Biological Characteristics and Optical Reflectance Spectroscopy of Human Placenta Derived Mesenchymal Stem Cells for Application in Regenerative Medicine

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Abstract

Introduction: The efficiency of stem cell isolation, culture, and biological characterization techniques for treatment is facing serious challenges. The purpose of this study was to provide a protocol for isolation and culture of three types of mesenchymal stem cells (MSCs) derived from the human placenta, amniotic membrane, and umbilical cord with high efficiency used for cell therapy.

Methods: During this experimental laboratory study, 10 complete placenta samples were prepared from cesarean section mothers. The protocol for isolation and culture of mesenchymal cells from the placenta tissue, umbilical cord, and amniotic membrane was enzymatically optimized. The morphological features of mesenchymal cells were investigated using an inverted microscope and their biological features were measured using flow cytometry. The differentiation potential of the cells was evaluated by measuring their differentiation capacity into osteocytes and adipocytes. The absorption and reflectance features of the cells were recorded by optical spectroscopy. Finally, the data were statistically analyzed.

Results: The expression of CD44, CD73, CD90 and CD29 markers in human placenta tissue-derived cells was significant. CD14, CD34 and CD45 markers were not expressed or were slightly expressed. These cells were highly viable and successfully differentiated into osteocytes and adipocytes. MSCs absorbed more light than visible light by showing light absorption peaks at wavelengths of about 435 and 550 nm.

Conclusion: The protocol used in this study for isolation and culture of human placenta tissue-derived MSCs had significant efficiency for the production of MSCs for use in cell therapy and tissue engineering.

Keywords: Mesenchymal stem cells; Placenta; Differentiation; Specific markers; Optical spectroscopy.

Introduction

In recent years the number of patients suffering from diabetic ulcers has increased. As a result, new treatments have been introduced as cellular therapy, laser therapy, and wound dressing. Skin fibroblast cells and mesenchymal stem cells (MSCs) are two of the main cells that play an important role in the healing process of diabetic wounds.1 MSCs have self-renewal potential and are differentiated into various mesenchymal lineages such as osteogenic, chondrogenic, adipogenic, neurogenic, and so on. This feature makes them suitable for cell therapy for various diseases.2-4 Bone marrow is a common tissue source for MSCs and its mesenchymal stromal cells are used for cell therapy.5,6 However, due to the limited number of bone marrow mesenchymal cells for autologous transplants (10 cells per million monocytes), cell death in the donor region, high viral infection rate, and reduced cell proliferation with increasing age, the need for an alternative source is a necessity.6

In recent years, it has been shown that umbilical cord blood stem cells and extra-embryonic tissues have emerged as a potential ‘half way house’ between embryonic cells and adult stem cells.5 The basic features of these cells are highly proliferative potential and lack

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of tumorigenesis, which make them a suitable option for restorative medicine in cell therapy and tissue engineering. In addition, the use of extra-embryonic tissues has fewer moral problems. These cells have the ability to differentiate into mesenchymal lineages such as bone, fat, cartilage, and hepatocytes. They do not express endothelial and hematopoietic markers such as CD45, CD34, CD14 and Von Willebrand Factor. However, CD29, CD73 (SH3, SH4), CD105 (SH2), and CD44 (HCAM1) stromal markers express the primary marker of bone marrow progenitor cells (thy-1, CD90) and extracellular matrix proteins Vimentin, Laminin, and fibronectin.6,9

Unlike adult bone marrow MSCs, extra-embryonic mesenchymal cells in the first trimester of pregnancy express markers of potent pleural stem cells: Oct-4, Nanog, Rex-1, SSEA-3, SSEA-4, TRA1-60 and TRA1-81.10 First-trimester extra-embryonic mesenchymal cells attain four times as many population doublings in 50 days compared to adult MSCs: 28 versus 7, a consequence of their much shorter doubling time (30 versus 80 hours) and at the same time their phenotype remains constant. For this reason, they are suitable for cell therapy and gene therapy before and after birth or tissue engineering.7 Their differentiation capacity does not only apply to the osteogenic lineage. In addition to differentiation down the osteogenic, chondrogenic, and adipogenic lineages, extra embryonic MSCs can also differentiate into muscle cells, hematopoietic and oligodendrocytes.11,12 In relation to cell therapy, these cells have other superior features over mature MSCs, that is their telomerase activity and expression of low levels of HLA A.13

Extra-embryonic MSCs express extra-embryonic α2, 4 α and 1 α5β integrin, which play an important role in replacing transplanted cells and binding more and better to extracellular matrix ligands than adult MSCs.14

The isolation techniques of MSCs derived from different tissues as well as investigations into the efficiency and health of these cells for tissue engineering and therapeutic purposes are some of the most important issues in working with stem cells. Although the investigation into surface antigens of MSCs is one of the common methods for proving the mesenchymal nature of these cells, there are still technically significant challenges in this regard.15,16 However, identifying these markers is still technically challenging. In this regard, achieving effective and common techniques and using reliable techniques to identify health, measure viability, and evaluate the proliferation ability of MSCs that could be used well and safely in laboratories are extremely valuable,16–21 although after many years, these techniques still occasionally do not function well or are associated with inconsistent results, which have caused serious contradictions among researchers.

Regarding the potential applications of MSCs in the clinical field and given that the placenta, amniotic membrane, and umbilical cord are among the most important sources of MSCs that are easily available, and on the other hand, although several studies have been conducted to identify and evaluate the methods of isolation, culture, and banking of stem cells, these studies are still ongoing in order to obtain accurate information and they are facing serious challenges; Therefore, the present study investigates the efficiency of isolation and culture of MSCs from human placenta tissue, amniotic membrane, and umbilical cord to implement the use of these techniques in the field of isolation and investigate the nature of MSCs derived from the placenta and introduce it as a routine technique (Figure 1).

Methods

Materials and Instruments

Phosphate-buffered saline (PBS, pH 7.4, Gibco™, 10010023), Collagenase (Type I, Gibco™, 17100017), Dulbecco’s Modified Eagle’s medium (DMEM, Glucose Concentration: 4500 mg/L, Gibco™, 11965118), Fetal bovine serum (FBS, Gibco™, 10099133), RPMI (Roswell Park Memorial Institute, Gibco™, 11875101), Trypsin/EDTA (Gibco™, 07901), Dimethyl sulfoxide (DMSO, Gibco™, 85190), Annexin V Apoptosis Detection Kit (ApoFlowEx FITC Kit, EXBIO, Reference: ED7044-T100), CD44 (FITC, BD Bioscience, 347943), CD73 (PE, BD Bioscience, 555596), CD90 (PE, BD Bioscience, 550257), CD90 (PE, BD Bioscience, 555596), CD29 (PE, BD Bioscience, 556049), CD14 (Dako, M0825), CD34 (Dako, M7165), CD45 (Dako, M0701), adipocyte differentiation medium (complete DMEM supplemented with 1 μM dexamethasone, 200 μM indomethacin, 500 μM 3-isobutyl-1-methylxantine and 10 μg/mL insulin), and osteocyte differentiation medium (composed of complete alpha MEM supplemented with 100 nM of dexamethasone, 200 μM of ascorbic acid and 10 mM of glycerol 2-phosphate).

Preparation of Placenta Tissue

Randomly, 10 donors were sampled. Placenta tissue was received in a sterile receiver in the operating room after cesarean section from women (20 to 40 years old). The sample containers were covered with sterile drapes and transferred to the laboratory. All isolation and culture stages were performed under a sterile air hood under sterile conditions according to good clinical practice (GCP) instructions in the culture room. In the laboratory, the tissue was placed on a sterile tray and its various components were isolated for cell culture in those areas and placed in a Falcon tube containing 30 cc of PBS buffer containing antibiotics. Peripheral blood samples of each volunteer were tested for human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), human papillomavirus virus (HPV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), Herpes simplex virus (HSV), Human T-lymphotropic virus (HTLV), parvovirus
B19, *Treponema pallidum*, *Toxoplasma*, and rubella.

**Isolation and Expansion of Placenta Mesenchymal Stem Cells**

The tissue of the placenta was cut into small pieces and placed in a 50 cc tube. PBS/EDTA was poured onto the tissue and centrifuged (1800 revolutions per minute [RPM] and 5 minutes) (Hettich, Germany). The supernatant was drained and collagenase (1 mg/mL) was poured onto the tissue and pipetted with a G17 or G16 needle. The sample was incubated (Memmert, Germany) at 37°C for 2 hours (it could also be overnight). It was removed from the incubator every 15 minutes and vortexed for 2-3 minutes. At the end of incubation, it was pipetted again by a needle. After this period, the enzyme activity was neutralized by adding an equal volume of 10% FBS culture medium. Centrifugation (1800 RPM and 5 minutes) was performed. PBS was poured onto the pellet, pipetted and centrifuged again. Cell pellets were cultured in the DMEM low glucose medium containing 20% FBS. The first change of media was performed 48 hours later. Then, it was done every 3-4 days. In the early days, the serum level reduced by 20% and in the following days the serum level reduced by 15% and then to 10% (red blood cells were isolated on the day of the first medium change by holding the flask slightly tilted, and the monocytes also survived only 7 days in culture and then died).

**Isolation and Expansion of Umbilical Cord Mesenchymal Stem Cells**

The umbilical cord tissue was completely cut by scissors in the plate and transferred to a 50 cc tube containing PBS. Centrifugation (1750 RPM and 7 minutes) was performed. The supernatant was drained and about 25 cc of collagenase (1 mg/mL) (2-3 times the tissue...
Investigation of Apoptosis and Necrosis
In this study, independent \( t \) test, one-way analysis of variance, and repeated measures analysis of variance were used. Data analysis was performed using SPSS software version 25 and the significance level of the tests was considered \( P < 0.05 \).

Results
Isolation, Culture, and Proliferation of Cells
Enzymatically, less time was required to reach the cells. RBCs were present in the media for up to a week, and since the half-life of RBCs is one week, there was no need to worry about their presence for a long time. By day 5, the cells were seen as round and then spindle-shaped. The infection was detectable in the culture medium for the first 24 to 48 hours. The morphology of the cells was spindle-shaped in dense and elongated as non-dense. The number

Investigation of the Cell Surface Markers
Fluorescent conjugated antibodies including CD44, CD73, CD90, CD29, CD14, CD34 and CD45 markers were used to evaluate the presence or absence of cluster of differentiation (CD) markers. After adding antibodies, the samples were refrigerated for 30 minutes and then analyzed using flow cytometry.

Differentiation into Adipocytes
In order to differentiate MSCs into adipocytes, the cells were cultured in two groups of under control and under differentiation, and when they reached a confluency of 40%-50%, they were placed in the adipose differentiation medium for 21 days. The differential culture medium was changed every 48 hours and on the 21st day, 1 cc of formalin was poured on the cells and placed at room temperature for 1 hour. Oil-red was then poured on the cells and after 15 minutes the sample was washed with a buffer.

Differentiation into Osteocytes
In order to differentiate MSCs from osteocytes, the cells were cultured in two control groups and when they reached a confluency of 40%-50%, they were placed in the osteocyte differentiation medium for 21 days. The differential culture medium was changed every 48 hours and on the 21st day, 1 cc of methanol was poured on the cells and placed at room temperature for 10 minutes. Alizarin-Red was then poured on the cells for 2-5 minutes and washed with a buffer.

Optical Reflective Spectroscopy
Using the optical spectroscopy system equipped with optical fiber (Ocean optics, USB2000), the absorption spectra of the extracted mesenchymal cell samples were recorded. The laboratory layout of this system is shown in Figure 1. In order to study the spectrum, a halogen-tungsten light source was used in the visible spectral region at a wavelength of 400-1000 nm. Source light was collected and sent by optical fiber to the evaluated cell samples (Figure 2).

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of umbilical cord matrix stem cells is small compared to bone marrow. On average, 500 000 cells could be cultured in a 50 cm umbilical cord. The longer the umbilical cord is delivered, the fewer cells would be cultured. The cells in the early cell passages were shaped like fibroblast bone marrow mesenchymal cells in the first week after culture. During the subsequent passages and in the second week, these stem cells became narrower. The cells that were in their growth phase were used to freeze the cells (Figures 3 and 4).

Viability and Apoptosis of Cells
Investigation of apoptosis in placenta-derived MSCs showed that a large number of cells were alive and only a small percentage of them had apoptosis (Table 1, Figure 5).

Flow Cytometry Analysis
Flow cytometry analysis showed that placenta, amniotic membrane and umbilical cord stem cells were positive for MSC markers such as CD73, CD29, CD90 and CD44 and negative for CD34, CD14 and CD45 markers (Table 2, Figure 6).

Differentiation Potential of Cells
Investigation of differentiation of placenta MSCs, amniotic membrane, and umbilical cord into adipocytes and osteocytes showed that these cells have the ability to differentiate into adipocytes and osteocytes. In this regard, the results of staining differentiated cells to osteocytes by Alizarin-Red showed a red mass and proved morphology of osteocytes, but in the control culture, no red mass was made. On the other hand, the results of staining all differentiated cell adipocytes with oil red indicated the presence of yellow fat vacuoles and proved morphology of adipocytes, while in the control culture no trace of such vacuoles was found (Figure 7).

Optical Reflectance Spectroscopy Analysis
Figure 8 shows the unique light spectra of the extracted cell samples, which show specific fingerprint features of these samples. Umbilical cord MSCs from other samples show less light absorption in the visible light region and higher light reflection in this region. However, MSCs of the amniotic sac and placenta absorb more light than visible light by showing light absorption peaks at wavelengths of about 435 and 550 nm (Figure 8).

Discussion
Stem cell isolation and characterization techniques face serious challenges, and the study results indicate that the techniques used are associated with significant weaknesses in significant cases, leading to serious doubts about their use on the cells available for tissue engineering and tissue repair. Also, there is no “routine and widely used technique” that could be used in a wide range. This study was designed and implemented to achieve a routine technique for the preparation of MSCs from human placenta tissue with high accuracy so that the obtained cells could be used in repairing skin lesions. The study results showed that by using optimized methods and techniques, MSCs were routinely isolated from human placenta tissue and subsequently cultured and propagated in a culture medium designed with the optimal formula, and subsequently “after proving their health in terms of mesenchymal nature, viability and proper proliferation of these cells” could be differentiated into adipocytes and osteocytes. Other studies consistent with the study results also show that mesenchymal cells can be isolated from different parts of human placenta tissue and differentiated into adult cells.24,25

In the present study, flow cytometry was used to investigate the mesenchymal nature of cells isolated from the placenta in terms of specialized markers. The results showed the expression of mesenchymal markers and lack of expression of endothelial and hematopoietic markers in the studied cells, which proved the mesenchymal nature of cells isolated from the placenta. Previous studies on specific biomarkers of mesenchymal cells were consistent with the results of the present study.26 Previous studies consistent with the study results have shown that mesenchymal cells express their specific biomarkers such as CD44, CD73, CD90 and CD105 markers and do not express other markers such as CD34 and CD45.27

Another feature of mesenchymal cells is their ability to differentiate into other adult cells, such as osteocytes.
and adipocytes. The study results showed that placenta-derived mesenchymal cells in the third passage could be successfully transformed into osteocytes and adipocytes in a specific culture medium. Previous studies have also shown that the maximum proliferation and differentiation of stem mesenchymal cells are between passages 2 and 6, and in higher passages differentiation reduces. On the other hand, studies indicate that MSCs are able to differentiate into different adult cells such as osteocytes, hepatocytes and epithelial cells.

The present study was conducted to investigate the efficiency of isolation, culture, and differentiation of MSCs derived from placenta tissue into other adult cells, and the interpretation of the results in this field could be explained. This study is limited in terms of patients. It is hoped that in the near future it would be possible to investigate the clinical trial level.

**Conclusion**

In general, the study results showed that the method used in this study is a reliable method for isolation, culture, and proliferation of MSCs derived from various components of placenta tissue and has the potential to be used as a routine method in research centers. Flow cytometry studies showed that placenta tissue-derived MSCs obtained from the method used in this study are mesenchymal in nature,
Table 1. Viability and apoptosis in placenta, amniotic membrane and umbilical cord mesenchymal stem cells.

|                      | Placenta MSCs Mean ± Sd | Umbilical cord MSCs Mean ± Sd | Amniotic membrane MSCs Mean ± Sd | P value |
|----------------------|-------------------------|-------------------------------|----------------------------------|---------|
| Viability (%)        | 86.10 ± 1.04            | 83.70 ± 1.13                 | 80.80 ± 1.59                    | 0.006   |
| Apoptosis (%)        | 7.25 ± 0.23             | 14.50 ± 0.50                 | 15.50 ± 0.50                    | <0.001  |

Table 2. Expression of positive and negative markers in placenta, amniotic membrane and umbilical cord mesenchymal stem cells.

| Marker  | Placenta MSCs Mean ± Sd | Umbilical cord MSCs Mean ± Sd | Amniotic membrane MSCs Mean ± Sd | P value |
|---------|-------------------------|-------------------------------|----------------------------------|---------|
| CD44    | 99.93 ± 0.11            | 95.89 ± 0.37                 | 99.47 ± 0.11                    | < 0.001 |
| CD73    | 99.87 ± 0.11            | 96.39 ± 0.43                 | 99.72 ± 0.02                    | < 0.001 |
| CD90    | 99.85 ± 0.05            | 98.17 ± 0.25                 | 99.76 ± 0.05                    | < 0.001 |
| CD29    | 99.78 ± 0.08            | 98.17 ± 1.04                 | 44.78 ± 0.19                    | < 0.001 |
| CD14    | 2.44 ± 0.01             | 7.52 ± 0.11                  | 0.793 ± 0.01                    | < 0.001 |
| CD45    | 5.90 ± 0.10             | 17.39 ± 0.08                 | 19.31 ± 0.59                    | < 0.001 |
| CD34    | 0.90 ± 0.10             | 1.17 ± 0.01                  | 0.310 ± 0.41                    | 0.012   |

Figure 5. A) Viability, B) apoptosis in placenta, amniotic membrane and umbilical cord mesenchymal stem cells.

Figure 6. Expression of positive and negative markers in placenta, amniotic membrane and umbilical cord mesenchymal stem cells.

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Conflict of Interests
The authors declare no conflict of interests.

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