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Isolation and Morphological Characterization of Ovine Adipose-Derived Mesenchymal Stem Cells in Scanning Electron Microscopy (SEM)

Research Article

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ABSTRACT

The main purpose of this study is to provide advanced insights into scanning electron microscopy (SEM) of adipose derived mesenchymal stem cells (oAD-MSCs). In this study after isolation and proliferation of AD-MSCs, their cell surface markers characterized using antibodies RT-PCR. SEM was used to study of ultra-structure of oAD-MSCs. Adipose tissue was obtained from tail fat of sheep. Surface markers (CD44, CD90, CD34, CD31) evaluated by RT-PCR. RT-PCR was done for CD44, CD90, CD34 and CD31 to identify of cells. Morphological characterization was done by inverted microscope and SEM. Cells was prepared by glutaraldehyde for first fixation and tetroxide osmium for second fixation and dehydration with different percent of ethanol. Finally, cells coated with gold and observed in SEM. Adipose derived mesenchymal stem cells (AD-MSCs) were isolated and proliferated. They were positive for CD44 and CD90 markers and negative for CD31 and Cd34 markers in RT-PCR technique. AD-MSCs showed a fibroblast-like, spindle-shaped morphology after they attached to the culture flasks observing in inverted microscope. Explanted specimens were imaged with scanning electron microscopy (SEM). SEM provides the main technology to visualize surface features. In SEM the outer surface of the mesenchymal cells could be observed; so, in this study, the pseudopods arising from each cell and extending through each other were clearly shown.

KEY WORDS adipose, mesenchymal stem cells, ovine, scanning electron microscopy.

INTRODUCTION

Mesenchymal stem cells (MSCs) were first identified in the pioneering studies of Baghaban Eslaminejad *et al.* (2007), who isolated bone-forming progenitor cells from rat marrow. They have the capacity to differentiate into different cells (Barry and Murphy, 2004). Adipose tissue-derived mesenchymal stem cells (ADMSCs) were first identified in the pioneering studies of Zuk *et al.* (2001). Although it is known that many tissues contain lineage-committed progenitor cells for tissue maintenance and repair, several studies have demonstrated the presence of uncommitted MSCs within the connective tissue matrices of several organs in birds, mice, rats, and rabbits. Furthermore, adipose tissue is derived from the embryonic mesoderm, like bone marrow, contains a heterogenous stromal cell population. These similarities, together with the identification of MSCs in several tissues, make plausible the concept that a stem cell population can be isolated from human adipose tissue. Therefore, in their study, they sought to determine if a population of multipotential stem cells could be isolated from human adipose tissue (Zuk *et al.* 2001). Adipose tissue-derived mesenchymal stem cells (AdMSCs) represent an attractive and ethical cell source for stem cell therapy. Another interesting characteristic of MSCs is their ability to mobilize to areas of tissue injury (Ra *et al.* 2011).

Neupane *et al*. (2008) for isolating and culturing of ADMSCs, collected adipose tissue from subcutaneous, omental, and inguinal fat depots of dogs, using standard surgical procedures. Neupane *et al*. (2008) weighed, and digested each adipose tissue sample overnight at 37 ˚C with collagenase type IA (1mg/mL) in D medium. Following centrifugation and washing of the pellet, cells were incubated (about 8 g of tissue/25 cm^2 flask) in D medium with 10% fetal bovine serum (FBS in incubator supplied with humidified air and 5% CO_2. Unattached cells were removed the next day by washing with phosphate buffered saline. Grzesiak *et al*. (2011) washed oAD-MSCs few times in solution and placed in digestion buffer, composed of 0.2% collagenase/0.25% trypsin dissolved in HBSS (sigma). Digestion process proceeded in humidified incubator, with 5% CO_2, 37 ˚C, for about forty minutes. Tubes with samples were shaked every five minutes during digestion. Next, tubes were centrifuged at 1.200 g for 10 minutes. After that, remaining undigested tissues were transferred to new buffer and digested again. Supernatants were discarded, and nucleated cells pellets, with some visible red blood cells (RBCs), were dissolved in DMEM/F12: Ham mixture with 10% FBS and 1% antibiotic/antimycotic solutions (sigma) and plated, every region separately, in T-25 culture flasks. Digestions were made three times. Culture vessels were properly signed and placed in 5% CO_2, 37oC, 100% humidity conditions for culture. After cells attached to surface, about 24 hours after, they were washed with HBSS with 2% FBS to remove RBCs and covered with fresh nutrient. Medium was changed two times a week, cultures were observed every day by inverted contrast-phase microscope. After adherent cells reached about 90% confluency, they were detached with EDTA/trypsin solution (sigma), counted in Thoma counting chamber and plated at 5 × 104 cells/cm2 in six well plate, with high glucose DMEM/10% FBS/1% antibiotic. Cells were passaged regularly before achieving full confluency.

MSC must be plastic-adherent when maintained in standard culture conditions. Second, MSC must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules (Dominci *et al*. 2006). Mesenchymal stem cells were recognized by immunophenotype using monoclonal antibodies specific for CD105 (endoglin), CD73, CD106 (VCAM-1), CD29, CD44, and CD90. In addition, they assessed the lack of endothelial cell (with anti- CD31 antibodies) and hematopoietic (with anti-CD45, anti-CD14, anti-CD11c, and anti-CD34 antibodies) marker expression (Rigotti *et al*. 2007).

The isolated cells expressed transcripts for markers CD29, CD73 and CD90, but failed to express the hematopoietic marker CD45 and expressed only low levels of CD105. The expression of CD34 was variable (Lyahyai *et al*. 2012).

AD-MSCs showed a fibroblast-like, spindle-shaped morphology after they attached to the culture flasks as Zuk *et al*. (2001) and Mehrabani *et al*. (2015) reported. The goal of the SEM is to scan a focused beam of primary electrons onto a sample, and to collect secondary electrons emitted from the sample to form an image. Modern SEMs involve 5 main components: An electron source (electron gun), Focusing and deflection optics (referred to as column), A specimen stage, A detection system, an image acquisition and control system.1-4 are contained within a vacuum system, 5 consists of a computer and a set of custom electronics. Three electron beam parameters determine sharpness, contrast, and depth of field of SEM images: Probe diameter – dp, Probe current – Ip, Probe convergence angle - αp. You must balance these three depending on your goals (Goldstein *et al*. 2018). Low magnification low voltage SEM enable stem cell biologists from all branches of Medical Sciences to bridge the gap in experimental approaches to understand cell shape, cell surface and attachment features, and is imperative before embarking on higher resolution technologies and further application (Joubert, 2010). Basically, by SEM, only the pseudopods arising from each cell and extending through each other were clearly shown in Ozen *et al*. (2013) study. Cell-to-cell interactions (signalization) play an important role in their production of cells within the flask, and it is believed that the cells communicate with each other; thus, monolayer confluence occurs (Ozen *et al*. 2013).

MATERIALS AND METHODS

Isolation and culture of adipose derived mesenchymal stem cells

The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee. Methods used to isolate MSCs from ovine adipose tissue were adapted to isolate MSCs from male lamb adipose tissue. This study carried out on six clinically healthy male lambs aged 3-6 months.

We used Grzesiak *et al*. (2011) methods with some modification. Briefly, adipose tissue was collected from tail fat depots of sheep, using standard surgical procedures. Each adipose tissue sample digested mechanically and then second digestion step performed 40min at 37 ˚C with 800λ collagenase type I (1mg/mL) (sigma) and 200λ trypsin 25% in DMEM medium. Following centrifugation and washing of the pellet, cells were cultured in culture medium (Dulbecco's Modified Eagle's Medium; DMEM; *bichrome*) supplemented with 5% fetal bovine serum (FBS; *Gibco-Thermo Fisher Scientific*, *Madrid*, *Spain*) and 1% penicil-

lin/streptomycin (P/S; *Gibco*) (5% FBS/DMEM) in incubator supplied with humidified air and 5% CO_2. Unattached cells were removed the next day by washing with phosphate buffered saline (sigma).

Identification of adipose derived mesenchymal stem cells

I: morphological characterization by inverted microscope

Inverted microscope was applied to see spindle-shaped morphology of AD-MSC after they attached to the culture flasks.

II: Realtime RT-PCR

Sampling and RNA extraction

In This study total RNA was isolated Abolghasemi *et al.* (2021) method with some modification from 1×10^6 undifferentiated oAD-MSCs during the 4th passages, isolation of total RNA was accomplished using Trizol Reagent. The desired sample was homogenized by adding 800 µL of Rouche Trizol solution. After complete homogenization of the cells, the solution was transferred to the microtube and the resulting solution was placed on ice for 10 min. Then about 300 µL of chloroform was added and allowed to stand on ice for 10 min. After wards, the tubes were gently shaken for 15 s. This process continued until the mixture was homogenized to full length and the 2 phases were mixed. It is not recommended at all to vortex the sample at this stage (this step is important for RNA extraction). The tube containing the sample was incubated again in ice for 15 min. Then samples were centrifuged at 13000 g for 15 min at 4 ˚C. The upper layer of 2 phases was transferred to a new RNase-free tube and 1 mL of cold isopropanol was added. The tube was slowly shaken and incubation was performed in the freezer for 45 min. The tube was centrifuged at 13000 g for 15 min at4 ˚C. The resulting RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at 8000 g for 10 min at 4 ˚C. The RNA pellet was air-dried and dissolved in 20 µL DEPC-treated water. Using dry bath, the precipitated RNA was placed at 56 ˚C for 10 min to dissolve in water. The purity of the extracted RNAs were analyzed by Nano Drop (Abolghasemi *et al.* 2021).

cDNA synthesis and real-time quantification of mRNA transcription

We had 10 µL ready vial and 0.5 µL oligo-dTand 0.5 µL random hexamer were added to synthesize complementary DNA (cDNA) from total RNA. So, we had 11 µL that was reached to 20 µL solution (RNA and water). 20 µL reaction mixture, carried out in a Thermal Cycler (PeQLab). The primers employed for the amplification reaction are listed in Table 1.

All samples were analyzed against glyceraldehydes-3-phosphatedehydrogenease (GAPDH) (housekeeping gene) as an internal control to confirm the progress of the RT reaction, and other pluripotency genes were confirmed with its specific primers.

RT-PCR with the Hyper Script RT master mix was carried out with 2 µL of the single-stranded cDNA sample for the confirmation of CD90 and CD44, CD34 and CD31 gene expression. The process proceeded with an initial denaturation at 94 ˚C for 10 min, PCR amplification was performed at 94 ˚C for 10 s, then, annealing temperature based on the type of primer (58 ˚C for CD90, CD34, CD31, CD44 and 59 ˚C for GAPDH) for 30 s, and 72 ˚C for 30 s at 45 cycles. Amplified PCR products were loaded and distinguished on a 1.5% agarose gel by electrophoresis and the bands were imaged by using dye and photographed with a gel doc.

SEM study of ovine adipose derived mesenchymal stem cells

At first for studying of Ultrastructure of oAD-MSC, medium from the dishes were decanted and washed with PBS, and the cells were fixed first time with freshly prepared 3% glutaraldehyde (Merck kGaA,8.20603.1000, Germany) in phosphate-buffered saline (PBS) (LOT 10650206, Germany) at 25 ˚C for 3minsand were transferred to 4 ˚C refrigerator for 30-40 mins. The dishes with fixed cells were washed twice with PBS and then placed in osmium tetroxide (R1015OsO_4) for 20 min for second fixation. Next, after washing in phosphate buffer (pH 7.4), cells were dehydrated.

Dehydration was carried out sequentially in the dishes with ethanol at concentrations of 30%, 50%, 70%, 80% and 90% for 5 min each, followed by an 90% ethanol then a 100% ethanol wash repeated three times. Cells were then dried in air and then stored at room temperature until SEM analysis was carried out. The surface of the samples was sputter-coated (EMI tech 550) in a vacuum with an electrically conductive 1Åthick layer of gold-palladium alloy with the Precession Etching Coating system. SEM images were then recorded with a scanning electron microscope at a lower voltage.

RESULTS AND DISCUSSION

Identification of oAD-MSCs was determined based on two ways: morphological characterization, and gene expression. First identification of AD-MSCs was determined on morphological characterization (Figure 1a, 1b, 1c). The gene expression was the most important way to identification of ovine cells. They were positive for CD44 and CD90 markers and negative for CD31 and Cd34 markers (Figure 2).

Table 1 Cell surface markers analyzed by RT-qPCR

Gene	Primer sequences	
	Forward (5'→ 3')	Reverse (5'→ 3')
CD90	CAGCAACTGGCTTCCATCCAC	GCCAAGACCGCTCCCCTTTT
CD34	CCTGCTGAGTCTGCTGCCTTC	GGACGTAGTTGTAGGGACAGG
CD31	ACGCAGAACTGATCGCCAAA	CAGAGCAGGAGTGTCAGCAGT
CD44	TGCGAACAACACAGGGGTTT	GGTGCCATCGCGGTTTACAAT
Housekeeping genes		
G6PDH	CAAGATCATCAGCAATGCCTCC	GCCATCACGCCACAGTTTCC

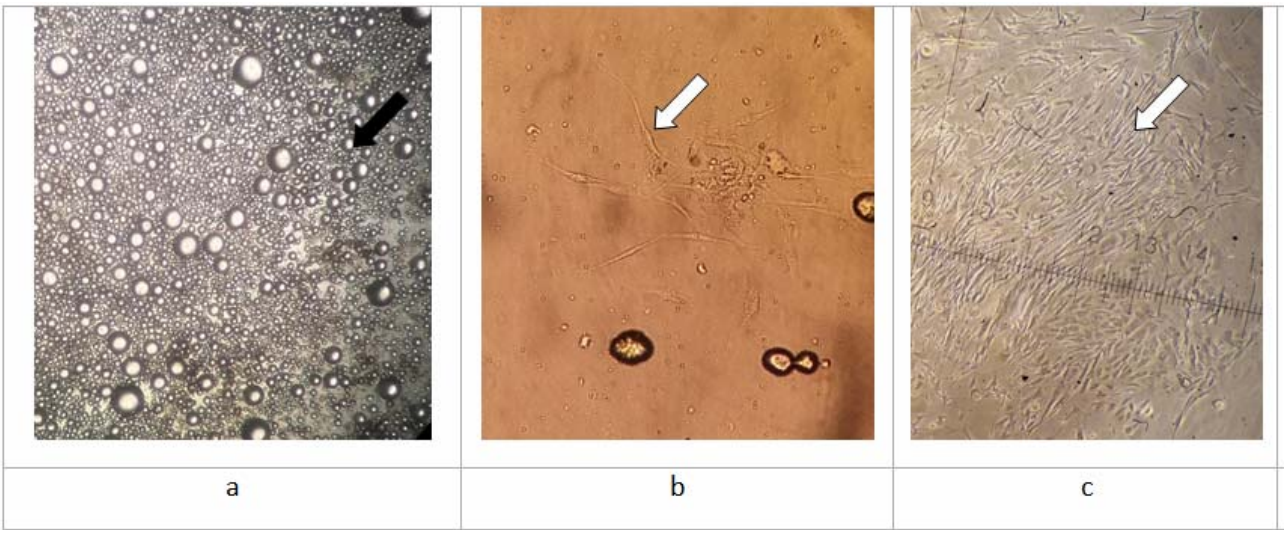

Figure 1 a: morphology of oAD-MSCs at primary culture after 1 hour (arrow shows fat drop.); b: cell culture after two days (arrow shows oAD-MSC) and c: spindle-shaped morphology of cells at passage 1 (arrow shows oAD-MSC)
The cells were studied with invert microscope

To characterize of oAD-MSCs, the expression of four cell surface markers specific for mesenchymal and hematopoietic cells were first analyzed at the transcript level by quantitative real time PCR (RTqPCR). All analyzed cultures were positive for CD90 (Thy-1) and CD44 (extracellular matrix receptor III), whereas analyzed cultures were negative for CD34 (mucosialin) and CD 31 (platelet endothelial cell adhesion molecule). We also did RT-PCR in this study and then did gel experiment to confident of non-specification. As it was shown the CD31 and CD34 band is lower than ladder band and less than 100 bp, so these markers are negative for oAD-MSCs.

Scanning electron microscopy (SEM) micrographs provide knowledge about the mineral structures put on the surface of MSCs, cell surface connections, and formation of cell surface structures. In this research, extensions of the cells through each other could be clearly visualized (Figure 3). By SEM, only the outer surface of the mesenchymal cells could be observed; thus, in this study, the micro processes sprouted by cell during adhesion and extending through each other were clearly shown.

Basically, by SEM, the pseudopods arising from each cell and extending through each other for Cell-to-cell interactions (signalization) were clearly shown.

AD-MSCs showed a fibroblast-like, spindle-shaped morphology after they attached to the culture flasks as Zuk *et al*. (2001) and Mehrabani *et al*. (2015) reported.

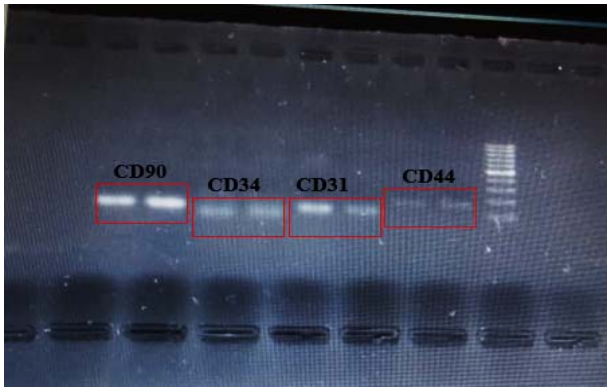

Figure 2a Cells were positive for CD44 and CD90 markers and negative for CD31 and Cd34 markers with RT-PCR technique

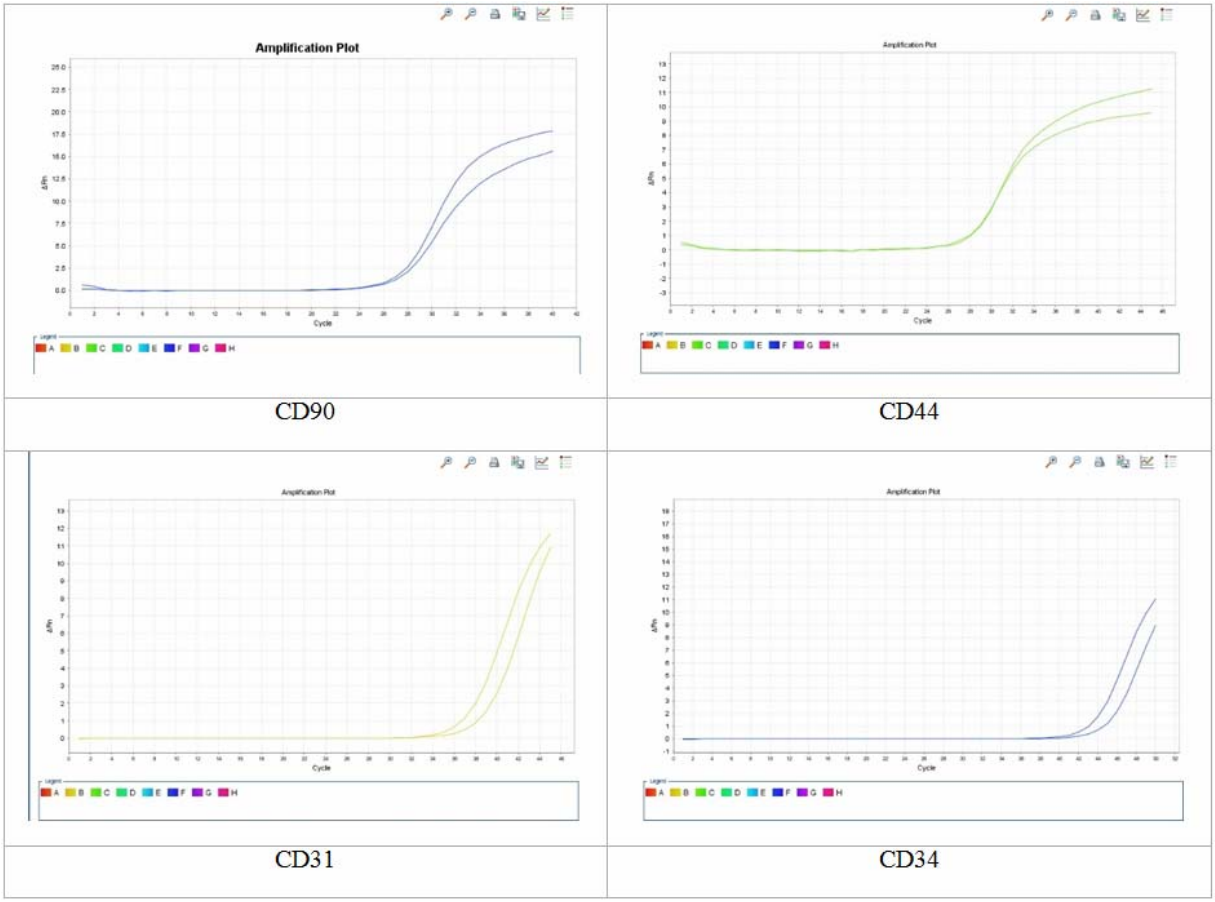

Figure 2b Cells were positive for CD44 and CD90 markers and negative for CD31 and Cd34 markers with Real time PCR technique

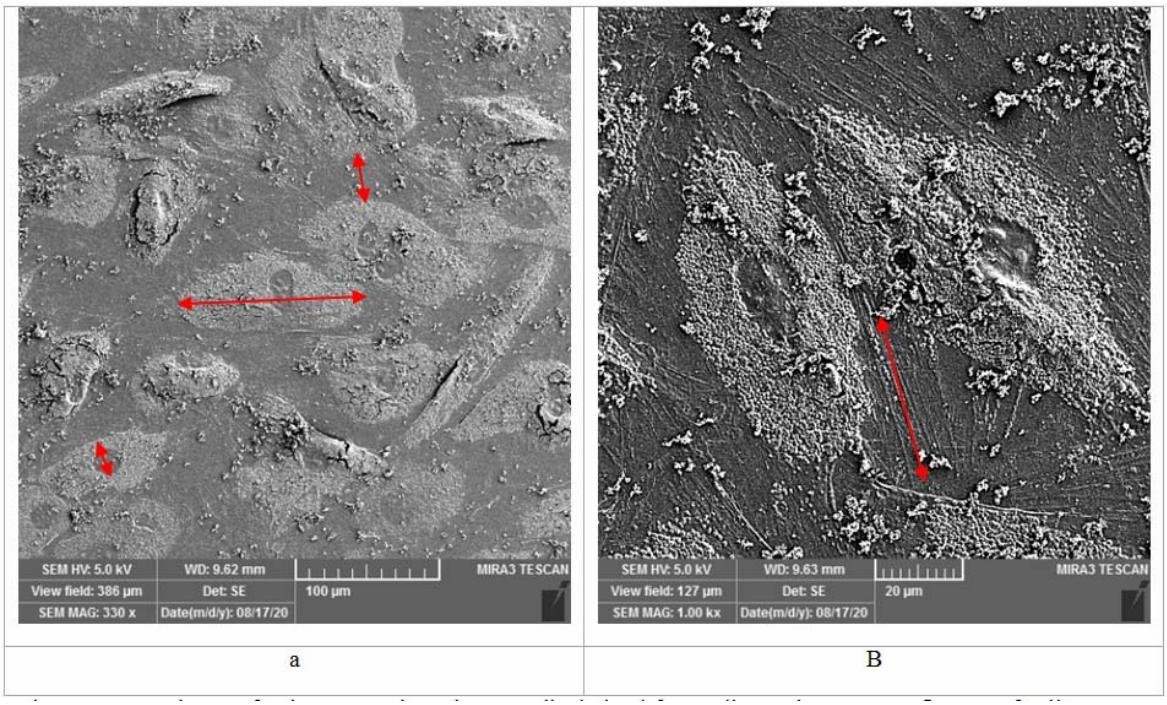

Figure 3 SEM photos of ovine mesenchymal stem cells derived from adipose tissue. a: confluency of cells was very high. From up to down respectively: distance between oAD-MSCs in culture, length of cells, nuclei diameter of cells and b: length of micro processes sprouted by cell during adhesion was visible clearly

In our study, plastic-adherent cells with a fibroblast-like morphology were obtained from all experimental sheep and were further analyzed to determine the expression of mesenchymal markers. Pham *et al*. (2013), Vahedi *et al*. (2016) reported CD90 and CD44 was positive for surface markers.

Previous studies suggested CD90 and CD44 as the most frequent markers for isolation of oAD-MSCs. AD-MSCs didn't express CD31 markers in study of Zuk *et al*. (2001), Zeng *et al*. (2013).

Recent studies indicated that the expression of CD34 was variable (Lyahyai *et al*. 2012). It is positive in study of Yoshimura *et al*. (2006) and didn't express in study of Zuk *et al*. (2001) and Pham *et al*. (2013). Vahedi *et al*. (2016) suggest that ASCs surface markers can be characterized by antihuman antibodies in sheep.

Moreover, most of the cell surface markers utilized to sort subpopulations of human MSC by flow cytometry have not been validated in sheep. Gene expression-based technologies may be useful for the identification of possible molecules described as MSC markers (Lyahyai *et al*. 2012).

Staining with antibodies against it didn't give effects equal with remaining markers. It could be caused by insufficient similarity between human and ovine CD90 aminoacids sequence (Grzesiak *et al*. 2011). So in this study we used RT-PCR.

In SEM, it was observed that small pseudopods were surrounding the oAD-MSCs. As in our study, it is believed and reported that small pseudopods visualized in MSCs allow the migration of cells within the tissue (Sarraf *et al*. 2011). Characterization of the microstructure of normal and differentiated stem cells and supplementation of the images obtained by phase-contrast and confocal immunofluorescent microscopy are possible through SEM analysis (Sathananthan and Nottola, 2007). Glutaraldehyde alone or in combination with other components such as formaldehyde, polyethyleneimine, lead acetate, malachite green, potassium dichromate, tannic acid, trinitro compounds, and uranyl acetate have been used for specimen preservation (Hayat, 1986). All the previous protocols used for SEM analysis have used osmium post fixation, as it is known to yield the best possible membrane preservation (Hayat, 1986; Danmark *et al*. 2010).

A number of previous studies have measured the interactions of electrons with organic matter at a variety of voltages. A compilation of results available from the literature is shown in Figure 3. For organic polymers, there is a general correlation that samples with higher thermal stability tend to be more resistant to electron beam damage. Most studies in the literature have been done at relatively high voltages (100-400 kV). However, recently data has been presented for beam damage at much lower voltages (0.2-1:0 kV) (Drummya *et al*. 2004).

We isolate and identify mesenchymal stem cells from adipose tissue using collagenase and trypsin and SEM. We offer using of AD-MSC because adipose tissue is an accessible, abundant, and reliable site for the isolation of adult stem cells suitable for tissue engineering and regenerative medicine applications. The MSCs comprise only a minor fraction of BM and other tissues, with bone marrow MSCs (BM-MSCs) constituting a mere 0.0001%–0.01% of all BM-nucleated cells. In contrast, adipose tissues contain 100.000 MSCs in each gram of fat. Further, the differential capacity of adipose tissue derived MSCs (AD-MSCs) is less affected by donor age. In this regard, the treatment efficacy of AD-MSCs for various diseases has been reported using animal models (Ra *et al*. 2011). We suggest that gene expression replace to flowcytometry in sheep studies because of rarely special ovine CD marker and high price of them.

CONCLUSION

Adipose derived mesenchymal stem cells (AD-MSCs) were isolated and proliferated. AD-MSCs were positive for CD90 and CD44 markers and were negative for CD31 and CD 34 in RT-PCR technique. AD-MSCs showed a fibroblast-like, spindle-shaped morphology after they attached to the culture flasks observing in inverted microscope. In SEM the outer surface of the mesenchymal cells could be observed. The pseudopods arising from each cell and extending through each other were clearly shown in this study.

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REFERENCES

Abolghasemi M., Poursaei E., Bornehdeli S., Shanehbandi D., Asadi M., Sadeghzadeh M. and Naghdi Sadeh R. (2021). Exploration of potential circulating micro-RNA as biomarker for Alzheimer's disease. *Meta Gene*. **30,** 1-6.

Baghaban Eslaminejad M.R., Taghiyar L, Kiani S. and Piryaee A. (2007). Subcutaneous transplantation of marrow–derived murine mesenchymal stem cells cultivated in alginate and their chondrogenesis. *Sci. J. Iranian Blood Transfus. Organ*. **4(2),** 105-114.

Barry F. and Murphy J.M. (2004). Mesenchymal stem cells: Clinical application and biological characterization. *J. Cell Biol*. **36,** 568-84.

Danmark S., Finne-Wistrand A., Wendel M., Arvidson K., Albertsson A.C. and Mustafa K. (2007). Osteogenic differentiation in rat bone marrow derived stromal cells on customized biodegradable polymer scaffolds. *J. Bioact. Compat. Polym*. **25,** 207-223.

Dominci M., Le Blanc K., Mueller I., Slaper-Cortenbach I., Marini F.C. and Krause D.S. (2006). Minimal criteria for defining multipotentmesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy*. **4,** 315-317.

Drummya L.F., Yangb J. and Martinc D.C. (2004). Low-voltage electron microscopy of polymer and organic molecular thin films. *Ultramicroscopy*. **99,** 247-256.

Goldstein J.I., Newbury D.E., Echlin P., Joy D.C., Romig A.D., Lyman C.E., Fiori C.E. and Lifshin E. (2018). Scanning Electron Microscopy and X-Ray Microanalysis. Plenum Press, New York.

Grzesiak J., Marycz K., Wrzeszcz K. and Czogała J. (2011). Isolation and morphological characterisation of ovine adipose-derived mesenchymal stem cells in culture. *Int. J. Stem Cells*. **4,** 99-104.

Hayat M.A. (1986). Glutaraldehyde: Role in electron microscopy. *Micron Microsc. Acta*. **17,** 115-135.

Joubert L.M. (2010). Scanning electron microscopy: Bridging the gap from stem cells to hydrogels. *Microsc. Microanal*. **16(2),** 596-597.

Lyahyai J., Mediano D.R., Ranera B., Sanz A., Remacha A.R., Bolea R, Zaragoza P., Rodellar C. and Martín-Burriel I. (2012). Isolation and characterization of ovine mesenchymal stem cells derived from peripheral blood. *BMC Vet. Res*. **8,** 169-175.

Mehrabani D., Hassanshahi M.A., Tamadon A., Zare S., Keshavarz S., Rahmanifar F., Dianatpour M., Khodabandeh Z., Razeghian Jahromi I., Tanideh N., Ramzi M., Aqababa H. and Kuhi-Hoseinabadi O. (2015). Adipose tissue-derived mesenchymal stem cells repair germinal cells of seminiferous tubules of busulfan-induced azoospermic rats. *J. Hum. Reprod. Sci*. **8(2),** 103-110.

Neupane M., Kiupel M. and Yuzbasiyan-Gurkan V. (2008). Isolation and characterization of canine adipose–derived mesenchymal stem cells. *Tissue Eng. Part A*. **14(6),** 1007-1014.

Ozen A., Sancak I.G., Tiryaki M., Ceylan A., Pinarli F.A. and Delibasi T. (2013). Mesenchymal stem cells (Mscs) in scanning electron microscopy (SEM). *Niche J*. **2,** 22-24.

Pham V.P., Bui K.H., Ngo D.Q., Vu N.B., Truong N.H., Phan N.L., Le D.M., Duong T.D., Nguyen T.D. and Le V.T. (2013). Activated platelet-rich plasma improves adipose-derived stem cell transplantation efficiency in injured articular cartilage. *Stem Cell Res. Ther*. **4,** 91-99.

Ra J.C., Shin I.S., Kim S.H., Kang S.K., Kang B.C., Lee H.Y., Kim Y.J., Jo J.Y., Yoon E.J., Choi H.J. and Kwon E. (2011). Safety of intravenous infusion of human adipose tissue-derived mesenchymal stem cells in animals and humans. *Stem Cells Dev*. **20 (8),** 1297-1309.

Rigotti G., Marchi A., Mirco G., Baroni G., Benati D., Krampera M., Pasini A. and Sbarbati A. (2007). Clinical treatment of radiotherapy tissue damage by lipoaspirate transplant: A healing process mediated by adipose-derived adult stem cells. *Plast. Reconst. Surg*. **119(5),** 1409-1422.

Sarraf C.E., Otto W.R. and Eastwood M. (2011). *In vitro* mesenchymal stem cell dif ferentiation after mechanical stimulation. Cell Prolif. **44,** 99-108.

Sathananthan A.H. and Nottola S.A. (2007). Stem Cell Assays, Humana, Totowa, New Jersey.

Vahedi P., Soleimanirad J., Roshangar L., Shafaei H., Jarolmasjed S.H. and Nozad Charoudeh H. (2016). Advantages of sheep infrapatellar fat pad adipose tissue derived stem cells in tissue engineering. *Adv. Pharm. Bull*. **6(1),** 105-110.

Yoshimura K., Shigeura T., Matsumoto D., Sato T., Takaki Y., Aiba-Kojima E., Sato K., Inoue K., Nagase T. and Koshima I. (2006). Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. *J. Cell. Physiol*. **208,** 64-76.

Zeng G., Lai K., Li J., Zou Y., Huang H., Liang J., Tang X., Wei J. and Zhang P. (2013). A rapid and efficient method for primary culture of human adipose-derived stem cells. *Organogenesis*. **9,** 287-295.

Zuk P.A., Zhu M., Mizuno H., Huang J., Futrell J.W. and Katz A.J. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng*. **7,** 211-228.