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Isolation, Proliferation, and Identification of Human Wharton's Jelly Mesenchymal Stem Cells (hWJMSCs)

Raed H. Ogaili[1,2], Elvy Suhana Mohd Ramli[2] ,Isma Liza Mohd Isa[2,3,*]

[1] Department of Anatomy, College of Veterinary Medicine, University of Kerbala, Iraq
[2]Department of Anatomy, Faculty of Medicine, Universiti Kebangsaan Malaysia, Cheras 56000, Kuala Lumpur, Malaysia
[3]CÚRAM Research Ireland Centre for Medical Devices, School of Medicine, University of Galway, Galway H91 W2TY, Ireland
*Corresponding author ismaliza.mohdisa@ukm.edu.my

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Abstract

In this research, we compared the biological characteristics and therapeutic potential of "human Wharton's jelly Mesenchymal Stem Cells (hWJMSCs)" isolated from various anatomical regions of the umbilical cord.". Cells were isolated from maternal, middle, and fetal segments of "umbilical cords" (n = 6) of six healthy donors who underwent elective cesarean delivery and systematically compared. Several analyses were used to characterize the cells, including growth kinetics, immunophenotyping, gene expression profiling, and differentiation potential assessment. Results showed substantial segment-specific variations, with yields and characteristics of "hWJMSCs" obtained from "maternal and fetal segments" superior to middle segment. Enhanced proliferation rates (population doubling times of 1.02 ± 0.09 and 1.21 ± 0.2 days compared with 1.86 ± 0.12 days), increased cell viability, and stronger expression of stemness markers and differentiation potential were observed in these cells.

On the other hand, reduced HLA-ABC expression in maternal and fetal segments indicates perhaps improved immune privilege. This research reveals strong evidence that the anatomical origin of "hWJMSCs" from the umbilical cord remarkably impacts biological characteristics and cytotherapeutic potential. Implications of these findings for regenerative medicine are significant and suggest that targeted harvesting from maternal and fetal segments has the potential to enhance therapeutic efficacy. This research lays the groundwork for standardizing cell-sourcing protocols and has implications for advancing cell-based therapy.

Keywords

hWJMSCs, Growth kinetics, Immunophenotyping, Gene expression profiling, Differentiation potential

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Introduction

"Human Wharton's jelly-derived Mesenchymal Stem Cells (hWJMSCs) are perinatal stem cells derived from "umbilical cords" of Wharton's jelly, a type of extra-embryonic tissue. They retain properties of both embryonic and adult stem cells" [1]

Proliferation and Multipotency: "hWJMSCs" exhibit high proliferation potential like embryonic stem cells and can differentiate into multiple cell lineages.

Immunomodulatory Properties: Similar to adult mesenchymal stem cells, they have immunomodulatory properties and are non-tumorigenic. [2] As "hWJMSCs" are derived from umbilical cord tissue after birth, their use avoids the ethical dilemmas that plague embryonic stem cells. [1–3]

"Mesenchymal stem cells (MSCs) are a heterogeneous population of multipotent stromal cells that possess self-renewal capacity and the ability to differentiate into various cell types, such as osteoblasts, chondrocytes, and adipocytes [4,5] These cells have shown strong immunomodulatory properties, which make them potential candidates in regenerative medicine applications [6] MSCs can be isolated from various tissue sources, including bone marrow, adipose tissue, and umbilical cord blood, each having its pros and cons" [7,8].

"Wharton's jelly", the thick substance inside the umbilical cord, has gained significant attention among these sources. "hWJMSCs" have received attention for their abundant availability, non-invasively obtained, and faster proliferative capability than MSCs from other origins [9,10]. In addition, "hWJMSCs" have low immunogenicity, which makes them more favorable for allologous transplantation.

In the clinical field, "hWJMSCs" are a promising cell type for therapeutic use. Several studies have demonstrated the ability to modulate immune responses and promote tissue regeneration [8, 9]. These advantages, however, warrant further investigation to untangle the mechanistic role of "hWJMSCs" differentiation, paracrine effects, and host-tissue interactions.

In this study, we explore the therapeutic potential of "hWJMSCs", focusing on their differentiation capacity, paracrine effects, and impact on host tissues. Our goal is to address existing knowledge gaps in the literature related to and assess their potential clinical applications.

Though the well-documented regenerative ability of "hWJMSCs" [10] has been highlighted in previous studies, sharing how they help the immune system and mediate tissue repair is still unclear. The present research studies that used "hWJMSCs" in regenerative medicine will enhance our knowledge of their use and provide an idea of their mechanism.

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Materials and Methods

Sample Collection and Processing

The study was completed in two phases. Phase 1: Cell isolation and identification in UKM Medical Centre with institutional ethical approval. Phase two (cell proliferation) was performed in the tissue culture lab, Anatomy Department, Faculty of Medicine, UKM.

All research methods followed the institution's guidelines. Informed consent was obtained from all participating patients before sample collection, guaranteeing voluntary participation and privacy. It also described the ethical framework concerning sample handling, storage, and research on umbilical cord tissues.

The research included six healthy patients (n=6) who met detailed inclusion criteria. All enrolled patients underwent elective cesarean delivery to maintain controlled conditions at the time of sample collection. The pregnancies were full-term, between 38–40 weeks of gestation, to keep the tissue maturation and development consistent. This deliberate selection process also helped minimise variables that could impact the quality and characteristics of the isolated mesenchymal stromal cells.

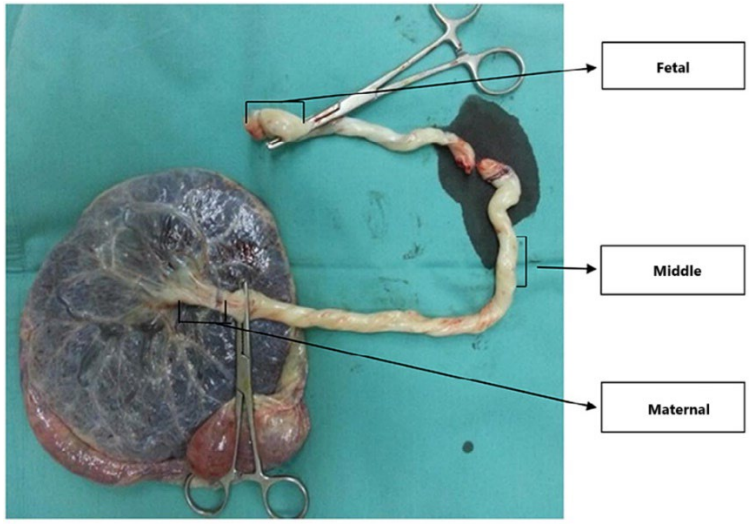

Figure 1. anatomical parts of human umbilical cord.[4]

Umbilical Cord Processing

The umbilical cord samples were processed within a critical three-hour window after collection to maintain tissue viability. Each umbilical cord, typically 50-60 cm in length, was systematically divided into three distinct anatomical segments: the maternal segment (3 cm from the placental end), the middle segment (3 cm from the center), and the fetal segment (3 cm from the fetal end). This standardized segmentation approach was crucial for studying potential differences in MSC characteristics based on their anatomical location within the cord. All segments were handled under sterile conditions and thoroughly rinsed with “Dulbecco's phosphate-buffered saline (DPBS)” to remove blood and potential contaminants before further processing.

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"hWJMSCs" Isolation and Culture

Initial Tissue Processing

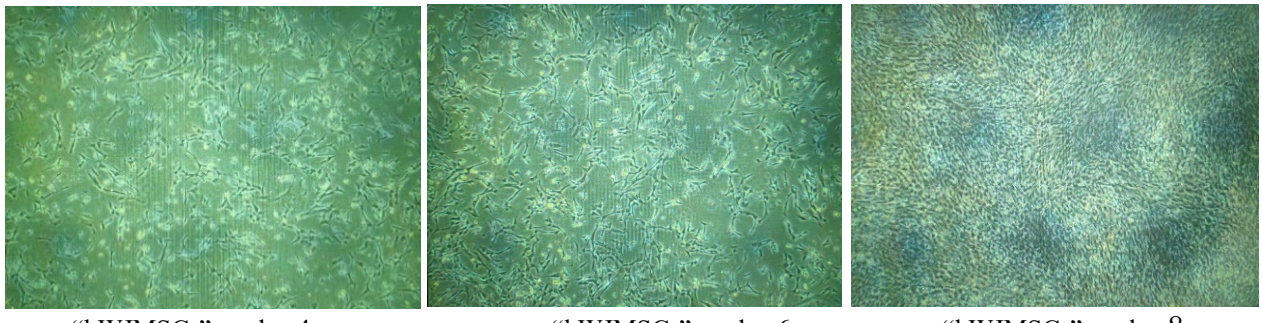
"hWJMSCs" were isolated by preparing the tissue under sterile conditions. The "umbilical cords" were rinsed with "Dulbecco's phosphate buffer saline" (DPBS; Gibco-Invitrogen) several times to eliminate residual blood and surface contaminants. The cord segments were anatomically dissected, during which the umbilical arteries and umbilical vein were stripped out to isolate the "Wharton's jelly" after washing. To enhance the efficiency of subsequent enzymatic dissociation, the isolated jelly tissue was mechanically minced into pieces of approximately 2 mm^2.

Enzymatic Digestion Protocol

The minced Wharton's jelly was subjected to enzymatic digestion using a well-tested solution containing "20 mL of 0.6% (w/v) collagenase type II (Worthington) in low-glucose DMEM media with 1% antibiotic-antimycotic. The digestion process was performed at 37°C in an incubator shaker (250 rpm/h) for one hour. After digestion, the tissue suspension was centrifuged for 5 minutes at 5000 rpm to collect the cell pellet, separating the isolated cells from the components of the digested matrix".

Culture Medium Composition

The separated cells were cultured in a custom-made medium to provide the optimal environment for the growth and fork stabilization of the MSC cells. "The media consisted of low-glucose DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% vitamin C (Sigma Aldrich),1% Glutamax (Gibco), 1% HEPES buffer solution (Gibco), and 1% antibiotic-antimycotic (Gibco)", and. This accurately designed media produced ideal circumstances under which cells could grow and be maintained, both when making primary cultures and even cell expansion later on.



"hWJMSCs" on day 4 "hWJMSCs" on day 6 "hWJMSCs" on day 8.

Figure 2 shows the stages of stem cell Proliferation

Growth Kinetic Analysis

We then systematically evaluated the growth characteristics of "hWJMSCs" using the trypan blue exclusion assay and hemocytometer counting. "Cells were passaged at a constant seeding density of 5000 cells/cm^2 for three passes. Specific formulas were applied for the calculation of parameters: population doubling (D) as D = Log C/Log 2 (where C is the final cell number/initial cell number), population doubling time as the number of days/D, and cell viability determined as percentage of live cells per total cell number". This additional data on proliferation rates and cell viability across the three cord segments was obtained from measurements taken at each passage once 90% confluency was achieved.

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Immunophenotypic Analysis

Cell surface markers were characterized in passage three, "hWJMSCs", using a comprehensive panel of antibodies to verify their mesenchymal stromal cell identity. The analysis included assessment of positive MSC markers "(CD13, CD29, CD44, CD73, CD90, CD105)", hematopoietic markers (CD14, CD34, CD45), and human leukocyte antigens (HLA-ABC, HLA-DR). This thorough phenotypic characterisation ensured the isolated cells met the minimal criteria for MSCs defined by the International Society for Cellular Therapy (ISCT).

Flow Cytometry

The cells from passage three (n=6) of each segment of cords were analyzed by "flow cytometry" using a FACS Calibur system with Cell Quest Pro software. Cells were treated with conjugated antibodies against cell surface markers, fixed with 4% paraformaldehyde, maintained at 4° C, and analyzed within 24 hrs. For each sample, we analysed a standardized count of 1×10^4 cells, which allows sufficient statistical confidence and consistency in quantifying the marker expression between the different cord segments.

Gene Expression Analysis

RNA was extracted and analyzed according to the manufacturer's protocol using the RNeasy Plus Mini kit. The EDC RNA was reverse-transcribed and amplified by PCR for the embryonic transcription factors Nanog, Sox2, Rex-1 and OCT-4, with GAPDH as the housekeeping gene. The PCR program was designed with 40 cycles (15 seconds at 94 °C, 30 seconds at 53 °C, 30 seconds at 72 °C) and melt curve analysis to ensure amplification specificity, giving us essential information on the cells' molecular features and stemness characteristics.

Immunofluorescence Analysis

Immunofluorescent staining was performed on passage one cells (n=6) to visualize the expression of embryonic markers. "The protocol involved fixing cells with 4% paraformaldehyde, permeabilizing with 0.1% Triton X-100, and blocking with 10% goat serum. Cells were then incubated with primary antibodies against TRA-1-60, Nanog, SSEA-4, SSEA-3, and Oct 3/4 (1:80 dilution), followed by fluorescent-conjugated secondary antibodies (AleXa Fluor 488 or 594; 1:150). Nuclear counterstaining was performed with DAPI, and imaging was conducted using a Nikon A1R confocal laser scanning microscope, providing detailed visual evidence of protein expression patterns".

RT-PCR Analysis

The RT-PCR analysis began with careful sample preparation using "hWJMSCs" at passage 3, with six samples (n=6) collected from each umbilical cord segment. RNA extraction was performed using the RNeasy Plus Mini kit (Qiagen, Germany), following a systematic protocol that began with cell lysis using RLT Plus Buffer. Before further analysis, the extracted RNA was assessed for quality using standard quality metrics to confirm high purity, as appropriate 260/280 ratios.

"Genomic DNA contamination was removed using a custom protocol that passed samples through a gDNA Eliminator spin column. Subsequently, the RNA was precipitated with 70% ethanol and purified using an RNeasy spin column (Qiagen) according to the manufacturer's instructions. Washed the purified RNA successively with RW1 Buffer and RPE Buffer and added 40 µL of RNase-free water for optimal quality during downstream applications.

cDNA synthesis was carried out using the QuantiTect Reverse Transcription kit (Qiagen), which includes a proprietary combination of Quantiscript Reverse Transcriptase, Quantiscript RT Buffer and RT Primer Mix. cDNA synthesis was performed using the reverse transcription reaction at 42°C for 20 min, an optimum temperature for efficient and maximum cDNA synthesis. Subsequently, PCR amplification was performed, and the Quantified SYBR Green Master mix, forward and reverse primers targeting selected embryonic markers (Nanog, Sox2, Rex-1, OCT-4), and the housekeeping gene (GAPDH) were used.

The cycles were run on a Bio-Rad I Cycler PCR machine (40 cycles, precise temperatures). Each cycle comprised 15 s denaturation at 94°C, 30 s annealing at 53 °C, and 30 s extension at 72°C. Custom-designed primers were derived from sequences available there using the primer 3 software (Palo Alto, CA, USA) so that amplification of target genes was specific. After amplification, a melt curve analysis was performed to verify the specificity of the PCR products".

Relative quantification methods were used to analyse data, and GAPDH served as the internal reference gene for normalisation. A careful examination of amplification curves and melt curve profiles confirmed the results' reliability and specificity. Overall, this RT-PCR protocol was a helpful addition to the study of "hWJMSCs" from three different segments of the umbilical cord and led to a better molecular understanding of hWJMSC.

Results

Isolation and Primary Culture of Cells

"hWJMSCs" were successfully isolated from 6 out of 6 available "umbilical cords" (n=6) with cells harvested from segment 2 (n=5), segment 1 (n=4), and segment 3 (n=4). The cell cultures showed homogenous, plastic-adherent, spindle-shaped cells that grew in monolayer formations. These properties were observed uniformly in all samples cultured in T175 flasks (seeded at 5000 cells/cm^2) and passaged three times. Isolated cells were confirmed to fulfil the essential MSC characteristics, such as adherence and morphological characteristics.

Growth Kinetics and Cell Viability

A significant difference in cellular behaviour was noted among the three portions. The maternal and fetal segments exhibited enhanced attachment capabilities, with cells sticking within one day of seeding, but cells from the middle segment necessitated 4 days for attachment. Analysis of cell production on day 4 indicated markedly elevated quantities in the fetal segment relative to the intermediate segment. Cells from the fetal section attained 85-95% confluency most swiftly (8 days), followed by the maternal segment, whereas the middle segment required the greatest time. Population doubling times at P1 exhibited notable disparities: maternal segment (24-48 hours) and fetal segment (24-36 hours) compared to the middle segment (36-48 hours). In passage 3, this pattern persisted, with maternal and fetal segments exhibiting markedly accelerated proliferation rates (0.78 ± 0.03 and 0.85 ± 0.04 days, respectively) in contrast to the middle segment (24-48 hours).

Immunophenotypic Profile

"flow cytometry" analysis demonstrated that cells from all three segments had significant numbers of typical MSC markers (CD13, CD29, CD44, CD73, CD90, CD105) exceeding 90%. The maternal and

fetal segments exhibited markedly reduced expression of HLA-ABC (MHC class I) compared to the intermediate segment, indicating a possible increase in immunological privilege. All segments exhibited little expression (<2%) of hematopoietic markers (CD34, CD45, CD14) and were negative for HLA-DR (MHC Class II), so affirming their MSC identity following ISCT criteria.

Embryonic Marker Expression

RT-PCR research revealed the expression of Nanog and OCT 3/4 in cells from all three segments; however, SOX-2 and REX-1 were absent. The expression of Nanog was significantly reduced in cells from the intermediate segment compared to those from the fetal and maternal segments. Immunofluorescence labelling corroborated these findings, demonstrating that SSEA-4 was only present in cells from the maternal and fetal segments, but SSEA-3 and TRA160 were lacking in all segments.

Differentiation Potential

The trilineage differentiation assays showed varying potentials among the segments. For osteogenic differentiation, all segments showed capability. Still, the maternal and fetal segments produced more bone nodules, with calcium deposition visible by day 19, compared to day 21 in the middle segment. In adipogenic differentiation, the maternal segment showed superior potential with higher lipid droplets and more significant optical density measurements. Chondrogenic differentiation was most pronounced in the fetal segment, though all segments demonstrated positive safranin O staining for glycosaminoglycans. These differences, while notable, were not always statistically significant due to the small sample size.

Statistical Analysis

All quantitative data were analysed using SPSS version 20, with results presented as mean ± SEM. The comparisons among the segments were conducted utilising one-way ANOVA, accompanied by Bonferroni post hoc testing. Statistical significance was established at $p<0.05$, indicating notable differences in growth kinetics and differentiation potential among the middle, maternal, and foetal segments.

Discussion

This study presents novel findings regarding the differential characteristics of “hWJMSCs” isolated from distinct anatomical segments of the umbilical cord. The maternal and fetal segments demonstrated superior characteristics to the middle segment, particularly in proliferation rates, cell viability, and stemness marker expression. These findings align with previous observations by [1,5], who reported that fetal-derived MSCs exhibit enhanced proliferation capabilities compared to adult sources. The enhanced expression of pluripotency markers (SSEA-4) and lower HLA-ABC levels in maternal and fetal segments suggest these regions harbour more primitive cell populations, supporting the observations of [6] regarding the relationship between SSEA-4 expression and MSC multipotency.

Identifying segment-specific variations in hWJMSC properties has significant implications for clinical applications. Our findings regarding superior osteogenic potential in maternal and fetal segments complement the work of [7], who demonstrated enhanced bone formation capabilities in “Wharton’s jelly”-derived MSCs. The lower HLA-ABC expression observed in these segments aligns with [8]

findings regarding the immunological properties of fetal MSCs, suggesting potential advantages for allogeneic transplantation. These characteristics support [9] assertion that "Wharton's jelly"-derived cells represent a primitive stromal cell population with unique therapeutic potential.

The systematic approach to comparing cells from defined anatomical segments provides robust evidence for optimal source selection, building upon the work of [10] in characterising umbilical cord MSCs. However, limitations include the small sample size (n=6) and the focus on standard "umbilical cords" only. As noted by [11], the relationship between MSC markers and functional properties requires further investigation. Our observations regarding segment-specific variations in differentiation potential contribute to the ongoing discussion initiated by [12] about optimising cell source selection for specific therapeutic applications.

These findings suggest several promising research avenues. Further investigation is needed into the molecular mechanisms underlying segment-specific differences, mainly focusing on the epigenetic regulation discussed by [13]. Studies examining therapeutic efficacy in various disease models would be valuable, following the approach of [14] in evaluating clinical applications. Future research should explore the impact of maternal and fetal health conditions on segment-specific cell properties, building on [15] work on placental MSCs. Additionally, long-term studies comparing post-transplantation behaviour and therapeutic efficacy would provide crucial information for clinical applications, as suggested by [16] in their comprehensive analysis of MSC properties.

This research advances our understanding of optimising cell source selection for regenerative medicine while highlighting the need for continued investigation to understand and fully utilise the unique properties of segment-specific "hWJMSCs". As emphasised by [17] in their landmark paper defining MSC characteristics, standardising cell preparation and characterisation remains crucial for therapeutic success.

Conclusion

The current study provides robust evidence that the anatomical niche of "hWJMSCs" is a key determinant of these cells' biological properties and therapeutic potential. Maternal and fetal segments performed equally well or better than the middle segment. MSCs derived from maternal and fetal segments exhibited higher proliferation rates, increased cell viability, stronger stemness marker expression, and improved differentiation potential. In addition, these segments exhibited reduced HLA-ABC expression, implying possibly improved immune privilege. This study has important implications for regenerative medicine, with selective harvesting from maternal and fetal segments that optimise therapeutic outcomes. This study provides a framework for standardising cell sourcing protocols and unlocks new strategies for enhancing cell therapy effectiveness. More studies are needed to elucidate the underlying mechanisms of these segment-specific differences. This study marks a significant advancement in regenerative medicine and provides a clear direction for optimising cell source selection in clinical applications.

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