine particle size and concentration of all samples using the NanoSight LM10 instrument (*Malvern Instruments Ltd*, UK) equipped with NTA 3.0 analytical software and an additional 488 nm laser. The samples were diluted in Hank's solution (*Biowest*, France) to an appropriate concentration before being analysed. At least five 60 s videos were recorded per sample in light scatter mode with a camera level of 13-16. Software settings were kept constant for all EV measurements (screen gain 3-10, detection threshold 2-5).

Protein quantification. Protein concentration in EV samples was measured using Pierce BCA Protein Assay Kit (*Thermo Fisher Scientific*, USA). The assay was performed according to the manufacturer's instructions in the working range 5-250 μ g/mL using standard protocol.

ELISA immunoassay. A detailed description of the method was presented in previous studies [43]. The presence of CD63 and CD81 in EV samples was measured using Human CD63 ELISA colorimetric kit and Human CD81 ELISA colorimetric kit (*Novus Biologicals*, USA). Both assays were performed according to the manufacturer's instructions. Plates were read on a microplate reader Multiskan SkyHigh (*Thermo Fisher Scientific*, USA).

Statistics. Statistical processing of the obtained data was carried out using MS Excel (*Microsoft*, USA) and Origin Pro (*OriginLab Corp.*, USA) software. Statistical analyses were performed using one-way analysis of variance (ANOVA) in Origin Pro software. Differences were considered to be statistically significant when $p < 0.05$. Data are presented as mean and standard deviation (M \pm SD).

RESULTS AND DISCUSSION

Confirmation of minimal criteria for human UC-MSCs. To confirm that the cells under investigation met the minimal criteria for mesenchymal stromal cells [48], we analyzed if cells corresponded to classical fibroblast-like morphology, the ability to form colony-forming units (CFUs) at clonogenic density, had a distinct MSC immunophenotype, and the ability to undergo directed differentiation into adipogenic and osteogenic lineages after passages 1-3.

Cell morphology. The enzymatic isolation method yielded a sufficient number of cells, which adhered evenly to the plastic surface, had a stellate shape, and demonstrated significant proliferative potential, with a large number of cells in mitosis. Overall, the condition of the umbilical cord MSC culture before cultivation in serum-free conditions met the basic criteria for passage 2: the morphology was classically fibroblast-like, and the number of actively proliferating cells, corresponding to 85-90 % confluence (Fig. 1).

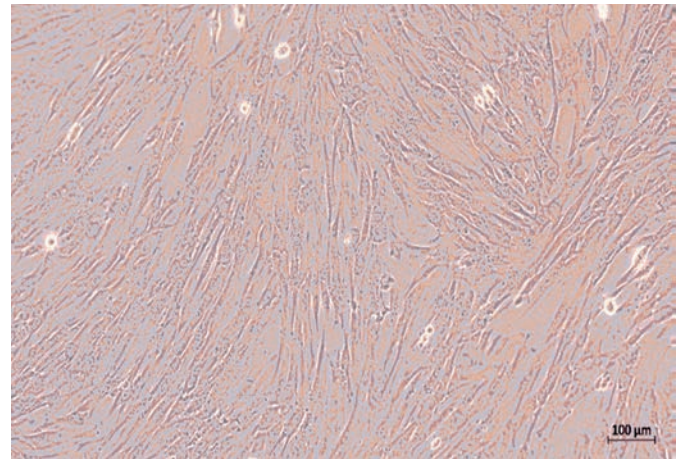


Fig. 1. The hUC-MSCs morphology at passage 2 before cultivation in serum-free conditions. Phase-contrast microscopy, scale bar – 100 μ m.

MSCs proliferative activity and of colony-forming ability. Proliferative activity and the ability to form colonies at clonogenic density are key indicators for assessing the stem cell nature of cells derived from biological materials. The proliferative activity (PDT) and colony-forming unit (CFU) assessment for hUC-MSCs were conducted at passages 1-3 for each donor with three repetitions. The summarized CFU and PDT data are presented in Table 1.

Table 1. CFU and PDT results for hUC-MSCs at passages 1-3 (n = 6, Mean \pm SD).

Passage	P1	P2	P3
CFU, %	20.6 \pm 2.2	32.8 \pm 0.5	39.1 \pm 1.4
PDT, hours	32.4 \pm 2.2	44.8 \pm 0.5	59.1 \pm 1.4

There was a noticeable dynamic increase in CFUs, with a characteristic peak at passage 3. Accordingly, hUC-MSCs demonstrated the ability to form colonies at clonogenic density, which is indicative of active proliferation and division. However, an increase in PDT values was observed compared to previous passages, which may indicate the onset of population aging and a decline in proliferative activity. Therefore, for the experiment on selecting the optimal conditions for obtaining extracellular vesicles, MSCs were used no later than passage 3.

Cell immunophenotype. The set of surface markers was assessed by flow cytometry (Fig. 2). hUC-MSCs were negative for hematopoietic markers CD34, CD45, and HLA-DR and positive for MSCs markers CD73, CD90, and CD105. The expression data for surface proteins confirm that the obtained population is a homogeneous MSC population and does not contain hematopoietic or endothelial-derived cells.

Table 2. Immunophenotype for hUC-MSCs at passage 2 (n = 6, Mean \pm SD).

CD marker	CD90	CD105	CD73	CD34	CD45	HLA-DR
Mean \pm SD	100 \pm 2.6 %	99.6 \pm 3.1 %	99.9 \pm 3.6 %	2.1 \pm 0.9 %	0.3 \pm 0.1 %	0.09 \pm 0.01 %

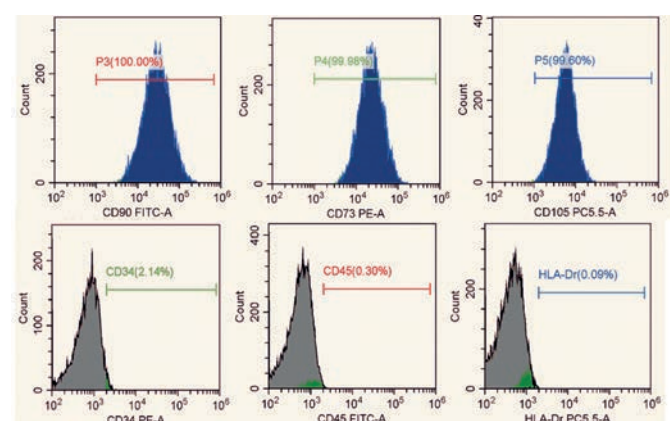


Fig. 2. Representative FACS histograms of the hUC-MSCs population at passage 2.

Adipogenic and osteogenic cell differentiation potential *in vitro*. According to the minimal criteria of the International Society for Cellular Therapy, multipotency is an important property of any MSCs [48] and is defined by their ability to undergo directed differentiation *in vitro* into derivatives of mesenchymal cells under specific conditions.

Fig. 3 shows the results of directed adipogenic and osteogenic differentiation of human hUC-MSCs at passage 2. Cytochemical detection of lipid inclusions in cells with Oil Red staining showed significant accumulation of cells localized in small groups. Cytochemical detection of calcium deposits in cells with Alizarin Red S staining revealed substantial accumulation of extracellular structures also localized in small groups. The cells in culture acquired characteristics typical for adipocytes and osteocytes. Therefore, cells derived from human hUC-MSCs are capable of directed differentiation into adipogenic and osteogenic lineages under the influence of inducers *in vitro*, as evidenced by the qualitative detection of lipid inclusions and calcium deposits, respectively.

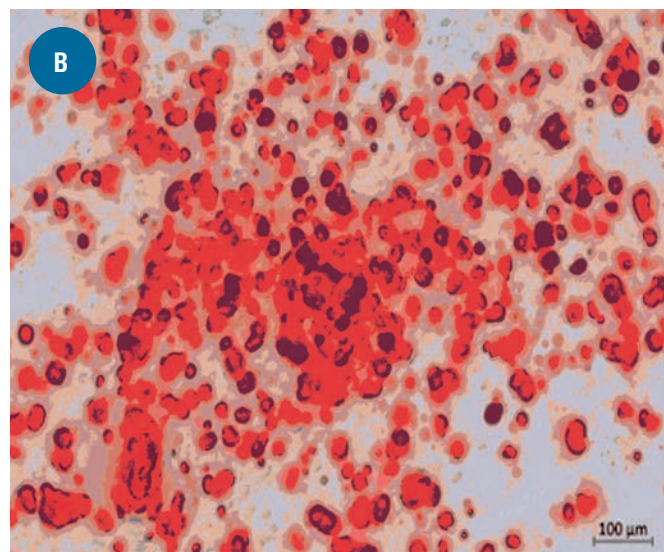
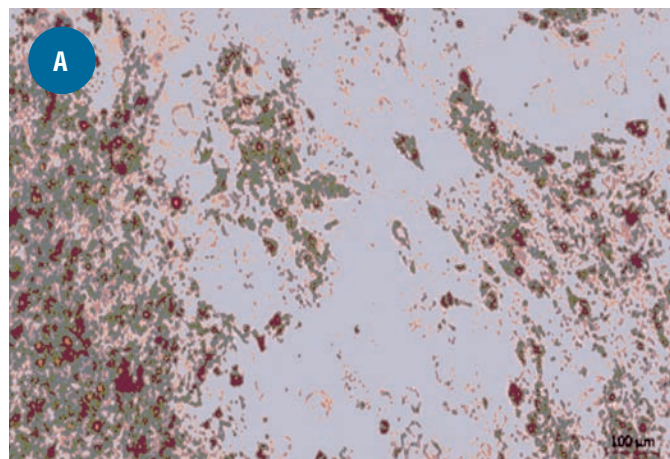
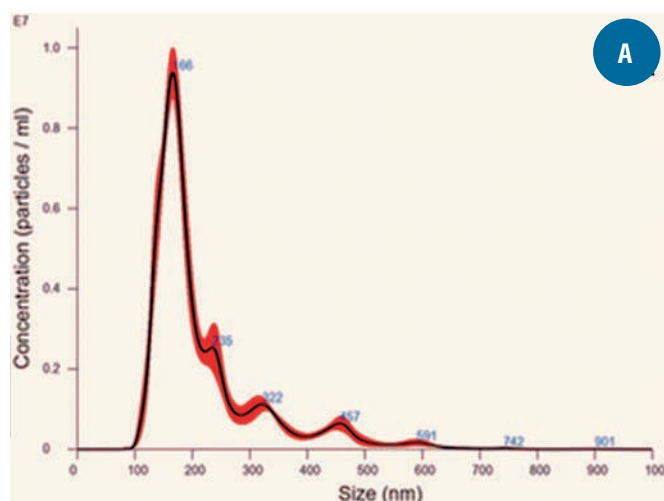


Fig. 3. The representative images of adipogenic (A) and osteogenic (B) differentiation of hUC-MSCs *in vitro*. Lipid droplets indicated by Oil Red staining and calcium deposits highlighted by Alizarin Red S staining, respectively. Scale bar = 100 μ m.

Characterization of EVs from conditioned medium. For the successful isolation and characterization of EVs from conditioned medium (CM), thorough characterization of the source cell culture is essential [49]. Quantitative analysis of extracellular vesicles obtained from the conditioned medium of hUC-MSCs was conducted using NTA analysis (Fig. 4). The peak concentration of EVs was found in the size range of 150–170 nm, which corresponds to the definition of “small vesicles”. Culture conditions and time are indicated in the caption of the Fig. 4. Additionally, protein concentration was determined for each sample using the Pierce method, which provided insights into the purity and yield of the EV preparations (Table 3). Furthermore, we performed ELISA analysis to detect the presence of CD63 and CD81 markers, which are characteristic of EVs (Table 3). Our results show high expression of CD63 and moderate levels of CD81 in the EVs culture conditions and time, which are indicated in the caption of the Table 3. Based on the cellular localization of tetraspanins, it has been speculated that tetraspanins such as CD63 are exclusively present on EVs of multivesicular bodies origin, whereas CD81, which is primarily localized on the cell surface, is preferentially sorted into microvesicles. This is reflected by the fact that CD63-positive vesicles are CD81 low or negative and vice versa [50].



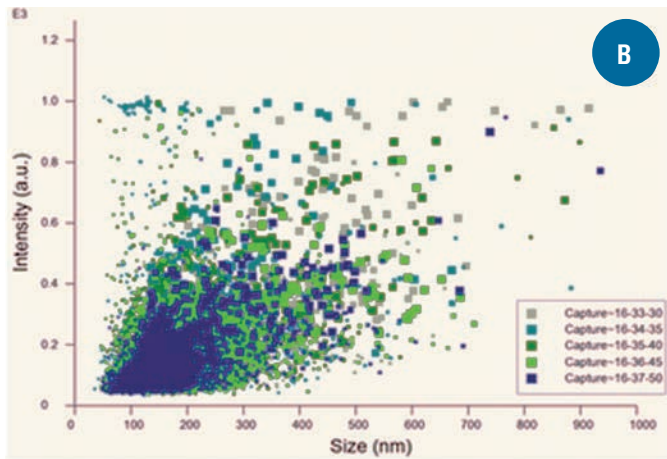


Fig. 4. Representative histograms of the quantitative Nanoparticle Tracking Analysis (NTA) of extracellular vesicles obtained from hUC-MSCs (culture media – Hanks without FBS, time – 48 hours): A – size and concentration distribution; B – size distribution.

Table 3. Total amount of protein (Pierce method) and expression of CD81, CD63 markers (ELISA) present in the EVs samples from hUC-MSCs (culture media – Hanks without FBS, time – 48 hours), n = 6, Mean ± SD.

Protein	CD63	CD81
192.0 ± 5.0 µg/mL	0.089 ± 0.006 ng/mL	0.034 ± 0.004 ng/mL

Extracellular vesicle concentration under changing cultivation conditions. A detailed description of the changes in the medium is provided in the Methods section. It is important to note that the cells were cultured in a multi-gas incubator at 37°C in a humidified atmosphere with 5% CO₂ and 5% O₂. Previous experiments with reduced concentrations of O₂ have confirmed higher MSC proliferation efficiency under these conditions [20, 35-39, 43-46].

In the first stage of the experiments, the conditions were sequentially changed, excluding certain variants (not representative or not reliable) at each stage of the analysis. The main parameter that we chose for the study was the quantitative assessment of the concentration of EVs (express NTA method). Total protein levels and specific markers for small EVs were determined only at the stage when the highest level of EVs concentration was obtained (48 hours).

Before we started the current study, the large work was done concerning optimization culture condition of no serum or growth factors. In previous experiments, we have shown that MSCs can be transferred from serum to serum-free medium while maintaining all the main morphofunctional indicators of culture at a high level. Therefore, in our experiments, we use serum-free medium, which removes many restrictions for the use of MSCs products for medical purposes. Hanks and MEM medium for MSCs cultivation are frequently used in culture experiments. In addition, if MSCs cultivation is carried out in serum-free conditions, it is necessary to have clear protocols taking into account all cultivation conditions. Our laboratory has a large pool of data on MSC cultivation in serum-free conditions based on MEM and Hanks medium. Therefore, we chose these two media for comparison.

Initially, we evaluated the effectiveness of replacing the culture medium one day before switching to serum-free medium. The results of this experiment showed that the concentration of extracellular vesicles was significantly higher when the growth medium in the umbilical cord MSC culture was replaced one day prior to switching to serum-free medium (Fig. 5).

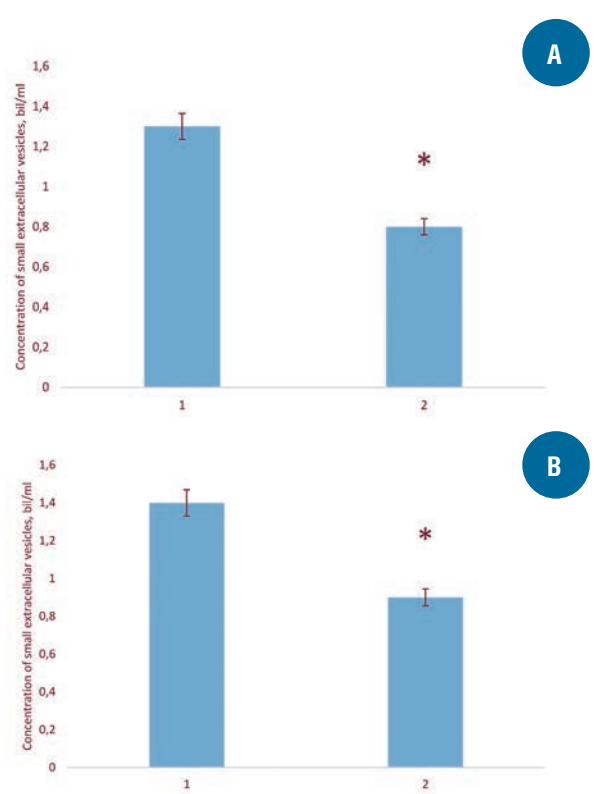


Fig. 5. Concentration of small extracellular vesicles in the conditioned medium after culturing (48 hours) hUC-MSCs with growth medium replacement 24 hours prior to switching to serum-free medium (1) and without additional replacement (2). A – cultivation in Hank's solution without FBS; B – cultivation in MEM without FBS (n = 6, Mean ± SD) Note: * – p < 0.05 compared to medium replacement 24 hours prior).

The next phase of the experiment focused on determining the optimal cultivation time for MSCs in serum-free medium to achieve the highest yield of small extracellular vesicles (with growth medium replacement 24 hours prior to switching to serum-free medium). The yield of extracellular vesicles was the highest after 48 and 72 hours of cultivation in both Hank's solution and MEM medium without FBS, but there was no significant difference depending on the composition of the serum-free medium (Fig. 6).

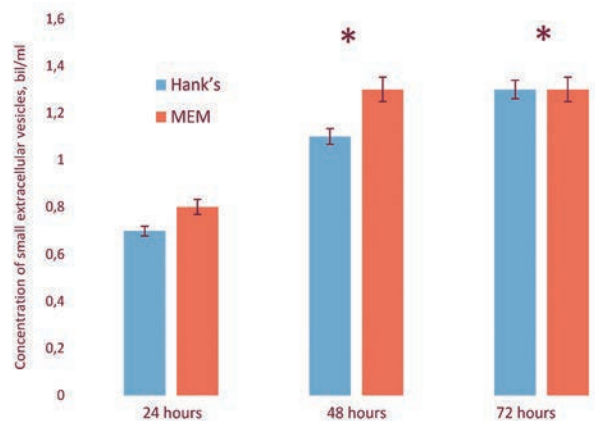


Fig. 6. Concentration of small EVs measured by NTA in the conditioned medium after culturing hUC-MSCs for various time periods in Hank's solution or α-MEM without FBS (n = 6, Mean ± SD). Note: * – p < 0.05 compared to 24 hours of cultivation.

Cultivation of human mesenchymal stem cells (MSCs) from various sources in serum-free medium has been extensively described and analyzed in the literature [30-42]. In most cases, serum-free medium has been found to be equally effective as traditional serum-containing protocols. For this study, we selected serum-free medium as the primary component to optimize conditions for the production of small extracellular vesicles from human umbilical cord MSCs.

The results of this experiment demonstrated a clear dependence of extracellular vesicle yield on the nature of the changes in the nutrient growth medium. Specifically, replacing the growth medium in the UC-MSC culture 24 hours before switching to serum-free medium led to a significantly higher yield of extracellular vesicles (Fig. 5). We hypothesize that this observation is related to the mitigation of stress within the culture when medium changes are made at regular intervals (in this case, every 72 hours).

Upon transitioning the culture to serum-free medium, we identified a dependency on both the composition of the serum-free medium and the duration of MSC cultivation in these conditions. The highest yield of extracellular vesicles was observed after 48 and 72 hours of cultivation, with no significant difference observed between the serum-free medium compositions (Fig. 6). We attribute this result to the adaptation period of the culture during the first 24 hours of serum-free cultivation. Given that no significant difference in extracellular vesicle yield was found between 48 and 72 hours, we propose 48 hours as the optimal cultivation time. This is because, beyond 72 hours, MSC populations in later passages may begin to lose their proliferative potential, which could negatively impact the yield of extracellular vesicles.

In our study, several factors present limitations, particularly regarding the question of why MSCs are not cultured in serum-free medium from the outset of donor material collection. In the current experimental design, the transition from conventional serum-supplemented medium occurs no later than passage 3. The primary constraints on the immediate use of serum-free medium are the significant costs associated with selecting appropriate components and optimizing culture conditions for MSCs. When considering the application of MSC-derived products in therapeutic and medical contexts, initiating culture in serum-free medium is not cost-effective, taking into account the factors of cost, quality, and production scalability. As such, the widely adopted practice in cellular cultivation involves a gradual transition to serum-free conditions. This approach not only mitigates potential complications arising from erroneous interpretations of experimental results but also eliminates the confounding influence of endogenous microvesicles present in animal sera, which can limit the applicability of these products in pharmaceutical and medical fields [37-42].

Our findings demonstrate that small extracellular vesicles can be successfully isolated from serum-free medium cultured MSCs, with no significant differences between the two types of media employed (Hank's and MEM). This approach addresses many of the challenges associated with the use of animal-derived sera, which are sources of xenogenic substances. These xenogenic components can impose limitations in pharmaceutical manufacturing and introduce variability into experimental results, particularly when protocols containing xenogenic additives are used. To further expand the therapeutic applications of extracellular vesicles, future research should focus on comparing the effectiveness of isolating extracellular vesicles from different MSC types cultured in serum-free conditions.

CONCLUSION

The findings of this study indicate that the optimal conditions for the cultivation of umbilical cord mesenchymal stem cells for the production of small extracellular vesicles in serum-free medium are as follows:

- *culturing hUC-MSCs in complete growth medium with regular medium exchanges every 2-3 days until the culture achieves 70 % confluence no later than passage 3;*
- *replacing the growth medium 24 hours prior to transitioning to serum-free medium;*
- *cultivating the UC-MSC culture for 48 hours in Hank's solution or MEM, without the addition of xenogeneic serum.*

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Оптимізація умов отримання малих позаклітинних везикул з мезенхімальних стовбурових клітин пуповини людини

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РЕЗЮМЕ

Позаклітинні везикули (ПВ), отримані з мезенхімальних стовбурових клітин (МСК), відіграють вирішальну роль у фізіологічних функціях МСК та їх терапевтичних ефектах. Порівняно з клітинними продуктами, склад ПВ, біологічні властивості, механізми дії та чудова стабільність роблять їх привабливими кандидатами для регенеративної медицини, що сповіщає про нову еру в клітинній терапії.

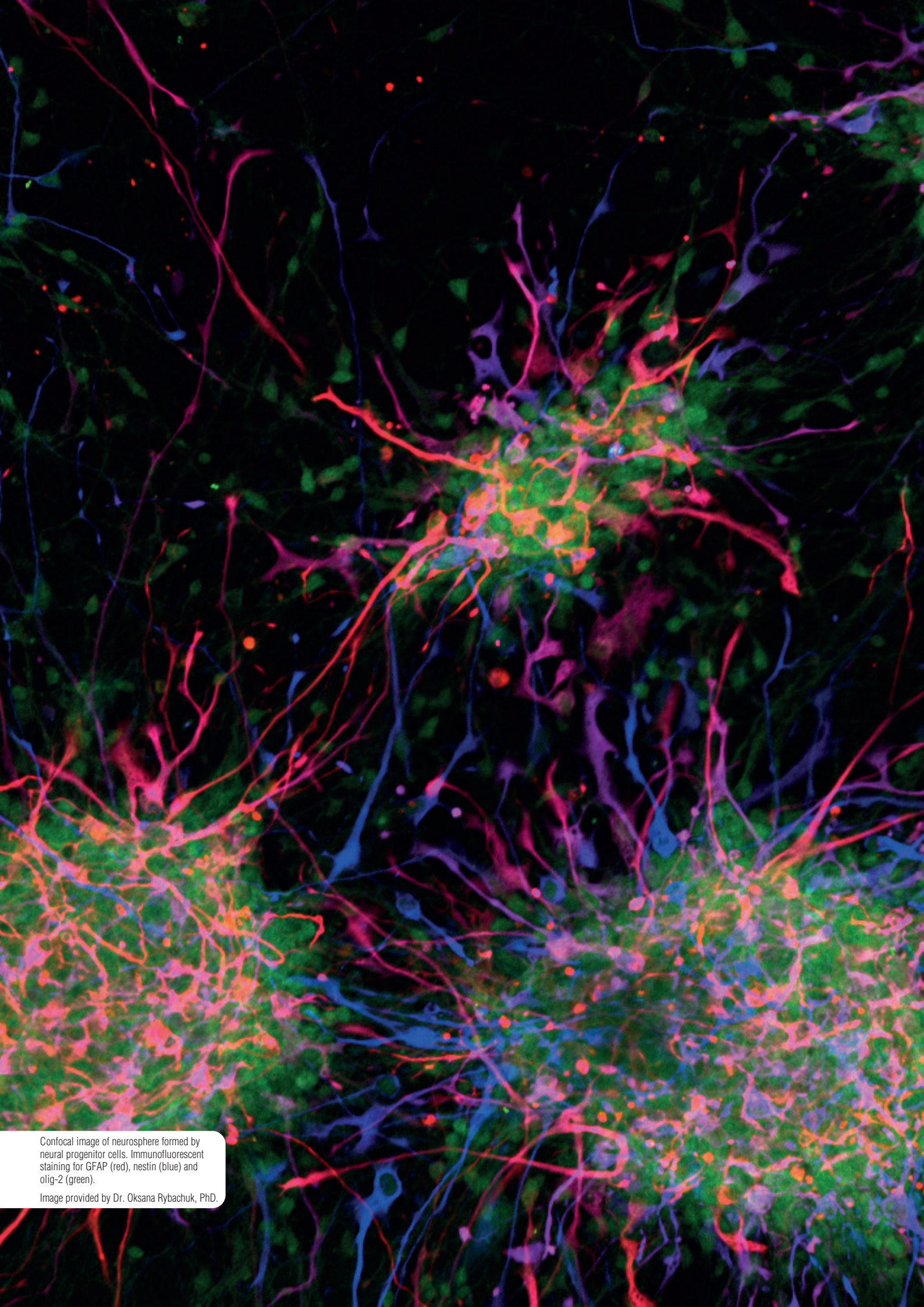
МЕТА – визначити оптимальні умови для отримання малих ПВ з культивованих мезенхімальних стовбурових клітин, отриманих з пуповини людини, на основі кількісних показників виходу ПВ та морфофункціональних характеристик культур МСК залежно від умов культивування.

МАТЕРІАЛИ ТА МЕТОДИ. МСК були виділені з пуповини людини шляхом ферментативного розщеплення. Процес оптимізації середовища культивування проводився наступним чином. Перший спосіб – середовище культивування замінювали свіжим середовищем та витримували протягом наступних 24 годин. Другий спосіб – культивування продовжували в незміненому середовищі. Потім їх змінювали на два середовища: розчин Хенкса та середовище MEM альфа, обидва без ксеногенної сироватки та екзогенних факторів росту. Культивування та утворення позаклітинних везикул з МСК-ПК в безсироваткових умовах проводили протягом 24, 48 та 72 годин. Усі везикули досліджували для підтвердження їхнього питомого розподілу за розмірами, визначеного за допомогою аналізу відстеження наночастинок (NTA), та наявності маркерів CD63 та CD81, оцінених за допомогою ELISA.

РЕЗУЛЬТАТИ. Заміна середовища росту в культурі МСК за 24 години до переходу на середовище без сироватки призвела до значно вищого виходу ПВ. Після переходу культури на середовище без сироватки ми виявили залежність як від складу середовища без сироватки, так і від тривалості культивування МСК в цих умовах. Найвищий вихід ПВ спостерігався після 48 та 72 годин культивування, при цьому суттєвої різниці між складами середовища без сироватки не спостерігалось. Ми пов'язуємо цей результат з періодом адаптації культури протягом перших 24 годин культивування без сироватки. Враховуючи, що суттєвої різниці у виході позаклітинних везикул не було виявлено між 48 та 72 годинами, ми пропонуємо 48 годин як оптимальний час культивування.

ВИСНОВКИ. Результати дослідження показують, що оптимальні умови для культивування мезенхімальних стовбурових клітин пуповини для отримання малих позаклітинних везикул у безсироватковому середовищі є такими: культивування МСК у повному середовищі для росту з регулярною заміною середовища кожні 2-3 дні, доки культура не досягне 70 % конфлюентності; заміна середовища для росту за 24 години до переходу на безсироваткове середовище; культивування культури МСК протягом 48 годин у розчині Хенкса або MEM без додавання ксеногенної сироватки.

КЛЮЧОВІ СЛОВА: мезенхімальні стовбурові клітини; позаклітинні везикули; екзосоми; безсироваткове середовище.



Confocal image of neurosphere formed by neural progenitor cells. Immunofluorescent staining for GFAP (red), nestin (blue) and olig-2 (green).

Image provided by Dr. Oksana Rybachuk, PhD.