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Comprehensive characterization of human Wharton's jelly mesenchymal stromal cells isolated by the combined explant-enzymatic method

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Abstract

Human mesenchymal stromal cells (MSCs) derived from Wharton's jelly (WJ) are a premier source for regenerative medicine. However, standardized isolation protocols that ensure both high cell yield and maintained proliferative potential remain a subject of active research.

***This study is aimed** to evaluate the efficiency of a combined explant-enzymatic method for human WJ-MSC isolation and characterize the resulting cell populations.*

***Materials and methods.** Cells were isolated using a combined explant-enzymatic method. Minced WJ fragments were cultured on gelatin-coated surfaces for 7 days, then explants underwent a brief (5–10 min) in situ enzymatic treatment with a trypsin-Versene solution, followed by further co-cultivation of the tissue fragments and released cells for an additional 7 days prior to the first passage. Cell characterization included colony-forming unit (CFU), immunophenotyping (CD29, CD90, CD73, CD105, CD34, CD45), directed differentiation (adipogenic and osteogenic), and secretory activity assays. Proliferative kinetics were assessed by calculating the population doubling time (PDT) across 5 passages. Secretory profile of WJ-MSCs (growth factors and cytokines regulating reparative-regenerative processes) was studied by enzyme-linked immunosorbent assay of conditioned media collected after 48 h cell incubation in minimal culture media.*

***Results.** The combined method ensured a stable cell yield with high reproducibility. Significant inter-donor variability in primary cell yield was noted. Isolated cells fulfilled the ISCT minimal criteria, exhibiting typical fibroblast-like morphology, characteristic immunophenotype, differentiation potential, and a high proportion of CFU, indicating the preservation of primitive progenitors. Proliferative analysis showed that PDT values remained comparable and did not change significantly between passages 1 and 3. Conditioned media from WJ-MSCs contained wide range of growth factors and cytokines, with inter-donor variability: VEGF, EGF, PDGF-BB, SCF, TNF-α, and TGF-β exhibited wide fluctuations, whereas NGF-β and FGF-β were relatively stable. Overall, WJ-MSC CM were characterized by very high levels of VEGF and EGF, high PDGF-BB, elevated TNF-α and TGF-β, and the lowest levels of FGF-β.*

Conclusions. *The combined explant-enzymatic method is an efficient and standardized protocol for human WJ-MSC isolation, preserving high functional potency of cells for clinical and biobanking purposes.*

Keywords: *mesenchymal stromal cells; Wharton's jelly of the umbilical cord; colony-forming activity; immunophenotype; directed differentiation; conditioned medium*

Introduction

Human mesenchymal stromal/stem cells (MSCs) of the umbilical cord, particularly those isolated from the Wharton's jelly (WJ), are currently considered the "gold standard" and the most promising cell type for applications in regenerative medicine [1, 2]. MSCs isolated from the WJ of umbilical cord (WJ-MSCs) possess several significant biological and clinical advantages over MSCs derived from bone marrow, skin, or adipose tissue. The umbilical cord is a routinely collected perinatal biomaterial; thus, obtaining umbilical cord tissue does not involve painful procedures for the donor, unlike bone marrow aspiration, which is invasive, or liposuction to obtain adipose tissue [3, 4]. Furthermore, the isolation of MSCs from the umbilical cord does not raise ethical controversies [5]. In addition, umbilical cord MSCs are at the early stages of ontogenetic development, resulting in high proliferative potential [6] and low immunogenicity [7, 8], which makes them highly attractive for allogeneic transplantation. They also exhibit superior differentiation potential [9] and stemness [10].

At the same time, the umbilical cord is a limited donor material, the availability of which depends on obtaining informed consent and specific biological material collection conditions. Consequently, optimizing isolation methods to increase the yield of MSCs obtainable from the Wharton's jelly of a single donor remains a critical issue. Methods for isolating MSCs from the umbilical cord WJ can be categorized into explant [11, 12, 13], enzymatic [13, 14], and combined approaches. Explant methods are based on the migration of cells onto the surface of a culture flask from tissue fragments (explants) placed upon it. The primary advantages of such approaches include the gentle adaptation of cells to culture conditions and a lower risk of damage to cell membranes and surface receptors due to the exclusion of enzymes. A comparative study [15] demonstrated that the explant method allows for the retrieval of MSCs with higher expansion potential. However, the dense extracellular matrix of the WJ tissue limits cell migration, resulting in the loss of a significant portion of cells remaining encapsulated within the tissue; consequently, the lag phase before the emergence of the first generation of cells is relatively long [11].

Enzymatic methods involve the use of enzymes such as collagenase, hyaluronidase, and trypsin, or their combinations, to degrade the extracellular matrix and release cells, followed by seeding the resulting cell suspension onto a culture surface. While enzymatic methods reduce the time required for cell isolation, they lack a dedicated adaptation phase for MSCs to culture conditions. This can lead to a lower cell yield due to decreased viability, the destruction of surface membrane structures, and a negative impact on functional properties. It has been shown [13] that the cell yield using enzymatic methods varies significantly between technical replicates; furthermore, cells may remain "trapped" within the jelly-like structure that forms after collagenase treatment. It was found that the enzymatic method significantly reduces the yield, viability, and downregulates genes related to mitosis in WJ-MSCs compared to the explant method [16].

Combined methods, which integrate both explant and enzymatic approaches, are considered the most promising, as they allow for cell adaptation to culture conditions while simultaneously accelerating cell migration from the explants due to the enzymatic treatment of the WJ matrix. This reduces the time required to establish a culture without a radical loss of viability. It has been shown [13, 17, 18] that combined methods significantly expedite the process and increase the yield of WJ-MSCs.

Despite considerable interest in the paracrine activity of MSCs, the majority of studies have focused on the secretome of cells derived from conventional sources (adipose tissue, bone marrow, and others). In contrast, WJ-MSCs remain insufficiently characterized, particularly regarding comprehensive analysis of their secretory profile. Conditioned media (CM) obtained during MSC cultivation represent a well-established, accessible, and highly convenient source of bioactive molecules. These media offer clear advantages over both cellular and cell-free preparations due to simplicity of their production and ability to preserve cellular material for subsequent use. When assessing the therapeutic potential of WJ-MSC CM, the greatest interest lies in bioactive factors responsible for reparative and regenerative processes. Accordingly, the following molecules were selected for the present study: Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF), Platelet-Derived Growth Factor (PDGF-BB), Nerve Growth Factor beta (NGF-β), Stem Cell Factor (SCF), Tumor Necrosis Factor alpha (TNF-α), basic Fibroblast Growth Factor (FGF-β), and Transforming Growth Factor beta (TGF-β). This panel includes factors known to stimulate tissue regeneration (EGF, NGF-β, FGF-β, PDGF), promote vascularization (VEGF), and serve as pro-inflammatory cytokines (TNF-α, TGF-β) that are essential for a complete reparative response, as well as SCF – a factor that indicates the presence of stem cells in culture. It has been shown [16] that the choice of isolation method can affect the secretory activity of WJ-MSCs, with higher FGF-β levels released after the explant method compared to lower levels observed with enzymatic isolation. Proteomic profiling [19] revealed that enzymatic isolation methods resulted in increased inflammatory signatures compared to explant methods.

Thus, the variability in the biological properties of MSCs depending on the isolation technique necessitates the optimization of isolation protocols to obtain standardized cell material. The choice of the isolation method for MSCs from Wharton's jelly determines the biological potential of the resulting culture. In this context, the detailed identification of the structural and functional profile of cells isolated using the combined enzymatic-explant method, which is considered one of the most promising approaches, is of particular importance. For practical applications in biotechnology and medicine, it is critical to clearly define the parameters of viability, proliferative capacity, immunophenotype, and functional activity of the culture obtained by this specific method, as these factors ensure its stability and therapeutic efficacy.

The aim of this study was to evaluate the yield, phenotype, growth and secretory patterns, and ability to multilineage differentiation of human WJ-MSCs obtained by the combined method to confirm their potency and suitability for further biomedical applications.

Materials and methods

Mesenchymal stromal/stem cells were isolated using a combined explant-enzymatic method [18] from the Wharton's jelly tissue (WJ) of umbilical cords obtained during elective cesarean sections. The material was collected from clinically healthy parturients (39-40 weeks of gestation) following their voluntary written informed consent. Due to institutional bioethics protocols aimed at maintaining donor anonymity, specific maternal age data were not recorded. The study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Bioethics Committee of the Institute for Problems of Cryobiology and Cryomedicine of the NAS of Ukraine (Protocol No. 6, dated November 28, 2023). Umbilical cord fragments (5-10 cm) were washed with Hanks' Balanced Salt Solution, HBSS (*Biowest*, France); subsequently, the WJ tissue was separated and minced into fragments of 0.5-2.0 mm. To ensure intra-experimental consistency, equal weights of the resulting tissue explants from each individual donor were transferred into 6-well culture plates or T-25 culture flasks (*TPP*, Switzerland), the adhesive surfaces of which had been pre-coated with gelatin (*Sigma*, USA). The total mass of usable WJ tissue obtained per umbilical

cord varied between donors (ranging from 0.06 g to 0.52 g) due to natural anatomical differences in cord thickness and the necessary excision of any areas containing hematomas. To initiate cell outgrowth, α-MEM medium (Biowest, France) was used, enriched with 20 % fetal bovine serum, FBS (*Biowest*, France) and supplemented with antibiotics (penicillin/streptomycin at 50 U/mL and 50 μg/mL, respectively). Cultivation was performed under standard conditions (37 °C, 5 % CO_2, 95 % humidity). On day 7, the explants underwent brief enzymatic disintegration using a mixture of 0.25 % trypsin in PBS (*Biowest*, France) and ready-to-use Versene (EDTA) solution (*Vetline Agroscience LLC*, Ukraine) at 1:4 ratio for 5-10 minutes. After neutralizing the enzyme action with complete medium and centrifugation, the cell suspension, along with the tissue fragments, was returned to the original culture flask for further proliferation for additional 7 days. Subsequently, the cells were detached from the culture surface (passage 0) and re-seeded onto fresh culture plates in complete medium containing 10 % FBS.

To evaluate the efficiency of MSC isolation from umbilical cord WJ, samples from five donors were analyzed. From each donor, two WJ samples were prepared and placed in independent culture flasks, subjected to identical cultivation and isolation conditions. Quantitative assessment of the cell yield was performed at the stage of obtaining passage 0 (P0) cells. The cell concentration in the suspension was determined using a hemocytometer. The total cell count was normalized to 1 g of the initial tissue weight.

The clonogenic capacity of MSCs was investigated at passage 4 using the colony-forming unit (CFU) assay. It was conducted for n = 5 independent biological donors, with each sample seeded in 2 flasks (2 technical replicate per donor). To determine colony-forming efficiency, a cell suspension was prepared in α-MEM supplemented with 20 % FBS and antibiotics, and cells were seeded into culture flasks at a low density (10 cells/cm^2 and 20 cells/cm^2). Cultivation was performed for 14 days under standard conditions (37 °C, 5 % CO_2), after which the cultures were fixed and stained with hematoxylin and eosin for colony counting. Aggregates containing 50 or more cells were considered colonies. The colony-forming efficiency was calculated using the following formula:

$$Colony\ forming\ efficiency = \left(\frac{a}{b}\right) \times 100\ \%,$$

where *a* is the number of colonies formed, and *b* is the total number of seeded cells.

To evaluate the growth kinetics of the obtained cell cultures, the population doubling time (PDT) was determined as previously described [20]. Briefly, to calculate PDT cells isolated from different donors were counted, plated, and passaged when the culture reached 70 % confluence. To standardize the PDT analysis and ensure a homogenous cell population, cells from the parallel cultures were pooled together within each individual donor. PDT was calculated using the formula:

$$PDT = \frac{t \times ln(2)}{ln(\frac{nt}{n0})},$$

where *t* is time in days, *n*0 is the number of seeded cells, *nt* is the number of harvested cells.

The surface marker profile of the WJ-MSCs was characterized at passages 4-5 using flow cytometry. A panel of fluorochrome-conjugated monoclonal antibodies was used: CD29-PE, CD45-PE, CD34-FITC, CD73-PE, CD90-FITC, and CD105-FITC (BD Biosciences, USA). Analysis was performed using a FACSCalibur flow cytometer (*Becton Dickinson*, USA). To ensure statistical reliability, at least 10,000 events were acquired per sample. Data were processed and quantitative fluorescence was analyzed using CELLQuest Pro (*BD Biosciences*, USA).

The ability of WJ-MSCs to undergo multilineage differentiation was assessed by culturing the cells in specific induction media based on α-MEM supplemented with 10 % FBS for 21 days (with medium changes every 3-4 days). The osteogenic induction medium [21] contained 100 nM dexamethasone, 10 mM β-glycerophosphate, and 0.2 mM L-ascorbic acid-2-phosphate (*Sigma-Aldrich*, USA). The adipogenic medium [22] consisted of 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 10 μg/mL insulin, and 100 μM indomethacin (*Sigma-Aldric*h, USA). Matrix mineralization and calcium accumulation were evaluated by staining fixed cultures (10 % formalin) with a 1 % Alizarin Red S solution (*Sigma-Aldrich*, USA). Analysis was performed using a CETI Inverso inverted microscope (*Medline Scientific*, Belgium). The presence of intracellular lipid inclusions was determined using Nile Red (*Sigma-Aldrich*, USA) staining. Stained samples were visualized using an LSM 510 META confocal laser scanning microscope (*Carl Zeiss*, Germany).

Conditioned media were obtained according to the method described by Acuto et al. [23]. Briefly, upon reaching 90 % confluence, the cell monolayer at passages 4-7 was washed twice with HBSS (*Biowest*, France). Subsequently, the complete culture medium was replaced with α-MEM medium without phenol red (*Gibco*, USA) and without the addition of FBS, using a volume equivalent to half of the standard complete culture medium volume at 7 mL for a T-75 flask (*TPP*, Switzerland). The cell cultures were incubated for 48 hours (37 °C, 5 % CO_2). Following incubation, the conditioned media were collected from the culture flasks, filtered through a 0.22 μm membrane filter (*Merck*, Germany), and stored at −80°C until further analysis. For each donor at each of the aforementioned passages CM was collected from three T-75 culture flasks. The CM samples obtained from passages 4 to 7 for each donor were pooled prior to the analysis to characterize the cumulative, representative secretory profile of the cells during this period of "cell expansion window".

For secretory profile analysis 4 mL of CM were concentrated and desalted using Amicon Ultra-4 Centrifugal Filter Units with Ultracel-3 membranes with nominal molecular weight cut-off of 3 kDa (*Millipore*, Ireland). At each stage of the procedure, sample volumes were determined by weighing empty and filled containers, thereby establishing the concentration factor required for subsequent calculations [24]. Total protein content in CM concentrates was quantified by the Lowry method in Miller modification [25], using bovine serum albumin as the standard.

Presence of growth factors and cytokines (VEGF, EGF, PDGF-BB, NGF-β, SCF, TNF-α, FGF-β, TGF-β) was determined by enzyme-linked immunosorbent assay using Human Growth Factor ELISA Strip II kit (*Signosis*, USA). This kit enables simultaneous measurement of specified bioregulators in samples, with each strip in the plate containing eight different types of antibodies against the target growth factors. Assays were performed on a Tecan Infinite 200Pro multimode plate reader (*Tecan Inc.*, Austria) in luminescence mode without filters. Raw data were subsequently normalized for total protein content (μg), concentration factors and experimental dilution factors, and expressed in normalized luminescence units (NLU).

Statistical processing of the obtained results was performed; the normality of the distribution was assessed using the Shapiro-Wilk test. Since the data followed a normal distribution, Student's t-test for independent samples was used for comparisons. The results are presented as the mean ± standard error of the mean (Mean ± SEM).

Results and discussion

Cells were isolated from the umbilical cord WJ tissue fragments (**Fig. 1, A**) using a combined explant-enzymatic method to ensure gentle cell adaptation to *in vitro* conditions and to create favorable circumstances for facilitated migration. This was achieved through controlled, short-term enzymatic treatment of the WJ fragments after 7 days of cultivation to loosen the connective tissue and further enhance cell migration. It was observed that the

onset and intensity of cell outgrowth from the WJ fragments varied depending on the individual characteristics of different donors. In most cases, fibroblast-like cells actively migrating from the WJ fragments were observed around days 5-7 of explant cultivation (**Fig. 1, B**). Notably, in samples from some donors, no cell outgrowth was observed after the initial 7 days of culture.

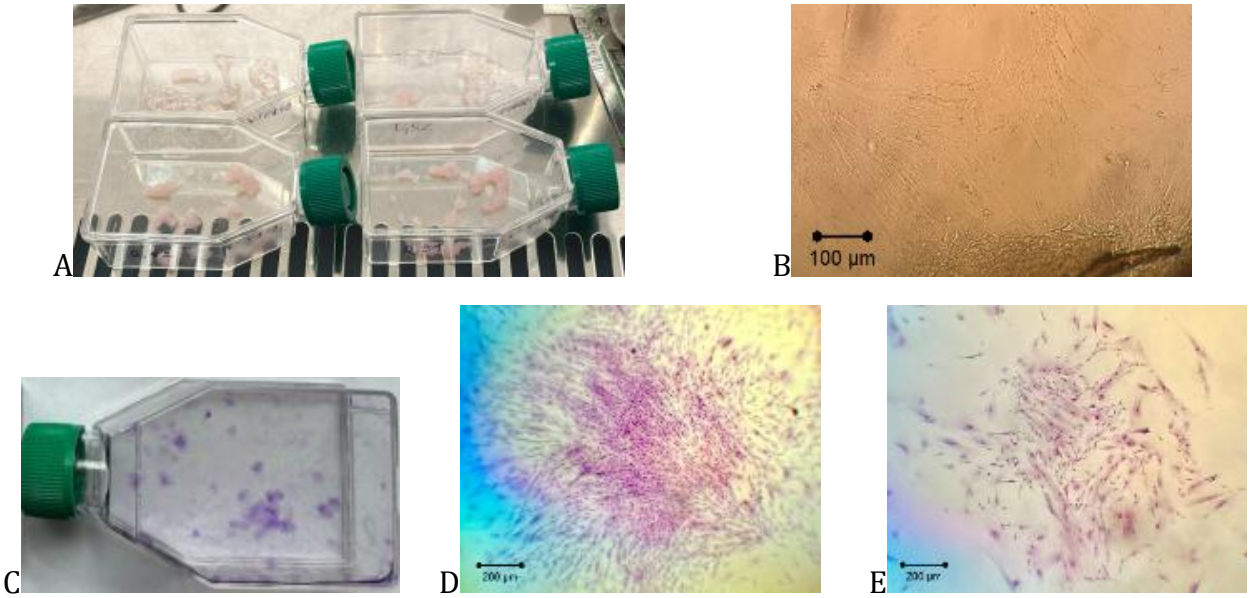

Fig. 1. *Macro- & microphotographs of cultures of human WJ-MSCs isolated by combined explant-enzymatic method: A – explants of WJ placed in culture flasks; B – fragment of umbilical cord WJ with cells migrating into the surface of culture flask; C-E – determination of clonogenic activity of WJ-MSCs. General view of the culture flask with cell colonies (C), "large" (D), and "small" (E) colonies; hematoxylin-eosin staining, light microscopy.*

In all cases, brief trypsinization of the WJ fragments on day 7, followed by their return to culture conditions, improved explant attachment and stimulated cell migration. For most donors, by day 14 after the initiation of culture, extensive areas of cell monolayers with a spindle-shaped morphology typical of MSCs and a confluence level of 70-100 % were obtained near the tissue fragments. At this stage, the cultures were passaged, cell yield was assessed, and the expansion procedure was initiated for further analysis of cell properties.

The quantitative assessment of cell yield from Wharton's jelly using the combined method demonstrated a high degree of reproducibility and minimal influence of technical factors on the results. Independent tissue samples from the same donor showed similar cell yield values for most analyzed donors (**Tab. 1**). However, significant inter-donor variability in MSC yield was observed. The cell yield ranged from 0.05×10^6 cells per g (Donor No. 5) to 1.0×10^6 cells per g (Donor No. 2). While the deviation in cell yield between independent samples from a single donor did not exceed one order of magnitude, inter-donor differences reached nearly a twenty-fold range. This fact allows us to conclude that the primary factor influencing the number of isolated cells is the biological characteristics of the donor material. Variations in the number of isolated cells depending on the specific WJ sample have also been reported by other researchers [11, 26]. It has been shown that MSC yield increases with younger maternal age, higher gestational age, and greater neonatal birth weight [26]. The high inter-donor variability of MSC yield from WJ may also be attributed to individual differences in cell density within the WJ tissue, the organization of the extracellular matrix, and varying numbers of progenitor cells in the umbilical cord connective tissue. It is known [27] that the density of the WJ extracellular matrix can significantly affect the efficiency of cell migration and release during cultivation and enzymatic treatment.

Table 1. *The yield of human mesenchymal stem cells from Wharton's jelly tissue of different donors.*

Donor, №	Sample №	Tissue weight, g	Count of cells isolated from a sample, ×10³	Cell yield (cells per g of tissue), ×10⁶
1	1-1	0.50	102.5	0.205
	1-2	0.52	135.0	0.260
2	2-1	0.25	315.0	1.260
	2-2	0.31	237.5	0.770
3	3-1	0.06	32.5	0.541
	3-2	0.09	30.0	0.333
4	4-1	0.18	17.5	0.097
	4-2	0.24	25.0	0.104
5	5-1	0.29	15.0	0.052
	5-2	0.25	12.5	0.050

Notes: n = 5 biological donors; two independent tissue samples processed per donor, making 10 samples total; for each flask, n = 3 technical measurement replicates (repeated hemocytometer counts) were performed to ensure precision, and the final data are presented as single absolute values per sample).

Comparison of the initial WJ sample weights with the number of obtained cells revealed no direct linear correlation between these parameters. Specifically, the cell yield from samples of comparable mass (0.25-0.31 g) differed by one order of magnitude between Donors No. 2 and 4, and by two orders of magnitude between Donors No. 2 and 5 (**Tab. 1**). These results confirm the necessity of normalizing cell yield to the initial tissue weight to ensure a correct comparison of isolation efficiency.

At passage 4, the clonogenic activity was evaluated, as it serves as a key functional criterion for assessing the quality and stemness of MSCs. Following the seeding of MSCs at low densities, colonies were calculated on the culture flask surface after 14 days (**Fig. 1, C**). These colonies were categorized into "large" (**Fig. 1, D**), primarily consisting of more than 100 small, densely packed cells, and "small" (**Fig. 1, E**), containing up to 100 cells of varying sizes. The colony-forming efficiency was 9.1 ± 1.0 % at a seeding density of 10 cells/cm² and 14.7 ± 0.8 % at 20 cells/cm². Notably, "large" colonies, derived from more primitive progenitor cells, accounted for over 70 % of the total colony count. These results indicate a higher clonogenic potential compared to WJ-MSCs isolated by standard enzymatic methods, which reported an efficiency of 13.4 % even at a higher seeding density of 50 cells/cm² [28].

During the subculturing of the obtained cells up to passages 4-5, no signs of senescence were observed; the cells maintained a fibroblast-like morphology typical of MSCs. We found that PDT values (**Fig. 2**) for the majority of examined samples (from different donors) remained comparable and did not change significantly within passages 1 to 3, ranging from 2.7 to 3.4 days. However, cells isolated from the WJ tissue of Donor No. 5 demonstrated a significantly prolonged PDT; therefore, these data were excluded from the average PDT calculations. A statistically significant decrease in proliferative activity was observed starting from passage 4 compared with the initial culture stage ($p < 0.05$), indicating a gradual decline in the regenerative potential of WJ-MSCs during *in vitro* expansion.

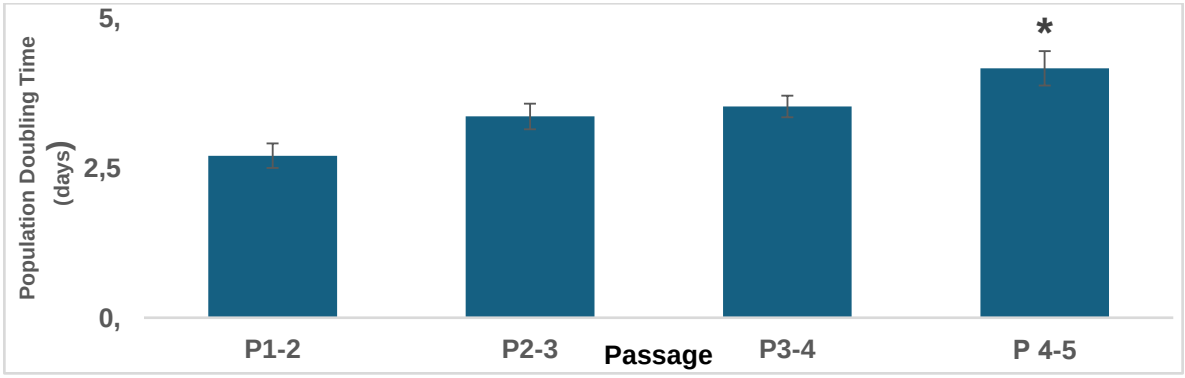

Fig. 2. *Population doubling time (PDT) of isolated human WJ-MCSs across different passages. Cells from parallel cultures of each individual donor were pooled to ensure population homogeneity. Data are presented as mean ± SEM (n = 4 biological donors).*
*Note: * p < 0.05 compared to PDT at passage 1 to passage 2.*

Analysis of the immunophenotype of WJ-MSCs obtained by the combined method from 5 independent biological donors analyzed separately at passages 4-5 (**Fig. 3**) revealed that cells from most donors expressed the following markers: CD29 (94.2 ± 0.7 %), CD73 (83.7 ± 1.7 %), CD90 (90.6 ± 2.5 %), and CD105 (79.3 ± 0.8 %). Simultaneously, the proportion of cells negative for the hematopoietic markers CD34 and CD45 was 95.6 ± 1.6 % and 96.8 ± 1.3 %, respectively (**Fig. 3**). These findings are consistent with data reported by other researchers regarding the immunophenotype of umbilical cord MSCs [11, 13, 28]. It should be noted that reduced [29] or absent [30] expression of CD105 has been previously described specifically for WJ-MSCs. Overall, the immunophenotype of the cells obtained in this study fulfills the criteria for defining MSCs established by the International Society for Cell & Gene Therapy (ISCT) [31].

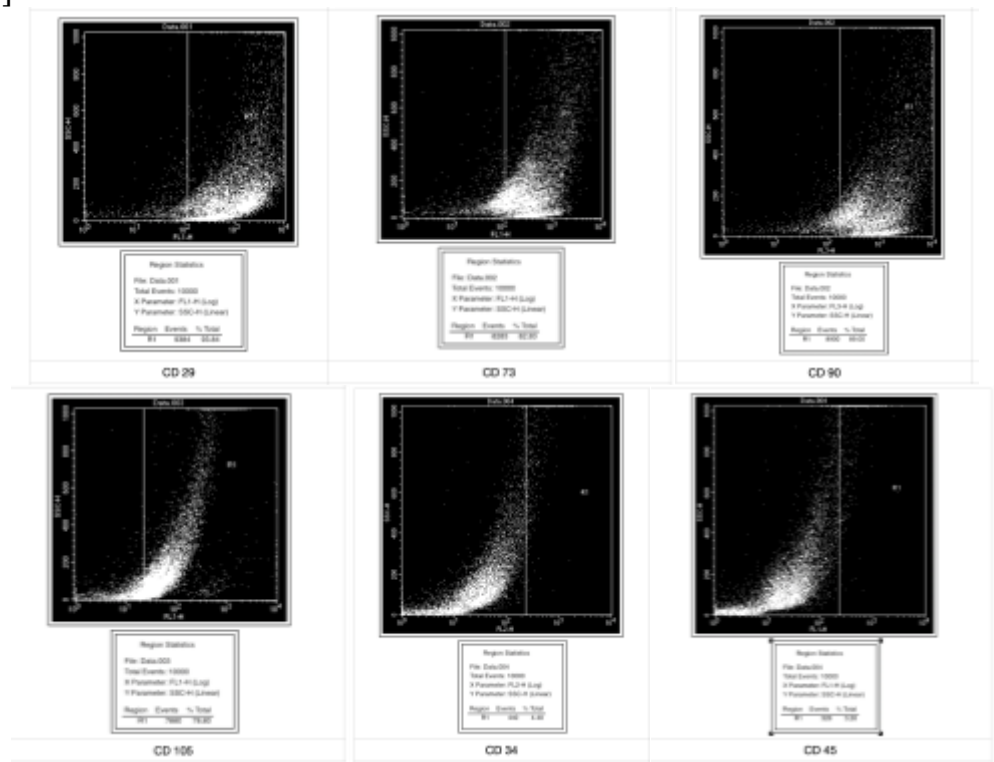

Fig 3. *Immunophenotypic analysis of human WJ-MSCs at passages 4-5 using flow cytometry. Representative dot plots showing positive expression of CD29, CD73, CD90, and CD105, and negative expression of CD34 and CD45.*

The capacity for directed multilineage differentiation is another essential criterion for defining MSCs [31]. The differentiation potential was assessed using WJ-MSCs at passage 4 through cultivation in media containing specific induction factors. On day 21 of adipogenic induction, cells with lipid inclusions that stained positive with Nile Red were detected in the cultures (**Fig. 4, A**). On day 21 of osteogenic induction, the deposition of a calcium-binding matrix was observed in the culture. The successful realization of osteogenic differentiation was confirmed by positive Alizarin Red S staining for bound calcium (**Fig. 4, B**).

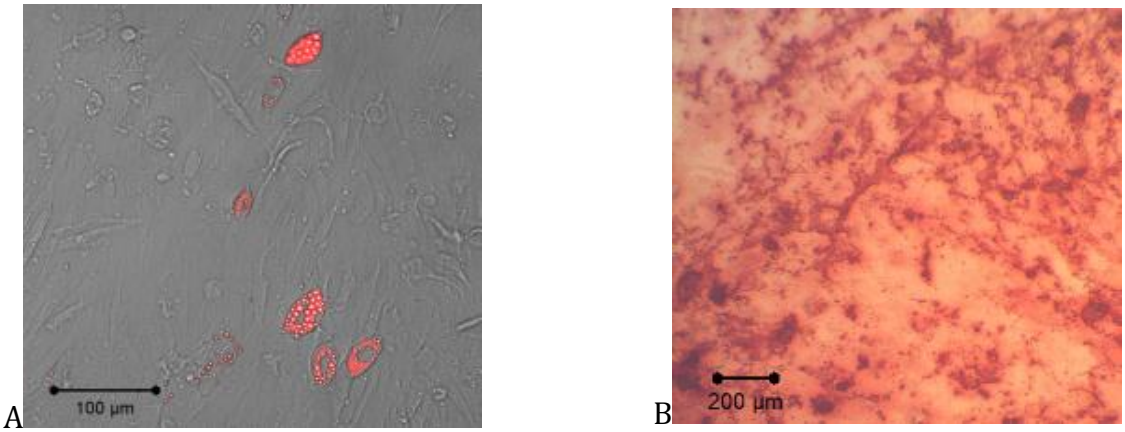

Fig 4. *Microphotographs of human WJ-MSCs isolated by combined explant-enzymatic method and cultivated in adipogeneic (A) or osteogeneic (B) inductive media for 21 days. A – fluorescence microscopy, staining with Nile Red; B – light microscopy, staining with Alizarin Red S.*

Pooled conditioned media from WJ-MSCs, harvested across passages 4 to 7 to evaluate the cumulative secretome during the cell expansion window, contained all the investigated growth factors and cytokines (**Fig. 5**). Marked inter-donor variability in their concentrations was observed, and the magnitude of these variations was clearly analyte-dependent. VEGF, EGF, PDGF-BB, SCF, TNF-α, and TGF-β showed large fluctuations in the thousands of units (6400–10200, 8800–12200, 2300–4000, 500–2000, 2200–5400, and 2800–7400 NLU, respectively). In contrast, NGF-β and FGF-β exhibited greater stability and lower variability (900–1200 NLU and 200–700 NLU, respectively). WJ-MSC CM were characterized by very high levels of VEGF and EGF, high levels of PDGF-BB, and unexpectedly high concentrations of the pro-inflammatory cytokines TNF-α and TGF-β, whereas FGF-β was present at the lowest level. Our findings regarding the secretome profile of WJ-MSCs align with recent studies [32, 33], confirming high angiogenic potential (high levels of VEGF, EGF).

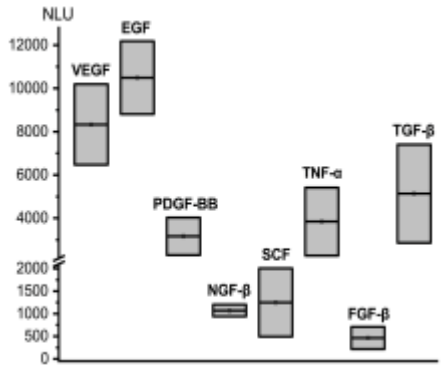

Fig. 5. *Secretory profile of CM obtained after 48 h of WJ-MSCs culturing without FBS. Growth factor quantification was performed using pooled conditioned media harvested across passages 4 to 7 from WJ-MSCs isolated from 5 distinct biological donors. Data are presented as mean ± SEM.*

Thus, the results of this study demonstrate that the isolation of cells from the connective tissue of Wharton's jelly using a combined explant-enzymatic method ensures a stable cell yield. The reproducibility of the proposed method is high, allowing for the minimization of technical factors such as non-uniform tissue processing and variable culture conditions. Following the expansion of the isolated cells, it was shown that they possess a high CFU-F capacity and fulfill the minimal criteria for defining MSCs established by the ISCT. Specifically, the cells adhered to the plastic surface, exhibited a fibroblast-like morphology, expressed characteristic surface markers (CD29, CD90, CD73, and CD105), were negative for hematopoietic markers (CD45 and CD34), and demonstrated the capacity for directed multilineage differentiation. Obtained results demonstrate that WJ-MSCs produce full spectrum of investigated growth factors and cytokines showing high inter-donor variability. WJ-MSC CM were characterized by very high concentrations of VEGF and EGF, high levels of PDGF-BB, and unexpectedly elevated amounts of the pro-inflammatory cytokines TNF-α and TGF-β. In contrast, FGF-β was consistently present at the lowest level, whereas NGF-β and FGF-β showed markedly greater stability across donors compared with the other factors.

A limitation of the present study is that the biological sex of the fetuses from whom the umbilical cords were obtained was not recorded or evaluated during data analysis due to institutional bioethical constraints regarding donor personal data. Emerging evidence suggests that fetal sex may influence the proliferation rate, differentiation capacity, and secretory activity of hWJ-MSCs [34, 35, 36]. While our strategy of pooling conditioned media helped mitigate potential individual donor-specific biases by providing a representative average profile, future studies with precisely documented donor demographics are warranted to investigate potential sex-related functional variations in hWJ-MSCs isolated by the combined method.

These findings confirm the functional integrity of human MSCs isolated from Wharton's jelly via the combined enzymatic-explant method. Consequently, this approach can be considered a promising standardized protocol for biobanking and cell therapy applications.

Conclusions

1. The proposed combined explant-enzymatic method provides a robust and reproducible approach for isolating MSCs from human Wharton's jelly, ensuring a stable cell yield while minimizing the impact of technical variability.
2. Individual biological characteristics of the donor material have significant impact on cell yield.
3. The isolated cell populations fully comply with the ISCT minimal criteria for MSCs, exhibiting typical fibroblast-like morphology, high clonogenic potential, a characteristic immunophenotype ($CD29^+$, $CD90^+$, $CD73^+$, $CD105^+$, $CD34^-$, $CD45^-$), and the capacity for directed multilineage differentiation.
4. Our findings confirm high secretory potential of human WJ-MSCs and highlight a distinctive secretory profile that is particularly enriched in key regulators of angiogenesis, cell proliferation, and tissue repair.
5. The functional integrity and standardized isolation protocol make these human WJ-MSCs a promising biological material for clinical applications in regenerative medicine and large-scale biobanking.

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AUTHOR CONTRIBUTIONS. *Conceptualization: O.P., R.O., N.T.; Methodology: O.P., R.O., N.T., O.L., D.C.; Investigation: R.O., N.T., O.R., D.C., P.Z.; Formal analysis: R.O., N.T., D.C., P.Z.; Writing – original draft: R.O., N.T.; Writing – review & editing: R.O., N.T., O.P. All authors approved the final manuscript.*

ARTIFICIAL INTELLIGENCE (AI) USAGE DECLARATION.
The authors declare that artificial intelligence (AI) tools were used during the preparation of this manuscript. Specifically, Gemini (2026 version, provided by Google) was used for scientific text refinement, language editing, and grammar correction. All AI-assisted outputs were carefully reviewed, verified, and revised by the authors to ensure scientific accuracy, integrity, and compliance with journal standards. The authors take full responsibility for the final content of the manuscript.

CONFLICT OF INTEREST STATEMENT. *The authors declare that they have no conflicts of interest related to the research, authorship, and/or publication of this article.*

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