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Isolation and Culture of Mesenchymal Stem Cells From Rabbit Scapular Subcutaneous Adipose Tissue and Their Ability to Differentiate Into Osteoblasts

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Abstract

Background: The objectives of this study were to separate and culture mesenchymal stem cells (MSCs) from adipose tissue, examine the expression of surface markers on these cells, and determine their ability to differentiate into osteoblasts in normal medium.
Objectives: The objectives of this study were to separate and culture mesenchymal stem cells (MSCs) from adipose tissue, examine the expression of surface markers on these cells, and determine their ability to differentiate into osteoblasts in normal medium.
Materials and Methods: Sterile adipose tissue was obtained from the scapular subcutaneous adipose tissue of two rabbits (average weight, 2.8 kg) for cultivation and differentiation by either liposuction with a blunt hallow tip cannula or by direct surgery. The morphology, differentiation, and expression of mesenchymal-specific surface markers of rabbit, such as CD90, CD45, CD73, CD44, and CD105, were examined in cells from the third passage by flow cytometry. The MSCs from adipose tissue were stained with a lentivirus genome for cell tracking. The differentiation of MSCs into osteoblasts was investigated using a specific histological stain, Alizarin red.
Results: The identity of adipose tissue cells was confirmed by oil-red O staining and examination under an optical microscope at both the initial stage and after differentiation into mesenchymal cells. The results demonstrated that cells derived from adipose tissue differentiated into mesenchymal cells. The nature of the mesenchymal cells was confirmed by the expression of specific surface markers, including CD90, CD45, CD44, CD73, and CD105, by flow cytometry. Finally, Alizarin red staining confirmed the differentiation of MSCs into osteoblasts.
Conclusions: Based on our findings, we conclude that the separation and reproduction of adipose tissue cells is an appropriate method for purification of MSCs in animal studies. Regarding the histomorphometric and flow cytometry analysis results, we demonstrated the differentiation ability of MSCs in normal medium and hope to employ these cells for the regeneration of damaged bone tissues in the future.

Keywords: Adipose Tissue, Mesenchymal Stem Cells, Differentiation, Surface Marker

1. Background

Bone loss can be the result of accidents or some diseases such as tumor, cancers, and skeletal or periodontal disease (1). Because the replacement of bone tissues is not an easy undertaking, the development of new strategies for bone regeneration is an international priority to allow the repair of disabilities resulting from skeletal injuries, particularly in the elderly (2). The current gold standard for bone regeneration is autologous bone transplantation, since the transplanted bone tissue has the same surface markers and the lowest immunogenicity (2, 3). However, in critical size defects, the current bone regeneration methods are insufficient to spontaneously heal the defects (4, 5).

Mesenchymal stem cells (MSCs) have high proliferative activity in culture media, are clonogenic, and maintain bone-formation ability even after several passages; these characteristics were demonstrated for the first time by Fridenstein (6, 7). MSCs have been shown to be important for improving hematopoiesis, bone regeneration, and the treatment of genetic diseases (8, 9).

Adipose tissue cells are an accessible source of MSCs that can be obtained with relative ease from any individual and can be stored as a personal stem cell bank. MSCs derived from adipose tissue could be a useful source of multipotent cells, which, in addition to their ability to differentiate into different categories of cells, including osteoblasts, chondroblasts, adipocytes, neurocytes, and myocytes, maintain the ability to divide for their entire

lifetime (4). In contrast to the cell division ability of other cell types, which declines over time, the cell division ability of MSCs usually increases as they age. These cells are considered a useful replacement cell source for cell therapy of severe injuries. The advantages of stem cells derived from adipose tissue over other stem cells, such as bone marrow-derived stem cells (BMDSCs), periodontal ligament stem cells (PDLSCs), and dental pulp stem cells (DPSCs), are that they include a greater amount of tissue, are easier to access, and can be obtained by using less invasive procedures.

Cellular and molecular biology, tissue engineering and scaffold design, cell conducting, and cell signaling applications face serious challenges, our objectives are to produce better stem cells that are a better match to target tissues, are derived from easier to access tissues, at lower cost, and are easier to maintain, with a goal to better serve clinical departments.

Another good source of stem cells is the umbilical cord; however, there are some limitations in using these cells, including and the special conditions required for their long-term storage. In contrast, stem cells derived from adipose tissues do not have such limitations (10). These stem cells have high cell division ability and can be found in various tissues and organs, such as adipose tissues, blood, umbilical cord, lung, liver, skin, periosteum, and skeletal muscle.

MSCs have the ability to differentiate into osteoblasts, which have the required osteogenic potential to restore and regenerate lost bone (11). The processes required to obtain these cells and induce their differentiation are relatively easy and inexpensive. In addition, these multipotent cells can be saved as a bank of personal cells for use in emergency cases.

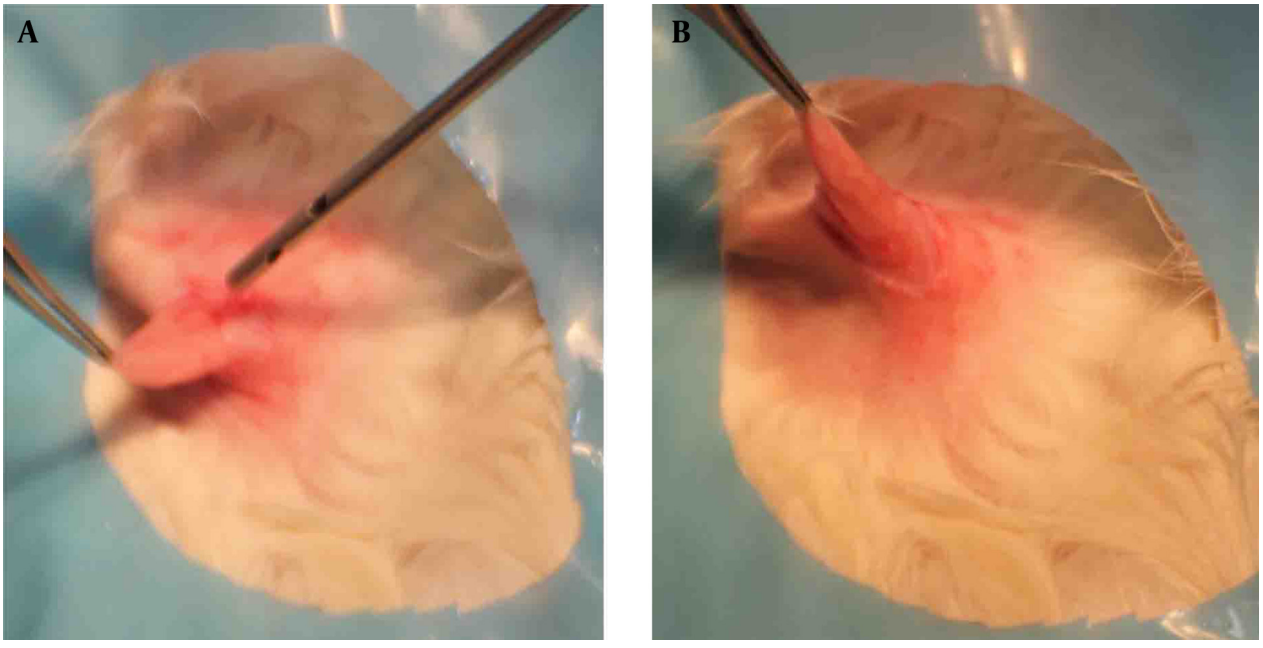
2. Objectives

The aim of our study was to isolate and culture MSCs from adipose tissue and evaluate their ability to differentiate into osteoblasts in normal medium by using various stains and optical microscopic examination, and to investigate the expression of specific mesenchymal surface CD markers on these cells.

3. Materials and Methods

Two rabbits (average weight, 2.8 kg) were selected and anesthetized by intraperitoneal injection of 40 - 50 mg/kg 10% ketamine (Alfasen, The Netherlands) and 5 - 7 mg/kg 2% xylazin. Then, after shaving the nape area and making a small incision, the required amount of tissue was removed from the scapular subcutaneous adipose tissue either by direct surgery or by using a hollow tip cannula that was connected to strong suction and a collector terminal. The tissue was then placed in PBS solution and transferred to the cultivation room (Figure 1).

Figure 1. Two Methods for Obtaining Adipose Tissue



A, using hallow tip cannula; B, direct surgery.

3.1. Culture of Rabbit Adipose Tissue Cells

3.1.1. Sample Transportation

Adipose tissue samples were transported from the operating room to the cultivation room in sterile tubes containing a 4 × antibiotic cocktail or PBS + Pen Strep. The tubes were then opened and emptied onto a plate under a laminar flow hood.

3.1.2. Plates and Procedures

In the cultivation room, various plates were used for cell cultivation in the following sequence: the first and second plates contained 4 × antibiotics, the third plate contained 1 × antibiotic, and the fourth plate contained DMEM-HIGH Glu.

On the first plate, we removed the unwanted tissue from the adipose sample and repeated this procedure on plates two and three. Then, finally, on the fourth plate, we cut the adipose tissue into 1 - 3-mm slices. The sliced adipose tissue was placed into 15-mL Falcon tubes containing 2 - 3 mL of type 1 collagenase mixture, and the tubes were incubated at 37°C for one hour. After one hour, the contents of the 15-mL Falcon tube were emptied onto a plate, and the tissue was sliced into small pieces (not so small that the cells would be damaged), and then the sliced tissue was finally transferred into a T25 flask containing 5 mL of culture medium (DMEM-HIGH Glc + 15% FBS + 2 - 4 mM L-Glutamine (Gibco, USA; CN: 2530-08), and incubated for 48 hours at 37°C.

After two days, the entire contents of the flask were centrifuged at 1800 rpm for 7 minutes (Eppendorf). Then, the adipose tissue with the digested texture, which is the topmost surface layer, was removed with a pipette and transferred into a T25 flask with new medium. The remaining content of the Falcon tube was removed by pipetting until approximately 1 mL was left, which was the cellular precipitate. Then, we mixed the cellular suspension and added it to the flask. The medium was replaced every other day so that the cells became confluent on the flask bottom. These cells were then passaged so that they had enough space for growth and proliferation.

Sliced adipose tissues will be put into a 15 mL falcon containing 2 to 3 mL collagenase type I and put into an incubator at 37°C for an hour. After one hour, all the contents of the 15 mL falcon have to be emptied into a plate and re-sliced into parts, not very small slices so the cells would not be damaged, and finally into a T25 flask containing 5 mL of culture media of DMEM-HIGH Glc + FBS 15% + L-Glutamine (Gibco, USA CN: 2530-08) 2 - 4 mM, and put into the incubator for 48 hours at 37°C. After two days, the entire contents of the flask have to be centrifuged with Eppendorf machine for 7 minutes, 1800 rpm. After that, the adipose digested texture which is the topmost surface layer is moved by a pipette into the previous flask but in a new media. All the rest of the content of the falcon is thrown away by a pipette till one mL left to the cellular precipitate. Then we will mix the cellular suspension well to be added to the flask. After this, we will replace the flask media every other day so the cells growth reaches a level to confluent the flask bottom. These cells are then passaged until they have enough space for growth and proliferation.

3.1.3. Cellular Passage of a Single T25 Flask Into Two T25 Flasks

The objective was to divide the contents of a single flask into two flasks, the original flask and another flask of the same size. To do this, we removed and discarded the supernatant from the primary flask, washed the cells in the flasks twice with 5 mL of PBS to remove the FBS, and then removed the wash with a pipette as FBS neutralizes trypsin. Then, we added 1 mL of Trypsin/EDTA (Gibco, USA; CN: 2530-081) and incubated the flask at 37°C for three minutes. We then immediately examined the contents of the flask under a microscope to confirm that the cells detached from the flask bottom. Adherent cells were released by tapping the flask walls and then suspended. To neutralize the trypsin, 5 mL of complete medium was added, and 2.5 mL of the call suspension was transferred to a new flask containing 2 mL of complete medium, and then 2 mL of complete medium was added to the first flask.

3.1.4. Cellular Passage of a T25 Flask Into a T75 Flask

To passage cells from a T25 flask into a T75 flask, we removed the supernatant from the primary (T25) flask, washed the flask twice with 5 mL of PBS, and then remove the wash with a pipette. Then, we added 1 mL of Trypsin/EDTA and incubated the flask at 37°C for 3 minutes. We then immediately examined the contents of the flask under a microscope to confirm that the cells were detached from the flask bottom. To neutralize the trypsin, 5 mL of complete medium was added, and the entire contents were quickly transferred to a new T75 flask containing 2 mL of complete medium. Since the cells might not have detached from the walls of the flask, we added 5 mL of complete medium to the primary flask to promote growth.

3.2. Differentiation of Adipose Mesenchymal Cells Into Osteoblasts

To promote the differentiation of adipose-derived MSCs (AD-MSCs) into osteoblasts, after the third passage, we added the following additives to the growth medium and replaced the medium every 48 hours for 21 days:

1- 50 μM ascorbate-2 phosphate (Sigma Aldrich, UK; PC: 289.54)

2- 10 mM β-glycerophosphate (Sigma Aldrich, UK; PC: 01203.4)

3- 0.1 μM dexamethasone (Gibco, USA; CN: Du908)

Fifteen to sixteen days later, the accumulation of mineralized calcium phosphate was observable in the cells, which was stained with Alizarin red.

3.3. Steps for Tracing Adipose Mesenchymal Cells with the Lentivirus Genome: Cell Tracking

Lentivirus is a retrovirus consisting of one or two copies of positive single-stranded RNA within a protein pyramidal head surrounded by a phospholipid cover (12).

The green florescent protein (GFP) solution is a mixture containing the virus, j-Red, which can be seen as red under the microscope, and a puromycin resistance marker , which allows the host cells to survive in medium containing puromycin longer than cells that are not resistant. Finally, GFP, which appears as green-colored cells under the dark field microscope.

For cell tracking in medium containing parenchymal adipose tissue with AD-MSCs, which reached about 100000 cells in Plate 6, we added two-thirds volume viral soup and one-third volume of new medium. For higher concentrations of virus-stained cells, polybrene, which was stored at −20°C, and was brought to 37°C in a water bath was added to the GFP solution 10 minutes before adding the viral soup to the AD-MSCs, and then, two-thirds volume of the soup and one-third volume of new medium were added. The 6-well plate was placed on a shaker and in an incubator for 8 - 12 hours. At this stage, approximately 20 - 25% of the cells would be coated. If a higher cellular concentration was preferred, 48 hours after removing the cells from the shaker, we added 2 μg/mL puromycin to the medium. Cells that received the puromycin resistance factor will survive, whereas those not coated with the virus will die. The whole process of staining cells takes 48 to 72 hours, during which, the medium was replaced every 12 h. If at the end of this process the adipose tissue cells did not appear to be adequately coated, the polybrene cycle was repeated. Then, the resulting cells can be differentiated or directly used in cell therapy.

3.4. Alizarin Red Staining

Alizarin Red staining, which stains osteoblasts and calcified cells, was incubated with differentiated MSCs. The staining process is shown below:

Cells were placed in basic culture medium plus 0.2 mol of L-ascorbic acid, 10 mMol of β-glycerophosphate, and 8 - 10 mol of dexamethasone, three additives that promote the differentiation of MSCs into osteoblasts. The cells were then washed once with PBS and fixed in phosphate-buffered formalin (FBS) for 20 minutes.

The fixed cells were washed once with distilled water and then stained with 1% Alizarin red (Sigma Aldrich UK) dissolved in distilled water for five minutes. The remaining stain was washed away twice with distilled water. Finally, the cells were air-dried and the stained cell image was obtained with an optical microscope and analysis software (Leica Microsystems GmbH Wetzlar, Germany).

3.5. Flow Cytometry Analysis

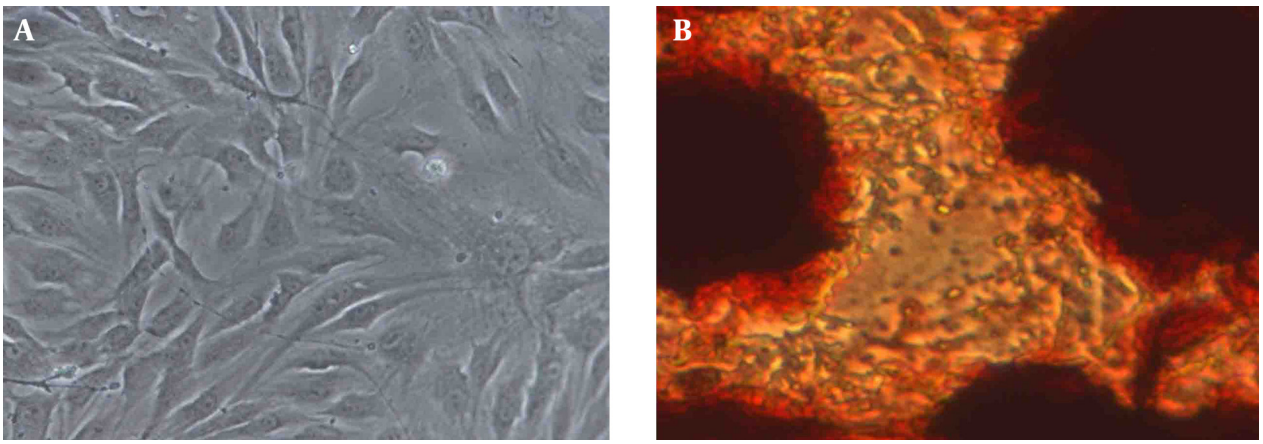
For the flow cytometry analysis, conjugated antibodies against CD45, CD44, CD73, CD90, and CD29 as well as a negative control (Dako corporation, Glostrup, Denmark) were used. For the analysis, isolated cells from the third passage were used. First, cells attached to the bottom of the flask were detached by Trypsin/EDTA, and then the cells were counted with the aid of Neubauer lam and Trypan blue solution.

At the next stage, in a dark environment, an appropriate concentration of the above-mentioned antibodies (1:10 dilution) along with the negative control was added, and then incubated at room temperature for 20 minutes. Next, the cells were washed with PBS and analyzed by flow cytometry using the fluorescein isothiocyanate antibody (FITC) method and a FACSCalibur (Becton Dickinson).

4. Results

Primary adipose tissue was stained with oil-red O. The mesenchymal cells were examined under an optical microscope (Figure 2).

Figure 2. Adipose-Derived Mesenchymal Stem Cells Observation



A, 100× optical microscope; B, Oil Red-O staining of adipose tissue samples.

The results of the flow cytometry analysis are shown in Table 1. The mesenchymal nature of the cells was confirmed by the presence of mesenchymal cell-specific surface markers (Figure 3).

For cell tracking, the AD-MSCs were stained with a lentivirus containing GFP prior to differentiation, and were tracked by immunofluorescence microscopy so that we could assess the cells before use in the in vivo applications (Figure 4).

Specific histological staining with Alizarin red confirmed that the AD-MSCs had differentiated into osteoblasts. Twenty-one days after the induction of the osteoblasts, secretion of mineralized matrix was demonstrated by Alizarin red staining (Figure 5).

Table 1. Flow Cytometry Analysis Results

Marker	Positive, %
CD44-FITC	96.7
CD45-PE	0.29
CD73-PE	65.7
CD90-PE	99.4
CD105-PE	99.8

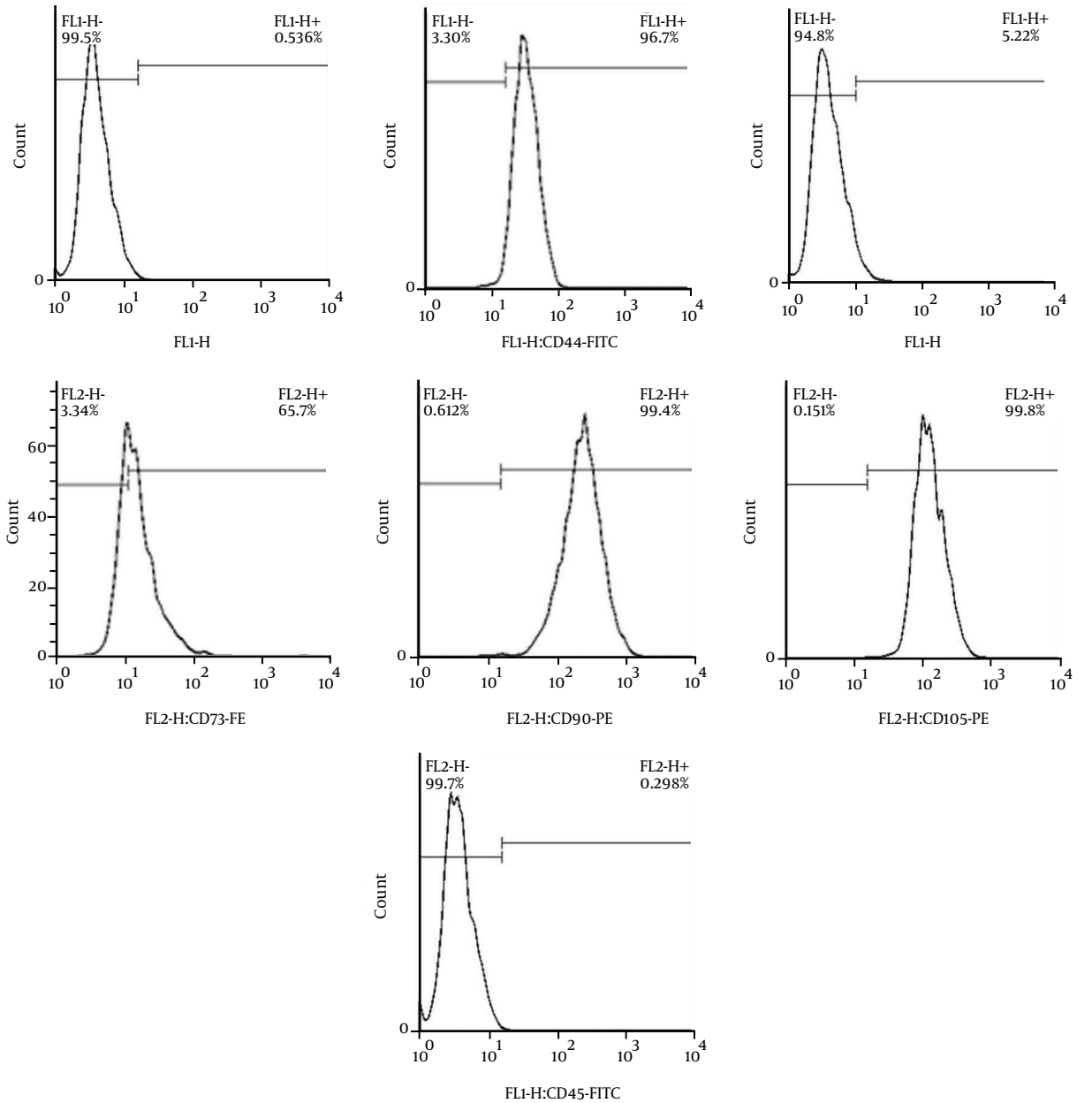


Figure 3. Flow Cytometry Analysis of Mesenchymal Cell Markers on Adipose-Derived MSCs

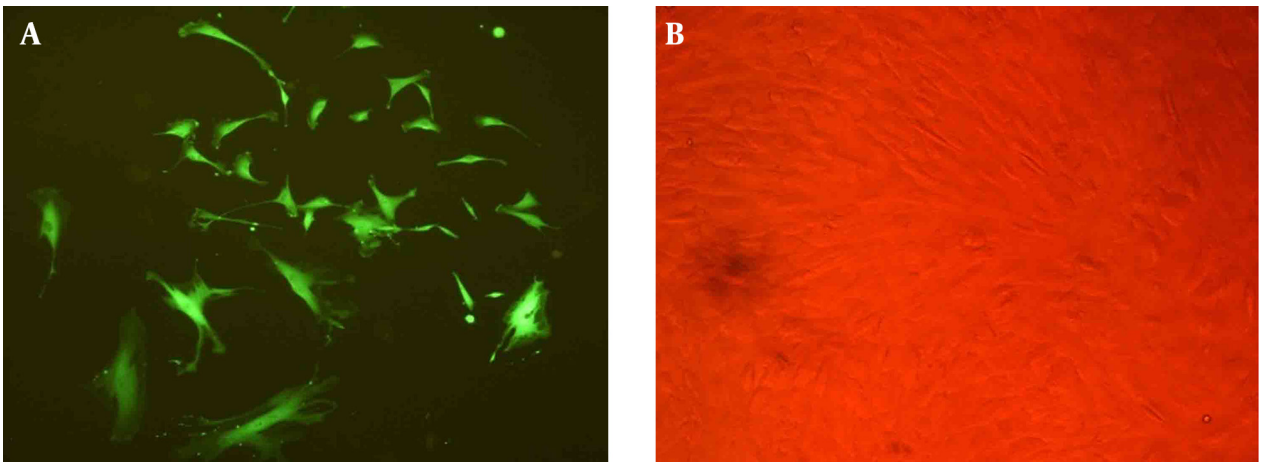
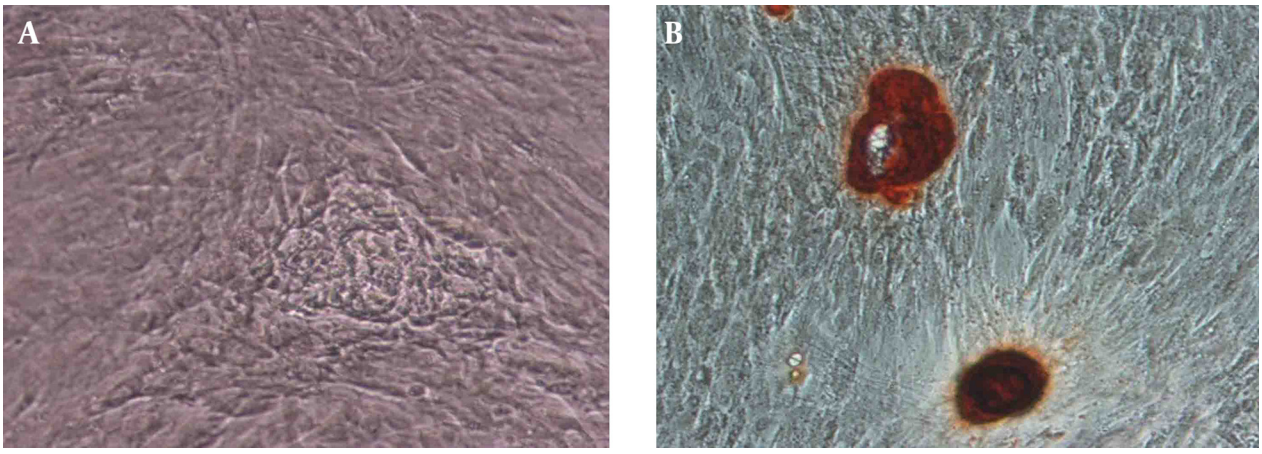


Figure 4. Immunofluorescence Microscope Images of Mesenchymal Cells Stained with Lentivirus

Figure 5. Optical Microscopy Images of MSCs Differentiated into Osteoblasts



A, Unstained; B, Alizarin red-stained osteoblasts.

5. Discussion

Obesity levels and life expectancy of human communities have increased owing to the increased welfare and decreased physical activities. In addition, the amount of adipose tissue extracted by liposuction and cosmetic surgeries is also increasing. These three sources of adipose tissue, which have a suitable amount of multipotent cells, have attracted the attention of experts, who propose to use this tissue, which is usually thrown away, for the direct repair of lost adipose tissue in other parts of the body and/or for storage in cell banks and then differentiating them into other cell types for clinical use in the repair of damaged tissues. Therefore, not only did the current study demonstrate the isolation and culture of MSCs from adipose tissue obtained from scapular subcutaneous adipose tissue (SCI) of rabbits, but it also showed the ability of these MSCs to differentiate into osteoblasts in normal medium. Tholpady et al. reported the differentiation ability of rat visceral fat-derived MSCs into osteoblasts after four weeks using both reverse transcriptase-polymerase chain reaction (RT-PCR) and von Kossa stain (13). However, in the present study, we demonstrated the ability of rabbit scapular subcutaneous AD-MSCs to differentiate into osteoblasts after three weeks using specific rabbit CD markers detected by flow cytometry analysis, cell tracking of MSCs by lentivirus genome staining, and mineralized matrix formation by Alizarin red staining.

As previously mentioned, adipose tissue contains many stromal stem cells, and obtaining them in large quantities is an easy task. Therefore, adipose tissue is an excellent source of stromal stem cells. Since Zuk et al. demonstrated stem cells in adipose tissue for the first time, and their potential as mesenchymal stem cells became known, new horizons in tissue regeneration using differentiated cells derived from this tissue have been opened

(14). Zuk et al. were the first to derive human stem cells from adipose tissue, and they examined the cells and described their characteristics. Friedenstein et al. also showed that, in the presence of appropriate media, these cells are able to differentiate into adipogenic, bone, cartilage, and myogenic lines (6, 7). Since then, various initiatives to regenerate lost tissue using stem cells from adipose tissue have been undertaken. This study is just one along the trail of converting mesenchymal cells derived from adipose tissue into osteoblasts (15).

Surgeons use different methods to extract and prepare adipose tissue in the operating room. Liposuction can be done by using standard techniques or vibrational techniques. It has been reported that liposuction using vibrational techniques is more affective due to a reduction in operation time and an increase in the amount of adipose tissue obtained (16).

The objective of the study by Zaminy et al. which was conducted in Iran, was to compare the effect of melatonin on the differentiation of bone marrow-derived and adipose-derived stem cells (17). The number of apoptotic cells was greater in the adipose-derived stem cells than in the bone marrow-derived stem cells. Flow cytometry analysis showed that the reduction in cell growth is due to a reduced number of cells in S phase of the cell cycle. The MMT assay showed that the number of viable cells was lower in the adipose-derived stem cells than in the bone marrow-derived stem cells. In the present study, the AD-MSCs had not lost their ability to proliferate up to the ninth passage.

Recently, several studies reported the differentiation ability of adipose tissue stem cells by using special structures or stimulating differentiation by various methods, for example, bone morphogenetic proteins (BMPs); however, in the present study, we evaluated the pure differentiation of MSCs into osteoblasts without using any specific structure or other stimulating factor for use in bone regeneration studies. For example, in a study by Chai et al. the objective was to determine the role of BMP-9 in the osteoblastic differentiation of MSCs derived from adipose tissue (18). In the study, adipose tissues of rabbits were obtained from the subcutaneous abdominal region and inguinal area, and the obtained cells were then subjected to in vitro cultivation and proliferation. The results showed that BMPs can affect the differentiation pathway of stem cells derived from adipose tissue. Chain reaction of polymer plays an important role in the BMP signaling pathway in bone differentiation. In the present study, no additional material or BMP was used, except the three differentiation ingredients in the differentiation cocktail. The adipocyte-derived mesenchymal cells differentiated into osteoblasts very well, as confirmed by Alizarin red staining.

In the study by Hosseinkhani et al. which was conducted in Japan, the osteogenic differentiation of MSCs into a nanofiber structure was demonstrated, which was also shown in the present study, even though in our study, the MSCs were differentiated into osteoblasts in normal medium without any specific structures (19).

This study successfully confirmed the presence of AD-MSCs by demonstrating the expression of rabbit-specific CD markers by flow cytometry, which can be a starting point for future studies. The results showed that adipose tissue contains a high percentage of MSCs that are visible in the third passage under an optical microscope and express surface markers that can be detected by flow cytometric analysis.

In present study, the levels of CD44, CD73, CD45, and CD90 were 96.7%, 65.7%, 99.7%, and 99.4%, respectively, which represents optimal expression in MSCs. In the next part of the study, these MSCs were successfully differentiated into osteoblasts, as confirmed by Alizarin red staining.

Based on our results, we concluded that adipose tissue is a common, accessible source of MSCs. We confirmed the ability of MSCs to differentiate into bone cells without a specific structure or well-known bone regeneration-stimulating factors, and these cells, with potential applications in tissue engineering and regenerative medicine, can be used for restoring hard tissue defects in clinical cases.

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Footnote

Authors' Contribution: Study concept and design: Hassan Semyari, Rajipour; acquisition of data: Rajipour, Bastami, Hossein Semyari; analysis and interpretation of data: Rajipour, Bastami, Hossein Semyari; drafting of the manuscript: Rajipour, Bastami; critical revision of the manuscript for important intellectual content: Hassan Semyari, Rajipour, Bastami; administrative, technical, and material support: Hassan Semyari, Rajipour; study supervision: Hassan Semyari, Rajipour, Bastami.

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