BUKTI KORESPONDENSI

Rapid isolation and characterization of Wharton's jelly-derived mesenchymal stem cells maintained in fresh-prepared human AB-serum

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1 message

Adi Hidayat <hidayat.adi@trisakti.ac.id> Thu, Dec 5, 2024 at 4:51 PM
To: Yudi Tjahyono <yudy.tjahjono@ukwms.ac.id>

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Isolation and Characterization of Wharton's Jelly-Derived Mesenchymal Stem Cells: ~~A~~ Foundational Protocol for Regenerative Medicine Applications at Gotong Royong Hospital

Isolation and Characterization of Wharton's Jelly-Derived Mesenchymal Stem Cells and maintain in ….

Sianty Dewi[1¶], Yudy Tjahjono[2*¶], Bernadette Dian Novita Dewi[1], Hendy Wijaya[1], Brilliant Dwi Putra[2], Teguh Widodo[2], Lucia Hendriati[2], Himawan Haryanto Jong[1], Franklin Malonda[1], and Suryo Kuncorojakti[3,4]

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ABSTRACT revised to around 250 words

Background

Mesenchymal stem cells (MSCs) are multipotent cells capable of differentiating into osteocytes, chondrocytes, and adipocytes, which makes them valuable for regenerative medicine. Isolation of post-partum umbilical cord Wharton's jelly stem cells has gained attention as an alternative source of the bone marrow. MSCs derived from Wharton's Jelly in the human umbilical cord (WJ-MSCs) are particularly promising due to non-invasive collection, high proliferation, and immunomodulatory properties. The aim of this study was to present the relevant methods for the isolation and characterization of Wharton`s jelly-derived MScs. ~~This study describes the first successful isolation and characterization of WJ-MSCs at Gotong Royong Hospital.~~

Methods

Human umbilical cords from three healthy donors were collected immediately post-cesarean section, meeting strict inclusion criteria. The WJ-MSCs were isolated using an explant culture technique in aseptic conditions, with cells adhering to T75 flasks pre-coated with 2% gelatin. Cultures were maintained in Dulbecco's modified Eagle medium (DMEM : brand, USA) supplemented with 10% Human AB serum, and growth was monitored for 21 days. Flow cytometry was conducted using BD FACSAria to assess expression of MSC markers CD105, CD73, CD90, and CD44, as well as the exclusion marker CD45, at Passages 1 and 5.

Results

The isolated WJ-MSCs displayed fibroblast-like morphology characteristic of MSCs by Passage 1, with consistent, robust proliferation over 21 days. Flow cytometry revealed that CD44 was the only highly expressed marker (~60%) at Passage 1, while CD105, CD73, and

CD90 became prominent by Passage 5. CD45 levels remained low, though detectable, suggesting minimal hematopoietic contamination. This expression pattern differs from MSCs derived from the cord-placental junction (CPJ), which tend to display higher and earlier marker expression.

Conclusions

The present study has revealed the feasibility of the culture medium with ……. in isolation and proliferation of WJ-MSCs.

~~This study successfully establishes a foundational protocol for WJ-MSC isolation and expansion at Gotong Royong Hospital, supporting local stem cell research initiatives~~. While effective, the protocol's 21-day outgrowth period may benefit from further optimization to improve scalability, such as adjustments in serum concentration, substrate coatings, and oxygen levels. The findings also indicate the potential advantage of CPJ-MSCs for applications requiring rapid cell expansion and robust marker profiles. ~~This protocol paves the way for future research and clinical applications of WJ-MSCs within the hospital's healthcare system.~~

Keywords: Wharton's jelly, mesenchymal stem cells, isolation, flow cytometry, regenerative medicine

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stromal cells capable of differentiating into various cell types, such as osteocytes, chondrocytes, and adipocytes, making them valuable for regenerative medicine. Mesenchymal stem cells are characterized by a specific set of surface markers that distinguish them from other cell types, particularly hematopoietic cells. According

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to the International Society for Cellular Therapy (ISCT), MSCs must express CD105, CD73, and CD90, which are markers associated with cell adhesion, growth, and differentiation potential. [1] CD105, or endoglin, plays a role in cell proliferation and vascular development, [2] while CD73 and CD90 contribute to immunomodulation and cell-cell interactions. [3,4] CD44, a receptor for hyaluronic acid, is also commonly expressed on MSCs and is involved in cell adhesion, migration, and homing, which are essential for therapeutic applications. [5] Importantly, MSCs are negative for CD45, a pan-leukocyte marker, which ensures the exclusion of hematopoietic lineage cells. [6] This unique combination of surface markers—CD105, CD73, CD90, and CD44, with the absence of CD45—confirms the purity and identity of MSCs, supporting their use in clinical and research settings. [7]

The potential of MSCs for regenerative medicine has led to extensive research on various sources of MSCs, including those derived from Wharton's Jelly of the human umbilical cord (WJ-MSCs). Wharton's Jelly of the human umbilical cord offer unique advantages, including non-invasive collection methods, high proliferation rates, and immunomodulatory properties, making them a promising cell type for therapeutic applications. [8] However, isolation and expansion methods must be refined to ensure the viability and consistency of WJ-MSCs.

Describe the results of previous similar studies. What was the results, consistent or inconclusive, Describe the difference between your study and the similar previous studies, This was the novelty of your study. You have to describe it. There were a lot of previous similar studies, you have to describe the difference between your study and the similar previous studies

Describe the objective of your study

A study has revealed the feasibility of the culture medium with high glucose and 15% FBS in isolation and proliferation of WJ-MSCs. When Wharton`s jelly pieces were put in the dry bottom of the flask, very effective separation of the MSCs was achieved. (Ranjbaran H,

Abediankenari S, Mohammadi M, et al. Wharton's jelly derived-mesenchymal stem cells: isolation and characterization. Acta Med Iran 2018;56:28-33.) Another study found that the isolation and characterization of UC-WJ cells in a serum-free condition and maintenance of primitive mesenchymal phenotype. The culture was stable under 60 consecutive passages with no genetic abnormalities and proliferating ratios. Taken together all results, it was possible to demonstrate an easy way to isolate and culture of bovine-derived UC-WJ cells under 2D and 3D serum-free condition, from fetal adnexa with a great potential in cell therapy and biotechnology. (Cardoso TC, Ferrari HF, Garcia AF, et al. Isolation and characterization of Wharton's jelly-derived multipotent mesenchymal stromal cells obtained from bovine umbilical cord and maintained in a defined serum-free three-dimensional system. BMC Biotechnology 2012, 12:18). A study found that the primary cultures exhibited success in isolating the umbilical cord Wharton's jelly mesenchymal stem cells, which maintained their tri-lineage differentiation potential, phenotypes and karyotype characteristics on further passage and expansion. (Abouelnaga H, El-Khateeb D, Moemen Y, El-Fert A, Elgazzar M, Khalil A.. Characterization of mesenchymal stem cells isolated from Wharton's jelly of the human umbilical cord. Egyptian Liver J 2022; 12:2. https://doi.org/10.1186/s43066-021-00165-w.)

~~In this study, we present the first successful isolation and characterization of WJ-MSCs conducted at Gotong Royong Hospital, demonstrating the feasibility of establishing a protocol for obtaining viable WJ-MSCs from human umbilical cords and expanding them in culture.~~

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~~The establishment of this protocol at Gotong Royong Hospital not only provides a new avenue for local research in stem cell therapy but also lays the groundwork for future clinical applications and cell-based treatments. Patient selection in this study involved strict criteria, ensuring that only umbilical cords from healthy pregnancies were used, thus optimizing the~~

~~quality and viability of the cells obtained. Ethical approval was secured, and careful steps were taken to remove blood vessels and isolate the Wharton's Jelly, maximizing MSC yield. This standardized approach enables a reliable source of WJ-MSCs, critical for any future applications in regenerative medicine and disease modelling~~.

~~Our results show that the WJ-MSCs isolated through this protocol display a fibroblast-like morphology characteristic of MSCs, with consistent morphology observed through the first passage. Using a detailed explant culture method, we were able to cultivate and expand WJ-MSCs, which adhered to the culture surface within days and proliferated significantly over 21 days. These cells retained their mesenchymal characteristics, evidenced by their elongated, spindle-shaped morphology and high proliferation rate. Such morphological consistency is essential for the continued use of these cells in therapeutic research, where cell integrity and stability are paramount.~~

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~~In addition to morphological assessment, flow cytometry analysis was employed to confirm the MSC phenotype, assessing the expression of surface markers CD105, CD73, CD90, and CD44, with CD45 as a hematopoietic exclusion marker. This phenotypic profile aligns with the International Society for Cellular Therapy (ISCT) guidelines for MSC characterization, confirming the cells' stem cell identity and purity across passages. This study thus establishes a foundational WJ-MSC isolation and expansion protocol at Gotong Royong Hospital, creating significant potential for future cell therapy research and applications within the hospital and the broader scientific community.~~

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~~MATERIAL~~ METHODS

Research design

Describe the research study, location and period of the study

Isolation of human umbilical cord-derived mesenchymal stem cells (WJ-MSCs)

Human umbilical cords were collected from 3 (three) patients in Gotong Royong Hospital, immediately following caesarean section, within 5 minutes of placental removal to ensure optimal cell viability. Patient selection included strict criteria: only umbilical cords from healthy pregnancies without maternal or fetal complications were used. Specifically, cases of preeclampsia, metabolic disorders, infections, or abnormal umbilical cord coiling were excluded. ~~The ethical approval was obtained.~~

Upon retrieval, each umbilical cord was placed in a sterile container with Dulbecco's Modified Eagle Medium (DMEM) containing 1% penicillin/streptomycin/amphotericin B and transported to the laboratory under aseptic conditions.

Preparation and culture of WJ-MSCs

In a laminar flow cell culture hood to maintain aseptic technique, each umbilical cord was first washed thoroughly with Hank's Balanced Salt Solution (HBSS) containing 1% penicillin/streptomycin/amphotericin B. Blood vessels were carefully removed from the Wharton's Jelly tissue to isolate the mesenchymal-rich regions, minimizing potential contamination, and promoting a higher yield of mesenchymal stem cells (MSCs). The remaining Wharton's Jelly tissue was cut into approximately 2 mm^2 pieces, which were then placed in T75 culture flasks precoated with 2% gelatin to enhance cellular adherence. [9]

The tissue explants were left undisturbed for 7 days to allow cells to attach to the surface. [10] The culture medium, composed of DMEM supplemented with 10% Human AB serum and 0.1% penicillin/streptomycin/amphotericin B, was carefully added to prevent dislodging the tissue fragments. Medium was replaced every 3 days, and cell outgrowth was monitored microscopically. After 21 days, successful outgrowth and proliferation of Passage 1 (P1) cells

were observed, characterized by the spindle-shaped, fibroblast-like morphology typical of MSCs.

Flow cytometry analysis

Flow cytometry was employed to phenotype Wharton's Jelly-derived mesenchymal stem cells (WJ-MSCs) isolated at Passage 2 and Passage 6 using a BD FACSAria flow cytometer (BD Biosciences). To assess the expression of key mesenchymal and hematopoietic markers, three antibody panels were prepared with mouse monoclonal antibodies specific to human antigens. Panel 1 included anti-human CD105 (clone 43A3, Alexa Fluor 488, BioLegend) and anti-human CD45 (clone H130, FITC, BioLegend); Panel 2 included anti-human CD73 (clone AD2, PE, BioLegend) and anti-human CD90 (clone 5E10, PE/Cy7, BioLegend); and Panel 3 included anti-human CD44 (clone IM7, PE, Elabscience). For each panel, 10^6cells in 100ul were aliquoted into individual tubes, and 2.5 µL of each antibody was added to each sample. The cells were incubated for 1 hour at 4°C in the dark to optimize antibody binding and prevent photobleaching. After incubation, cells were washed twice with staining buffer (PBS with 1% BSA) to remove unbound antibodies and resuspended in 500 µL of buffer for analysis. Gating parameters was set to exclude debris and non-viable cells based on forward and side scatter properties. Unstained controls were applied to correct for spectral overlap among fluorophores. Expression of CD105, CD73, CD90, and CD44 was assessed to confirm MSC phenotype, while CD45 was used to exclude hematopoietic contamination. [(11)] Data were analyzed to determine the percentage of cells expressing each marker, providing a comprehensive phenotypic profile of WJ-MSCs across passages.

Ethical clearance

The ethical clearance for collecting the human tissue was obtained from medical Faculty, Universitas Hang Tuah (**E/006/UHT.KEPK.03/II/2024**)

Statistical analysis

GraphPad Prism version 8.0.1 was used for data presentation. Unpaired t-tests were employed to compare relative intensity differences between specific groups. A p-value < 0.05 is considered significant. All data were presented as mean ± standard deviation (SD).

RESULTS

Isolation and culture of Wharton's jelly-derived MSCs

The isolation of Wharton's Jelly-derived mesenchymal stem cells (WJ-MSCs) was achieved through the explant culture method, allowing MSCs to be successfully obtained from human umbilical cord tissue. Following isolation, WJ-MSCs adhered to the culture surface within the first few days, and after 21 days in culture, Passage 1 (P1) showed significant cell proliferation. The MSCs exhibited a characteristic fibroblast-like morphology (see figure 1), typical of mesenchymal cells, with elongated, spindle-shaped bodies and a uniform, flattened appearance. Cells were tightly adherent, forming a monolayer with parallel, aligned arrangements. The morphology of the cells remained stable through Passage 14 (P14), showing no signs of senescence or morphological abnormalities. This consistent fibroblast-like morphology, with the presence of prominent cell projections and well-defined cellular boundaries, suggests that the WJ-MSCs maintained their mesenchymal characteristics and were successfully expanded in primary culture.

Phenotypic characterization of WJ-MSCs by flow cytometry

To confirm the MSC phenotype, we performed flow cytometric analysis on WJ-MSCs passage 5 (P5), assessing the expression of key surface markers (see figure 2). The gating strategy effectively isolated MSC populations, and the analysis revealed high expression levels of CD73 and CD90, alongside positive populations for CD105. Additionally, the cells were negative for CD45, confirming the absence of hematopoietic contamination (Figure 2). Positive CD44 expression further validated the MSC phenotype, affirming the cells' stem cell characteristics and purity.

Comparative analysis of surface markers at different passages

Comparisons of WJ-MSC surface markers between P1 and P5 revealed significant differences in marker expression (see figure 3). At Passage 1, the MSCs demonstrated a statistically significant increase in pluripotency surface markers compared to the negative phenotype CD45 ($p = 0.01$). By P5, a dramatic increase in CD105, CD90, CD73, and CD44 expression was observed, with statistical significance ($p < 0.0001$) (Figure 3). These findings indicate robust MSC proliferation and stable expression of essential stem cell markers across passages, supporting the successful expansion and maintenance of MSC characteristics through successive passages.

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DISCUSSION

This study successfully demonstrates the isolation and characterization of Wharton's Jelly-derived mesenchymal stem cells (WJ-MSCs) from human umbilical cords collected ~~at Gotong Royong Hospital. Our findings confirm the feasibility of establishing a reliable protocol for obtaining viable WJ-MSCs locally, marking the first successful effort at this institution.~~ These cells exhibited typical MSC morphology and a stable phenotype through early passages, supporting their potential for future research and clinical applications. [12]. However, despite

overall success, the detection of low levels of CD45 expression indicates potential hematopoietic contamination. [11] This may be attributed to residual blood cells within the Wharton's Jelly, as complete removal of all blood vessels can be challenging in clinical settings. [13] Future refinements could include additional washes or enzymatic treatments to further minimize CD45-positive cells, improving the MSC purity and strengthening the clinical potential of these cells. [14]

Interestingly, recent studies have highlighted the potential of MSCs derived specifically from the cord-placental junction, which may offer advantages over WJ-MSCs in terms of cellular yield, differentiation potential, and immunomodulatory properties. [15] MSCs from the cord-placental junction have shown enhanced proliferation rates and greater multilineage differentiation capabilities compared to WJ-MSCs, likely due to their proximity to the placenta and the rich microenvironmental cues present there .[16] Exploring the isolation of MSCs from this region could expand the scope of stem cell research at Gotong Royong Hospital, potentially yielding MSCs with more robust properties for regenerative therapies.

Our analysis of MSC surface marker expression showed unique patterns across different passages, specifically with P1 exhibiting only high CD44 expression (approximately 60%) while other markers, including CD105, CD73, and CD90, were undetectable. This selective expression at P1 could reflect an early stage in MSC culture, where cells prioritize adhesion and migration functions, as CD44 is a receptor for hyaluronic acid involved in cell adhesion and motility. [15,17] successive passages, notably by P5, all MSC markers (CD105, CD73, CD90, and CD44) reached high expression levels, suggesting that these cells may require additional time or specific culture conditions to fully upregulate markers associated with mesenchymal identity and functionality. CD45, as expected, remained low across passages, though its continued detectability indicates potential residual hematopoietic contamination, a finding that aligns with reports from other studies on WJ-MSCs. [11] Interestingly, this marker pattern differs

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from MSCs derived from the cord-placental junction (CPJ-MSCs), which tend to express high levels of all MSC markers, including CD105, CD73, and CD90, and lower CD45. These differences may arise from microenvironmental factors unique to CPJ-MSCs that accelerate marker expression and MSC commitment earlier in culture, highlighting the potential value of CPJ as an alternative MSC source for applications requiring rapid cell expansion and stable marker profiles. [9]

Another area for improvement in our protocol is the lengthy outgrowth period, as cell attachment and expansion required 21 days before reaching significant confluence. Long outgrowth times can limit scalability and delay potential downstream applications. [18] Optimizing the initial explant culture conditions could reduce this outgrowth period, thereby increasing efficiency. This might include using higher serum concentrations, pre-coating culture surfaces with adhesion-promoting molecules such as fibronectin, or adjusting oxygen levels to more closely mimic the native environment of umbilical cord MSCs. [19,20] Reducing the outgrowth time will not only streamline the MSC isolation process but also decrease the potential for cell senescence and culture-induced variations, ultimately improving the consistency and reliability of the cells obtained for research and therapeutic use.

Describe the limitation of the study, clinical implication of the study and future directions

CONCLUSION

~~This study establishes a foundational protocol for WJ-MSC isolation at Gotong Royong Hospital, creating new opportunities for cell-based therapies and research within the local healthcare system.~~

This study demonstrated that ……this in only an example the isolation and characterization of WJ-MSCs was achieved with high purity and obtained at a more suitable time. This research showed that the culture with high glucose and 15%FBS with putting pieces of Wharton`s Jelly to the dried bottom flask achieved a very effective separation of the MSCs.

~~Although minor challenges, such as the presence of CD45-positive cells and prolonged outgrowth, remain, the success of this protocol provides a strong basis for further innovation and optimization. Future work could build upon this study by investigating MSCs from the cord-placental junction, refining isolation techniques, and reducing culture times, all of which would enhance the therapeutic applicability of MSCs derived at Gotong Royong Hospital. This research not only contributes to the field of regenerative medicine but also has the potential to open new pathways for personalized cellular therapies.~~

Authors Contribution.

SD, YT, and BDNDewi designed the experiments, carried out the experiments, and prepared the manuscript. BDP, KF, LH, HW, HHJ, FM, and SK carried out the experiments and analyzed the data. Who revised the manuscript . All authors have read and approved the final manuscript

Conflict of interest

All authors declare no conflict of interest.

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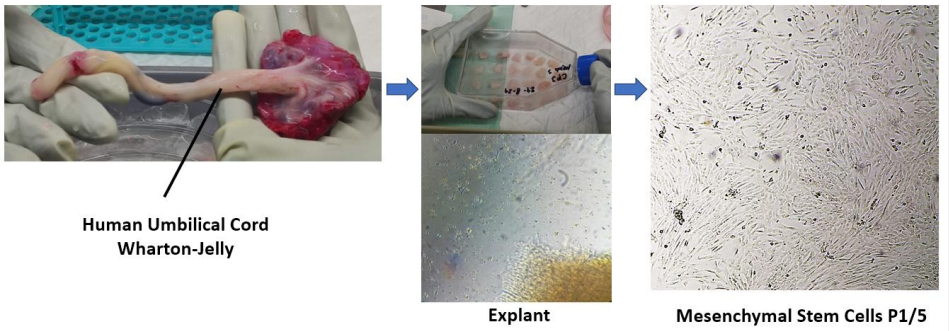

Figure 1. **Explant culture of Wharton's jelly-derived MSCs**
The initial explant culture of Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) was isolated from human umbilical cords. The explant technique and subsequent cell morphology are shown, with primary cultures demonstrating successful MSC adherence and proliferation.

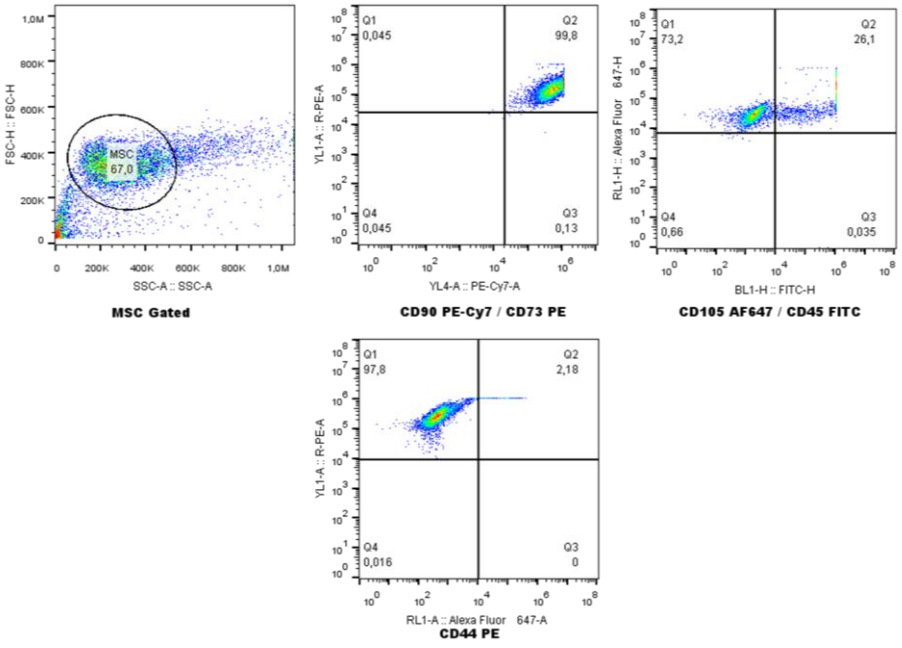

Figure 2. **Representative flow cytometric analysis of WJ-MSC surface markers**
The flow cytometric gating strategy assesses key surface markers on WJ-MSCs, confirming their stem cell characteristics (upper left). The expression levels of positive markers are shown in the upper middle (CD73, CD90), with CD105-positive and CD45-negative populations (Q1

for CD105; Q2 for CD45; upper right), as well as CD44-positive populations (lower graphic), verifying the MSC phenotype.

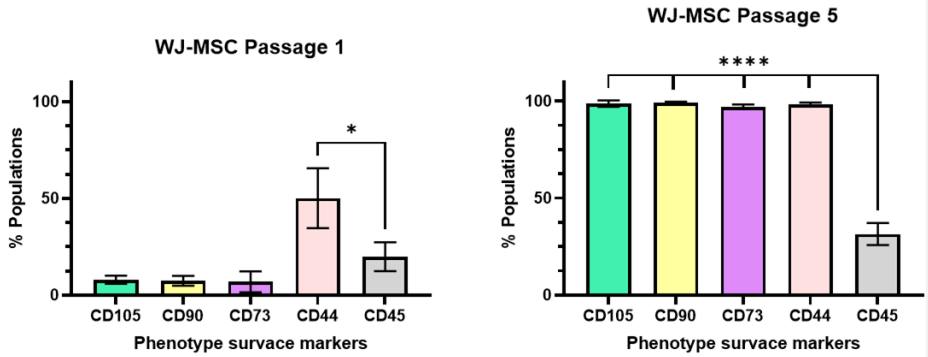

Figure 3. **Comparative cell surface markers of WJ-MSCs at passage 1 and passage 5**
Compared to the negative phenotype (CD45), WJ-MSCs demonstrate a significant increase in surface markers at passage 1 (p=0.01). However, a dramatic increase was observed in CD105, CD90, CD73, and CD44 surface markers at passage 5 ($p < 0.000$), indicating successful MSC proliferation and phenotype stability across passages.
Statistical analysis was conducted using one-way ANOVA with post-hoc tests to determine significance between groups. p-values were calculated using GraphPad Prism, with significance levels denoted as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.000$ (****).

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Please address all correspondence concerning this manuscript to me at yudy.tjahjono@ukwms.ac.id.
Thank you for your consideration of this manuscript.
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Dr. Yudy Tjahjono, B.Sc.Biol., M.Sc.Biol.
Faculty of Pharmacy; Widya Mandala Catholic University Surabaya

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TRACK CHANGES FOR REBUTTAL 1

Isolation and Characterization of Wharton's Jelly-Derived Mesenchymal Stem Cells: A Foundational Protocol for Regenerative Medicine Applications at Gotong Royong Hospital

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ABSTRACT

Background:
Mesenchymal stem cells (MSCs) are multipotent cells capable of differentiating into osteocytes, chondrocytes, and adipocytes, which makes them valuable for regenerative medicine. MSCs derived from Wharton's Jelly in the human umbilical cord (WJ-MSCs) are particularly promising due to non-invasive collection, high proliferation, and immunomodulatory properties. This study describes the first successful isolation and characterization of WJ-MSCs at Gotong Royong Hospital.
Methods:
Human umbilical cords from three healthy donors were collected immediately post-cesarean section, meeting strict inclusion criteria. The WJ-MSCs were isolated using an explant culture technique in aseptic conditions, with cells adhering to T75 flasks pre-coated with 2% gelatin. Cultures were maintained in DMEM supplemented with 10% Human AB serum, and growth was monitored for 21 days. Flow cytometry was conducted using BD FACSAria to assess expression of MSC markers CD105, CD73, CD90, and CD44, as well as the exclusion marker CD45, at Passages 1 and 5.
Results:
The isolated WJ-MSCs displayed fibroblast-like morphology characteristic of MSCs by Passage 1, with consistent, robust proliferation over 21 days. Flow cytometry revealed that CD44 was the only highly expressed marker (~60%) at Passage 1, while CD105, CD73, and CD90 became prominent by Passage 5. CD45 levels remained low, though detectable, suggesting minimal hematopoietic contamination. This expression pattern differs from MSCs derived from the cord-placental junction (CPJ), which tend to display higher and earlier marker expression.
Conclusions:
This study successfully establishes a foundational protocol for WJ-MSC isolation and expansion at Gotong Royong Hospital, supporting local stem cell research initiatives. While effective, the protocol's 21-day outgrowth period may benefit from further optimization to improve scalability, such as adjustments in serum concentration, substrate coatings, and oxygen levels. The findings also indicate the potential advantage of CPJ-MSCs for applications requiring rapid cell expansion and robust marker profiles. This protocol paves the way for future research and clinical applications of WJ-MSCs within the hospital's healthcare system.

Keywords: Wharton's Jelly; Mesenchymal Stem Cells; Isolation; Flow Cytometry; Regenerative Medicine

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stromal cells capable of differentiating into various cell types, such as osteocytes, chondrocytes, and adipocytes, making them valuable for regenerative medicine. MSCs are characterized by a specific set of surface markers that distinguish them from other cell types, particularly hematopoietic cells. ~~According to the International Society for Cellular Therapy (ISCT), MSCs must express CD105, CD73, and CD90, which are markers associated with cell adhesion, growth, and differentiation potential (Dominici *et al*., 2006). CD105, or endoglin, plays a role in cell proliferation and vascular development (Kauer,J. *et al*., 2019). while CD73 and CD90 contribute to immunomodulation and cell-cell interactions (Chen,S. *et al*., 2019;Xia,C. *et al*., 2023). CD44, a receptor for hyaluronic acid, is also commonly expressed on MSCs and is involved in cell adhesion, migration, and homing, which are essential for therapeutic applications (Mattheolabakis, G., *et al*. 2015). Importantly, MSCs are negative for CD45, a pan-leukocyte marker, which ensures the exclusion of hematopoietic lineage cells (Ghanialvar, H. *et al*., 2018). This unique combination of surface markers—CD105, CD73, CD90, and CD44, with the absence of CD45—confirms the purity and identity of MSCs, supporting their use in clinical and research settings (He, H. *et al*., 2015).~~According to the International Society for Cellular Therapy (ISCT), MSCs must express CD105, CD73, and CD90, which are markers associated with cell adhesion, growth, and differentiation potential (1). CD105, or endoglin, plays a role in cell proliferation and vascular development(2). while CD73 and CD90 contribute to immunomodulation and cell-cell interactions (3,4). CD44, a receptor for hyaluronic acid, is also commonly expressed on MSCs and is involved in cell adhesion, migration, and homing, which are essential for therapeutic applications (5). Importantly, MSCs are negative for CD45, a pan-leukocyte marker, which ensures the exclusion of hematopoietic lineage cells (6). This unique combination of surface markers—CD105, CD73, CD90, and CD44, with the absence of CD45—confirms the purity and identity of MSCs, supporting their use in clinical and research settings (7).

The potential of mesenchymal stem cells (MSCs) for regenerative medicine has led to extensive research on various sources of MSCs, including those derived from Wharton's Jelly of the human umbilical cord (WJ-MSCs). WJ-MSCs offer unique advantages, including non-invasive collection methods, high proliferation rates, and immunomodulatory properties, making them a promising cell type for therapeutic applications ~~(Kamal, M.M. *et al*., 2020).~~(8). However, isolation and expansion methods must be refined to ensure the viability and consistency of WJ-MSCs. In this study, we present the first successful isolation and characterization of WJ-MSCs conducted at Gotong Royong Hospital, demonstrating the feasibility of establishing a protocol for obtaining viable WJ-MSCs from human umbilical cords and expanding them in culture.

The establishment of this protocol at Gotong Royong Hospital not only provides a new avenue for local research in stem cell therapy but also lays the groundwork for future clinical applications and cell-based treatments. Patient selection in this study involved strict criteria, ensuring that only umbilical cords from healthy pregnancies were used, thus optimizing the quality and viability of the cells obtained. Ethical approval was secured, and careful steps were taken to remove blood vessels and isolate the Wharton's Jelly, maximizing MSC yield. This standardized approach enables a reliable source of WJ-MSCs, critical for any future applications in regenerative medicine and disease modelling.

Our results show that the WJ-MSCs isolated through this protocol display a fibroblast-like morphology characteristic of MSCs, with consistent morphology observed through the first

passage. Using a detailed explant culture method, we were able to cultivate and expand WJ-MSCs, which adhered to the culture surface within days and proliferated significantly over 21 days. These cells retained their mesenchymal characteristics, evidenced by their elongated, spindle-shaped morphology and high proliferation rate. Such morphological consistency is essential for the continued use of these cells in therapeutic research, where cell integrity and stability are paramount.

In addition to morphological assessment, flow cytometry analysis was employed to confirm the MSC phenotype, assessing the expression of surface markers CD105, CD73, CD90, and CD44, with CD45 as a hematopoietic exclusion marker. This phenotypic profile aligns with the International Society for Cellular Therapy (ISCT) guidelines for MSC characterization, confirming the cells' stem cell identity and purity across passages. This study thus establishes a foundational WJ-MSC isolation and expansion protocol at Gotong Royong Hospital, creating significant potential for future cell therapy research and applications within the hospital and the broader scientific community.

MATERIAL METHODS

Isolation of Human Umbilical Cord-Derived Mesenchymal Stem Cells (WJ-MSCs)

Human umbilical cords were collected from 3 (three) patients in Gotong Royong Hospital, immediately following caesarean section, within 5 minutes of placental removal to ensure optimal cell viability. Patient selection included strict criteria: only umbilical cords from healthy pregnancies without maternal or fetal complications were used. Specifically, cases of preeclampsia, metabolic disorders, infections, or abnormal umbilical cord coiling were excluded. The ethical approval was obtained.

Upon retrieval, each umbilical cord was placed in a sterile container with Dulbecco's Modified Eagle Medium (DMEM) containing 1% penicillin/streptomycin/amphotericin B and transported to the laboratory under aseptic conditions.

Preparation and Culture of WJ-MSCs

In a laminar flow cell culture hood to maintain aseptic technique, each umbilical cord was first washed thoroughly with Hank's Balanced Salt Solution (HBSS) containing 1% penicillin/streptomycin/amphotericin B. Blood vessels were carefully removed from the Wharton's Jelly tissue to isolate the mesenchymal-rich regions, minimizing potential contamination, and promoting a higher yield of mesenchymal stem cells (MSCs). ~~The remaining Wharton's Jelly tissue was cut into approximately 2 mm² pieces, which were then placed in T75 culture flasks precoated with 2% gelatin to enhance cellular adherence (Todtenhaupt, P. *et al.*, 2023).~~The remaining Wharton's Jelly tissue was cut into approximately 2 mm² pieces, which were then placed in T75 culture flasks precoated with 2% gelatin to enhance cellular adherence (9).

~~The tissue explants were left undisturbed for 7 days to allow cells to attach to the surface (Hendijani, F. *et al.*, 2017).~~The tissue explants were left undisturbed for 7 days to allow cells to attach to the surface (10). The culture medium, composed of DMEM supplemented with 10% Human AB serum and 0.1% penicillin/streptomycin/amphotericin B, was carefully added to prevent dislodging the tissue fragments. Medium was replaced every 3 days, and cell outgrowth was monitored microscopically. After 21 days, successful outgrowth and proliferation of Passage 1 (P1) cells were observed, characterized by the spindle-shaped, fibroblast-like morphology typical of MSCs.

Flow cytometry analysis

Flow cytometry was employed to phenotype Wharton's Jelly-derived mesenchymal stem cells (WJ-MSCs) isolated at Passage 2 and Passage 6 using a BD FACSAria flow cytometer (BD Biosciences). To assess the expression of key mesenchymal and hematopoietic markers, three antibody panels were prepared with mouse monoclonal antibodies specific to human antigens. Panel 1 included anti-human CD105 (clone 43A3, Alexa Fluor 488, BioLegend) and anti-human CD45 (clone H130, FITC, BioLegend); Panel 2 included anti-human CD73 (clone AD2, PE, BioLegend) and anti-human CD90 (clone 5E10, PE/Cy7, BioLegend); and Panel 3 included anti-human CD44 (clone IM7, PE, Elabscience). For each panel, 10^6cells in 100ul were aliquoted into individual tubes, and 2.5 µL of each antibody was added to each sample. The cells were incubated for 1 hour at 4°C in the dark to optimize antibody binding and prevent photobleaching. After incubation, cells were washed twice with staining buffer (PBS with 1% BSA) to remove unbound antibodies and resuspended in 500 µL of buffer for analysis. Gating parameters was set to exclude debris and non-viable cells based on forward and side scatter properties. Unstained controls were applied to correct for spectral overlap among fluorophores. ~~Expression of CD105, CD73, CD90, and CD44 was assessed to confirm MSC phenotype, while CD45 was used to exclude hematopoietic contamination (Pham, H. *et al*., 2018).~~Expression of CD105, CD73, CD90, and CD44 was assessed to confirm MSC phenotype, while CD45 was used to exclude hematopoietic contamination (11). Data were analyzed to determine the percentage of cells expressing each marker, providing a comprehensive phenotypic profile of WJ-MSCs across passages.

Ethical clearance

The ethical clearance for collecting the human tissue was obtained from medical Faculty, Universitas Hang Tuah (**E/006/UHT.KEPK.03/II/2024**)

Statistical analysis

GraphPad Prism version 8.0.1 was used for data presentation. Unpaired t-tests were employed to compare relative intensity differences between specific groups. A p-value < 0.05 is considered significant. All data were presented as mean ± standard deviation (SD).

RESULTS

Isolation and Culture of Wharton's Jelly-Derived MSCs

The isolation of Wharton's Jelly-derived mesenchymal stem cells (WJ-MSCs) was achieved through the explant culture method, allowing MSCs to be successfully obtained from human umbilical cord tissue. Following isolation, WJ-MSCs adhered to the culture surface within the first few days, and after 21 days in culture, Passage 1 (P1) showed significant cell proliferation. The MSCs exhibited a characteristic fibroblast-like morphology (see figure 1), typical of mesenchymal cells, with elongated, spindle-shaped bodies and a uniform, flattened appearance. Cells were tightly adherent, forming a monolayer with parallel, aligned arrangements. The morphology of the cells remained stable through Passage 14 (P14), showing no signs of senescence or morphological abnormalities. This consistent fibroblast-like morphology, with the presence of prominent cell projections and well-defined cellular boundaries, suggests that the WJ-MSCs maintained their mesenchymal characteristics and were successfully expanded in primary culture.

Phenotypic Characterization of WJ-MSCs by Flow Cytometry

To confirm the MSC phenotype, we performed flow cytometric analysis on WJ-MSCs passage 5 (P5), assessing the expression of key surface markers (see figure 2). The gating strategy effectively isolated MSC populations, and the analysis revealed high expression levels of CD73

and CD90, alongside positive populations for CD105. Additionally, the cells were negative for CD45, confirming the absence of hematopoietic contamination (Figure 2). Positive CD44 expression further validated the MSC phenotype, affirming the cells' stem cell characteristics and purity.

Comparative Analysis of Surface Markers at Different Passages

Comparisons of WJ-MSC surface markers between P1 and P5 revealed significant differences in marker expression (see figure 3). At Passage 1, the MSCs demonstrated a statistically significant increase in pluripotency surface markers compared to the negative phenotype CD45 ($P = 0.01$). By P5, a dramatic increase in CD105, CD90, CD73, and CD44 expression was observed, with statistical significance denoted as ***$P < 0.0001$ (Figure 3). These findings indicate robust MSC proliferation and stable expression of essential stem cell markers across passages, supporting the successful expansion and maintenance of MSC characteristics through successive passages.

DISCUSSION

This study successfully demonstrates the isolation and characterization of Wharton's Jelly-derived mesenchymal stem cells (WJ-MSCs) from human umbilical cords collected at Gotong Royong Hospital. Our findings confirm the feasibility of establishing a reliable protocol for obtaining viable WJ-MSCs locally, marking the first successful effort at this institution. ~~These cells exhibited typical MSC morphology and a stable phenotype through early passages, supporting their potential for future research and clinical applications (Suyama, T. *et al*., 2022). However, despite overall success, the detection of low levels of CD45 expression indicates potential hematopoietic contamination (Pham, H. *et al*., 2018). This may be attributed to residual blood cells within the Wharton's Jelly, as complete removal of all blood vessels can be challenging in clinical settings (Niknam, B. *et al*., 2023). Future refinements could include additional washes or enzymatic treatments to further minimize CD45-positive cells, improving the MSC purity and strengthening the clinical potential of these cells (Zheng, S.*et al*., 2022).~~These cells exhibited typical MSC morphology and a stable phenotype through early passages, supporting their potential for future research and clinical applications (12). However, despite overall success, the detection of low levels of CD45 expression indicates potential hematopoietic contamination (11). This may be attributed to residual blood cells within the Wharton's Jelly, as complete removal of all blood vessels can be challenging in clinical settings (13). Future refinements could include additional washes or enzymatic treatments to further minimize CD45-positive cells, improving the MSC purity and strengthening the clinical potential of these cells (14).

~~Interestingly, recent studies have highlighted the potential of MSCs derived specifically from the cord-placental junction, which may offer advantages over WJ-MSCs in terms of cellular yield, differentiation potential, and immunomodulatory properties (Beeravolu, N. *et al*., 2017). MSCs from the cord-placental junction have shown enhanced proliferation rates and greater multilineage differentiation capabilities compared to WJ-MSCs, likely due to their proximity to the placenta and the rich microenvironmental cues present there (Bharti,D. *et al*., 2017). Exploring the isolation of MSCs from this region could expand the scope of stem cell research at Gotong Royong Hospital, potentially yielding MSCs with more robust properties for regenerative therapies.~~

~~Our analysis of MSC surface marker expression showed unique patterns across different passages, specifically with P1 exhibiting only high CD44 expression (approximately 60%)~~

~~while other markers, including CD105, CD73, and CD90, were undetectable. This selective expression at P1 could reflect an early stage in MSC culture, where cells prioritize adhesion and migration functions, as CD44 is a receptor for hyaluronic acid involved in cell adhesion and motility (Beeravolu, N. *et al*., 2017; Cao, Y. *et al*., 2024). Over successive passages, notably by P5, all MSC markers (CD105, CD73, CD90, and CD44) reached high expression levels, suggesting that these cells may require additional time or specific culture conditions to fully upregulate markers associated with mesenchymal identity and functionality. CD45, as expected, remained low across passages, though its continued detectability indicates potential residual hematopoietic contamination, a finding that aligns with reports from other studies on WJ-MSCs (Pham, H. *et al*., 2018). Interestingly, this marker pattern differs from MSCs derived from the cord-placental junction (CPJ-MSCs), which tend to express high levels of all MSC markers, including CD105, CD73, and CD90, and lower CD45. These differences may arise from microenvironmental factors unique to CPJ-MSCs that accelerate marker expression and MSC commitment earlier in culture, highlighting the potential value of CPJ as an alternative MSC source for applications requiring rapid cell expansion and stable marker profiles (Todtenhaupt, P. *et al*., 2023).~~

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Another area for improvement in our protocol is the lengthy outgrowth period, as cell attachment and expansion required 21 days before reaching significant confluence. Long outgrowth times can limit scalability and delay potential downstream applications ~~(Bodiou, V. I*et al*., 2024).~~(18). Optimizing the initial explant culture conditions could reduce this outgrowth period, thereby increasing efficiency. This might include using higher serum concentrations, pre-coating culture surfaces with adhesion-promoting molecules such as fibronectin, or adjusting oxygen levels to more closely mimic the native environment of

umbilical cord MSCs ~~(Moniz, I. *et al*., 2022;Kasten, A. *et al*., 2014).~~(19,20). Reducing the outgrowth time will not only streamline the MSC isolation process but also decrease the potential for cell senescence and culture-induced variations, ultimately improving the consistency and reliability of the cells obtained for research and therapeutic use.

In conclusion, this study establishes a foundational protocol for WJ-MSC isolation at Gotong Royong Hospital, creating new opportunities for cell-based therapies and research within the local healthcare system. Although minor challenges, such as the presence of CD45-positive cells and prolonged outgrowth, remain, the success of this protocol provides a strong basis for further innovation and optimization. Future work could build upon this study by investigating MSCs from the cord-placental junction, refining isolation techniques, and reducing culture times, all of which would enhance the therapeutic applicability of MSCs derived at Gotong Royong Hospital. This research not only contributes to the field of regenerative medicine but also has the potential to open new pathways for personalized cellular therapies.

Authors contribution.
Sianty Dewi, Yudy Tjahjono, and Bernadette Dian Novita Dewi designed the experiments, carried out the experiments, and prepared the manuscript. Brilliant Dwi Putra, Kuncoro Foe, Lucia Hendriati, Hendy Wijaya, Himawan Haryanto Jong, Franklin Malonda, and Suryo Kuncorojakti carried out the experiments and analyzed the data.

Conflict of interest
All authors declare no conflict of interest.

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We would like to thank Department of Obstetric and Gynacology Gotong Royong Hospital ,Surabaya-Indonesia for the support. The work was supported by the following grants: Research and Community Service Institute of Widya Mandala Catholic University, Surabaya, Indonesia (3172/WM01/N/2024 to Sianty Dewi)

FIGURE

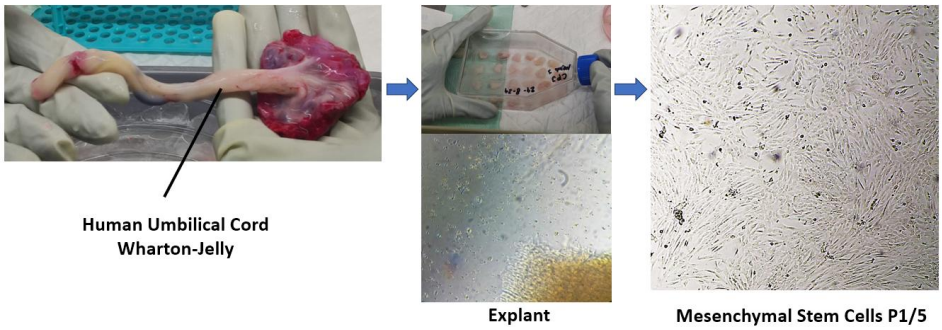

Figure 1: **Explant Culture of Wharton's Jelly-Derived MSCs**

The initial explant culture of Wharton's Jelly-derived mesenchymal stem cells (WJ-MSCs) was isolated from human umbilical cords. The explant technique and subsequent cell morphology are shown, with primary cultures demonstrating successful MSC adherence and proliferation.

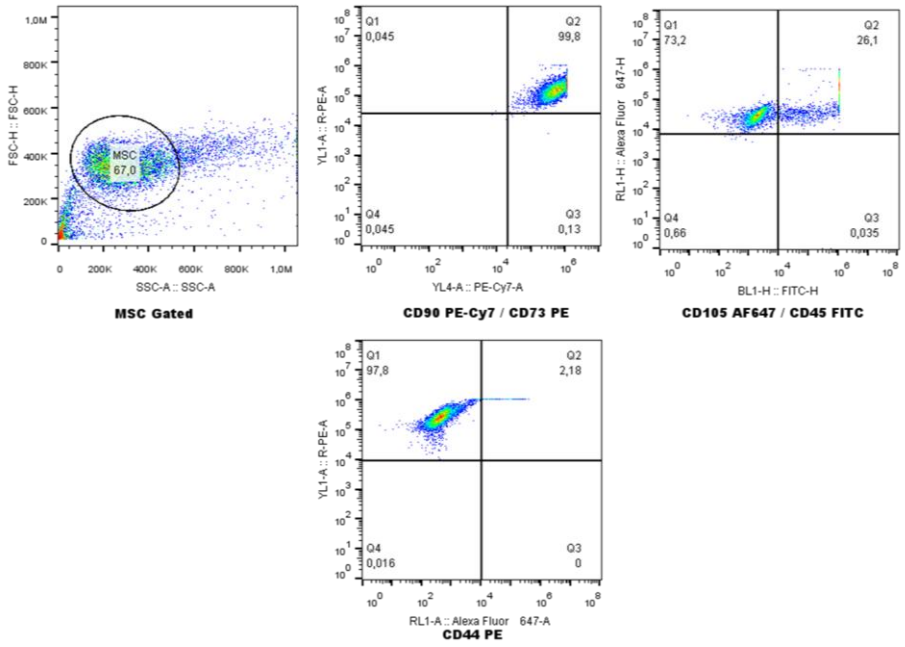

Figure 2: **Representative Flow Cytometric Analysis of WJ-MSC Surface Markers**
The flow cytometric gating strategy assesses key surface markers on WJ-MSCs, confirming their stem cell characteristics (upper left). The expression levels of positive markers are shown in the upper middle (CD73, CD90), with CD105-positive and CD45-negative populations (Q1 for CD105; Q2 for CD45; upper right), as well as CD44-positive populations (lower graphic), verifying the MSC phenotype.

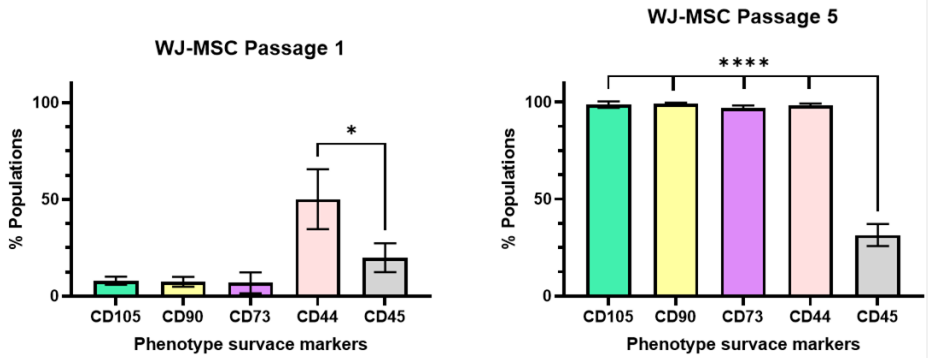

Figure 3: **Comparative Cell Surface Markers of WJ-MSCs at Passage 1 and Passage 5**

Compared to the negative phenotype (CD45), WJ-MSCs demonstrate a significant increase in surface markers at Passage 1 (*P=0.01). However, a dramatic increase was observed in CD105, CD90, CD73, and CD44 surface markers at Passage 5 (****P < 0.0001), indicating successful MSC proliferation and phenotype stability across passages.
Statistical analysis was conducted using one-way ANOVA with post-hoc tests to determine significance between groups. P-values were calculated using GraphPad Prism, with significance levels denoted as follows: P < 0.05 (*), P < 0.01 (**), P < 0.001 (***), and P < 0.0001 (****).

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EDITORIAL DECISION 2: MINOR REVISION

Messages

Note	From
Dear Author, We have reached a decision regarding your submission to Universa Medicina Journal, entitled **Rapid isolation and characterization of Wharton's jelly-derived mesenchymal stem cells mantained in fresh-prepared human AB-serum** **Editor decision : revision required (minor revision)** Please download files from the reviewers/Editors. Revisions can be made from the Editor's' articles. When submitting a revised manuscript, please include a COVER LETTER outlining, point by point, the revisions made in response to the reviewers' comments and guidelines. Besides, please use the TRACK CHANGES feature in MS Word or highlight the text to indicate which sections have been revised. Once revised, please upload the manuscript in MS Word format to our website (Login > Authors > Submissions > Review > Editor's Decision > Upload Author Version). We appreciate your contribution to the Universa Medicina Journal and look	bunga_melati 2025-03-10 07:30 PM

Yudi Tjahyono (ytjahyono)

Messages

Note	From
Dear Author, We have reached a decision regarding your submission to Universa Medicina Journal, entitled **Rapid isolation and characterization of Wharton's jelly-derived mesenchymal stem cells mantained in fresh-prepared human AB-serum** **Editor decision : revision required (minor revision)** Please download files from the reviewers/Editors. Revisions can be made from the Editor's' articles. When submitting a revised manuscript, please include a COVER LETTER outlining, point by point, the revisions made in response to the reviewers' comments and guidelines. Besides, please use the TRACK CHANGES feature in MS Word or highlight the text to indicate which sections have been revised. Once revised, please upload the manuscript in MS Word format to our website (Login > Authors > Submissions > Review > Editor's Decision > Upload Author Version). We appreciate your contribution to the Universa Medicina Journal and look forward to receiving your revised manuscript within 2	bunga_melati 2025-03-10 07:30 PM

Rapid isolation and characterization of Wharton's jelly-derived mesenchymal stem cells mantained in fresh-prepared human AB-serum

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Running title:
"Characterization of WJ-MSCs in human AB-serum"

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ABSTRACT 248 words

Background

Mesenchymal stem cells (MSCs) are valued in regenerative medicine for their multipotency, proliferation capacity, and immunomodulatory properties. Wharton's Jelly-derived MSCs (WJ-MSCs) from the umbilical cord offer a non-invasive, promising source for clinical applications. Because easy isolation, lack of ethical concerns, and the presence of both embryonic and adult stem cells have made them a valuable source for use in therapeutic applications and regenerative medicine. This study aims to optimize WJ-MSC isolation and characterization methods.

Methods

Human umbilical cords from three healthy donors were collected post-caesarean under strict inclusion criteria. WJ-MSCs were isolated using the explant culture method, with cells adhering to T75 flasks pre-coated with 2% gelatin. Cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% freshly prepared Human AB serum and monitored for 21 days. Flow cytometry (BD FACSAria) was performed at Passages 1 and 5 to assess MSC markers CD105, CD73, CD90, and CD44, alongside the exclusion marker CD45.

Results

WJ-MSCs exhibited fibroblast-like morphology by Passage 1 and showed robust proliferation. Flow cytometry revealed high CD44 expression (~60%) at Passage 1, while CD105, CD73,

and CD90 became prominent by Passage 5. CD45 remained low, suggesting minimal hematopoietic contamination.

Conclusions

This study confirms the feasibility of isolating and expanding WJ-MSCs using DMEM with 10% human AB serum. While consistent cell growth was achieved, the 21-day culture period may require optimization for scalability, including serum concentration, substrate coatings, and oxygen levels. CPJ-MSCs may be preferable for applications demanding rapid expansion and early marker expression.

Keywords: Wharton's jelly, mesenchymal stem cells, isolation, flow cytometry, regenerative medicine

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stromal cells capable of differentiating into various cell types, such as osteocytes, chondrocytes, and adipocytes, making them valuable for regenerative medicine. Mesenchymal stem cells are characterized by a specific set of surface markers that distinguish them from other cell types, particularly hematopoietic cells. [(1)] According to the International Society for Cellular Therapy (ISCT), MSCs must express CD105, CD73, and CD90, which are markers associated with cell adhesion, growth, and differentiation potential. [(2)] CD105, or endoglin, plays a role in cell proliferation and vascular development, [(3)] while CD73 and CD90 contribute to immunomodulation and cell-cell interactions. [(4,5)] CD44, a receptor for hyaluronic acid, is also commonly expressed on MSCs and is involved in cell adhesion, migration, and homing, which are essential for therapeutic applications. [(6)] Importantly, MSCs are negative for CD45, a pan-leukocyte marker, which ensures the exclusion of hematopoietic lineage cells. [(7)] This unique combination of surface

markers—CD105, CD73, CD90, and CD44, with the absence of CD45—confirms the purity and identity of MSCs, supporting their use in clinical and research settings. [8]

The potential of MSCs for regenerative medicine has led to extensive research on various sources of MSCs, including those derived from Wharton's jelly of the human umbilical cord (WJ-MSCs). Wharton's Jelly of the human umbilical cord offer unique advantages, including non-invasive collection methods, high proliferation rates, and immunomodulatory properties, making them a promising cell type for therapeutic applications. [9] However, isolation and expansion methods must be refined to ensure the viability and consistency of WJ-MSCs.

Previous works on the isolation and characterization of Wharton's jelly derived mesenchymal stem cells (WJ-MSCs) have produced different yields, markers and abilities to differentiate. For instance, results by Ranjbaran et al., [10] were able to isolate WJ-MSCs in a high glucose culture medium containing 15% fetal bovine serum (FBS), and separated MSCs efficiently by placing Wharton's jelly pieces at the bottom of the flask. Likewise, Cardoso et al. [11] cultured WJ-MSCs in a serum-free condition and the cells displayed typical mesenchymal stem cell phenotype up to 60 passages without having any genetic disorders. Abouelnaga et al.[12] also reported that the primary cultures exhibited success in isolating the umbilical cord Wharton's jelly mesenchymal stem cells, which maintained their tri-lineage differentiation potential, phenotypes and karyotype characteristics on further passage and expansion.

The originality of our work is based on the approach ~~that was developed at the Gotong Royong Hospital~~, which aims at improving the isolation and growth of WJ-MSCs in a 2% gelatine coated flask and a culture medium containing 10% human AB serum. Previous studies used fetal bovine serum (FBS), but we used human serum in our experiment to avoid immune reaction in future clinical use. Furthermore, the current study also revealed the distinct pattern of surface markers (CD44, CD105, CD73, and CD90) during the passage, and CD44 was found to be most expressive at Passage 1 while the others at Passage 5.

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The primary objective of this study is to establish a standardized protocol for the isolation and characterization of Wharton's Jelly-derived mesenchymal stem cells (WJ-MSCs) from human umbilical cords ~~at Gotong Royong Hospital~~. The study aimed to optimize the culture conditions for WJ-MSCs, including the use of 2% gelatin-coated flasks and a culture medium supplemented with 10% Human AB serum, to ensure high cell viability, proliferation, and purity. Additionally, the study sought to characterize the phenotypic profile of WJ-MSCs using flow cytometry to assess the expression of key surface markers (CD105, CD73, CD90, and CD44) and the absence of hematopoietic markers (CD45). ~~The findings from this study provide a foundational protocol for future research and clinical applications of WJ-MSCs in regenerative medicine at the hospital.~~

METHODS

Research design

This research was conducted in Gotong Royong Hospital in Surabaya in collaboration with the Faculty of Medicine and Faculty of Pharmacy of Widya Mandala Catholic University Surabaya. The research period spanned from June 2024 to December 2024. ~~during which umbilical cords were collected from healthy donors. This study focused on the isolation culture and characterization of Wharton's Jelly derived mesenchymal stem cell (WJ-MSCs) using an explant culture technique. It was conducted in a controlled laboratory environment which following to strict aseptic conditions and ethical guidelines approved by the Medical Faculty of Universitas Hang Tuah Surabaya (Ethical Clearance No. E/006/UHT.KEPK.03/II/2024).~~

Isolation of human umbilical cord-derived mesenchymal stem cells (WJ-MSCs)

Human umbilical cords were collected from 3 (three) patients in Gotong Royong Hospital, immediately following caesarean section, within 5 minutes of placental removal to ensure optimal cell viability. Patient selection included strict criteria: only umbilical cords from

healthy pregnancies without maternal or fetal complications were used. Specifically, cases of preeclampsia, metabolic disorders, infections, or abnormal umbilical cord coiling were excluded. Upon retrieval, each umbilical cord was placed in a sterile container with Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) containing 1% penicillin/streptomycin/amphotericin B and transported to the laboratory under aseptic conditions.

In addition to morphological assessment, flow cytometry analysis was employed to confirm the MSC phenotype, assessing the expression of surface markers CD105, CD73, CD90, and CD44, with CD45 as a hematopoietic exclusion marker. This phenotypic profile aligns with the International Society for Cellular Therapy (ISCT) guidelines for MSC characterization, confirming the cells' stem cell identity and purity across passages. This study thus establishes a foundational WJ-MSC isolation and expansion protocol at Gotong Royong Hospital, creating significant potential for future cell therapy research and applications within the hospital and the broader scientific community.

Preparation and culture of WJ-MSCs

In a laminar flow cell culture hood to maintain aseptic technique, each umbilical cord was first washed thoroughly with Hank's Balanced Salt Solution (HBSS) containing 1% penicillin/streptomycin/amphotericin B (Service Bio,China). Blood vessels were carefully removed from the Wharton's Jelly tissue to isolate the mesenchymal-rich regions, minimizing potential contamination, and promoting a higher yield of mesenchymal stem cells (MSCs). The remaining Wharton's Jelly tissue was cut into approximately 2 mm^2 pieces, which were then placed in T75 culture flasks precoated with 2% gelatin to enhance cellular adherence. [13]

The tissue explants were left undisturbed for 7 days to allow cells to attach to the surface. [14]

The culture medium, composed of DMEM supplemented with 10% human AB serum and 0.1%

penicillin/streptomycin/amphotericin B, was carefully added to prevent dislodging the tissue fragments. Medium was replaced every 3 days, and cell outgrowth was monitored microscopically. After 21 days, successful outgrowth and proliferation of Passage 1 (P1) cells were observed, characterized by the spindle-shaped, fibroblast-like morphology typical of MSCs.

Flow cytometry analysis

Flow cytometry was employed to phenotype Wharton's Jelly-derived mesenchymal stem cells (WJ-MSCs) isolated at Passage 2 and Passage 6 using a BD FACSAria flow cytometer (BD Biosciences). To assess the expression of key mesenchymal and hematopoietic markers, three antibody panels were prepared with mouse monoclonal antibodies specific to human antigens. Panel 1 included anti-human CD105 (clone 43A3, Alexa Fluor 488, BioLegend) and anti-human CD45 (clone H130, FITC, BioLegend); Panel 2 included anti-human CD73 (clone AD2, PE, BioLegend) and anti-human CD90 (clone 5E10, PE/Cy7, BioLegend); and Panel 3 included anti-human CD44 (clone IM7, PE, Elabscience). For each panel, 10^6cells in 100ul were aliquoted into individual tubes, and 2.5 µL of each antibody was added to each sample. The cells were incubated for 1 hour at 4°C in the dark to optimize antibody binding and prevent photobleaching. After incubation, cells were washed twice with staining buffer (PBS with 1% BSA) to remove unbound antibodies and resuspended in 500 µL of buffer for analysis. Gating parameters was set to exclude debris and non-viable cells based on forward and side scatter properties. Unstained controls were applied to correct for spectral overlap among fluorophores. Expression of CD105, CD73, CD90, and CD44 was assessed to confirm MSC phenotype, while CD45 was used to exclude hematopoietic contamination. [15] Data were analyzed to determine the percentage of cells expressing each marker, providing a comprehensive phenotypic profile of WJ-MSCs across passages.

Ethical clearance

The ethical clearance for collecting the human tissue was obtained from medical Faculty, Universitas Hang Tuah (**E/006/UHT.KEPK.03/II/2024**)

Statistical analysis

GraphPad Prism version 8.0.1 was used for data presentation. Unpaired t-tests were employed to compare relative intensity differences between specific groups. One way ANOVA ??? A p-value < 0.05 is considered significant. All data were presented as mean ± standard deviation (SD).

RESULTS

Isolation and culture of Wharton's jelly-derived MSCs

Our results show that the WJ-MSCs isolated through this protocol display a fibroblast-like morphology characteristic of MSCs, with consistent morphology observed through the first passage. Using a detailed explant culture method, we were able to cultivate and expand WJ-MSCs, which adhered to the culture surface within days and proliferated significantly over 21 days. These cells retained their mesenchymal characteristics, evidenced by their elongated, spindle-shaped morphology and high proliferation rate. Such morphological consistency is essential for the continued use of these cells in therapeutic research, where cell integrity and stability are paramount.

The isolation of WJ-MSCs was achieved through the explant culture method, allowing MSCs to be successfully obtained from human umbilical cord tissue. Following isolation, WJ-MSCs adhered to the culture surface within the first few days, and after 21 days in culture, Passage 1 (P1) showed significant cell proliferation. The MSCs exhibited a characteristic fibroblast-like

morphology (see Figure 1), typical of mesenchymal cells, with elongated, spindle-shaped bodies and a uniform, flattened appearance. Cells were tightly adherent, forming a monolayer with parallel, aligned arrangements. The morphology of the cells remained stable through Passage 14 (P14), showing no signs of senescence or morphological abnormalities. This consistent fibroblast-like morphology, with the presence of prominent cell projections and well-defined cellular boundaries, suggests that the WJ-MSCs maintained their mesenchymal characteristics and were successfully expanded in primary culture.

Phenotypic characterization of WJ-MSCs by flow cytometry

To confirm the MSC phenotype, we performed flow cytometric analysis on WJ-MSCs passage 5 (P5), assessing the expression of key surface markers (see Figure 2). The gating strategy effectively isolated MSC populations, and the analysis revealed high expression levels of CD73 and CD90, alongside positive populations for CD105. Additionally, the cells were negative for CD45, confirming the absence of hematopoietic contamination (Figure 2). Positive CD44 expression further validated the MSC phenotype, affirming the cells' stem cell characteristics and purity.

Comparative analysis of surface markers at different passages

Comparisons of WJ-MSC surface markers between P1 and P5 revealed significant differences in marker expression (see Figure 3). At Passage 1, the MSCs demonstrated a statistically significant increase in pluripotency surface markers compared to the negative phenotype CD45 ($p = 0.010$). By P5, a dramatic increase in CD105, CD90, CD73, and CD44 expression was observed, with statistical significance ($p < 0.0001$) (Figure 3). These findings indicate robust MSC proliferation and stable expression of essential stem cell markers across passages,

supporting the successful expansion and maintenance of MSC characteristics through successive passages.

DISCUSSION

This study successfully demonstrates the rapid isolation and characterization of WJ-MSCs from human umbilical cords using freshly prepared human AB-serum. These cells exhibited typical MSC morphology and a stable phenotype through early passages, supporting their potential for future research and clinical applications. [16]. However, despite overall success, the detection of low levels of CD45 expression indicates potential hematopoietic contamination. [15] This may be attributed to residual blood cells within the Wharton's Jelly, as complete removal of all blood vessels can be challenging in clinical settings. [17] Future refinements could include additional washes or enzymatic treatments to further minimize CD45-positive cells, improving the MSC purity and strengthening the clinical potential of these cells. [18]

Interestingly, recent study have highlighted the potential of MSCs derived specifically from the cord-placental junction, which may offer advantages over WJ-MSCs in terms of cellular yield, differentiation potential, and immunomodulatory properties. [19] MSCs from the cord-placental junction have shown enhanced proliferation rates and greater multilineage differentiation capabilities compared to WJ-MSCs, likely due to their proximity to the placenta and the rich microenvironmental cues present there .[20] Exploring the isolation of MSCs from this region could expand the scope of stem cell research at Gotong Royong Hospital, potentially yielding MSCs with more robust properties for regenerative therapies.

Our analysis of MSC surface marker expression showed unique patterns across different passages, specifically with P1 exhibiting only high CD44 expression (approximately 60%) while other markers, including CD105, CD73, and CD90, were undetectable. This selective expression at P1 could reflect an early stage in MSC culture, where cells prioritize adhesion

and migration functions, as CD44 is a receptor for hyaluronic acid involved in cell adhesion and motility. [19,21] ……..successive passages, notably by P5, all MSC markers (CD105, CD73, CD90, and CD44) reached high expression levels, suggesting that these cells may require additional time or specific culture conditions to fully upregulate markers associated with mesenchymal identity and functionality. CD45, as expected, remained low across passages, though its continued detectability indicates potential residual hematopoietic contamination, a finding that aligns with reports from other study on WJ-MSCs. [15] Interestingly, this marker pattern differs from MSCs derived from the cord-placental junction (CPJ-MSCs), which tend to express high levels of all MSC markers, including CD105, CD73, and CD90, and lower CD45. These differences may arise from microenvironmental factors unique to CPJ-MSCs that accelerate marker expression and MSC commitment earlier in culture, highlighting the potential value of CPJ as an alternative MSC source for applications requiring rapid cell expansion and stable marker profiles. [13]

Another area for improvement in our protocol is the lengthy outgrowth period, as cell attachment and expansion required 21 days before reaching significant confluence. Long outgrowth times can limit scalability and delay potential downstream applications. [22] Optimizing the initial explant culture conditions could reduce this outgrowth period, thereby increasing efficiency. This might include using higher serum concentrations, pre-coating culture surfaces with adhesion-promoting molecules such as fibronectin, or adjusting oxygen levels to more closely mimic the native environment of umbilical cord MSCs. [23,24] Reducing the outgrowth time will not only streamline the MSC isolation process but also decrease the potential for cell senescence and culture-induced variations, ultimately improving the consistency and reliability of the cells obtained for research and therapeutic use.

The limitations of this study include, first, the contamination of CD45, which is still detected in antibody expressions. This may result from incomplete removal of blood vessels during the

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isolation process, potentially affecting the purity of WJ-MSCs and necessitating further refinement of the isolation protocol. Second, the prolonged outgrowth period limits the scalability and efficiency of the protocol for large-scale applications.

Despite these limitations, this study successfully isolated and characterized WJ-MSCs, providing a foundation for future clinical applications in regenerative medicine, including tissue repair, immunomodulation, and cell-based therapies. The use of human AB serum instead of fetal bovine serum (FBS) reduces the risk of immune reactions, making the protocol more suitable for clinical use.

Looking ahead, several future directions could build on this research. First, optimizing the isolation process by carefully extracting arteries and veins could minimize CD45 contamination and improve MSC purity. Second, reducing the outgrowth period by optimizing culture conditions—such as using higher serum concentrations, adhesion-promoting molecules like fibronectin, or adjusting oxygen levels to better mimic the native environment of umbilical cord MSCs—could enhance efficiency and applicability.

CONCLUSION

This study successfully demonstrates the isolation and characterization of Wharton's jelly (WJ-MSC) using a culture medium supplemented with 10% Human AB serum and high glucose, combined with a 2% gelatine-coated flask. This protocol achieved high cell viability and proliferation, with WJ-MSCs exhibiting fibroblast like morphology and stable expression of MSC key markers (CD105, CD44, CD90 and CD73) by passage 5. Low level of CD45 expression indicating minimal hematopoietic contamination, confirming the purity of the isolated cells.

The results highlight the feasibility of this protocol for obtaining viable WJ-MSCs, providing a path for future research and clinical applications in regenerative medicine. However, the 21-

day outgrowth period and the presence of residual CD45 positive suggesting for further optimization, such as refining the isolation techniques, reducing culture times and exploring alternative MSC sources.

Authors Contribution

SD, YT, and BDN designed the experiments, carried out the experiments, and prepared the manuscript. BDP, KF, BDP, LH, HW, HHJ, FM, and SK carried out the experiments and analyzed the data. YT, SD and BDF revised the manuscript. All authors have read and approved the final manuscript.

Conflict of interest

All authors declare no conflict of interest.

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Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article

Declaration the Use of AI in Scientific Writing

During the preparation of this work, the authors used ChatGPT (OpenAI) to assist in language refinement, grammar correction, and improving technical clarity. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

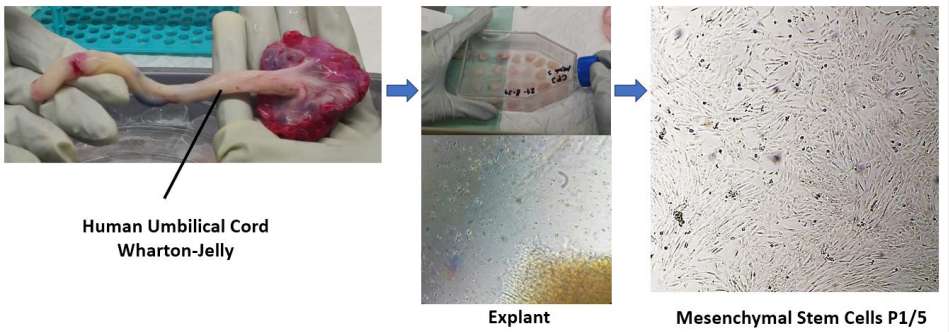

Figure 1. **Explant culture of Wharton's jelly-derived MSCs**
The initial explant culture of Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) was isolated from human umbilical cords. The explant technique and subsequent cell morphology are shown, with primary cultures demonstrating successful MSC adherence and proliferation.

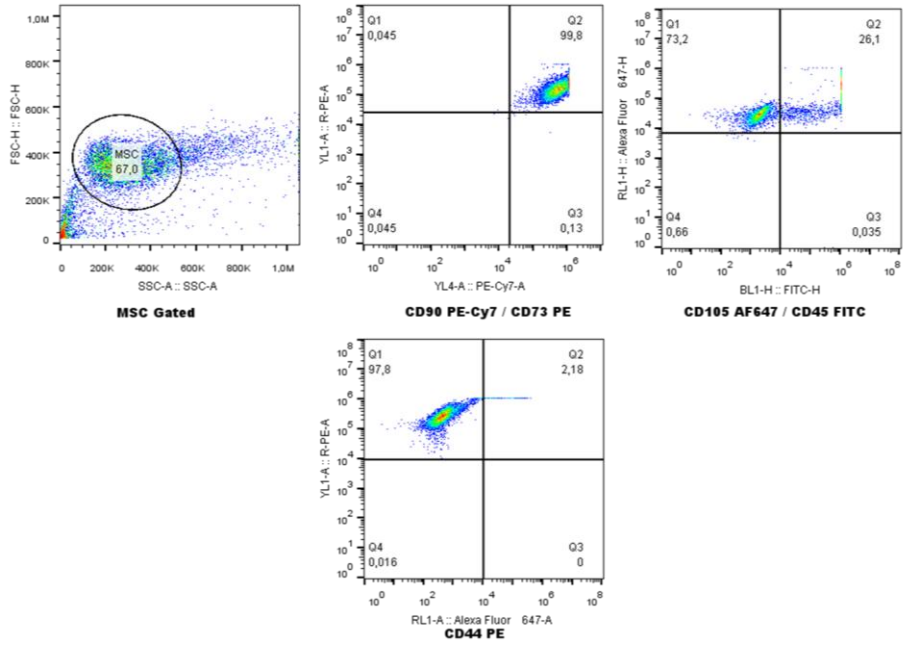

Figure 2. **Representative flow cytometric analysis of WJ-MSC surface markers**
The flow cytometric gating strategy assesses key surface markers on WJ-MSCs, confirming their stem cell characteristics (upper left). The expression levels of positive markers are shown in the upper middle (CD73, CD90), with CD105-positive and CD45-negative populations (Q1 for CD105; Q2 for CD45; upper right), as well as CD44-positive populations (lower graphic), verifying the MSC phenotype.

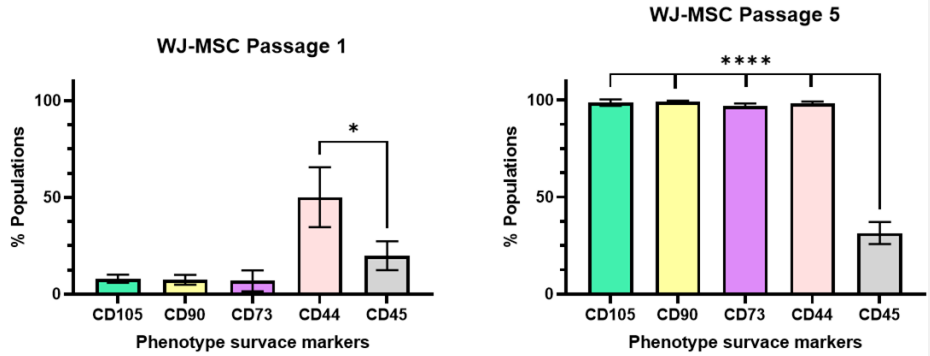

Figure 3. **Comparative cell surface markers of WJ-MSCs at passage 1 and passage 5**
Compared to the negative phenotype (CD45), WJ-MSCs demonstrate a significant increase in surface markers at passage 1 ($p=0.010$). However, a dramatic increase was observed in CD105, CD90, CD73, and CD44 surface markers at passage 5 ($p < 0.000$), indicating successful MSC proliferation and phenotype stability across passages.
Statistical analysis was conducted using one-way ANOVA with post-hoc tests to determine significance between groups. p-values were calculated using GraphPad Prism, with significance levels denoted as follows: $p < 0.05$ (*), $p < 0.010$ (**), $p < 0.001$ (***), and $p < 0.000$ (****).

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Thank you for your consideration of this manuscript.

Sincerely,

Dr. Yudy Tjahjono, B.Sc.Biol., M.Sc.Biol.

Faculty of Pharmacy; Widya Mandala Catholic University Surabaya

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~~Isolation and Characterization of Wharton's Jelly-Derived Mesenchymal Stem Cells: A Foundational Protocol for Regenerative Medicine Applications at Gotong Royong Hospital~~

Rapid isolation and characterization of Wharton's jelly-derived mesenchymal stem cells mantained in fresh-prepared human AB-serum

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"Characterization of WJ-MSCs in human AB-serum"

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ABSTRACT 248 words

Background

Mesenchymal stem cells (MSCs) are ~~multipotent cells capable of differentiating into osteocytes, chondrocytes, and adipocytes, which makes them valuable for~~ valued in regenerative medicine~~. MSCs derived from Wharton's Jelly in the human umbilical cord (WJ-MSCs) are particularly promising due to non-invasive collection, high~~ for their multipotency, proliferation capacity, and immunomodulatory properties. Wharton's Jelly-derived MSCs (WJ-MSCs) from the umbilical cord offer a non-invasive, promising source for clinical applications. Because easy isolation, lack of ethical concerns, and the presence of both embryonic and adult stem cells have made them a valuable source for use in therapeutic applications and regenerative medicine. This study ~~describes the first successful~~aims to

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optimize WJ-MSC isolation and characterization ~~of WJ-MSCs at Gotong Royong Hospital~~methods.

Methods~~:~~

Human umbilical cords from three healthy donors were collected ~~immediately post-cesarean section, meeting~~post-caesarean under strict inclusion criteria. ~~The~~ WJ-MSCs were isolated using ~~an~~the explant culture ~~technique in aseptic conditions~~method, with cells adhering to T75 flasks pre-coated with 2% gelatin. Cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% freshly prepared Human AB serum~~,~~ and ~~growth was~~ monitored for 21 days. Flow cytometry ~~was conducted using~~ (BD FACSAria) was performed at Passages 1 and 5 to assess ~~expression of~~ MSC markers CD105, CD73, CD90, and CD44, ~~as well as~~alongside the exclusion marker CD45~~, at Passages 1 and 5~~.

Results~~:~~

~~The isolated~~ WJ-MSCs ~~displayed~~exhibited fibroblast-like morphology ~~characteristic of MSCs~~ by Passage 1~~, with consistent,~~ and showed robust proliferation~~ over 21 days.~~. Flow cytometry revealed ~~that~~high CD44 ~~was the only highly expressed marker~~expression (~60%) at Passage 1, while CD105, CD73, and CD90 became prominent by Passage 5. CD45~~ levels~~ remained low, ~~though detectable,~~ suggesting minimal hematopoietic contamination. ~~This expression pattern differs from MSCs derived from the cord-placental junction (CPJ), which tend to display higher and earlier marker expression.~~

Conclusions~~:~~

This study ~~successfully establishes a foundational protocol for WJ-MSC isolation and expansion at Gotong Royong Hospital, supporting local stem cell research initiatives. While~~

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~~effective~~confirms the feasibility of isolating and expanding WJ-MSCs using DMEM with 10% human AB serum. While consistent cell growth was achieved, the ~~protocol's~~ 21-day ~~outgrowth~~culture period may ~~benefit from further~~require optimization ~~to improve~~for scalability, ~~such as adjustments in~~including serum concentration, substrate coatings, and oxygen levels. ~~The findings also indicate the potential advantage of~~ CPJ-MSCs may be preferable for applications ~~requiring~~demanding rapid ~~cell~~ expansion and ~~robust~~early marker ~~profiles. This protocol paves the way for future research and clinical applications of WJ-MSCs within the hospital's healthcare system~~expression.

Keywords: Wharton's ~~Jelly; Mesenchymal Stem Cells; Isolation; Flow Cytometry; Regenerative Medicine~~jelly, mesenchymal stem cells, isolation, flow cytometry, regenerative medicine

INTRODUCTION

~~Mesenchymal stem cells (MSCs) are multipotent stromal cells capable of differentiating into various cell types, such as osteocytes, chondrocytes, and adipocytes, making them valuable for regenerative medicine. MSCs are characterized by a specific set of surface markers that distinguish them from other cell types, particularly hematopoietic cells. According to the International Society for Cellular Therapy (ISCT), MSCs must express CD105, CD73, and CD90, which are markers associated with cell adhesion, growth, and differentiation potential (1). CD105, or endoglin, plays a role in cell proliferation and vascular development(2). while CD73 and CD90 contribute to immunomodulation and cell-cell interactions (3,4). CD44, a receptor for hyaluronic acid, is also commonly expressed on MSCs and is involved in cell adhesion, migration, and homing, which are essential for therapeutic applications (5). Importantly, MSCs are negative for CD45, a pan-leukocyte marker, which ensures the exclusion of hematopoietic lineage cells (6). This unique combination of surface markers~~

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~~CD105, CD73, CD90, and CD44, with the absence of CD45—confirms the purity and identity of MSCs, supporting their use in clinical and research settings (7).~~

Mesenchymal stem cells (MSCs) are multipotent stromal cells capable of differentiating into various cell types, such as osteocytes, chondrocytes, and adipocytes, making them valuable for regenerative medicine. Mesenchymal stem cells are characterized by a specific set of surface markers that distinguish them from other cell types, particularly hematopoietic cells. [1] According to the International Society for Cellular Therapy (ISCT), MSCs must express CD105, CD73, and CD90, which are markers associated with cell adhesion, growth, and differentiation potential. [2] CD105, or endoglin, plays a role in cell proliferation and vascular development, [3] while CD73 and CD90 contribute to immunomodulation and cell-cell interactions. [4,5] CD44, a receptor for hyaluronic acid, is also commonly expressed on MSCs and is involved in cell adhesion, migration, and homing, which are essential for therapeutic applications. [6] Importantly, MSCs are negative for CD45, a pan-leukocyte marker, which ensures the exclusion of hematopoietic lineage cells. [7] This unique combination of surface markers—CD105, CD73, CD90, and CD44, with the absence of CD45—confirms the purity and identity of MSCs, supporting their use in clinical and research settings. [8]

The potential of ~~mesenchymal stem cells (~~MSCs~~)~~ for regenerative medicine has led to extensive research on various sources of MSCs, including those derived from Wharton's jelly of the human umbilical cord (WJ-MSCs). Wharton's Jelly of the human umbilical cord ~~(WJ-MSCs). WJ-MSCs~~ offer unique advantages, including non-invasive collection methods, high proliferation rates, and immunomodulatory properties, making them a promising cell type for therapeutic applications. ~~(8).~~[9] However, isolation and expansion methods must be refined to ensure the viability and consistency of WJ-MSCs. ~~In this study, we present the first successful isolation and characterization of WJ-MSCs conducted~~

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Previous works on the isolation and characterization of Wharton's jelly derived mesenchymal stem cells (WJ-MSCs) have produced different yields, markers and abilities to differentiate. For instance, results by Ranjbaran et al., [10] were able to isolate WJ-MSCs in a high glucose culture medium containing 15% fetal bovine serum (FBS), and separated MSCs efficiently by placing Wharton's jelly pieces at the bottom of the flask. Likewise, Cardoso et al. [11] cultured WJ-MSCs in a serum-free condition and the cells displayed typical mesenchymal stem cell phenotype up to 60 passages without having any genetic disorders. Abouelnaga et al.[12] also reported that the primary cultures exhibited success in isolating the umbilical cord Wharton's jelly mesenchymal stem cells, which maintained their tri-lineage differentiation potential, phenotypes and karyotype characteristics on further passage and expansion.

The originality of our work is based on the approach, which aims at improving the isolation and growth of WJ-MSCs in a 2% gelatine coated flask and a culture medium containing 10% human AB serum. Previous studies used fetal bovine serum (FBS)[13,14], but we used human serum in our experiment to avoid immune reaction in future clinical use. Furthermore, the current study also revealed the distinct pattern of surface markers (CD44, CD105, CD73, and CD90) during the passage, and CD44 was found to be most expressive at Passage 1 while the others at Passage 5.

The primary objective of this study is to establish a standardized protocol for the isolation and characterization of Wharton's Jelly-derived mesenchymal stem cells (WJ-MSCs) from human umbilical cords ~~at Gotong Royong Hospital, demonstrating the feasibility of establishing a protocol for obtaining viable WJ-MSCs from human umbilical cords and expanding them in culture.~~. The study aimed to optimize the culture conditions for WJ-MSCs, including the use of 2% gelatin-coated flasks and a culture medium supplemented with 10% Human AB serum, to ensure high cell viability, proliferation, and purity. Additionally, the study sought to characterize the phenotypic profile of WJ-MSCs using flow cytometry to assess the expression

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of key surface markers (CD105, CD73, CD90, and CD44) and the absence of hematopoietic markers (CD45).

METHODS

~~The establishment of this protocol at Gotong Royong Hospital not only provides a new avenue for local research in stem cell therapy but also lays the groundwork for future clinical applications and cell-based treatments. Patient selection in this study involved strict criteria, ensuring that only umbilical cords from healthy pregnancies were used, thus optimizing the quality and viability of the cells obtained. Ethical approval was secured, and careful steps were taken to remove blood vessels and isolate the Wharton's Jelly, maximizing MSC yield. This standardized approach enables a reliable source of WJ-MSCs, critical for any future applications in regenerative medicine and disease modelling.~~

~~Our results show that the WJ-MSCs isolated through this protocol display a fibroblast-like morphology characteristic of MSCs, with consistent morphology observed through the first passage. Using a detailed explant culture method, we were able to cultivate and expand WJ-MSCs, which adhered to the culture surface within days and proliferated significantly over 21 days. These cells retained their mesenchymal characteristics, evidenced by their elongated, spindle-shaped morphology and high proliferation rate. Such morphological consistency is essential for the continued use of these cells in therapeutic research, where cell integrity and stability are paramount.~~

~~In addition to morphological assessment, flow cytometry analysis was employed to confirm the MSC phenotype, assessing the expression of surface markers CD105, CD73, CD90, and CD44, with CD45 as a hematopoietic exclusion marker. This phenotypic profile aligns with the International Society for Cellular Therapy (ISCT) guidelines for MSC characterization, confirming the cells' stem cell identity and purity across passages. This study thus establishes a foundational WJ-MSC isolation and expansion protocol at Gotong Royong Hospital, creating significant potential for future cell therapy research and applications within the hospital and the broader scientific community.~~

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~~MATERIAL METHODS~~

~~Isolation of Human Umbilical Cord-Derived Mesenchymal Stem Cells~~Research design

This research was conducted in Gotong Royong Hospital in Surabaya in collaboration with the Faculty of Medicine and Faculty of Pharmacy of Widya Mandala Catholic University Surabaya. The research period spanned from June 2024 to December 2024.

Isolation of human umbilical cord-derived mesenchymal stem cells (WJ-MSCs)

Human umbilical cords were collected from 3 (three) patients in Gotong Royong Hospital, immediately following caesarean section, within 5 minutes of placental removal to ensure optimal cell viability. Patient selection included strict criteria: only umbilical cords from healthy pregnancies without maternal or fetal complications were used. Specifically, cases of preeclampsia, metabolic disorders, infections, or abnormal umbilical cord coiling were excluded. ~~The ethical approval was obtained.~~

Upon retrieval, each umbilical cord was placed in a sterile container with Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) containing 1% penicillin/streptomycin/amphotericin B and transported to the laboratory under aseptic conditions.

In addition to morphological assessment, flow cytometry analysis was employed to confirm the MSC phenotype, assessing the expression of surface markers CD105, CD73, CD90, and CD44, with CD45 as a hematopoietic exclusion marker. This phenotypic profile aligns with the International Society for Cellular Therapy (ISCT) guidelines for MSC characterization, confirming the cells' stem cell identity and purity across passages. This study thus establishes a foundational WJ-MSC isolation and expansion protocol at Gotong Royong Hospital, creating significant potential for future cell therapy research and applications within the hospital and the broader scientific community.

Preparation and ~~Culture~~culture of WJ-MSCs

~~In a laminar flow cell culture hood to maintain aseptic technique, each umbilical cord was first washed thoroughly with Hank's Balanced Salt Solution (HBSS) containing 1% penicillin/streptomycin/amphotericin B. Blood vessels were carefully removed from the~~

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~~Wharton's Jelly tissue to isolate the mesenchymal-rich regions, minimizing potential contamination, and promoting a higher yield of mesenchymal stem cells (MSCs). The remaining Wharton's Jelly tissue was cut into approximately 2 mm² pieces, which were then placed in T75 culture flasks precoated with 2% gelatin to enhance cellular adherence (9).~~
In a laminar flow cell culture hood to maintain aseptic technique, each umbilical cord was first washed thoroughly with Hank's Balanced Salt Solution (HBSS) containing 1% penicillin/streptomycin/amphotericin B (Service Bio,China). Blood vessels were carefully removed from the Wharton's Jelly tissue to isolate the mesenchymal-rich regions, minimizing potential contamination, and promoting a higher yield of mesenchymal stem cells (MSCs). The remaining Wharton's Jelly tissue was cut into approximately 2 mm² pieces, which were then placed in T75 culture flasks precoated with 2% gelatin to enhance cellular adherence. (14)

The tissue explants were left undisturbed for 7 days to allow cells to attach to the surface. ~~(10).~~ ~~The culture medium, composed of DMEM supplemented with 10% Human AB serum and 0.1% penicillin/streptomycin/amphotericin B, was carefully added to prevent dislodging the tissue fragments.~~(15) The culture medium, composed of DMEM supplemented with 10% human AB serum and 0.1% penicillin/streptomycin/amphotericin B, was carefully added to prevent dislodging the tissue fragments. Medium was replaced every 3 days, and cell outgrowth was monitored microscopically. After 21 days, successful outgrowth and proliferation of Passage 1 (P1) cells were observed, characterized by the spindle-shaped, fibroblast-like morphology typical of MSCs.

Flow cytometry analysis

Flow cytometry was employed to phenotype Wharton's Jelly-derived mesenchymal stem cells (WJ-MSCs) isolated at Passage 2 and Passage 6 using a BD FACSAria flow cytometer (BD Biosciences). To assess the expression of key mesenchymal and hematopoietic markers, three antibody panels were prepared with mouse monoclonal antibodies specific to human antigens. Panel 1 included anti-human CD105 (clone 43A3, Alexa Fluor 488, BioLegend) and anti-

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human CD45 (clone H130, FITC, BioLegend); Panel 2 included anti-human CD73 (clone AD2, PE, BioLegend) and anti-human CD90 (clone 5E10, PE/Cy7, BioLegend); and Panel 3 included anti-human CD44 (clone IM7, PE, Elabscience). For each panel, 10^6cells in 100ul were aliquoted into individual tubes, and 2.5 µL of each antibody was added to each sample. The cells were incubated for 1 hour at 4°C in the dark to optimize antibody binding and prevent photobleaching. After incubation, cells were washed twice with staining buffer (PBS with 1% BSA) to remove unbound antibodies and resuspended in 500 µL of buffer for analysis. Gating parameters was set to exclude debris and non-viable cells based on forward and side scatter properties. Unstained controls were applied to correct for spectral overlap among fluorophores. ~~Expression of CD105, CD73, CD90, and CD44 was assessed to confirm MSC phenotype, while CD45 was used to exclude hematopoietic contamination (11).~~Expression of CD105, CD73, CD90, and CD44 was assessed to confirm MSC phenotype, while CD45 was used to exclude hematopoietic contamination. (16) Data were analyzed to determine the percentage of cells expressing each marker, providing a comprehensive phenotypic profile of WJ-MSCs across passages.

Ethical clearance

The ethical clearance for collecting the human tissue was obtained from medical Faculty, Universitas Hang Tuah (**E/006/UHT.KEPK.03/II/2024**)

Statistical analysis

GraphPad Prism version 8.0.1 was used for data presentation. ~~Unpaired t-tests were employed to compare relative intensity differences between specific groups.~~The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) when the data followed normality and homogeneity assumption of parametric analysis

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(otherwise using nonparametric Kruskal-Wallis followed by Dunn's test). Normality and homogeneity were assessed using the Shapiro-Wilk and Brown-Forsythe tests, respectively. A p-value < 0.05 is considered significant. All data were presented as mean ± standard deviation (SD).

RESULTS

Isolation and ~~Culture~~culture of Wharton's ~~Jelly Derived~~ jelly-derived MSCs

Our results show that the WJ-MSCs isolated through this protocol display a fibroblast-like morphology characteristic of MSCs, with consistent morphology observed through the first passage. Using a detailed explant culture method, we were able to cultivate and expand WJ-MSCs, which adhered to the culture surface within days and proliferated significantly over 21 days. These cells retained their mesenchymal characteristics, evidenced by their elongated, spindle-shaped morphology and high proliferation rate. Such morphological consistency is essential for the continued use of these cells in therapeutic research, where cell integrity and stability are paramount.

The isolation of ~~Wharton's Jelly-derived mesenchymal stem cells (WJ-MSCs)~~WJ-MSCs was achieved through the explant culture method, allowing MSCs to be successfully obtained from human umbilical cord tissue. Following isolation, WJ-MSCs adhered to the culture surface within the first few days, and after 21 days in culture, Passage 1 (P1) showed significant cell proliferation. The MSCs exhibited a characteristic fibroblast-like morphology (see ~~figure~~Figure 1), typical of mesenchymal cells, with elongated, spindle-shaped bodies and a uniform, flattened appearance. Cells were tightly adherent, forming a monolayer with parallel, aligned arrangements. The morphology of the cells remained stable through Passage 14 (P14), showing no signs of senescence or morphological abnormalities. This consistent fibroblast-like morphology, with the presence of prominent cell projections and well-defined cellular

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boundaries, suggests that the WJ-MSCs maintained their mesenchymal characteristics and were successfully expanded in primary culture.

Phenotypic ~~Characterization~~characterization of WJ-MSCs by ~~Flow Cytometry~~flow cytometry

To confirm the MSC phenotype, we performed flow cytometric analysis on WJ-MSCs passage 5 (P5), assessing the expression of key surface markers (see ~~figure~~Figure 2). The gating strategy effectively isolated MSC populations, and the analysis revealed high expression levels of CD73 and CD90, alongside positive populations for CD105. Additionally, the cells were negative for CD45, confirming the absence of hematopoietic contamination (Figure 2). Positive CD44 expression further validated the MSC phenotype, affirming the cells' stem cell characteristics and purity.

Comparative ~~Analysis~~analysis of ~~Surface Markers~~surface markers at ~~Different Passages~~different passages

Comparisons of WJ-MSC surface markers between P1 and P5 revealed significant differences in marker expression (see ~~figure~~Figure 3). At Passage 1, the MSCs demonstrated a statistically significant increase in pluripotency surface markers compared to the negative phenotype CD45 (~~P~~p = 0.~~01~~010). By P5, a dramatic increase in CD105, CD90, CD73, and CD44 expression was observed, with statistical significance ~~denoted as ***P~~(p < 0.0001) (Figure 3). These findings indicate robust MSC proliferation and stable expression of essential stem cell markers across passages, supporting the successful expansion and maintenance of MSC characteristics through successive passages.

DISCUSSION

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~~This study successfully demonstrates the isolation and characterization of Wharton's Jelly-derived mesenchymal stem cells (WJ-MSCs) from human umbilical cords collected at Gotong Royong Hospital. Our findings confirm the feasibility of establishing a reliable protocol for obtaining viable WJ-MSCs locally, marking the first successful effort at this institution. These cells exhibited typical MSC morphology and a stable phenotype through early passages, supporting their potential for future research and clinical applications (12). However, despite overall success, the detection of low levels of CD45 expression indicates potential hematopoietic contamination (11). This may be attributed to residual blood cells within the Wharton's Jelly, as complete removal of all blood vessels can be challenging in clinical settings (13). Future refinements could include additional washes or enzymatic treatments to further minimize CD45-positive cells, improving the MSC purity and strengthening the clinical potential of these cells (14).~~

~~Interestingly, recent studies have highlighted the potential of MSCs derived specifically from the cord-placental junction, which may offer advantages over WJ-MSCs in terms of cellular yield, differentiation potential, and immunomodulatory properties (15). MSCs from the cord-placental junction have shown enhanced proliferation rates and greater multilineage differentiation capabilities compared to WJ-MSCs, likely due to their proximity to the placenta and the rich microenvironmental cues present there (16). Exploring the isolation of MSCs from this region could expand the scope of stem cell research at Gotong Royong Hospital, potentially yielding MSCs with more robust properties for regenerative therapies.~~

~~Our analysis of MSC surface marker expression showed unique patterns across different passages, specifically with P1 exhibiting only high CD44 expression (approximately 60%) while other markers, including CD105, CD73, and CD90, were undetectable. This selective expression at P1 could reflect an early stage in MSC culture, where cells prioritize adhesion and migration functions, as CD44 is a receptor for hyaluronic acid involved in cell adhesion and motility (15,17). successive passages, notably by P5, all MSC markers (CD105, CD73, CD90, and CD44) reached high expression levels, suggesting that these cells may require additional time or specific culture conditions to fully upregulate markers associated with mesenchymal identity and functionality. CD45, as expected, remained low across passages, though its continued detectability indicates potential residual hematopoietic contamination, a finding that aligns with reports from other studies on WJ-MSCs (11). Interestingly, this marker pattern differs from MSCs derived from the cord-placental junction (CPJ-MSCs), which tend to express high levels of all MSC markers, including CD105, CD73, and CD90, and lower CD45. These differences may arise from microenvironmental factors unique to CPJ-MSCs that accelerate marker expression and MSC commitment earlier in culture, highlighting the potential value of CPJ as an alternative MSC source for applications requiring rapid cell expansion and stable marker profiles (9).~~

~~Another area for improvement in our protocol is the lengthy outgrowth period, as cell attachment and expansion required 21 days before reaching significant confluence. Long outgrowth times can limit scalability and delay potential downstream applications (18). Optimizing the initial explant culture conditions could reduce this outgrowth period, thereby increasing efficiency. This might include using higher serum concentrations, pre-coating culture surfaces with adhesion-promoting molecules such as fibronectin, or adjusting oxygen levels to more closely mimic the native environment of umbilical cord MSCs (19,20). Reducing the outgrowth time will not only streamline the MSC isolation process but also decrease the potential for cell senescence and culture-induced variations, ultimately improving the consistency and reliability of the cells obtained for research and therapeutic use.~~

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~~In conclusion, this study establishes a foundational protocol for WJ-MSC isolation at Gotong Royong Hospital, creating new opportunities for cell-based therapies and research within the local healthcare system. Although minor challenges, such as the presence of CD45-positive cells and prolonged outgrowth, remain, the success of this protocol provides a strong basis for further innovation and optimization. Future work could build upon this study by investigating MSCs from the cord-placental junction, refining isolation techniques, and reducing culture times, all of which would enhance the therapeutic applicability of MSCs derived at Gotong Royong Hospital. This research not only contributes to the field of regenerative medicine but also has the potential to open new pathways for personalized cellular therapies.~~

This study successfully demonstrates the rapid isolation and characterization of WJ-MSCs from human umbilical cords using freshly prepared human AB-serum. These cells exhibited typical MSC morphology and a stable phenotype through early passages, supporting their potential for future research and clinical applications. (17) However, despite overall success, the detection of low levels of CD45 expression indicates potential hematopoietic contamination. (16) This may be attributed to residual blood cells within the Wharton's Jelly, as complete removal of all blood vessels can be challenging in clinical settings. (18) Future refinements could include additional washes or enzymatic treatments to further minimize CD45-positive cells, improving the MSC purity and strengthening the clinical potential of these cells. (19)

Interestingly, recent study have highlighted the potential of MSCs derived specifically from the cord-placental junction, which may offer advantages over WJ-MSCs in terms of cellular yield, differentiation potential, and immunomodulatory properties. (13) MSCs from the cord-placental junction have shown enhanced proliferation rates and greater multilineage differentiation capabilities compared to WJ-MSCs, likely due to their proximity to the placenta and the rich microenvironmental cues present there .[20] Exploring the isolation of MSCs from this region could expand the scope of stem cell research at Gotong Royong Hospital, potentially yielding MSCs with more robust properties for regenerative therapies.

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while other markers, including CD105, CD73, and CD90, were undetectable. This selective expression at P1 could reflect an early stage in MSC culture, where cells prioritize adhesion and migration functions, as CD44 is a receptor for hyaluronic acid involved in cell adhesion and motility. (13,21) Figure 3 demonstrated a successive passage, notably by P5, all MSC markers (CD105, CD73, CD90, and CD44) reached high expression levels. This finding suggest that these cells may require additional time or specific culture conditions to fully upregulate markers associated with mesenchymal identity and functionality. CD45, as expected, remained low across passage 5, though its continued detectability indicates potential residual hematopoietic contamination, aligns with reports from other study. (16) Interestingly, this marker pattern differs from MSCs derived from the cord-placental junction (CPJ-MSCs), which tend to express high levels of all MSC markers, including CD105, CD73, and CD90, and lower CD45. These differences may arise from microenvironmental factors unique to CPJ-MSCs that accelerate marker expression and MSC commitment earlier in culture, highlighting the potential value of CPJ as an alternative MSC source for applications requiring rapid cell expansion and stable marker profiles. (14)

Another area for improvement in our protocol is the lengthy outgrowth period, as cell attachment and expansion required 21 days before reaching significant confluence. Long outgrowth times can limit scalability and delay potential downstream applications. [22] Optimizing the initial explant culture conditions could reduce this outgrowth period, thereby increasing efficiency. This might include using higher serum concentrations, pre-coating culture surfaces with adhesion-promoting molecules such as fibronectin, or adjusting oxygen levels to more closely mimic the native environment of umbilical cord MSCs. [23,24] Reducing the outgrowth time will not only streamline the MSC isolation process but also decrease the potential for cell senescence and culture-induced variations, ultimately improving the consistency and reliability of the cells obtained for research and therapeutic use.

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The limitations of this study include, first, the contamination of CD45, which is still detected in antibody expressions. This may result from incomplete removal of blood vessels during the isolation process, potentially affecting the purity of WJ-MSCs and necessitating further refinement of the isolation protocol. Second, the prolonged outgrowth period limits the scalability and efficiency of the protocol for large-scale applications.

Despite these limitations, this study successfully isolated and characterized WJ-MSCs, providing a foundation for future clinical applications in regenerative medicine, including tissue repair, immunomodulation, and cell-based therapies. The use of human AB serum instead of fetal bovine serum (FBS) reduces the risk of immune reactions, making the protocol more suitable for clinical use.

Looking ahead, several future directions could build on this research. First, optimizing the isolation process by carefully extracting arteries and veins could minimize CD45 contamination and improve MSC purity. Second, reducing the outgrowth period by optimizing culture conditions—such as using higher serum concentrations, adhesion-promoting molecules like fibronectin, or adjusting oxygen levels to better mimic the native environment of umbilical cord MSCs—could enhance efficiency and applicability.

CONCLUSION

This study successfully demonstrates the isolation and characterization of Wharton's jelly (WJ-MSC) using a culture medium supplemented with 10% Human AB serum and high glucose, combined with a 2% gelatine-coated flask. This protocol achieved high cell viability and proliferation, with WJ-MSCs exhibiting fibroblast like morphology and stable expression of MSC key markers (CD105, CD44, CD90 and CD73) by passage 5. Low level of CD45 expression indicating minimal hematopoietic contamination, confirming the purity of the isolated cells.

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The results highlight the feasibility of this protocol for obtaining viable WJ-MSCs, providing a path for future research and clinical applications in regenerative medicine. However, the 21-day outgrowth period and the presence of residual CD45 positive suggesting for further optimization, such as refining the isolation techniques, reducing culture times and exploring alternative MSC sources.

Authors ~~contribution.~~Contribution

~~Sianty Dewi, Yudy Tjahjono, and Bernadette Dian Novita Dewi~~SD, YT, and BDN designed the experiments, carried out the experiments, and prepared the manuscript. ~~Brilliant Dwi Putra, Kuncoro Foe, Lucia Hendriati, Hendy Wijaya, Himawan Haryanto Jong, Franklin Malonda, and Suryo Kuncorojakti~~BDP, KF, BDP, LH, HW, HHJ, FM, and SK carried out the experiments and analyzed the data. YT, SD and BDF revised the manuscript. All authors have read and approved the final manuscript.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgement

We would like to thank Department of ~~Obstetric~~Obstetrics and Gynacology Gotong Royong Hospital~~,~~ Surabaya-Indonesia for the support. ~~The work was supported by~~

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~~FIGURE~~ **Data Availability Statement**

The authors confirm that the data supporting the findings of this study are available within the article

Declaration the Use of AI in Scientific Writing

During the preparation of this work, the authors used ChatGPT (OpenAI) to assist in language refinement, grammar correction, and improving technical clarity. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

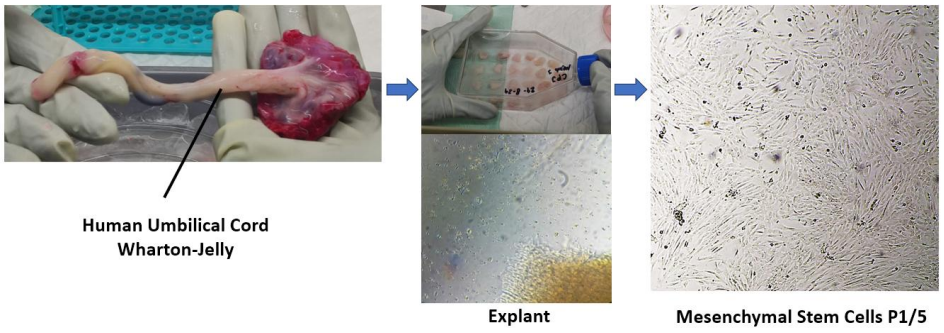

Figure 1~~:~~. **Explant ~~Culture~~culture of Wharton's ~~Jelly-Derived~~jelly-derived MSCs**
The initial explant culture of Wharton's ~~Jelly~~jelly-derived mesenchymal stem cells (WJ-MSCs) was isolated from human umbilical cords. The explant technique and subsequent cell morphology are shown, with primary cultures demonstrating successful MSC adherence and proliferation.

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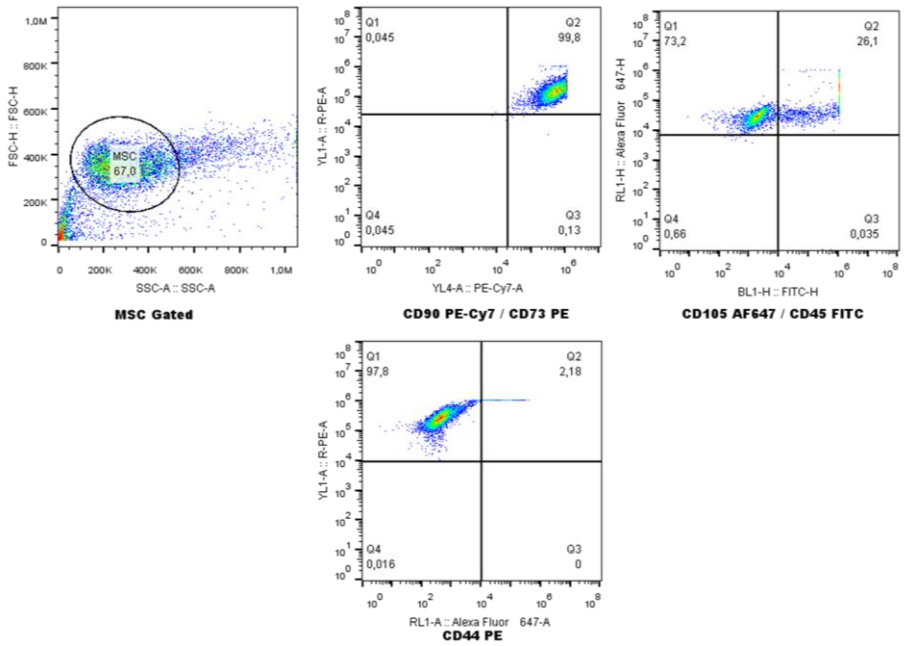

Figure 2~~:~~. **Representative ~~Flow Cytometric Analysis~~flow cytometric analysis of WJ-MSC ~~Surface Markers~~surface markers**
The flow cytometric gating strategy assesses key surface markers on WJ-MSCs, confirming their stem cell characteristics (upper left). The expression levels of positive markers are shown in the upper middle (CD73, CD90), with CD105-positive and CD45-negative populations (Q1 for CD105; Q2 for CD45; upper right), as well as CD44-positive populations (lower graphic), verifying the MSC phenotype.

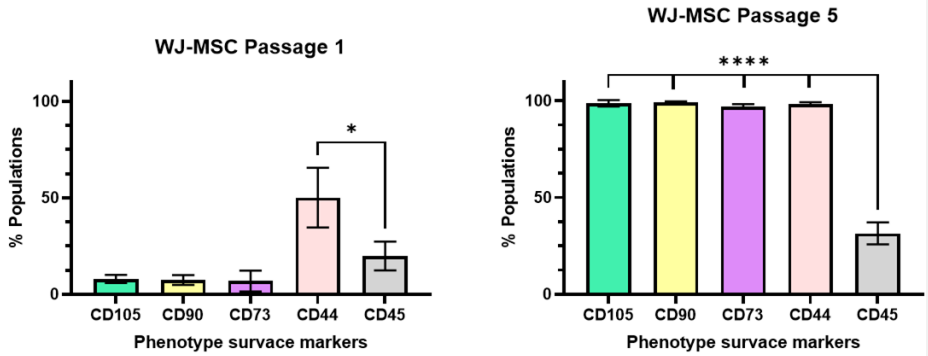

Figure 3~~:~~. **Comparative ~~Cell Surface Markers~~cell surface markers of WJ-MSCs at ~~Passage~~passage 1 and ~~Passage~~passage 5**

Compared to the negative phenotype (CD45), WJ-MSCs demonstrate a significant increase in surface markers at ~~Passage~~passage 1 ~~(*P~~(p=0.~~01~~010). However, a dramatic increase was observed in CD105, CD90, CD73, and CD44 surface markers at ~~Passage~~passage 5 ~~(****P~~(p < 0.~~0001~~000), indicating successful MSC proliferation and phenotype stability across passages. Statistical analysis was conducted using one-way ANOVA with post-hoc tests to determine significance between groups. ~~P~~p-values were calculated using GraphPad Prism, with significance levels denoted as follows: ~~P~~p < 0.05 (*), ~~P~~p < 0.~~01 (**), P~~010 (**), p < 0.001 (***), and ~~P~~p < 0.~~0001~~000 (****).

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Gmail ACCEPTANCE LETTER/EMAIL **Yudy Tjahjono <yudy.tjahjono@ukwms.ac.id>**

Editor Decision

1 message

do-not-reply@univmed.org <do-not-reply@univmed.org> Mon, Apr 7, 2025 at 10:35 AM
Reply-To: Pusparini Pusparini <pusparini@trisakti.ac.id>
To: Yudi Tjahyono <yudy.tjahjono@ukwms.ac.id>

Yudy Tjahjono, Sianty Dewi, Bernadette Dian Novita Dewi, Hendy Wijaya, Brilliant Dwi Putra, Suryo Kuncorojakti, Lucia Hendriati, FX Himawan Haryanto Jong, Teguh Widodo, Franklin Vincentius Malonda:

We have reached a decision regarding your submission to Universa Medicina, "Isolation and Characterization of Wharton's Jelly-Derived Mesenchymal Stem Cells: A Foundational Protocol for Regenerative Medicine Applications at Gotong Royong Hospital".

Our decision is to: Accept Submission

Editor Decision

1 message

do-not-reply@univmed.org <do-not-reply@univmed.org> Thu, Apr 10, 2025 at 9:54 AM
Reply-To: Pusparini Pusparini <pusparini@trisakti.ac.id>
To: Yudi Tjahyono <yudy.tjahjono@ukwms.ac.id>

Yudy Tjahjono, Sianty Dewi, Bernadette Dian Novita Dewi, Hendy Wijaya, Brilliant Dwi Putra, Suryo Kuncorojakti, Lucia Hendriati, FX Himawan Haryanto Jong, Teguh Widodo, Franklin Vincentius Malonda:

The editing of your submission, "Isolation and Characterization of Wharton's Jelly-Derived Mesenchymal Stem Cells: A Foundational Protocol for Regenerative Medicine Applications at Gotong Royong Hospital," is complete. We are now sending it to production.

Submission URL: https://univmed.org/ejurnal/index.php/medicina/authorDashboard/submission/1678

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ORIGINAL ARTICLE

Rapid isolation and characterization of Wharton's jelly-derived mesenchymal stem cells maintained in fresh-prepared human AB-serum

Sianty Dewi[1], Yudy Tjahjono[2]*, Bernadette Dian Novita[1], Hendy Wijaya[1], Brilliant Dwi Putra[2], Teguh Widodo[2], Lucia Hendriati[2], Himawan Haryanto Jong[1], Franklin Malonda[1], and Suryo Kuncorojakti[3,4]

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ABSTRACT

BACKGROUND

Mesenchymal stem cells (MSCs) are valued in regenerative medicine for their multipotency, proliferative capacity, and immunomodulatory properties. Wharton's jelly-derived MSCs (WJ-MSCs) from the umbilical cord offer a non-invasive, promising source for clinical applications, because easy isolation, lack of ethical concerns, and the presence of both embryonic and adult stem cells have made them a valuable source for use in therapeutic applications and regenerative medicine. This study aimed to optimize WJ-MSC isolation and characterization methods.

METHODS

Human umbilical cords from three healthy donors were collected post-cesarean under strict inclusion criteria. WJ-MSCs were isolated using the explant culture method, with cells adhering to T75 flasks pre-coated with 2% gelatin. Cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% freshly prepared Human AB serum and monitored for 21 days. Flow cytometry (BD FACSAria) was performed at passages 1 and 5 to assess MSC markers CD105, CD73, CD90, and CD44, alongside the exclusion marker CD45.

RESULTS

WJ-MSCs exhibited fibroblast-like morphology by passage 1 and showed robust proliferation. Flow cytometry revealed high CD44 expression (~60%) at passage 1, while CD105, CD73, and CD90 became prominent by passage 5. CD45 remained low, suggesting minimal hematopoietic contamination.

CONCLUSIONS

This study confirms the feasibility of isolating and expanding WJ-MSCs using DMEM with 10% human AB serum. While consistent cell growth was achieved, the 21-day culture period may require optimization for

scalability, including serum concentration, substrate coatings, and oxygen levels. CPJ-MSCs may be preferable for applications demanding rapid expansion and early marker expression.

Keywords: Wharton's jelly, mesenchymal stem cells, isolation, flow cytometry, regenerative medicine

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stromal cells capable of differentiating into various cell types, such as osteocytes, chondrocytes, and adipocytes, making them valuable for regenerative medicine. Mesenchymal stem cells are characterized by a specific set of surface markers that distinguish them from other cell types, particularly hematopoietic cells.[1] According to the International Society for Cellular Therapy (ISCT), MSCs must express CD105, CD73, and CD90, which are markers associated with cell adhesion, growth, and differentiation potential, respectively.[2] CD105, or endoglin, plays a role in cell proliferation and vascular development,[3] while CD73 and CD90 contribute to immunomodulation and cell-cell interactions, respectively.[4,5] Additionally, CD44, a receptor for hyaluronic acid, is also commonly expressed on MSCs and is involved in cell adhesion, migration, and homing, which are essential for therapeutic applications.[6] Importantly, MSCs are negative for CD45, a pan-leukocyte marker, which ensures the exclusion of hematopoietic lineage cells.[7] This unique combination of surface markers—CD105, CD73, CD90, and CD44, with the absence of CD45—confirms the purity and identity of MSCs, supporting their use in clinical and research settings.[8]

The potential of MSCs for regenerative medicine has led to extensive research on various sources of MSCs, including those derived from Wharton's jelly of the human umbilical cord (WJ-MSCs). The use of Wharton's jelly of the human umbilical cord as a source of MSCs offers unique advantages, including non-invasive collection methods, high proliferation rates, and immunomodulatory properties, making them a promising cell type for therapeutic applications.[9] However, isolation and expansion methods must be refined to ensure the viability and consistency of WJ-MSCs.

Previous works on the isolation and characterization of Wharton's jelly derived mesenchymal stem cells (WJ-MSCs) have produced different yields, markers and abilities to differentiate. For instance, Ranjbaran et al.[10] were able to isolate WJ-MSCs in 10cm^2 plates containing high glucose-Dulbecco`s Modified Eagle Medium-F12 (HG-DMEM-F12), supplemented with 15% fetal bovine serum (FBS), and to separate the MSCs efficiently by placing pieces of Wharton's jelly at the bottom of the plates. Similarly, Cardoso et al.[11] cultured bovine WJ-MSCs in a serum-free condition and the cells displayed the typical mesenchymal stem cell phenotype up to 60 passages without having any genetic abnormalities. Abouelnaga et al.[12] also reported the successful isolation of umbilical cord Wharton's jelly mesenchymal stem cells in primary cultures, after which the cells maintained their tri-lineage differentiation potential, phenotypes, and karyotype characteristics on further passage and expansion.

The originality of our work is based on the approach, which aims at improving the isolation and growth of WJ-MSCs in a 2% gelatine coated flask and a culture medium containing 10% human AB serum. Previous studies used fetal bovine serum (FBS)[13,14], but we used human serum in our experiment to avoid immune reactions in future clinical use. Furthermore, the current study also revealed the distinct pattern of surface markers (CD44, CD105, CD73, and CD90) during the passage, with CD44 being most expressive at passage 1 and the other markers at passage 5.

The primary objective of this study was to establish a standardized protocol for the isolation and characterization of WJ-MSCs from human umbilical cords. The study aimed to optimize the culture conditions for WJ-MSCs, including the use of 2% gelatin-coated flasks and a culture medium supplemented with 10% human AB serum, to ensure high cell viability, proliferation, and purity. Additionally, the study sought to characterize the phenotypic profile of WJ-MSCs using flow cytometry to assess the expression of key surface markers (CD105, CD73, CD90, and CD44) and the absence of hematopoietic markers (CD45).

METHODS

Research design

This research was conducted in Gotong Royong Hospital in Surabaya in collaboration with the Faculty of Medicine and Faculty of Pharmacy of Widya Mandala Catholic University Surabaya. The research period was from June 2024 to December 2024.

Isolation of human umbilical cord-derived mesenchymal stem cells (WJ-MSCs)

Human umbilical cords were collected from 3 patients in Gotong Royong Hospital, immediately following cesarean section, within 5 minutes of placental removal to ensure optimal cell viability. Patient selection included strict criteria: only umbilical cords from healthy pregnancies without maternal or fetal complications were used. Specifically, cases of preeclampsia, metabolic disorders, infections, or abnormal umbilical cord coiling were excluded. Upon retrieval, each umbilical cord was placed in a sterile container with Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) containing 1% penicillin/streptomycin/amphotericin B and transported to the laboratory under aseptic conditions. In addition to morphological assessment, flow cytometry analysis was employed to confirm the MSC phenotype, assessing the expression of surface markers CD105, CD73, CD90, and CD44, with CD45 as a hematopoietic exclusion marker. This phenotypic profile aligns with the International Society for Cellular Therapy (ISCT) guidelines for MSC characterization, confirming the cells' stem cell identity and purity across passages.

Preparation and culture of WJ-MSCs

In a laminar flow cell culture hood to maintain aseptic technique, each umbilical cord was first washed thoroughly with Hank's Balanced Salt Solution (HBSS) containing 1% penicillin/ streptomycin/amphotericin B (Service Bio, China). Blood vessels were carefully removed from the Wharton's Jelly tissue to isolate the mesenchymal-rich regions, minimizing potential contamination, and promoting a higher yield of mesenchymal stem cells (MSCs). The remaining Wharton's Jelly tissue was cut into approximately 2 mm^2 pieces, which were then placed in T75 culture flasks precoated with 2% gelatin to enhance cellular adherence.[14] The tissue explants were left undisturbed for 7 days to allow cells to attach to the surface. [15] The culture medium, composed of DMEM supplemented with 10% human AB serum and 0.1% penicillin/streptomycin/amphotericin B, was carefully added to prevent dislodging the tissue fragments. Medium was replaced every 3 days, and cell outgrowth was monitored microscopically. After 21 days, successful outgrowth and proliferation of passage 1 (P1) cells were observed, characterized by the spindle-shaped, fibroblast-like morphology typical of MSCs.

Flow cytometry analysis

Flow cytometry was employed to determine the phenotype of WJ-MSCs isolated at passages 2 and 6 using a BD FACSAria flow cytometer (BD Biosciences). To assess the expression of key mesenchymal and hematopoietic markers, three antibody panels were prepared with mouse monoclonal antibodies specific to human antigens. Panel 1 included anti-human CD105 (clone 43A3, Alexa Fluor 488, BioLegend) and anti-human CD45 (clone H130, FITC, BioLegend); Panel 2 included anti-human CD73 (clone AD2, PE, BioLegend) and anti-human CD90 (clone 5E10, PE/Cy7, BioLegend); and Panel 3 included anti-human CD44 (clone IM7, PE, Elabscience). For each panel, 10^6 cells in 100 µL were aliquoted into individual tubes, and 2.5 µL of each antibody was added to each sample. The cells were incubated for 1 hour at 4°C in the dark to optimize antibody binding and prevent photobleaching. After incubation, cells were washed twice with staining buffer (PBS with 1% BSA) to remove unbound antibodies, and were resuspended in 500 µL of buffer for analysis. Gating parameters were set to exclude debris and non-viable cells based on forward and side scatter properties. Unstained controls were applied to correct for spectral overlap among fluorophores. Expression of CD105, CD73, CD90, and CD44 was assessed to confirm MSC phenotype, while CD45 was used to exclude hematopoietic contamination.[16] Data were analyzed to determine the percentage of cells expressing each marker, providing a comprehensive phenotypic profile of WJ-MSCs across passages.

Ethical clearance

The ethical clearance for collecting the human tissue was obtained from Medical Faculty, Universitas Hang Tuah (E/006/UHT.KEPK.03/II/2024).

Statistical analysis

GraphPad Prism version 8.0.1 was used for data presentation. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) when the data followed normality and homogeneity assumption of parametric analysis (otherwise using nonparametric Kruskal-Wallis followed by Dunn's test). Normality and homogeneity were assessed using the Shapiro-Wilk and Brown-Forsythe tests, respectively. A p-value <0.05 was considered significant. All data were presented as mean ± standard deviation (SD).

RESULTS

Isolation and culture of Wharton's jelly-derived MSCs

Our results show that the WJ-MSCs isolated through this protocol display a fibroblast-like morphology characteristic of MSCs, with consistent morphology observed through the first passage. The isolation of WJ-MSCs was achieved through the explant culture method, allowing MSCs to be successfully obtained from human umbilical cord tissue. Following isolation, WJ-MSCs adhered to the culture surface within the first few days, and after 21 days in culture, passage 1 (P1) showed significant cell proliferation. The MSCs exhibited a characteristic fibroblast-like morphology (Figure 1), typical of mesenchymal cells, with elongated, spindle-shaped bodies and a uniform, flattened appearance. Cells were tightly adherent, forming a monolayer with parallel, aligned arrangements. The morphology of the cells remained stable through passage 14 (P14), showing no signs of senescence or morphological abnormalities. This consistent fibroblast-like morphology, with the presence of prominent cell projections and well-defined cellular boundaries, suggests that the WJ-MSCs maintained their mesenchymal characteristics as evidenced by their elongated spindle-shaped morphology and high proliferation rate, and were successfully expanded in primary culture. Such morphological consistency is essential for the continued use of these cells in therapeutic research, where cell integrity and stability are paramount.

Phenotypic characterization of WJ-MSCs by flow cytometry

To confirm the MSC phenotype, we performed flow cytometric analysis on WJ-MSCs passage 5 (P5), assessing the expression of key surface markers (Figure 2). The gating strategy effectively isolated MSC populations, and the analysis revealed high expression levels of CD73 and CD90, alongside positive populations for CD105. Additionally, the cells were negative for CD45, confirming the absence of hematopoietic contamination (Figure 2). Positive CD44 expression further validated the MSC phenotype, affirming the cells' stem cell characteristics and purity.

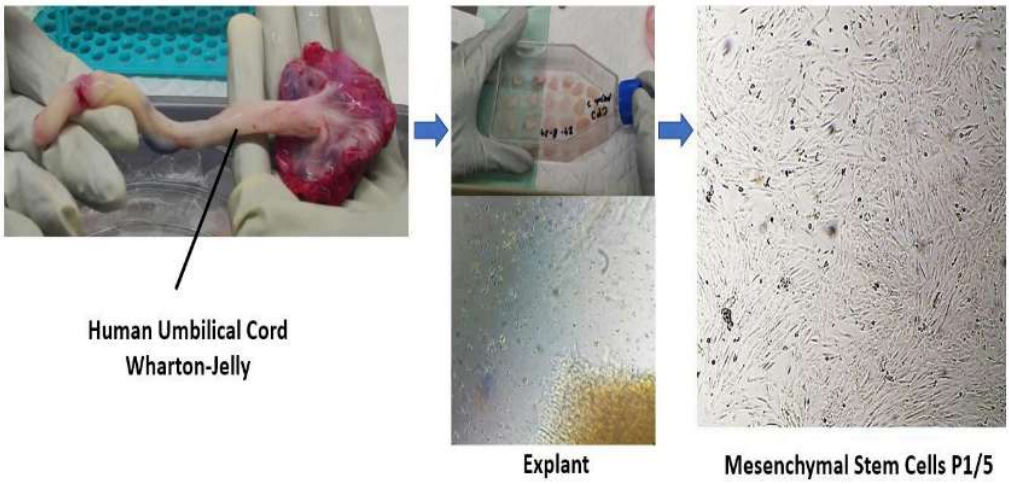

Figure 1. Explant culture of Wharton's jelly-derived MSCs
The initial explant culture of Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) was isolated from human umbilical cords. The explant technique and subsequent cell morphology are shown, with primary cultures demonstrating successful MSC adherence and proliferation.

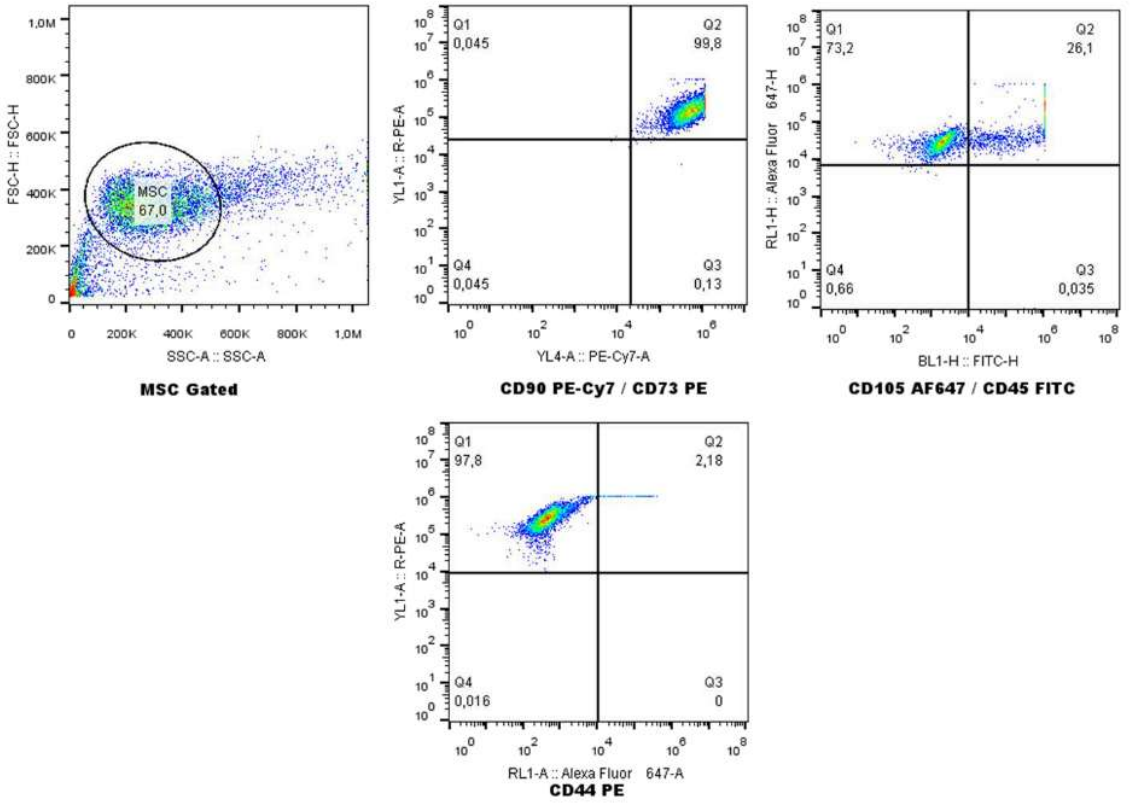

Figure 2. Representative flow cytometric analysis of WJ-MSC surface markers
The flow cytometric gating strategy assesses key surface markers on WJ-MSCs, confirming their stem cell characteristics (upper left graph). The expression levels of positive markers are shown in the upper middle graph (CD73, CD90), with CD105-positive and CD45-negative populations (Q1 for CD105; Q2 for CD45; upper right), as well as CD44-positive populations (lower middle graph), verifying the MSC phenotype.
Note: Figures with decimal commas should be read as having decimal points.

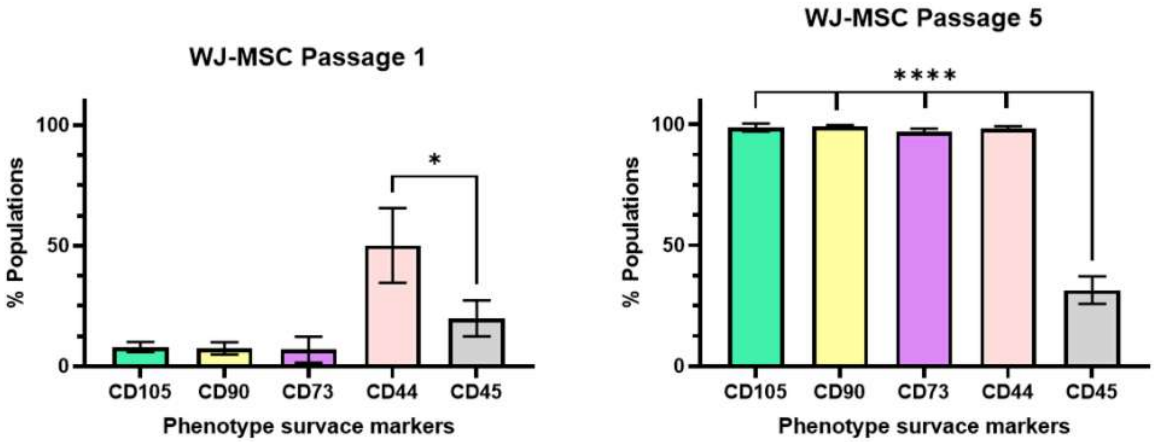

Figure 3. **Comparative cell surface markers of WJ-MSCs at passages 1 and 5**
Compared to the negative phenotype (CD45), WJ-MSCs demonstrate a significant increase in surface markers at passage 1 ($p=0.010$). Additionally, a dramatic increase was observed in CD105, CD90, CD73, and CD44 surface markers at passage 5 ($p < 0.000$), indicating successful MSC proliferation and phenotype stability across passages. Statistical analysis was conducted using one-way ANOVA with post-hoc tests to determine significance between groups. p-values were calculated using GraphPad Prism, with significance levels denoted as follows: $p<0.05$ (*), $p<0.010$ (**), $p<0.001$ (***), and $p<0.000$ (****)

Comparative analysis of surface markers at different passages

Comparisons of WJ-MSC surface markers between P1 and P5 revealed significant differences in marker expression (Figure 3). On passage 1, the MSCs demonstrated a statistically significant increase in pluripotency surface markers compared to the CD45-negative phenotype ($p = 0.010$). By P5, a dramatic increase in CD105, CD90, CD73, and CD44 expression was observed, with statistical significance ($p < 0.001$) (Figure 3). These findings indicate robust MSC proliferation and stable expression of essential stem cell markers across passages, supporting the successful expansion and maintenance of MSC characteristics through successive passages.

DISCUSSION

This study successfully demonstrates the rapid isolation and characterization of WJ-MSCs from human umbilical cords using freshly prepared human AB-serum. These cells exhibited typical MSC morphology and a stable phenotype through early passages, supporting their potential for future research and clinical applications.[17] However, despite overall success, the detection of low levels of CD45 expression indicates potential hematopoietic contamination.[16] This may be attributed to residual blood cells within the Wharton's Jelly, as complete removal of all blood vessels can be challenging in clinical settings.[18] Future refinements could include additional washes or enzymatic treatments to further minimize CD45-positive cells, improving the MSC purity and strengthening the clinical potential of these cells.[19]

The MSCs present in Wharton's jelly tend to have similar characteristics, irrespective of umbilical cord regions (maternal, fetal, or central attachment).[20] Interestingly, a recent study has highlighted the potential of MSCs derived specifically from the cord-placental junction, which may offer advantages over WJ-MSCs in terms of cellular yield, differentiation potential, and immunomodulatory properties. These cord-placental junction MSCs have shown enhanced proliferation rates and greater multilineage differentiation capabilities compared to WJ-MSCs, likely due to their proximity to the placenta and the presence of rich microenvironmental cues.[13]

Our analysis of MSC surface marker expression showed unique patterns across different passages, specifically with P1 exhibiting only high CD44 expression (approximately 60%) while other markers, including CD105, CD73, and CD90, were undetectable. This selective expression at P1 could reflect an early stage in MSC culture, where cells prioritize adhesion and migration functions, as CD44 is a receptor for hyaluronic acid involved in cell adhesion and motility.[13,21] Figure 3 demonstrates a successive passage, notably by P5, when all MSC markers (CD105, CD73, CD90, and CD44) reached high expression levels. This finding suggests that these cells may require additional time or specific culture conditions to fully upregulate markers associated with mesenchymal identity and functionality. CD45, as expected, remained low across passage 5, although its continued detectability indicates potential residual hematopoietic contamination, which aligns with reports from another study.[16] Interestingly, this marker pattern differs from MSCs derived from the cord-placental junction (CPJ-MSCs), which tend to express high levels of all MSC markers, including CD105, CD73, and CD90, and low CD45 levels. These differences may arise from microenvironmental factors unique to CPJ-MSCs that show marker expression and MSC commitment earlier in culture, highlighting the potential value of CPJ as an alternative MSC source for applications requiring rapid cell expansion and stable marker profiles.[14]

Another area for improvement in our protocol is the lengthy outgrowth period, as cell attachment and expansion required 21 days before reaching significant confluence. Long outgrowth times can limit scalability and delay potential downstream applications.[22] Optimizing the initial explant culture conditions could reduce this outgrowth period, thereby increasing efficiency. This might include using higher serum concentrations, pre-coating culture surfaces with adhesion-promoting molecules such as fibronectin, or adjusting oxygen levels to more closely mimic the native environment of umbilical cord MSCs.[23,24] Reducing the outgrowth time will not only streamline the MSC isolation process but also decrease the potential for cell senescence and culture-induced variations, ultimately improving

the consistency and reliability of the cells obtained for research and therapeutic use.

The limitations of this study include, first, the contamination with CD45, which is still detected in antibody expressions. This may result from incomplete removal of blood vessels during the isolation process, potentially affecting the purity of WJ-MSCs and necessitating further refinement of the isolation protocol. Second, the prolonged outgrowth period limits the scalability and efficiency of the protocol for large-scale applications.

Despite these limitations, this study successfully isolated and characterized WJ-MSCs, providing a foundation for future clinical applications in regenerative medicine, including tissue repair, immunomodulation, and cell-based therapies. The use of human AB serum instead of fetal bovine serum (FBS) reduces the risk of immune reactions, making the protocol more suitable for clinical use.

Looking ahead, several future directions could be built on this research. First, optimizing the isolation process by carefully extracting arteries and veins could minimize CD45 contamination and improve MSC purity. Second, reducing the outgrowth period by optimizing culture conditions—such as using higher serum concentrations, adhesion-promoting molecules like fibronectin, or adjusting oxygen levels to better mimic the native environment of umbilical cord MSCs—could enhance efficiency and applicability.

CONCLUSION

This study successfully demonstrates the isolation and characterization of Wharton's jelly derived MSCs (WJ-MSC) using a culture medium supplemented with 10% human AB serum and high glucose, combined with a 2% gelatine-coated flask. This protocol achieved high cell viability and proliferation, with WJ-MSCs exhibiting fibroblast-like morphology and stable expression of MSC key markers (CD105, CD44, CD90 and CD73) by passage 5, as well as low levels of CD45 expression indicating minimal hematopoietic contamination and confirming the purity of the isolated cells.

The results highlight the feasibility of this protocol for obtaining viable WJ-MSCs, providing a path for future research and clinical applications in regenerative medicine. However, the 21-day outgrowth period and the presence of residual CD45 positivity suggest further optimization, such as refining the isolation techniques, reducing culture times, and exploring alternative MSC sources.

Authors Contribution

SD, YT, and BDN designed the experiments, carried out the experiments, and prepared the manuscript. BDP, TW, LH, HW, HHJ, FM, and SK carried out the experiments and analyzed the data. YT, SD and BDP revised the manuscript. All authors have read and approved the final manuscript.

Conflict of Interest

All authors declare no conflict of interest.

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Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article.

Declaration the Use of AI in Scientific Writing

During the preparation of this work, the authors used ChatGPT (OpenAI) to assist in language refinement, grammar correction, and improving technical clarity. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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