ORIGINAL ARTICLE

GMJ.2017;6(3):217-25
www.gmj.ir

Received 2016-07-05
Revised 2017-05-09
Accepted 2017-06-10

A Rapid and Cost-Effective Protocol for Isolating Mesenchymal Stem Cells from the Human Amniotic Membrane

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Abstract

Background: Regarding the role of mesenchymal stem cells (MSCs) in regenerative medicine, many studies have been conducted to isolate these cells from various sources. In this study, a method was developed which will use only one enzyme and in the shortest time MSCs will be isolated from the amniotic membranes and expanded. **Materials and Methods:** The amniotic membrane (AM) was mechanically separated from the underlying membrane called chorionic. Then, the AM was sliced into tiny pieces and to isolate MSCs, it was digested only using collagens I instead of applying various enzymes. The isolated cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum. The expression profiles of several markers at isolated cells were determined by flow cytometry. To assess the differentiation potential, the isolated cells were cultured in osteogenic and adipogenic induction media. **Results:** The results indicated that cells isolated from the AM expressed markers of CD44, CD105 and CD166 mesenchymal cells, but did not express CD34 and CD45 hematopoietic markers. The osteoblastic differentiation of the isolated cells was proven by alizarin red and alkaline phosphatase staining methods, whereas the adipogenic differentiation of the isolated cells was proven by Oil Red-O staining. **Conclusion:** The results of the study indicated that the isolated cells were of the MSCs family. Furthermore, it was demonstrated that MSCs can be obtained easily only by spending a short time and using one enzyme.
[GMJ.2017;6(3):217-25] DOI: 10.22086/gmj.v0i0.670

Keywords: Mesenchymal Stem Cell; Amniotic Membrane; Protocol

Introduction

Mesenchymal stem cells (MSCs) are able to differentiate into other cell types with mesenchymal origin, including adipocytes, osteoblasts, cartilage, muscle and tendon [1]. In addition to the ability to differentiate into mesenchymal-origin tissue, they have the power of self-renewal; these two properties together make them suitable for regenerative medicine [2]. According to previous studies, different sources can be used to separate MSCs. Some of these sources include: umbilical cord [3], placenta [4], connective tissue

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of adult humans [5], and adipose tissue [6]. Among these sources, human amniotic mesenchymal stromal cell (hAMSCs) have advantages compared to other sources. Human amniotic membrane is readily available after each delivery and usually discarded as medical waste. Unlike other human mesenchymal cells, hAMSCs has a high immunological tolerance [7]. Studies have shown that hAMSCs can differentiate into various organs such as cardiomyocytes [7] and neurons [8]. According to the features listed, hAMSCs have a special place in regenerative medicine. To date, a number of techniques have been developed to obtain hAMSCs, which in most cases are isolated in subsequent enzymatic digestions and scrolling successive stages and consumes a lot of time [9, 10]. For the reasons listed, separation costs are very high; therefore, it seems that providing a cost-effective new method is necessary for the isolation of hAMSCs. In this study, a novel protocol for isolating mesenchymal cells derived from amniotic membrane was introduced, in which the number of procedures for isolation as well as the used enzymes is reduced and each step is shortened. The purpose of this study was to introduce a simple, quick, affordable and convenient technique which involves using one enzyme to isolate mesenchymal stem cells from the amniotic membrane.

Materials and Methods

Study Design and Setting

The protocol of the study was approved by The Ethical Committee of Tehran University of Medical Sciences. The placentas from which amniotic membrane was used in the study were obtained from natural parturition and from delivery by Cesarean section. The placentas from pregnancies with normal growth and development in the absence of ruptured membranes or chromosomal abnormalities were obtained from the Hospital of Gynecology Akbarabadi (Tehran, Iran). The amniotic membrane tissues were collected in sterile conditions in the operating room of mothers who had signed consent forms.

Donor Selection

The pregnant women who were selected to participate in this study had volunteered. Those who had severe systemic diseases such as diabetes and obstetric disorders such as pre-eclampsia or eclampsia were excluded from this study. In addition, those with communicable and infectious diseases were excluded from the study, such as those with human immunodeficiency virus (HIV), toxoplasmosis, cytomegalovirus (CMV) infection and rubella. A total of four pregnant women were selected for this study. A series of tests were performed on them, which were regarding issues they had encountered during the course of pregnancy as well as their medical histories.

Isolation and Culture of MSCs from the Amniotic Membrane

Of placentas which their amniotic membranes were used in the study, three were from natural parturition and one from a delivery by cesarean section.Avascular transparent amniotic membranes were mechanically separated from the underlying opaque membrane called chorionic membrane (Figure-1A ,B). To clean up the drops of blood and probable contamination, the amniotic membrane was washed three times with phosphate buffered saline (PBS) containing 200 U/mL penicillin, 200 µg/mL streptomycin and 0.5 µg/mL amphotericin B. Thereafter, the amniotic membrane was transported in cold PBS solution with a biological safety box to the research laboratory and preparations began instantly.

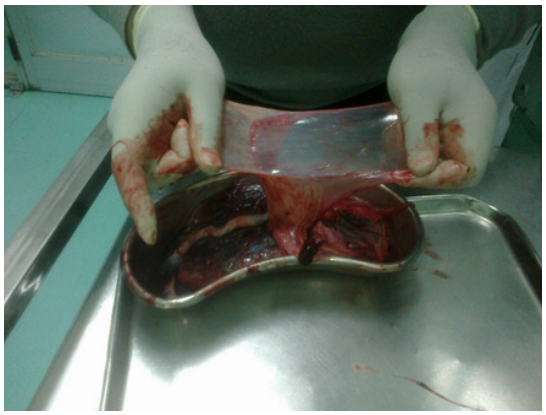

Figure 1. Amniotic membrane was mechanically peeled off from the underlying chorionic membrane

At first, under laminar hood, the amniotic membrane was sliced into tiny pieces (1 × 1 cm) and was washed again with PBS. Approximately 15 g of the fragment pieces of amniotic membranes were incubated for about 60 minutes with 0.75 mg/mL collagens I (Gibco, USA) solution in low glucose Dulbecco's modified Eagle's medium (DMEM-LG; Gibco) in 37°C and 5% CO_2. Every 10 minutes, the enzyme and tissue suspension were brought out of the incubator and after vigorous shaking, they were returned to the incubator again. After centrifugation (300 g, 5 minutes, 24°C), the cell suspension was washed twice with PBS and then filtered through a 70 μm falcon cell strainer (Falcon, USA). The cells that were passed through the filter were collected by centrifugation (300 g, 5 minutes, 24°C). The collected cells were seeded in 25 cm^2 Cole flasks composed of DMEM-LG plus medium supplemented with 20% fetal bovine serum (FBS), 100 U/mL penicillin, 100 g/mL streptomycin and 2 mm L-glutamine (Sigma-Aldrich) and were incubated at 37°C in a humidified atmosphere with 5% CO_2. The MSCs derived from the amniotic membrane were attached to the bottom of the flask and were spindle shaped. After 24 hours with replaced culture medium, the floating cells (non-MSCs) were removed from the environment and MSCs were bound to the bottom of the flask; they formed colonies and reproduced. When more than 80% - 90% of the flask was covered by cells, using 0.25% trypsin-EDTA (Gibco) solutions, the cells were harvested from the bottom of the flask and 5 × 105 cells were cultured again in a new flask; then, the medium (with 10% FBS) was replaced every four days. Therefore, successive passages of the cells were obtained. During the study, subsequent passages after freezing and thawing of the cells were carried out. Since the beginning of primary culture, the flasks containing cells were checked daily for growth and morphology as well as for possible contamination of the cells by inverted microscope.

Immunophenotyping Analysis of Isolated Cells

To confirm the origin of mesenchymal cells, flow cytometric analysis was used to assess surface markers and identify the phenotype of the stem cells isolated from the amniotic membrane. In flow cytometry, for each marker, a sample of the cells of passage 3 or 5 was prepared. To prepare stem cells for flow cytometry, the following steps were used: 50 μL of the cell suspension was prepared and 5 μL of fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated monoclonal antibodies specific for human markers were incubated for 30 minutes at 4°C in the dark. Then, the prepared samples were fixed with 4% paraformaldehyde (PFA). Finally, the samples were analyzed using a flow cytometer (Partec PAS III). The following antibodies were used: CD 44–FITC (BD Pharmingen; Clone: 5E 10) ،CD 34–FITC، CD 45–FITC، CD 166-FITC، and CD 105–PE. Control samples were incubated in PBS without primary antibody.

In Vitro Differentiation Studies

At passage 3, hAMSCs were induced to differentiate into two different types of cells including adipocytes and osteoblasts cells.

Osteogenic and Adipogenic Differentiation

To assess the differentiation potential of the isolated cells from hAMSCs, the surface of a six-well plate was treated by 4% collagen solution and was washed twice with PBS solution. Thereafter, the 4 × 103 cells from passage 3 were seeded on each well with medium containing DMEM-LG with 10% FBS and cultured at 37°C under humidified atmosphere of 5% CO2 in air. When 80% of the well areas were coated with stem cells, the culture medium was replaced with osteogenic or adipogenic induction medium. There are different media for inducing osteogenic and adipogenic differentiation of MSCs in vitro [11, 5].

Osteogenic Differentiation

Osteogenic induction medium consisted of high glucose DMEM, 10% FBS, 10-7 M dexamethasone (Sigma), 10 mM β–glycerophosphate (Merck, Darmstadt, Germany), and 0.5 mM ascorbic acid 2-phosphate (Sigma) [12]. The hAMSCs were maintained in osteogenic induction medium for up to four weeks, with change of media every three days. Control cultures were grown in DMEM contain-

ing 10% FBS. Osteogenic differentiation was confirmed by alizarin red and alkaline phosphatase staining [13].

Adipogenic Differentiation

Adipogenic induction medium consisted of DMEM containing 10% FBS, 106 M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 200 µM indomethacin (Sigma), and 10 µg/mL insulin (Gibco) [14]. Control cultures were grown in DMEM containing 10% FBS. The hAMSCs were maintained in adipogenic induction media for up to three weeks, with fresh media changed every three days. The morphology of the cells was studied using inverted microscope each day. After three weeks of culture, cytoplasmic inclusions of neutral lipids were stained with Oil Red O (Sigma) [13].

Results

Isolation of hAMSCs from Amniotic Membrane

In this study, we succeeded in isolating and culturing mesenchymal stem cells from the human amniotic membrane.The cell bonding occurred about two days after the start of the primary culture. At first, all the cells isolated from the amniotic membrane were seen as spherical and floating in the culture medium (Figure-2A). Over time, some of them

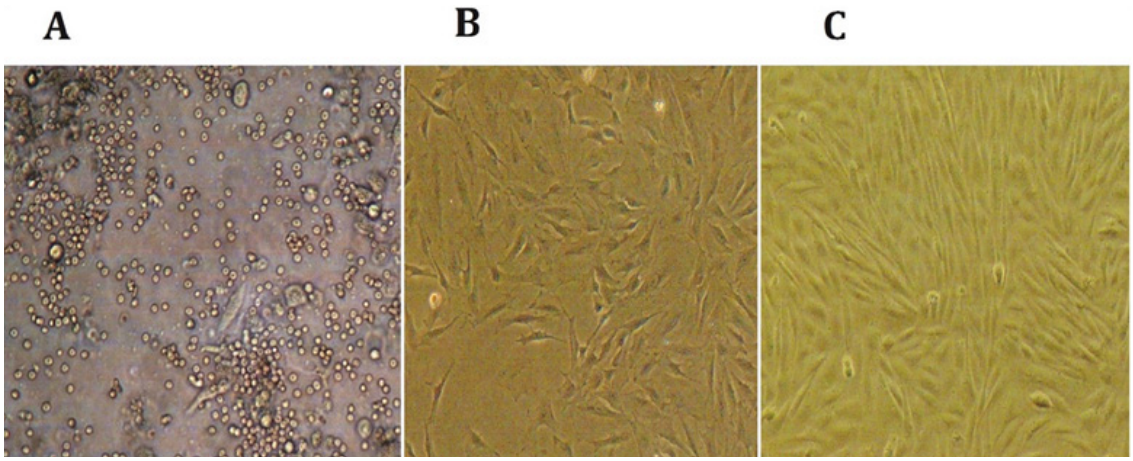

Figure 2. Morphology of hAMSCs. (A) hAMSCs one day after isolation (200x magnification); (B) adherent hAMSCs 15 days after isolation (200x magnification); (C) adherent hAMSCs 21 days after isolation (200x magnification); hAMSCs reached subconfluence at day 21

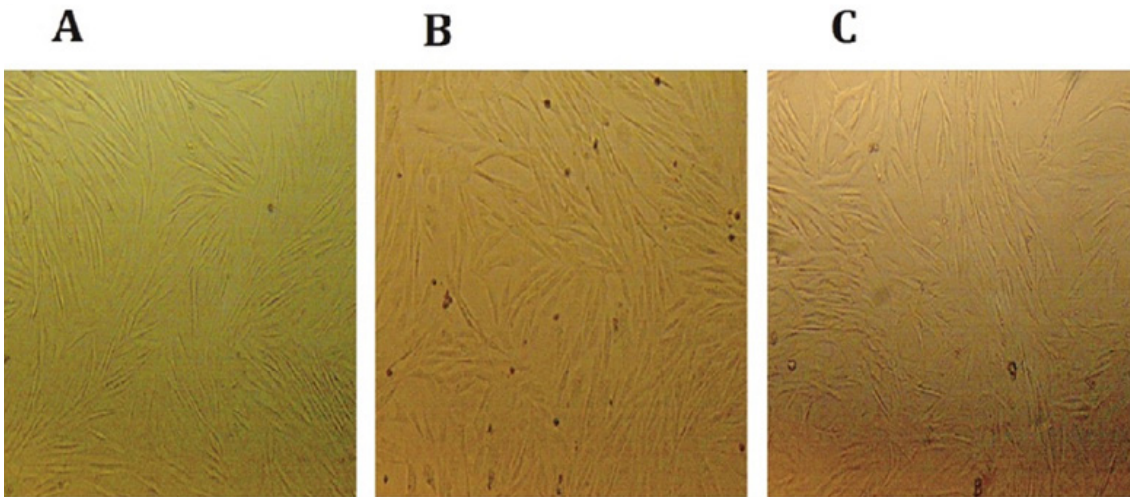

Figure 3. Morphology of hAMSCs. (A) hAMSCs in the 8th passage (100x magnification); (B) hAMSCs in the 11th passage (100x magnification); (C) hAMSCs in the 15th passage (100x magnification

were stuck in the bottom of the flask, as only a small number of the cells were attached to the culture flasks and the rest remained floating. After two days, the culture medium was replaced and the floating cells were removed from the flask; only the cells attached to the bottom remained, which were a population consisting of a few fusiform-shape fibroblastoid cells. At the beginning the isolated cells had circular shapes and with passage of time they became elongated, lastly acquiring unusual fibroblastic morphology (Figure-2B). These cells were introduced as MSCs and after 15 days of culture, they became the main cell type in the flasks (Figure-2B), readily sticking to the bottom of the flask and proliferating. The isolated cells reached 70–80% confluence after three weeks from harvesting and the first passage was done (Figure- 2C). Following passages were conducted when the cell density reached 80-90% confluence. At the subsequent passages, the MSC cultures appeared with higher growth and replicative potential, reaching 80–90% confluence after a week or even earlier.In this study, it was observed that the cultured cells maintained their growth and replication potency up to the 15th and 17th passages, respectively (Figure-3A, B, C). At 17^{th} passage, the cells showed signs of aging and slowing down the growth and proliferation and morphology of the cells was flatter and longer; this change was similar to malformations of the Hayflick model of cellular aging [15]. The cells lost their shape and size and their cytoplasm started to become granular form; wreckages were also formed in the medium.

Immunophenotype of hAMSCs

Immunophenotype of the isolated cells was designated by flow cytometry using phycoerythrin (PE)-conjugated or fluorescein isothiocyanate (FITC)-conjugated antibodies against CD 34, CD 44, CD 45, CD 105 and CD 166. The cells isolated were expressed for mesenchymal stem cell markers of CD44 (99.66%), CD105 (97.91%) and CD166 (26%), whereas hematopoietic lineage markers CD34 (3.64%) and the leukocyte common antigen marker CD45 (10%) were not expressed (Figure-4).

Osteogenic Differentiation Potential of hAMSCs

After three weeks of osteogenic induction, precipitation of calcium in the cells and the formation of mineralization in their matrix were examined. Staining with alkaline phosphatase and alizarin red demonstrated the presence of osteogenic differentiation in the induced MSC culture (Figure-5A, 5C), such that the cells expressed alkaline phosphatase activity and showed positive reaction for alizarin red staining. The cells cultured in the control medium did not respond to alizarin red staining (Figure-5B) and did not show alkaline phosphatase activity (Figure-5D).

Adipogenic Differentiation Potential of hAMSCs

Twenty one days after the hAMSCs culture in adipogenic differentiation medium, the spindle-shaped cells became round and multiple lipid vacuoles in their cytoplasm appeared, which were confirmed by positive Oil Red O staining (Figure-6A); whereas in the untreated control cells, no lipid droplets were seen in their cytoplasm (Figure-6B).

Discussion

In this study, using a new protocol with only one enzyme called collagenase I in the shortest possible time, the mesenchymal cells were isolated from the amniotic membrane and their identity was determined as MSCs by flow cytometry; they were also induced to differentiate into two different types of cells including adipocytes and osteoblasts. The use of stem cells has attracted much attention and they are considered as a potential source of cells to repair damaged organs such as heart and brain. MSCs have been recognized as a valuable resource in regenerative medicine and cell transplantation [16, 17]. Among them, hAMSCs, according to their attributes, have recently created much attention in these fields [7]. Given that previous reports about the isolation of mesenchymal stem cells from amniotic membrane tissue were performed by multiple enzymes and isolation stages took a very long time [10, 8, 14, 18], this study is in-

troducing a new protocol for the isolation and expansion of hAMSCs.Most studies conducted for isolating hAMSCs have used different concentrations of collagenase I or IV and trypsin-EDTA and some of them have used DNAse and dispase [10, 9, 19]. For example, the method performed by Soncini *et al*. [9] to isolate stem cells from the amniotic membrane involved the use of 2.4 U/mL dispase, 0.75 mg/mL collagenase, 20 µg/mL DNAse and 0.25% trypsin enzymes, whereas in this study, only one enzyme called collagenase I was used at a concentration of 0.75 mg/mL. In the protocol used in this study, the time spent for isolation of mesenchymal cells was about 1.5 hours; however, in studies by Soncini *et al*. [9], and Alviano *et al*. [14], due to various stages involved in the process of isolation, it seems that the time spent was much more than 1.5 hours. One of the advantages of the present method was that unlike some previous studies [9, 8], trypsin-EDTA was not used. In a study by Mihu *et al*. [10], it was noted that the use of trypsin in the process of isolating the stem cells for than 5 minutes can had adverse effects on isolated cells, which can cause cell destruction and a lower viability in culture.

After a long time and several passages, in the 15-17th passages , hAMSCs showed abnormality characteristic of the Hayflick model of cellular aging in the culture [15]. In a study by Lisi *et al*. [20], hAMSCs were expanded up to the 10th passage without any morphological changes. This study confirmed the investigation conducted by Alviano *et al*. [14], in which cells did not show any morphological

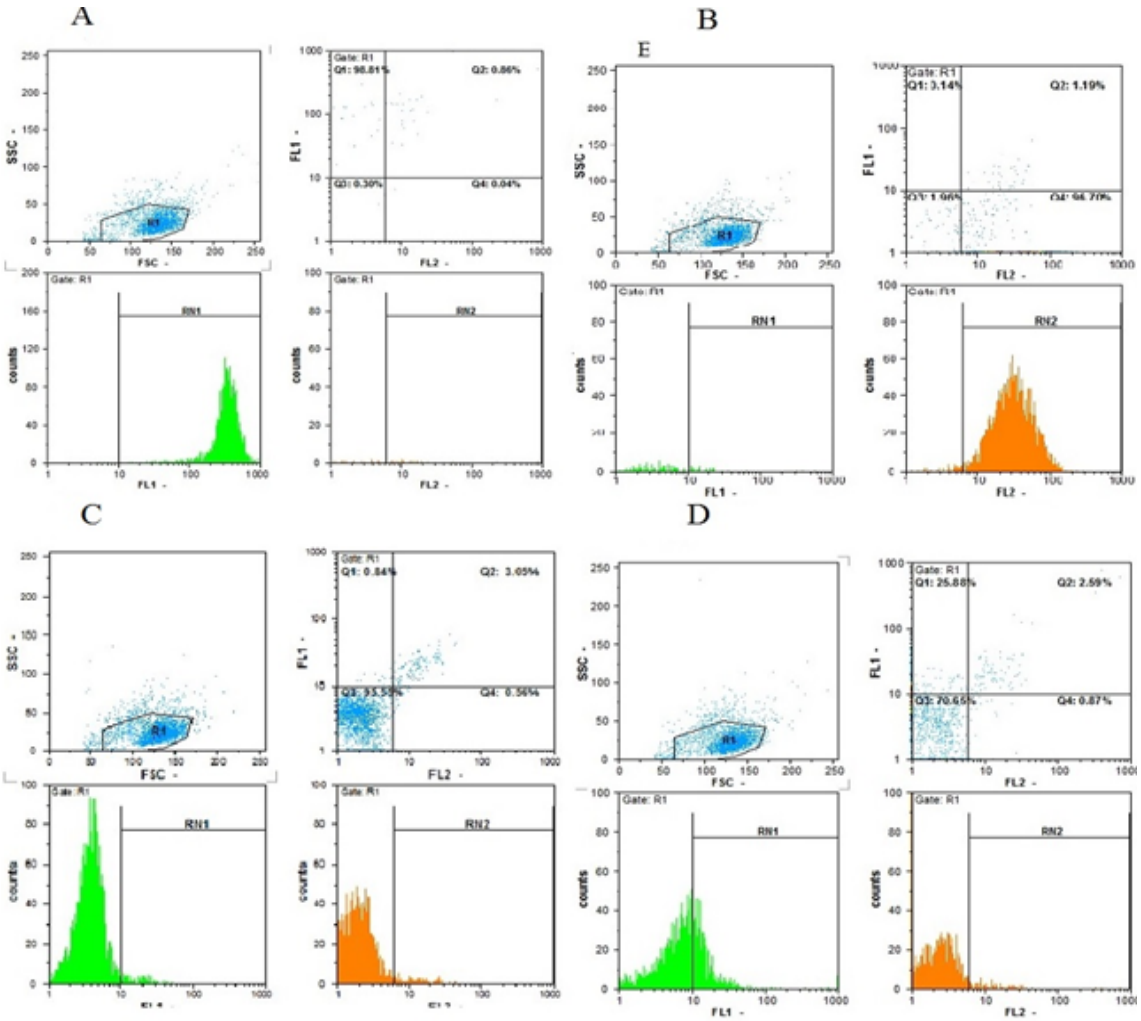

Figure 4. Immunophenotypical characterization of amniotic membrane-derived cells. Isolated cells expressed mesenchymal stem cell markers CD44 (A) and CD166 (B), while CD34 (C) and CD45 (D) were negative

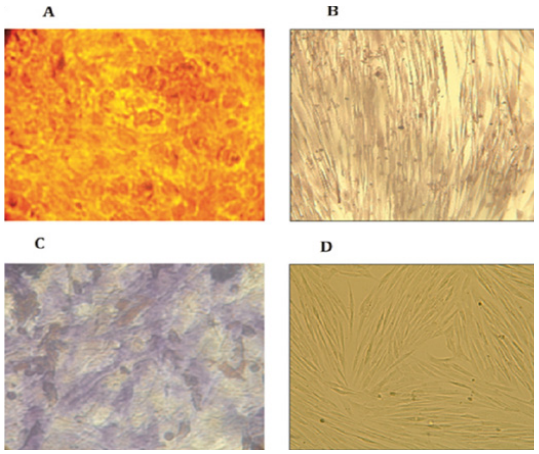

Figure 5. Osteogenic differentiation of hAMSCs. **A and C:** osteogenic differentiation was evident both in the formation of vast extracellular calcium deposits (alizarin red staining-positive) and the formation of alkaline phosphatase-positive aggregates in cytoplasm; **B and D:** negative control (200x magnification)

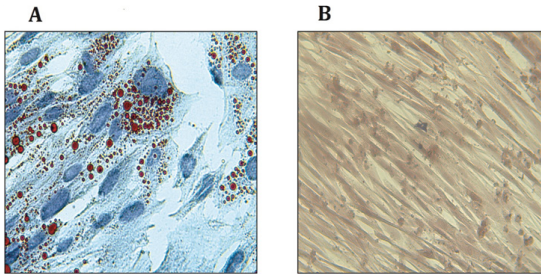

Figure 6. Adipogenic differentiation of hAMSCs. (A) Adipogenic differentiation was evident by the formation of lipid droplet (Oil Red O-positive) in cytoplasm; (B) negative control (200x magnification)

alterations up to the 15th passage. One of the ways that allows rapid identification of a population of cells is evaluating the expression of the cell surface antigens by flow cytometry. This method was used in this study. Previous studies have shown that mesenchymal cells are positive for CD44, CD105 and CD166 and negative for CD 34 and 45 [21, 2, 22]. The cells isolated from the amniotic membrane in this study showed high levels of expression of MSCs markers such as CD44, CD105 and CD166, while CD34 (marks primitive hematopoietic progenitors and endothelial cells) and CD45 (a pan-leukocyte marker) markers were not expressed. These results confirmed the findings of studies conducted by Dominici *et al*. [21], Lee *et al*. [22] and Feng *et al*. [2] in regards with the expression of CD 90, CD44, CD105 and CD166 markers and non-expression of CD34 and CD45 markers. Therefore, the results of flow cytometry showed that the method offered in this study can isolate MSCs without any contamination to hematopoietic and endothelial cells. Indeed, the amniotic membrane is usually devoid of vasculature and hematopoietic and endothelial cells which are usually derived from placenta [8]. MSCs are adult stem cells which have the potential to develop into osteoblasts, chondrocytes and adipocytes [1]. Hence, it must be demonstrated that the isolated cells are capable of differentiating into osteoblasts and adipocytes using typical in vitro tissue culture-differentiating media.

Conclusion

In this study, a new protocol for isolating hAMSCs was used. Immunophenotyping, morphological and differentiation characteristics of the isolated cells showed that they were of mesenchymal lineage cells. The present protocol is different from other previous protocols used to separate the MSCs, because it is simple, effective and fast. In addition, this observation indicates that the amniotic membrane can be a significant source of MSCs which is easily available and its use has no legal problem.

Acknowledgements

The authors wish to thank the physicians and midwives of the Hospital of Gynecology Akbarabadi (Tehran, Iran). The authors also appreciate the efforts of Dr. Masoud Hamidi (Guilan University of Medical Sciences). This article was written based on a data set of a PhD thesis, registered at Tehran University of Medical Sciences. This work has been supported financially under the PhD thesis proposal, submitted at No. 23061 at Tehran University of Medical Sciences, Tehran, Iran.

Conflict of Interest

The authors report no competing interests.

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