Main features and isolation technique mesenchymal stem cells from wharton's jelly

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Abstract

Background: Wharton's jelly-derived mesenchymal stem cells are highly homogeneous cells population, easily expanded, especially they can bring potential applications. However, their yield depends on the method of cells extraction conducted. Our research includes two purposes: 1/Procedure for isolating and differentiating mesenchymal stem cells from Wharton's jelly, 2/Determining the main features of mesenchymal stem cells isolated from Wharton's jelly. **Materials and methods:** Umbilical cords (UCs) were harvested from the department of Pediatrics at Sassari Hospital, Italy, from healthy full-term pregnant women who did not undergo cesarean section. After isolating Wharton's jelly from UCs, the samples were cultured in a medium containing DMEM 78%, Serum 18%, L-glutamine 2%, Pen/strept 2%. The fibroblast-like cells obtaining after isolation were identified by markers on the surface which are special markers of mesenchymal stem cells. Besides, they were cultured to differentiate into osteocytes, chondrocytes and adipocytes. After differentiation, we used special chemicals to recognize each of line. **Results:** After 2 weeks, fibroblast-like cells started to attach to bottom of the flasks. They can differenciate into osteocytes, chondrocytes and adipocytes. The majority of isolated Wharton's jelly-mesenchymal stem cells (WJ-MSCs) population showed positivity for CD34 and CD117 surface markers, while a little percentage of cells showed positivity for CD45 marker. The 95.8% of all stem cells analyzed (CD34+/CD117+) were alive. **Conclusions**: Fibroblast-like cells isolating from Wharton's jelly which can differenciate into adipocytes, osteocytes, chondrocytes and show positivity for CD34, CD117 are mesenchymal stem cells.

Key words: *Mesenchymal stem cells (MSCs), umbilical's cord, Wharton's jelly, CD34, CD45, CD117.*

1. BACKGROUND

Stem cells derived from umbilical cord have significant potential applications in regenerative medicine and disease treatment. They includes three main types: (1) Mesenchymal stem cells (MSCs): found in Wharton's jelly, these cells can differentiate into bone, cartilage, fat, and other tissues. Therefore, they can applied for repairing damaged tissues and treating immune disorders. (2) Hematopoietic stem cells (HSCs): found in cord blood, they can generate blood cells such as red blood cells, white blood cells, and platelets, commonly used in treating blood-related diseases like leukemia and anemia; (3) Epithelial stem cells: located in the umbilical cord lining, these cells are promising for regenerating skin, cornea, and connective tissues. WJ-MSCs are highly homogeneous population of cells, a unique feature of somatic stem cell populations [1].

WJ-MSCs differ from other umbilical cord-derived stem cells in several key aspects. Firstly, related to origin: They are derived from the connective tissue in Wharton's jelly, while HSCs come from cord blood, and epithelial stem cells are sourced from the umbilical cord lining. Secondly, about differentiation potential: WJ-MSCs can differentiate into a wide range of cells, including bone, cartilage, fat, muscle, and nerve cells, whereas HSCs primarily form blood cells, and epithelial stem cells focus on regenerating skin and connective tissues. Thirdly, related to immunological properties: WJ-MSCs have superior immunomodulatory properties, making them less likely to trigger immune rejection [2], unlike HSCs, which require HLA matching for transplantation. Especially, about applications: WJ-MSCs are widely used in regenerative medicine to treat neurological, cardiovascular, and musculoskeletal diseases, while

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DOI: 10.34071/jmp.2024.6.24
Received: 10/5/2024; Accepted: 24/11/2024; Published: 25/12/2024

HSCs are primarily used for blood-related conditions, and epithelial stem cells are applied in skin and corneal regeneration. In summary, WJ-MSCs stand out for their versatility, low immunogenicity, and broader clinical applications.

WJ-MSCs have shown promising applications in both animal models and clinical trials due to their regenerative capabilities and immunomodulatory properties. Neurological diseases: Studies on animal models with Parkinson's and Alzheimer's diseases demonstrated reduced inflammation and enhanced neural regeneration [3]; Liver diseases: acute liver injury [4]; Autoimmune diseases: WJ-MSCs improved symptoms in patients with lupus and rheumatoid arthritis [5]; Covid-19: Trials revealed reduced lung inflammation and mortality in severe COVID-19 patients [6]; Skin injuries: WJ-MSCs enhanced recovery in burn patients by promoting tissue regeneration [7]; These studies highlight WJ-MSCs' potential applications, including organ transplantation, cancer therapy, and gene editing, paving the way for next-generation cell-based therapies.

Recent research has explored various methods to isolate WJ-MSCs, focusing on optimizing yield and maintaining cell quality. The two primary techniques are enzymatic digestion and explant culture, each with unique advantages and limitations. Enzymatic digestion uses enzymes like collagenase to dissociate the tissue rapidly, enabling faster cell proliferation. It provides a shorter culture duration and ensures uniform cell growth. However, it can result in slightly lower cell yield compared to explant methods and may risk enzyme-related damage to cells; While, explant culture involves placing tissue fragments in culture media, allowing cells to migrate out over time. It generally yields a slightly higher number of cells and avoids exposure to enzymes, preserving cell integrity. Additionally, isolating only Wharton's jelly can gain more homogeneous cell populations than isolating not removal vessel, amniotic membrane [8].

WJ-MSCs are characterized by the expression of specific molecular markers. They are positive for surface markers such as CD29, CD44, CD73, CD90, and CD105, which are standard for mesenchymal stem cells. They also exhibit some pluripotency-associated markers like NANOG, OCT-4, and SOX2, although at lower levels compared to embryonic stem cells. These markers indicate their self-renewal capacity and potential for multi-lineage differentiation while lacking hematopoietic markers like CD34 and CD45, confirming their non-hematopoietic nature [9].

In conclusion, Wharton's jelly is the simplest human connective tissue which provides an more homogeneous harvested cell population than other sources. Their potential applications make scientists and clinicians interested in there cells. Thus, there is an urgent need to understand more fully about the derivation, functional phenotypes, characteristics and especially develop strategies to implement isolation of these cells with higher cell yield. Our research includes two purposes: 1/Procedure for isolating and differentiating mesenchymal stem cells from Wharton's jelly, 2/Determining the main features of mesenchymal stem cells isolated from Wharton's jelly.

2. MATERIALS AND METHODOLOGY

2.1. Materials

After Ethical Committee approval, the umbilical cords were obtained from healthy full-term women who will not undergo an elective cesarean section at Pediatric department-Sassari hospital-Italy.

Mediums using in my research are as follows:

- Collecting umbilical cord medium: PBS, Pen/strept: 1%.

- Culturing stem cells medium: DMEM 78%, Serum 18%, L-glutamin 2%, Pen/strept 2%.

2.2. Methodology

2.2.1. Isolation and culture

The umbilical cords were collected in sterile bottles containing PBS supplemented penicillin/streptomycin 1% and kept in cool refrigerator compartment (4°C). The sample was rinsed repeatedly with 20ml of collecting medium in order to remove the blood cells. Then, it was cut into shorter pieces about 5-6 cm and put into 10 cm petri dish for handle. Firstly, three vessels (two arteries and one vein) must be cleanly removed [10].

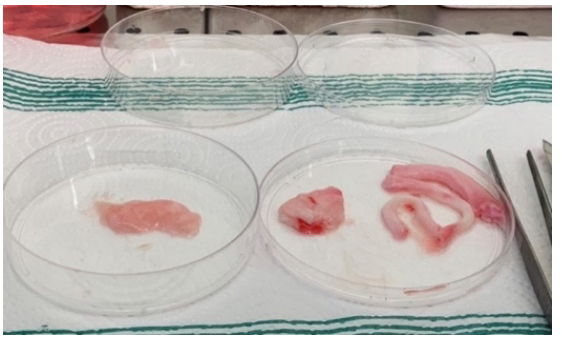

Fig 1. Vessels were cleanly removed.

The Wharton's jelly was minced into 0.2 mm pieces by forceps and scalpels. All of them were put and extended on the surface of dry culture bottle, 25 cm^2. Then 5ml of medium for culturing was added slowly to avoid detaching the pieces of Wharton's jelly from the surface of the bottle and forming bubbles. The cell culture bottles were placed into CO_2 incubator (37°C, CO_2 5%) within 10-15 days until observe the fibroblast-like cells attach on the surface of culture bottles. Then, the Wharton's jelly pieces were removed gently. The medium was replaced every 2-3 days until the surface of culture bottles become confluent cells, they were detached with prewarmed trypsin (Gibco) and split to other flasks. The reaching confluences were monitored by microscopes.

2.2.2. Identification of Wharton's jelly-derived mesenchymal stem cells

The fibroblast-like cells after isolating from Wharton's jelly were cultured to differentiate into osteogenic, chondrogenic and adipogenic phenotypes in tissue culture 6-wells plate (BD-falcon) with specific osteogenic, chondrogenic, adipogenic differentiation medium or only basic growing medium. They are osteogenesis differentiation kit, chondrogenesis differentiation kit and adipogenic differentiation kit which provide standardized culture workflow solution for MSCs isolation, expansion, and differentiation into mineralized matrix-producing osteocytes, collagen matrix-producing chondrocytes and adipogenic.

The medium was changed every three days. 21 days later, all the wells of plate were stained to recognize osteogenic, chondrogenic, adipogenic phenotypes by Alizarin red, Toluidine blue and Oil red [11].

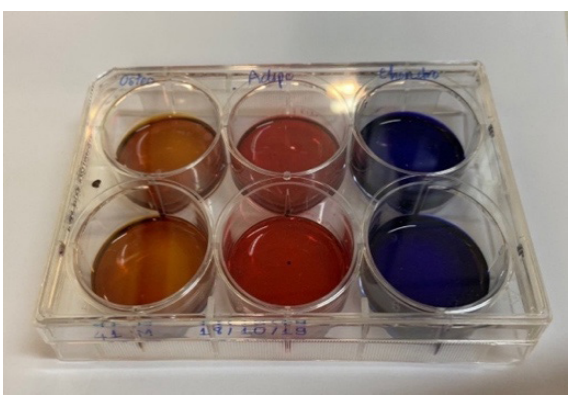

Fig 2. Osteogenic, chondrogenic, adipogenic phenotypes after 21 days cultured with specific differentiation mediums were stained with Alizarin red, Toluidine blue and Oil red

2.2.3. Cell characterization by flow-cytometry

Flow cytometry analysis was used to evaluate the percentage of mesenchymal stem cells markers of the isolated population. WJ-MSCs were detached from flasks' surface by using 0.25% Trypsip-EDTA for 5 minutes and centrifuged for 5 minutes. Collected cells were washed by PBS. Then, about $5x10^5$ cells were incubated with primary conjugated antibodies CD34-APC, CD45-FITC, CD117-PE and 7AAD-PerCP for 30 min at 4°C in the dark to evaluate cell viability [12]. Operating principle of flow cytometer (FACS, BD Bioscience) was to collect 10 000 events and FACSDiva Software was used to analyze gained datas.

3. RESULTS

3.1. Isolation and differentiation

3.1.1. Isolation and culture

It was observed that the spindle-shaped and fibroblast-like cells adhered on the surface of plastic culture flasks, they are homogeneous cell population.

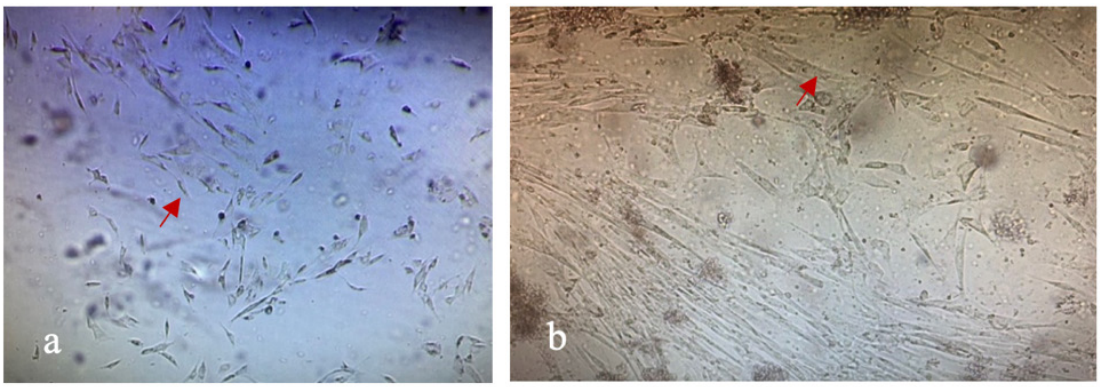

Fig 3. Fibroblast-like cells began to attach on the surface of flasks after 19 days (a: X20; b: X40)

3.1.2. Cell differentiation

The differentiation into osteogenic, chondrogenic and adipogenic phenotypes were performed during 14 days with special medium for each of phenotype.

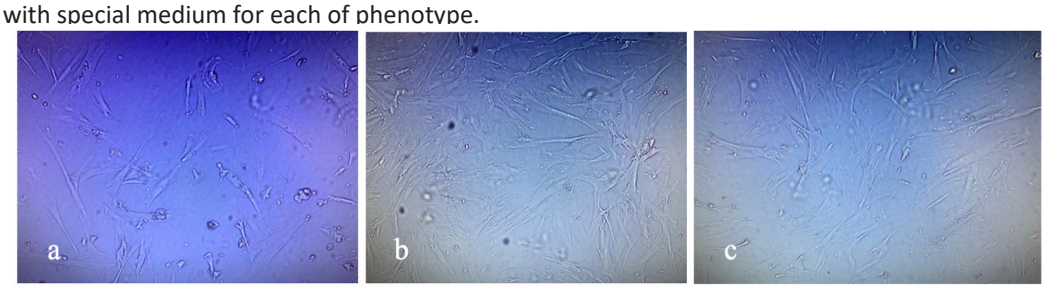

Fig 4. Osteogenic (a), chondrogenic (b), adipogenic (c) differentiations after 7 days. X40

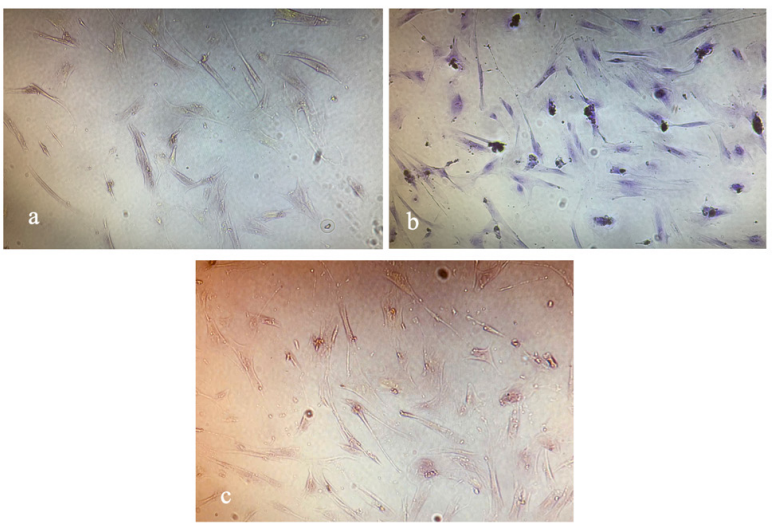

Fig 5. WJ- MSCs differentiation after 21 days. Osteogenic (a), chondrogenic (b), adipogenic (c) phenotypes were evident in treated cells. X20

3.3. Cell characterization

The majority of isolated WJ-MSCs population showed positivity for CD34 and CD117 surface markers, while a little percentage of cells was positivity for CD45 marker. The 95.8% of all analyzed (CD34+/CD117+) stem cells were alive.

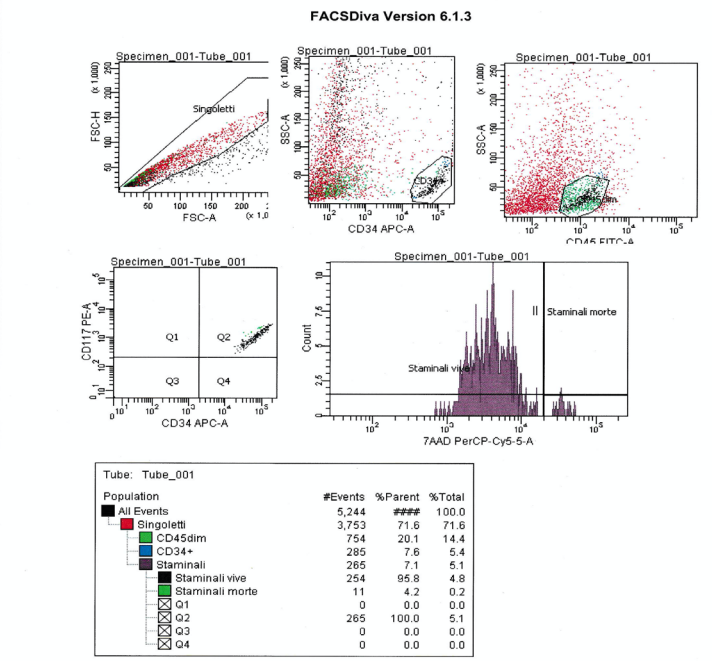

Fig 6. Characterization of WJ-MSCs population by flow cytometry.
The sample has 71.6% singlets, 20.1% cells with CD45+ and 7.6% cells with CD34+. Stem cells account for 7.1% of the population, with a high viability rate (95.8%).

4. DISCUSSION

4.1. Isolation and differentiation

4.1.1. Isolation and culture of stem cells from Wharton's jelly

Umbilical cords were collected from healthy full-term women who did not undergo an elective cesarean section. After handling these samples by many steps following to protocol, the Wharton's jelly was minced and put gently into plastic culture flasks. After two to four weeks, the spindle-shaped and fibroblast-like cells adhered on the surface of plastic culture flasks when observing by inverted phase microscopes. Indeed, adherence to the plastic flask bottom is the main feature of MSCs.

Related to the protocols used to isolate WJ-MSCs, some authors remove umbilical cord vessels, scrape off WJ with a scalpel and then treat the tissue enzymatically. Others cut UC into smaller segments, strip the vessels and then directly immerse the remaining UC to an enzymatic solution [13]. The enzymes used for incubation period haven't been standardized. Besides, instead of enzymatic digestion, several groups dissect the cord segment into very small pieces, with or without discarding the cord vessels and the segments are used as explants from which cultures are subsequently established [13]. In our research, three vessels (two arteries and one vein) were be cleanly removed and we didn't use enzyme to avoids exposure to enzymes.

For the growth of WJ-MSCs, Fatemeh Ahangari et al., (2023) used medium enriched with 15-20% FBS [14]; Nekoei et al., (2015) applied 10% FBS and high glucose-DMEM [15]; Fong et al., (2007) used high glucose-DMEM medium supplemented 15% FBS [16]. Our research used the concentration of FBS 18% and high glucose-DMEM, the result was that WJ-MSCs could grow. Therefore, we can conclude that the 18% FBS concentration and high glucose-DMEM are suitable for culture WJ-MSCs.

4.1.2. Cell differentiation

Stem cells committee in 2006 proposed three minimum criteria for human-derived MSCs experiments: (1) Under standard culture conditions, MSCs must have attachment properties to plastic

substrates; (2) The positive rate of CD105, CD73 and CD90 expression in MSCs population was greater than or equal to 95% by flow cytometry, and the negative expression rate of CD45, CD34, CD14 or CD11b, CD79a or CD19, HLA - DR greater than 98%; (3) In vitro induction by standard methods, MSCs must be able to differentiate into osteoblasts, chondrocytes and adipocytes [17].

In the differentiation into phenotypes, we used osteocyte/chondrocyte differentiation basal medium (StemPro® osteogenesis differentiation kit, Gibco, USA), chondrogenesis supplement (StemPro™ chondrogenesis differentiation kit, COD A1007101, Gibco, Invitrogen, USA), adipogenic differentiation medium (Adipogenic differentiation medium bulletkit, COD PT-3004, Lonza, USA) to differentiate into osteogenic, chondrogenic, adipogenic phenotypes. Then, we used 10% Alizarin red solution, Toluidine blue, Oil red to stain each of line. Alizarin red solution is a dye that binds to calcium salts. Toluidine blue is a basic thiazine metachromatic dye with high affinity for acidic tissue components. Using toluidine blue in tissue is to highlight components, such as mast cells granules, mucins, and cartilage. Oil red can used to stain the adipocytes. In the Fig 3.3, adipocytes are bigger than osteocytes and lipid droplets can be observed beginning to form in the cytoplasm of the cell. Consequently, fibroblast-like cells can differentiate into osteogenic, chondrogenic and adipogenic phenotype. Therefore, we can conclude that they are mesenchymal stem cells.

4.2. Cell characterization

Related to CD34, MSCs from adipose tissue or umbilical cord could express CD34 together with other markers. A portion of tested population in our research was positive to this marker. In 1991, Simmon and Torok-Storb published a paper titled "CD34 expression by stromal precursors in normal human adult bone marrow" [12]. Moreover, MSCs were confirmed by differentiation into phenotypes: osteoblasts, chondrocytes and adipocytes. Importantly, after in vitro expansion, all of the cells that originated from the CD34+ fraction became CD34-, so they became CD34- in cell culture [18]. Besides, CD34 is one of the most frequently used markers for the recognition of MSCs associated with the vasculature [19]. In our study, the majority of isolated cell population showed positivity for CD34. Additionally, they are always mesenchymal stem cells because they can differentiate into ostegenic, chondrogenic and adipogenic phenotypes.

CD45 is present on the surface of all hematopoietic stem cells. In our end result, 20,1% cells showed positivity for CD45. This is explained that during the process isolating WJ-MSCs, a small number of hematopoietic stem cells were able to be remained and adhere on the surface of the flasks.

CD117 is a cytokine receptor expressed on the surface of hematopoietic stem cells as well as other cell types [20]. CD117 is a receptor tyrosine kinase type III, which binds to stem cell factor (a substance that causes certain types of cells to grow) and it plays a role in cell survival, proliferation, and differentiation. This marker was first described by the German biochemist Axel Ullrich in 1987. It is typically expressed in MSCs. Cytometry results showed that MSCs were positive for both CD34 and CD117. Therefore, the fibroblast-like cells derived from WJ were mesenchymal stem cells.

7-AAD-PerCP, the viability dye, is used for live/dead cell discrimination during flow cytometric analysis. In our research, 95.8% of stem cells analyzed (CD34+/CD117+) were alive.

Therefore, growth conditions of analyzed stem cells populations was optimized.

5. CONCLUSIONS

Stem cells is one of the most fascinating field of researchers nowadays, especially stem cells from the umbilical cords because they are young, potent and viable. There are various health professionals and scientists believe in the potential of stem cells from umbilical cords' blood and tissue will be important therapeutic options in future medicine. Wharton's jelly derived stem cells are easy to obtain with a high yield and high propagation potential. Moreover, collecting stem cells from the wharton's jelly is less invasive than doing from the bone marrow or other sources.

Our research showed that mesenchymal stem cells isolated from Wharton's jelly are fibroblast-like cells, positive for CD34 and CD117 surface markers, especially they can differentiate into osteogenic, chondrogenic and adipogenic phenotypes. To get this outcome, Wharton's jelly were minced after removing cleanly three vessles of umbilical cords and put into plastic flasks with DMEM high-glucose medium implemented FBS 18% in CO_2 incubators. With this result, we hope that WJ-MSCs will contribute to various applications which can tackle many health issues in the future.

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