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Original paper

Investigation of Biological Properties of Mesenchymal Stem Cells Derived from Placenta Tissue

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Abstract

Background and Aim: Much information has been provided on the biological properties of MSCs. Investigation of biological properties and biological properties of placenta-derived mesenchymal cells is very limited, especially at the molecular level. Accordingly, the present study deals with the properties of placenta-derived mesenchymal stem cells at the cellular and molecular levels.

Method: In this experimental-in-vitro study placental tissue was obtained from 30 women (mean age 20-40) and maintained in standard conditions. Cells were cultured after explantation using explant method. Biological characteristics including surface markers, the ROS system, and apoptosis were determined to investigate the properties of placenta-derived mesenchymal stem cells (MSCs) at the cellular and molecular levels. Surface markers were measured using flow cytometry. The expression of specific markers indicative of mesenchymal stem cells, such as CD73, CD90, and CD105, was assessed. To evaluate the ROS system, the levels of reactive oxygen species in the placenta-derived MSCs were measured using fluorometric assays. Apoptosis was assessed by flow cytometry. The obtained data were subjected to statistical analysis using t-test or analysis of variance (ANOVA).

Results: The analysis of surface markers confirmed the mesenchymal nature of the cells and their ability for self-renewal and differentiation. The well-regulated ROS system observed in these cells suggests their potential for various cellular processes. Additionally, the low levels of apoptosis indicate the robustness and viability of placenta-derived MSCs for potential therapeutic applications.

Conclusion: Overall, the findings of this study contribute to the understanding of placenta-derived MSCs and their potential utility in regenerative medicine and cell-based therapies. Further investigations are warranted to explore their specific differentiation capabilities, their therapeutic efficacy in various disease models, and their potential for immunomodulatory effects.

Keywords: *Mesenchymal stem cells, Placenta tissue, Cell surface markers, ROS, Apoptosis*

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Introduction

Mesenchymal stem cells (MSCs) are a type of multipotent stromal cells that possess the ability to differentiate into various cell lineages, including bone cells, cartilage cells, muscle cells, and fat cells. These cells have small cell bodies and thin, long appendages and are derived from mesoderm tissue. They can be found in various sources such as bone marrow, umbilical cord, amniotic fluid, and fat tissue. MSCs have differentiation capacity and antimicrobial properties, and they also play a crucial role in regulating the immune system [1].

In addition to their differentiation capacity and antimicrobial properties, MSCs possess specific biological characteristics such as CD surface markers, ROS, and apoptotic properties. CD markers, which are used to recognize and differentiate cell surface molecules, play a crucial role in identifying MSCs. These markers are responsible for the receptor and ligand on the cell surface [2].

Reactive oxygen species (ROS) are formed as a byproduct of oxidative phosphorylation and can oxidize proteins, lipids, and DNA. The ROS system takes place inside organelles such as mitochondria, peroxisomes, and endoplasmic reticulum, and it plays a vital role in cell signaling and homeostasis [3].

Apoptosis is a physiological and biological process of active and normal growth that occurs in multicellular organisms. It helps maintain homeostasis by triggering different pathways, some of which are activated by ligands binding to cell surface receptors while others are triggered by the lack of certain growth factors. Severe damage to the genetic material of the cell can also activate various pathways that lead to apoptosis, eventually resulting in cell death [4].

Research shows that stem cells possess different biomarkers such as CD159, CD9, and Thy-1 [5], as well as surface markers including CD29, CD24, and CD59 [6]. Studies have also shown that mesenchymal stem cells have various biomarkers such as CD73, CD105, Class-I HLA, and c-kit [7], [8], as well as surface markers such as CD146 and CD271 [9], [10]. Furthermore, stem cells and mesenchymal stem cells have been found to possess the ROS system [11].

However, some studies have suggested that ROS have a dual role. Although they can create oxidative stress and be destructive, they can also act as messenger molecules and stimulate several physiological pathways. ROS are also involved in the aging process of mesenchymal stem cells [12].

Research indicates that stem cells undergo cell death [13] and apoptosis [14], [15], and similarly, mesenchymal stem cells also undergo cell death [16] and apoptosis [17].

Mesenchymal stem cells (MSCs) are known for their undifferentiated and versatile nature. They have been found to have potential in eliminating tumors by inhibiting the proliferation of tumor cells and inducing apoptosis, as well as preventing tumorigenesis, angiogenesis, and metastasis. However, some studies have shown that MSCs may also promote tumor growth and inhibit apoptosis through signaling pathways, making them a double-edged sword in terms of their potential therapeutic applications [18].

With the increasing use of MSCs in both research and clinical fields, it is important to fully understand their characteristics and potential risks associated with their use. Further research is needed to elucidate the exact mechanisms of action of MSCs in tumor growth and to optimize their therapeutic potential. Nonetheless, the versatility and regenerative potential of MSCs make them a promising area of research for future therapeutic applications [19].

It is important to examine their biological characteristics such as surface markers (CD), specific oxidation reaction (ROS), and programmed cell death.

Despite their potential, there are limited studies on the biological characteristics of mesenchymal

stem cells. Therefore, the present research aims to examine these characteristics by studying mesenchymal stem cells derived from placental tissue.

Material and Methods

Ethical Approval and Consent

This project obtained ethics committee approval for biomedical research from the Research and Technology Deputy Organization of Tehran University of Medical Sciences with ID IR.TUMS.VCR.REC.1397.506 on 7/26/1397, as well as from the country's Forensic Medicine Organization with ID IR.LMO.REC.1397.021 on 12/8/2017. The research team corresponded with hospitals and clinics to obtain umbilical cord tissue samples, and written consent was obtained from the tissue donor prior to sampling placenta tissue.

Collection of Placental Tissue Samples

This experimental-laboratory research utilized mesenchymal cells derived from placenta tissue. Placental tissue samples were collected from 30 women aged between 20 and 40, with an average gestational age of 39 weeks (ranging from 41 to 47 weeks). The samples were collected randomly after cesarean section and placed in sterile containers covered with sterile cloth. The containers were then transferred to the laboratory.

In the laboratory, the tissue was placed on a sterile tray, and its various components were separated to culture the cells from those areas. The cells were placed in a falcon tube containing 30 cc of PBS with antibiotics (2X or 3X) (100mg/ml). The placental tissue samples were obtained from Akbarabadi and Mahdieh hospitals in Tehran and were transported to the laboratory within 6 hours of collection. All isolation and culture steps were performed under sterile conditions in a culture room with an air flow hood, according to GCP instructions.

Isolation of Placental Mesenchymal Stem Cells

The placental tissue was cut into small pieces and placed in a Falcon 50 tube. To prevent tissue aggregation, some PBS/EDTA was added, and the tube was centrifuged at 1800 RPM for 5 minutes. The supernatant was discarded, and some collagenase (1 mg/mL) was added to the tissue. The sample was then pipetted with a G17 or G16 needle and placed in a 37-degree incubator for 2 hours (or overnight). The sample was vortexed every 15 minutes during the incubation period.

After the incubation period, the sample was pipetted again with a needle tip, and the enzyme activity was neutralized by adding an equal volume of 10% culture medium. The contents of the Falcon tube were then passed through a gas sterilized strainer, and some PBS was added to the strainer to collect all the cell contents. The tube was centrifuged at 1800 RPM for 5 minutes, and some PBS was added to the cell pellet. The sample was then pipetted and centrifuged again.

The resulting cell pellet was cultured in DMEM low glucose containing 20% FBS serum.

Cell Culture Maintenance

The first change of the culture medium was performed 48 hours after the initial culture, followed by changes every 3-4 days. The culture environment was gradually decreased to 20% during the first few days, and the amount of serum was reduced from 20% to 15% and then to 10% in the following days. Red blood cells were isolated on the day of the first medium change by tilting the flask slightly, and monocytes survived for only 7 days in culture before dying.

Isolation and Cultivation of Cells by Tissue Explant Method

In the tissue explant method, the desired tissue was washed and divided into small pieces measuring 0.1 to 0.2 mm. 3-4 pieces of tissue were then placed in a 6-well plate and incubated for 15 minutes. Next, a drop of serum was added to each piece, and the plate was incubated overnight. The following day, a drop of culture medium was added to each piece of tissue, and on the second

day, 500 microliters of culture medium were added to each well.

Cell Counting and Determination of Living Cell Percentage

Cell counting was necessary for passage, freezing, transplantation, and culture on hydrogel. We used a slide hemocytometer (Neobar) and trypan blue (%) solution to count cells and determine the percentage of living cells. Trypan blue is a vital dye used to measure the percentage of living cells. To prepare the trypan blue solution, 0.4 grams of trypan blue dye was dissolved in 100 ml of physiological serum.

For counting, 50 microliters of the cell suspension that was well dispersed and spread with the sampler was mixed with the same amount of trypan blue dye and placed on the hemocytometer. The hemocytometer was then placed between coverslips and counted under a microscope with 10x magnification. Dead cells lost their selective permeability due to cell death, which allowed the trypan blue dye to penetrate into them. These cells were observed in blue color and could be counted. Living cells, on the other hand, did not receive the dye and appeared transparent under the microscope.

Regarding the cells that were placed on the border lines of the houses, conventionally only cells on the lower and right sides were counted. The number of cells was counted in 4 houses of 16 cells (for white blood cells), and the total was divided by 4. Then, the total number of cells was calculated based on the following formula: Volume of suspension $\times 10^4 \times 2$ (dilution factor) $\times$ number of cells in a square of 16.

The percentage of living cells was calculated based on the following formula:

Viability percentage = (number of live cells + number of dead cells / numbers of live cells) x 100

Cell Passage, Freezing, and Thawing

The passage process involved pouring culture medium and some PBS onto the cells in the flask or drain plate. About 1-2 cc of 0.25% trypsin enzyme was added, and the mixture was incubated for 5 minutes. Trypsin enzyme activity was neutralized by adding a few cc of culture medium containing 10% serum. After pipetting, the contents of the flask were transferred into a Falcon 15 tube and centrifuged at 1200RPM for 5 minutes. The top culture medium was removed, and some culture medium was added to the bottom of the cell pellet, which was then transferred to a flask 25. In subsequent passages, the cells were transferred to a flask 75. The cells were passaged three times and frozen at passage 3 before passage 4.

The freezing process involved quickly transferring the cell pellet to freezing medium (90% serum and 10% DMSO) and transferring it to a 1.5cc cryovial. The vial was labeled and coded and transferred to a cryobox (containing propanol alcohol) and placed in a -70-degree freezer for 1 day. The cryovial was then transferred to a -196-degree nitrogen tank for long-term storage.

Thawing of frozen cells was performed by quickly transferring the cryovial to a 37-degree water bath and swirling it until the contents thawed. The contents were then transferred to a Falcon tube containing warm culture medium and centrifuged at 1200RPM for 5 minutes. The top culture medium was removed, and some culture medium was added to the bottom of the cell pellet, which was then transferred to a flask for further culture.

The freezing process involved quickly transferring the vial to a 37-degree water bath to melt its contents. The contents were then transferred to a Falcon tube containing some culture medium and centrifuged at 800 RPM for 3 minutes. After pipetting only 2-3 times, the cell pellet was transferred to a flask containing culture medium and placed in the incubator. The following day, the culture medium was changed.

Examination of Cells

Since the isolated cells were used in transplantation, their health was evaluated in terms of cell

characteristics, cell identity, physical characteristics of cells, and microbial contamination. By examining the cells, it is possible to ensure that they are healthy, free from contamination, and suitable for transplantation. Factors that are evaluated include cell characteristics, cell identity, physical characteristics of cells, and microbial contamination. This thorough examination is essential to ensure the safety and efficacy of cell transplantation.

Examining Cell Characteristics: Apoptosis and Necrosis

To analyze cell characteristics, the Annexin V kit was used in this study. Cells from flask 25 (passage 3, density 80%, and 10^6 cells) were sent to the flow cytometry laboratory to check the amount of apoptosis and necrosis. The sample preparation process involved the following steps:

Preparation of Annexin binding buffer solution X1.

Separation of the cells from the flask, followed by washing once in PBS and centrifugation (5 minutes at 300 g).

Removal of the supernatant and addition of 100 microliters of Annexin binding buffer to the cells.

Addition of Annexin V and PI and incubation in the dark for 15 minutes at room temperature.

Addition of 400 microliters of binding buffer to the tubes and reading the results using the device.

This technique was performed on extraembryonic mesenchymal cells and fat cells to evaluate the amount of apoptosis and necrosis. Apoptosis is a programmed cell death that occurs as a part of normal physiological processes, whereas necrosis is the death of cells caused by external factors such as injury or infection. By analyzing the amount of apoptosis and necrosis in cells, it is possible to gain insights into their health and viability.

Checking the Identity of Cells

To ensure the exact identity of cells in passage 3, confirmatory evaluations were conducted. These evaluations included the examination of surface markers, differentiation potential, and reflectance spectroscopy of cells.

Investigation of Surface Markers

The cells from flask 25 (passage 3, density 80%, and 10^6 cells) were sent to the flow cytometry laboratory to investigate surface markers. In the laboratory, five glass tubes were prepared, and antibodies conjugated with fluorescent dyes were added to each tube according to the table below:

Table1. Flow Cytometry Analysis of Cell Surface Markers in Different Tube Conditions

Tube	Antibodies
1	CD34-PE (negative marker) and CD45-FITC (negative marker)
2	CD44-FITC (positive marker) and CD73-PE (positive marker)
3	CD29-PE (positive marker)
4	CD166-PE (positive marker)
5	No antibodies added

The cells were trypsinized and suspended in 1 cc of serum-free culture medium, and then transferred to a microtube. From the microtube, 70 microliters of the cell suspension were added to each of the five glass tubes. The tubes were mixed by votexing, and aluminum foil was wrapped around them. The tubes were then placed in the fridge door for 30 minutes before analysis.

The flow cytometry machine was used to analyze the cells in each tube, and the results were used to confirm the identity of the cells. By examining surface markers, it is possible to identify specific cell types and confirm their identity.

Checking the Identity of Cells

To ensure the exact identity of cells in passage 3, confirmatory evaluations were conducted. This

included the examination of surface markers, differentiation potential, and reflectance spectroscopy of cells.

Examination of Differentiation Potential - Differentiation into Fat Cells

To examine the differentiation potential of the cells into fat cells, they were cultured in two wells of a six-well plate in passage 3 (one control sample and one sample for differentiation). When the cells reached a density of 40-50%, they were cultured in differentiation medium for 15 days with the addition of adipose tissue. The differentiating culture medium was changed every two days.

On day 16, 1 cc of formalin was poured on the cells and left at room temperature for 1 hour. Then Oil-Red dye was added to the cells for 15 minutes and washed with PBS 2-3 times. The cells were evaluated and photographed using an inverted microscope.

By examining the differentiation potential of the cells, it is possible to confirm their identity as specific cell types. In this case, the cells were examined for their potential to differentiate into fat cells, which can provide important insights into their identity and function.

Checking the Identity of Cells

To ensure the exact identity of cells in passage 3, confirmatory evaluations were conducted. This included the examination of surface markers, differentiation potential, and reflectance spectroscopy of cells.

Examination of Differentiation Potential - Differentiation into Bone Cells

To examine the differentiation potential of the cells into bone cells, they were cultured in two wells of a six-well plate in passage 3 (one control sample and one sample for differentiation). When the cells reached a density of 40-50%, they were cultured in tissue differentiation medium for 15 days with the addition of bones. The differentiation culture medium was changed every two days.

On day 16, 1% methanol was poured on the cells and left at room temperature for 10 minutes. Alizarin-Red dye was then added to the cells for 2-5 minutes and washed with PBS 2-3 times. The cells were evaluated and photographed using an inverted microscope.

By examining the differentiation potential of the cells, it is possible to confirm their identity as specific cell types. In this case, the cells were examined for their potential to differentiate into bone cells, which can provide important insights into their identity and function.

Flow Cytometry Analysis

Flow cytometry is a powerful and fast method used to identify particles, such as cells, and evaluate their characteristics. In this research, the cells in flask 25 (passage 3, density 80% and 10^6 cells) were sent to the flow cytometry laboratory.

In the flow cytometry laboratory, five glass tubes were prepared, and antibodies conjugated with fluorescent dyes were added to each tube according to the following table:

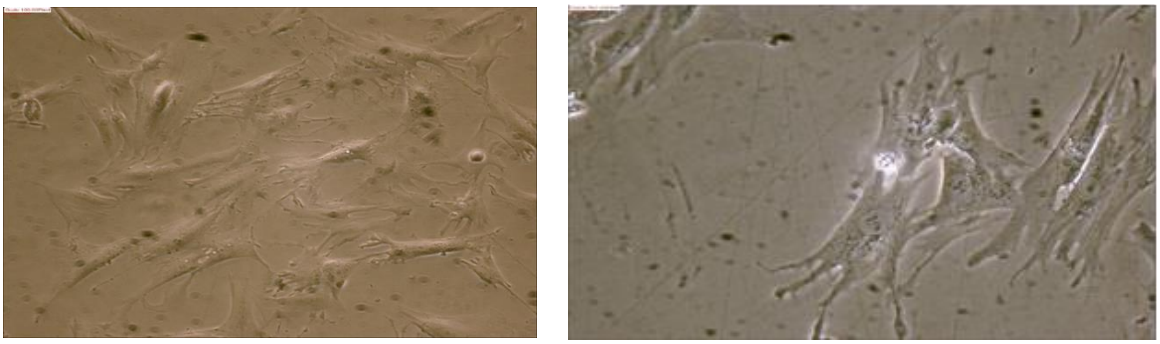
Table 2. Expression of Cell Surface Markers in Various Flow Cytometry Tube Configurations

Tube	Antibodies
1	CD34-PE (negative marker) and CD45-FITC (negative marker)
2	CD44-FITC (positive marker) and CD73-PE (positive marker)
3	CD29-PE (positive marker)
4	CD166-PE (positive marker)
5	No antibody added (control)

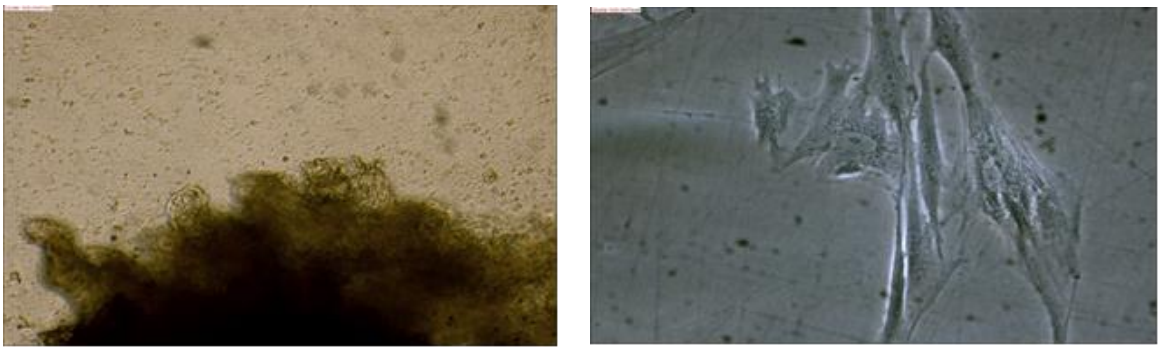
To examine each cell separately, 70 microliters of the cell suspension from the microtube containing the cells were added to each of the five tubes. The cells were trypsinized, suspended in 1 cc of serum-free culture medium, and transferred to the microtube.

The cells were then examined using a flow cytometry device. The cells were suspended in a liquid and passed through a narrow hole at a speed of 5 to 50 meters per second. As the cells passed in front of a narrow beam of laser light, their characteristics were evaluated based on the fluorescent signals generated by the antibodies conjugated with fluorescent dyes.
Flow cytometry analysis is a useful tool for identifying specific cell types and confirming their identity by examining the presence or absence of certain markers.
5 tubes were mixed by vortex and aluminum foil was wrapped around it and placed in the refrigerator door for 30 minutes and finally read by flow cytometry. Data analysis was done using the $SPSS_{25}$ software and the Kolmogorov-Smirnov test for the normal distribution of the data. And data analysis is also expressed experimentally-laboratory.

Results



Passage1- 20X



9 Day After Explant X20-3 Passage

Figure 1. Placenta MSCs Cells Morphology

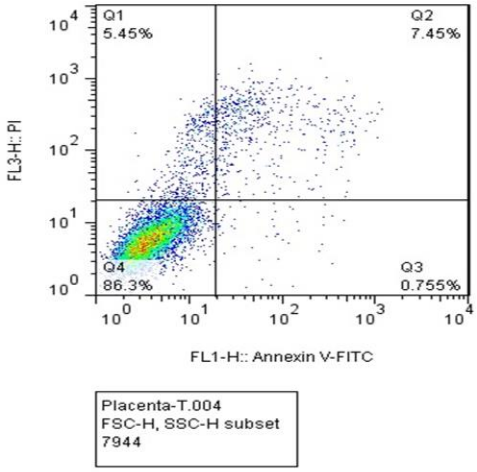


Figure 2. Apoptosis and necrosis in the cell

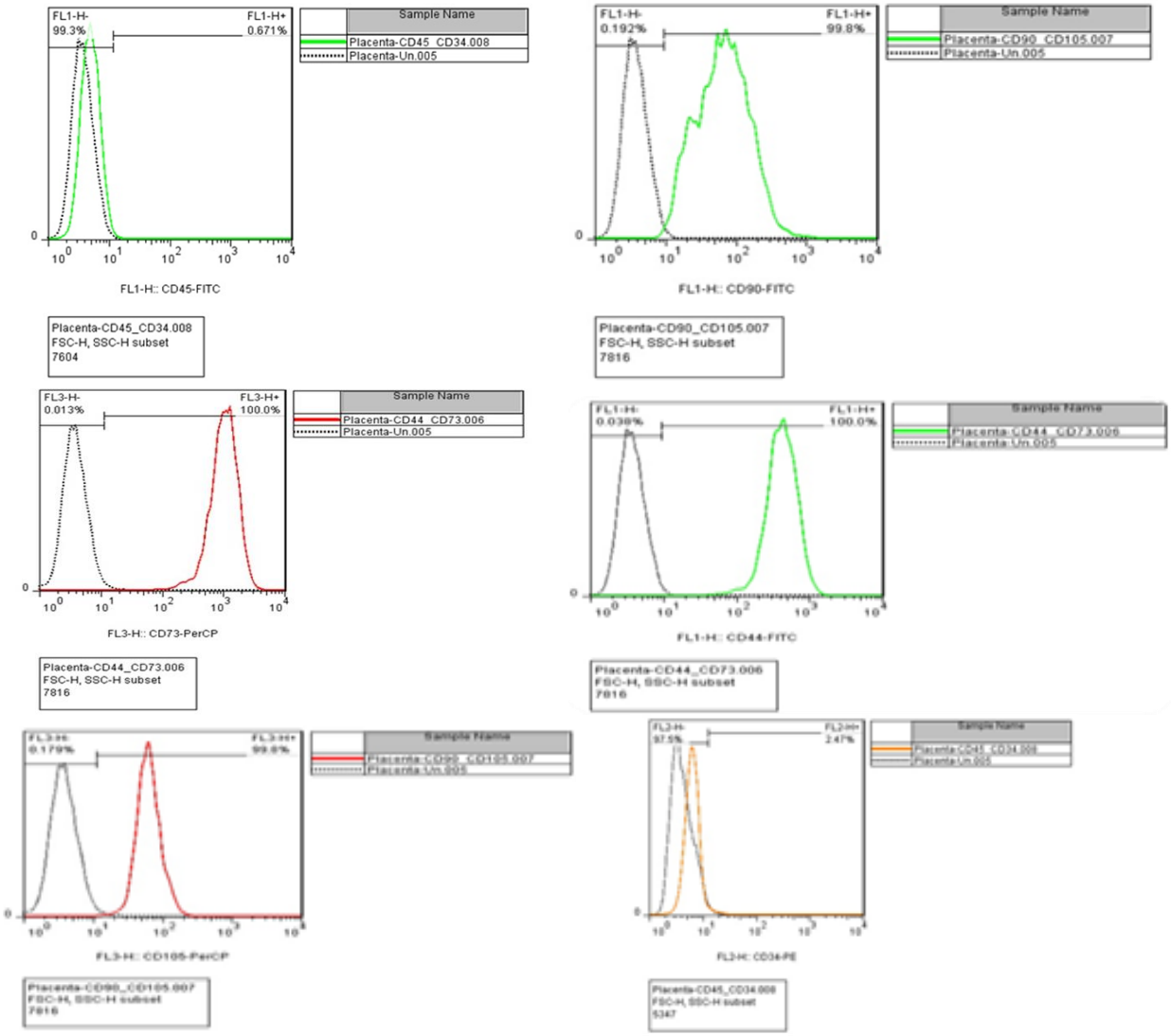


Figure 3. Surface markers (CD) in mesenchymal stem cells (MSC)

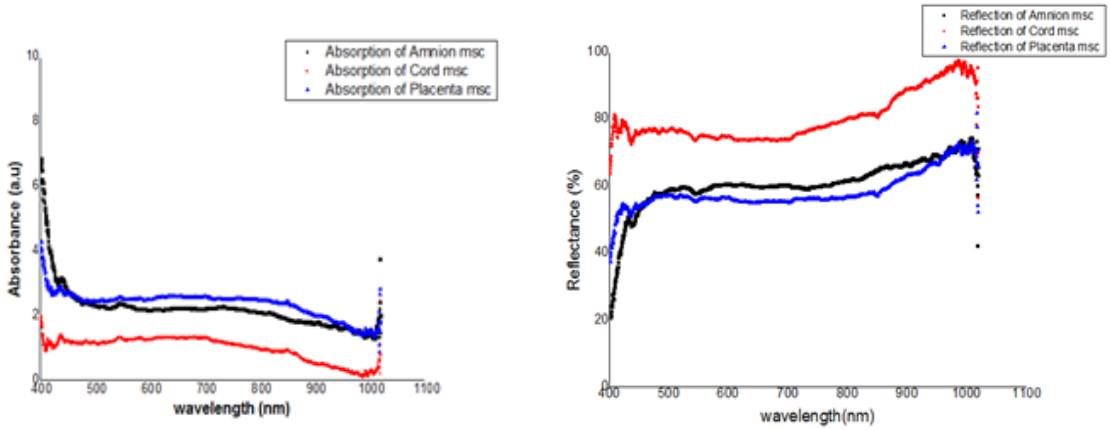
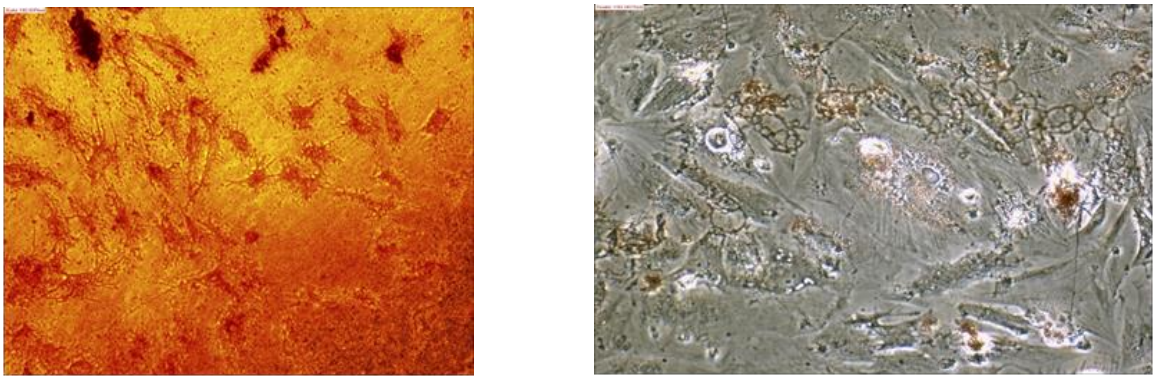


Figure 4. Reflectance Spectroscopy Results



Differentiation potential of bone cells

Differentiation potential to fat cells

Figure 5. Differentiation potential

Discussion

In the present study, we isolated the population of placental tissue and mesenchymal stem cells from different parts. All isolated mesenchymal stem cells had typical fibroblastic morphology, expressed cell surface markers in a mesodermal lineage, and each part of the placenta showed the same immunophenotype.

Further, the results of this research have shown that mesenchymal stem cells have CD44+, CD73+ and CD166+ surface markers and lack CD45+ and CD34+ surface markers.

And several studies have shown that in addition to the above surface markers, MSCs have surface markers (Thy-1) (CD90) and CD105 (SH2). And also the surface markers of blood and endothelial cells such as CD14, CD31 and CD11b are negative [20]. Also, the results of this research have shown that mesenchymal stem cells have programmed death (apoptosis) and necrosis. Figure y-represents 0.755% of primary apoptotic cells and 7.45% of completely apoptotic and necrotic cells, and they are mesenchymal stem cells with a survival percentage of 86.3.

Other researches have also shown that mesenchymal stem cells have the property of apoptosis [21]. On the other hand, some researches have shown that MSCs can increase tumor growth, inhibit apoptosis, angiogenesis in place and metastasis, which they do by activating NF-kb pathway signals through the AMPK/mTOR pathway. The AMPK/mTOR pathway activates the NF-kb pathway and thus the dual role of mesenchymal stem cells as a therapeutic agent is proposed [22]. Further, studies show that MSCs have a ROS system, which was evaluated in this study by flow

cytometry analysis, and also the exact level of regulation of the ROS system is very important for the differentiation of MSCs [23]. In line with other research, it is also indicated that mesenchymal stem cells have ROS system, on the other hand, some researches have shown that ROS has a dual role, so that by creating oxidative stress, they have a destructive role and also in the process of aging and programmed cell death. Mesenchymal stem cells are involved, or as messenger molecules, they start many physiological pathways and act as a stimulus [24].
Limitations: The investigation of the biological characteristics of mesenchymal stem cells derived from the placenta tissue was carried out in the cell culture environment and the change of the results in this area can be observed. Due to the limited financial support and available facilities, certain aspects of the research were not investigated. It is hoped that more complete research will be done in the future.

Conclusion

This study has successfully isolated mesenchymal stem cells from different parts of the placenta and demonstrated their typical fibroblastic morphology and immunophenotype. The results confirm that MSCs express mesodermal lineage surface markers, lack blood and endothelial cell surface markers, and have CD44+, CD73+, CD166+ surface markers. The study also revealed the presence of the ROS system in MSCs and its importance in their differentiation. However, due to limitations in the study's resources, it was not possible to investigate this issue at a microscopic and more advanced cellular and molecular level. Overall, these findings contribute to the understanding of the biological characteristics of MSCs derived from placental tissue and their potential therapeutic applications. Further studies with more specialized surface markers and advanced gene expression analyses can provide more insights into these cells' functions and potential applications.

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Conflict of interests

The authors state that there are no conflicts of interests regarding the publication of this article.

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