



An Inexpensive and Simple Method for Isolation Mesenchymal Stem Cell of Human Amnion Membrane

Ali Shadmanesh^{1,2}, Hassan Nazari^{3*}, Abolfazl Shirazi⁴, Ebrahim Ahmadi³, Naser Shams-Esfandabadi^{3,5}

¹ Department of Veterinary Medicine, Islamic Azad University, Eghlid Branch, Iran

² PhD student, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran

³ Research Institute of Animal Embryo Technology, Shahrekord University, Shahrekord, Iran

⁴ Reproductive Biotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran

⁵ Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran

*Corresponding Author E-mail: hnazari@sku.ac.ir

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ABSTRACT

Background: Background: Human-amnion membrane mesenchymal stem cells (hAMSCs) are the accessible cells that can be grown in *in vitro* condition to produce a great number of cells required for cell therapy in medicine. Several protocols have been proposed for isolation of hAMSCs, but the most of them are multi-step and expensive. The purpose of this article was to introduce a one-step, inexpensive protocol.

Methods: In this study human amnion membrane (hAM) was obtained from selected caesarean-sectioned births. The AM was sliced into small pieces and to isolate MSCs, it was digested only with one step instead of multi-step. Isolated cells were cultured in Dulbecco's Modified Eagle's Medium-Low Glucose (DMEM-LG) with 10% fetal bovine serum (FBS) without adding growth factors. After 80-90% confluency, the adherent cells were characterized by flow cytometry and multi-potentiality differentiation toward adipocyte-, and osteocyte-like cells.

Results: The results showed that hAMSCs isolated from hAM expressed CD105, CD90 and CD73 such as other MSCs, but did not express CD34 and CD45 hematopoietic markers. The osteogenic and adipogenic differentiation of the isolated cells were proven by Alizarin Red and Oil-Red-O staining, respectively.

Conclusions: The results showed that the stem cells derived from the AM belonged to the mesenchymal stem cells family. Furthermore, this method made it easier and cheaper to obtain this type of stem cells.

Keywords: hAMSC, Amnion membrane, Isolation of cell, stem cell

1. Introduction

Mesenchymal stem cells (MSCs) are non-hematopoietic multipotent stem

cells that, in addition to their power of self-renewal, can be differentiated from other cell types including osteocytes,

adipocytes, cartilage, nerve, and so on [1]. Due to the spread of diseases and recent studies, they are suitable for cell therapy [2-4]. According to studies, MSCs can be separated from different sources, including bone marrow [5], umbilical cord [6], placenta [7-9], connective tissue of adult human [10], adipose tissue [11] and Amnion membrane [12-14].

Despite some limitations, bone marrow is commonly used in adults as a tissue source for mesenchymal stem cells. Some of the most important limitations are the accessibility, the invasive and painful required procedure to obtain this kind of tissue, the possibility of donor site morbidity, low number of derived MSCs, and attenuation of the proliferation and differentiation potential of these cells simultaneously with donor's growing older. Therefore, the identification of other sources of MSCs would be beneficial for both research and therapeutic purposes.

Unlike other sources (especially bone marrow) the human amniotic membrane is readily available after each delivery and usually discarded as medical wastage. On the other hand, it is easier to extract MSCs as it has a high immunological tolerance [15]. Several studies have shown that human amnion membrane-derived mesenchymal stem cells (hAMSCs) can be differentiated from various cells, including adipocytes, osteocytes, cardiomyocyte, cartilage, nerve, and so on [15]. Therefore, it has a special place in cell therapy [16].

The human amnion membrane (hAM) contains two different types of cells of different embryological origins: Human amnion epithelial cells (hAECs) derived from embryonic ectoderm, which forms a continuous monolayer that is in contact with the amniotic fluid, and hAMSCs derived from embryonic mesoderm which are sparsely distributed in the stroma underlying the amnion epithelium.

It had been reported that hAMSCs are differentiated into adipogenic, osteogenic, chondrogenic, and myogenic tissues. In addition, Alviano et al. showed that hAMSCs are differentiated into endothelial cells in the presence of vascular endothelial growth factor (VEGF) in angiogenic experiments [17]. AMSCs have the higher chemotactic and angiogenic properties compared with adipose tissue-derived MSCs (Ad-MSCs) [18]. This property suggests that hAMSCs may be used as a new candidate in the cell therapy for vascular disease. Clinical and empirical studies have shown that transplantation of the amnion membrane causes recurrent epithelialization, reduction of inflammation, fibrosis [19] and regulation of angiogenesis [20]. Several amnion membrane-produced factors such as TNF- α stimulated gene/protein 6 (TSG-6) and Prostaglandin E2 are involved in these processes and are also able to suppress T-cell proliferation [4]. hAMSCs can be used as a valuable tool for the treatment of multiple diseases, and bone and cartilage repair.

In addition to mechanical techniques for stem cell separation, different enzymes and growth factors are used to digest tissue and prevent the differentiation of isolated cells. Adding these factors to the culture medium increases the cost of stem cell separation. So, one of the requirements is to propose a cheaper and easier technique for stem cell separation. 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 was reduced and each step was shortened. The purpose of this study was to introduce a simple, quick, affordable and convenient technique which involved using two enzymes to isolate mesenchymal stem cells from the amniotic membrane and without using

growth factors in culture medium of isolated MSCs.

2. Materials and Methods

Except for a few cases, all chemicals were purchased from the Sigma (St. Louis, MO, USA).

2.1. Harvest and preparation of hAM

The study protocol was approved by the Ethics Committee of Zanjan University of Medical Sciences, IR-ZUMS.REC.398.485.

The amnion membrane used in this study was obtained from the hospital of Ayatollah Mousavi (Zanjan, Iran) in completely sterile conditions, after a written consent from a 35-year-old woman undergoing cesarean following the similar criteria for donation reported by Chávez-García [21]. The amnion membrane was transferred to Research Institute of Animal Embryo Technology of Shahrekord University in a sterile container which was filled with phosphate buffered saline (PBS), and immediately after the sample arrived, preparation and isolation of MSCs were performed on it.

2.2. Isolation and culture of hAMSCs

During the cesarean section, the amniotic membrane was separated from the other membranes of placenta by the surgeon. It was sent to the laboratory under sterile conditions. The amniotic membrane is a very thin and transparent membrane. A slice of amniotic membrane was removed by sterile scissors and placed in a Petri dish and washed twice with PBS (PH=7.2) to remove torn pieces, blood clots, and cellular debris. The separated slice was first mechanically chopped into disintegrating pieces by a sterile surgical blade and then transferred into a 15 mL falcon conical tube containing 5 mL pre-warm digestion medium (DMEM-LG

containing 1 mg/mL collagenase IV (Gibco), 1 mg/mL trypsin, 50 IU/mL DNase I, 100 IU/mL penicillin, and 100 µg/mL streptomycin) for enzymatic digestion by incubation at 30 °C for 35 min with gentle shaking. After enzymatic digestion, the falcon conical tube was centrifuged for 1 min the least turn per minute, and the supernatant was transferred to another tube and centrifuged again at 400g for 10min. The collected cells were cultured in DMEM-LG supplemented with 20% FBS and incubated at 37 °C in a humidified atmosphere with 5% CO₂. After 7 days of culture, non-adherent cells were removed and the medium was refreshed every 2 days up to 80-90% confluency. At desired confluency, the cells were isolated by incubation in 0.25% Trypsin/EDTA for 10 min and were then plated at 2×10^4 cells/mL.

2.3. *In vitro* differentiation

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

2.4. A. Adipogenic differentiation

After culture of hAMSCs until 80% confluency, adipogenic differentiation medium (Bioidea, Iran, Cat.No: B1-1101) containing DMEM-LG supplemented with dexamethasone, IBMX (3-isobutyl-1-methyl xanthine), insulin and 10% FBS was added and Cultured for 3 weeks with change of media every three days. To confirm the differentiation of mesenchymal stem cells into adipocytes, cells were stained with 0.5% Oil Red O dye following the manufacturer's instructions.

2.5. B. Osteogenic differentiation

Osteogenesis was induced in 80% confluent cultured hAMSCs using osteogenic differentiation medium

(Bioidea, Iran, Cat.No: B1-1102) including DMEM-LG containing dexamethasone, beta-glycerol phosphate, sodium pyruvate, ascorbic acid and 10% FBS, for 3 weeks. This culture medium was changed every 3 days. To evaluate osteogenesis, the presence of calcium deposits in the cultured cells was determined using 2% alizarin red stain according to a standard protocol.

2.6. hAMSCs immuno-phenotyping using flow cytometry

The purity and phenotyping characteristics of freshly isolated hAMSCs from passage 2 were determined using flow cytometry. The cells (4×10^5) were stained with different antibodies containing conjugated Anti-Human-CD105-PE, Anti-Human-CD90-FITC, Anti-Human-CD73-PERCP, Anti-Human-CD45 and Anti-Human-CD34-PE was prepared from eBiosciences, Exbio, Biolegend, BD Bioscience and Exbio, respectively, or conjugated- isotype control matched-isotype control IgG at 4 °C for 45 min. To prepare stem cells for flow cytometry, 50 μ l of the cell suspension was incubated with 5 μ l of fluorescein isothiocyanate (FITC), Peridinin-chlorophyll protein complex (PERCP) or phycoerythrin (PE)-conjugated monoclonal antibodies specific for human markers for 30 min 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 (BD FACs Calibur, BD biosciences, San Jose, CA, USA). The data was acquired using a FACSCalibur system (Becton–Dickinson, CA) and analyzed using CellQuest software (Becton–Dickinson, CA).

3. Results

In this study, Mesenchymal stem cells were successfully isolated and cultured from the human amniotic membrane. The observations showed early cells that were seen small, spherical and floating in the culture medium, and began to adhere to the bottom of the flask after two days of culture. A uniform spindle-like population of mesenchymal stem cells similar to fibroblast cells was observed after about 5-6 days of culture. These cells were appeared on the bottom of the flask and reached 80% confluency during 14 days of culture (Figure 1A). With each change of medium and passage, the purity of the spindle cells (AMSCs) increased. Passage of cells up to sixth passage was performed to evaluate stability of cells and no change in cell shape was observed (Figure 1C).



Figure 1. Human amnion membrane-derived mesenchymal stem cells (hAMSCs); A, on day 14 (initial cultivation); B, on passage 1; C, on passage 6

3.1. Flow cytometry

The results of flow cytometer analysis showed that the expression levels of

hematopoietic markers CD₃₄ and CD₄₅ were negative and expression levels of CD₁₀₅, CD₉₀, CD₇₃ markers were positive (Dominici et al., 2006) with highly

expression in hAMSCs (Figure 2). Therefore, these markers could be used to confirm hAMSCs.

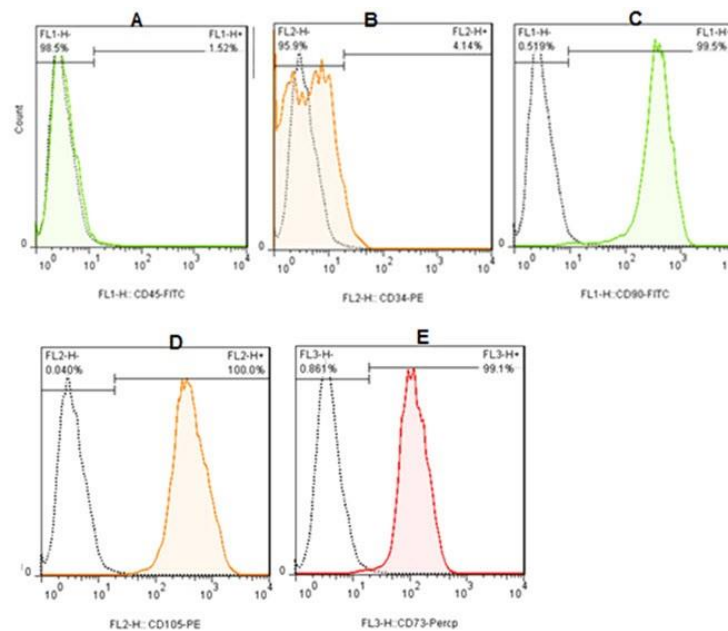


Figure 2. Flow cytometric histogram of immuno-phenotype of hAMSCs; A and B, more than 98.5% and 95.5% of cells were negative for CD₄₅ and CD₃₄ surface markers, respectively, and did not express these markers

3.2. *In vitro* 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 3A).

After three weeks of osteogenic induction, precipitation of calcium in the cells and the formation of mineralization in their matrix were examined. Staining with alizarin red demonstrated osteogenesis in hAMSCs (Figure 3B).

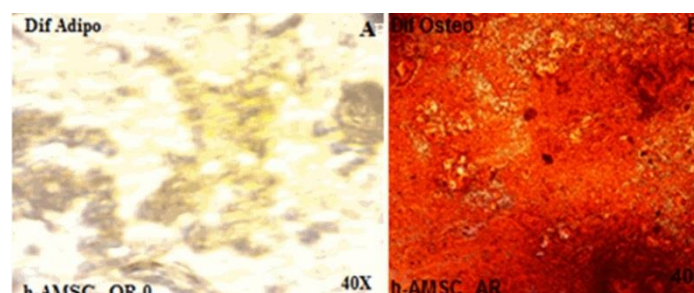


Figure 3. Differentiation of hAMSCs into adipose and bone cells; A shows fat clones after staining with oil red; B shows calcium deposits after staining with alizarin red

4. Discussion

hAMSCs are a type of stem cells isolated from the amnion membrane of the placenta. In addition to stem cell-Like properties, which proposed hAMSCs as a

potential candidate for regenerative medicine and cell transplantation [2, 22, 23], it has been shown that they can be used as an immune-modulatory agent in treatment of diseases with immune pathophysiology.

In this study, we isolated mesenchymal stem cells from human amniotic membrane by simple mechanical shredding and one-step enzymatic digestion, which was easier, faster and cheaper than the methods used in other studies [3, 12, 24, 25], being with similar results of the separation and confirmation of the cells as mesenchymal stem cells by flow cytometry, and differentiation tests. Therefore, this study showed that it is not necessary to digest several stages of tissue to separate the stem cells from the amniotic membrane.

There is a little variability in the purity, yield and viability of hAMSCs isolation using previous protocols [26]. These variations might be related to several factors including the size and quality of the placenta, affected by duration of the time between the delivery and hAMSCs isolation, mother related parameters, e.g. age, delivery type, and gestational week, transportation condition such as cold chain, residual blood on the tissue in spite of extensive washing, type and concentration of the enzyme solution, and enzymatic digestion time [27]. However, in this study, despite the long transfer time of the sample to the laboratory (about 48 hours), no effect was observed on the rate of stem cell isolation, which could be due to the amniotic membrane being separated from the placenta at parturition. The time spent for isolation of mesenchymal cells in this study was about 1 hour; however, in other studies due to various stages involved in the process of isolation, the time spent was seemingly much more than 1 hours [24, 25].

In this study, the growth factors such as epidermal growth factor (EGF), basic-fibroblast growth factor (bFGF), etc. were not considered for culturing cells in different passages, and passage of cells continued without problem until the

sixth passage. While most studies have used at least one growth factor in their culture medium for culturing AMSCs from different sources.

The flow cytometry results from this study show that similar to other MSCs derived from other sources, hAMSCs expressed Cd105, Cd90 and Cd73 molecules at their cellular surface, while CD34 (marks primitive hematopoietic progenitors and endothelial cells) and CD45 (a pan-leukocyte marker) markers were not expressed [8, 14]. These results confirmed the findings of studies conducted by Razavi et al, Diaz-Prado et al, and Carmen Mihaela Miha et al regarding the expression of CD 90, CD73, and CD105 markers and non-expression of CD34 and CD45 markers [3, 12, 14, 28, 29]. Therefore, the results of flow cytometry showed that the method offered in this study can isolate AMSCs without any contamination to hematopoietic and endothelial cells.

Placental MSCs have been shown to be differentiated into chondrogenic, osteogenic, endothelial, hepatocytic, myogenic, and neurogenic lineages, with appreciable differences in differentiation capability depending on the different fetal sources, placenta, chorion, or amnion [12, 14, 30, 31]. In this study, it was confirmed that hAMSCs isolated by this protocol can be differentiated into osteocytic and adipocytic lineages, indicating the presence of mesenchymal progenitors.

In summary, by using the new modified protocol a high hAMSCs yield with high viability and purity was obtained, and the cells kept their proliferation ability until passage 6.

Conflict of interest

None of the authors have any conflict of interest to declare.

Consent for publications

All authors approved the final manuscript for publication.

Availability of data and material

Data are available on request from the authors.

Authors' contributions

In this study Hassan Nazari presented the design, supervised, and wrote the manuscript; Ali Shadmanesh carried out the experiment, wrote the original draft; Abolfazl Shirazi went over the presented design and supervised the experiment; Ebrahim Ahmadi carried out the experiment; and Naser Shams-Esfandabadi carried out the formal and statistical analysis.

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Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of Zanjan University of Medical Sciences, IR-ZUMS.REC.398.485.

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