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Differentiation Potential and Culture Requirements of Mesenchymal Stem Cells from Ovine Bone Marrow for Tissue Regeneration Applications

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Abstract

Objectives- To isolate, culture-expand and differentiate mesenchymal stem cells from ovine bone marrow and determine their culture requirements for high expansion rate.
Design- Experimental study.
Animals- Five Shal sheep.
Procedures- In this study, ovine marrow cells were plated and culture expanded through 3 successive subcultures. The resultant cells were then plated at differentiating conditions into bone, cartilage and adipose cell lineages in order to verify their MSC nature. Furthermore, we determined the culture requirements of the cells in terms of FBS (fetal bovine serum) concentration and initiating cell seeding density. In this study, also population doubling time (PDT) was determined for the isolated cells.
Results- According to our observations, MSCs from ovine marrow appeared to be fibroblastic in appearance. They were easily able to differentiate into bone, cartilage and adipose cell lineages as it was evident in RT-PCR analysis of specific gene expression and specific staining of differentiated cells. According to our findings, the cells indicated extensive proliferation when being cultivated at 100 cells/cm^2 in a medium with 15% FBS. Ovine MSCs possessed a relatively short population doubling time (24.94±2.67 hours).
Conclusions and Clinical Relevance- Fibroblastic cells from ovine bone marrow are able to undergo extensive proliferation and capable of differencing into three skeletal lineages, hence they are MSCs that are appropriate for cell therapy experimentation.

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Introduction

Mesenchymal stem cells are a kind of adult stem cells that were first isolated from the bone marrow tissue. These cells have been described as the cells that can adhere on culture surfaces producing colonies consisting of several fibroblastic cells.[1] Plastic adherence is the property that has been used to isolate and culture expand MSCs from several other species.[2-6] Further investigation has revealed that MSCs possessed two fundamental properties as the capacity of extensive replication and the potential of differentiation into skeletal cell lineages. These characteristics along with their easy accessibility have rendered them as a suitable cellular candidate for clinical applications.[7-9] By date, the efficiency of the cells has been indicated in curing osteogenesis imperfecta, regenerating bone and cardiac muscle and resurfacing articular cartilage as well as restoring hematopoiesis in patients receiving chemotherapy.[10-16] Furthermore MSCs are appropriate cellular materials for tissue engineering applications as well.[17-21]
Despite to the established significance of MSCs in tissue regeneration, the safety of the cells for human applications has yet remained to be clarified. This subject needs more investigations especially in animal models. A large animal as sheep which is genetically more closely related to human is less being the subject of MSCs investigations. Most experiments employing sheep as animal model have directly transplanted isolated MSCs into defect site and have ignored to determine their in vitro characteristics. In a study by Chen et al MSCs from ovine bone marrow were loaded onto PLGA scaffolds and transplanted into joint cavity of the same animal to evaluate their chondrogenic differentiation.[22] In the other investigation, by Frosch et al in 2006, to regenerate the cartilage damage, MSCs isolated from ovine marrow were used to coat the titanium which implanted into surface lesions in articular cartilage of knee joint.[23] Rhodes et al have isolated MSCs from ovine bone marrow to evaluate their proliferation potential in vitro.[24] None of these studies considered MSCs in vitro differentiation potential into skeletal cell lineage as bone, cartilage and adipose cells. MSCs committee of international society for cell therapy has proposed that the cells in question can be termed as MSCs if they are able to undergo differentiation into bone, cartilage and adipose cell lineages.[25] In the present study, this subject was explored. In addition to examination of differentiation potential, culture requirements of MSCs isolated from the ovine marrow were investigated as well. Study like this, which deals with MSCs differentiation capacity as well as their culture needs, would help to prepare putative ovine MSCs in a sufficient number for conducting any experiment in the field of reconstructive and tissue replacement surgeries.

Materials and Methods

Bone marrow cell culture

Four sheep of Shal strains with about 12 months age were used in this study, before experiments; approval of animal use was obtained from the Royan institute ethic committee. The animals were kept in the facility of Royan institute (Karaj, Iran) till become acclimatized to housing and diet. Under general anesthesia by Ketamine (22 mg/kg) and xylazine (0.2 mg/kg) IM injection, bone marrow aspirate (about 10 ml) were drawn from the humerous, collected into 50 ml tube

containing 7500 unit heparin and shipped on the ice to cell culture facility of Royan Institute. Bone marrow aspirates were added with 5 ml DMEM medium (Dulbeccoo's modified eagle medium, sigma, USA) supplemented with 100 IU/ml penicillin (Sigma, USA), 100 IU/ml streptomycin (Sigma, USA), 10% FBS (Fetal Bovine Serum) and centrifuged at 300 g for 5 minutes. Supernatant was discarded, cell pellet resuspended in 1 ml DMEM medium and plated at 5×10^5 cells/cm^2 in 75-cm^2 culture flasks in an atmosphere of 5% CO2 and 37 °C. About 3-4 days after culture initiation, culture medium was replaced with fresh DMEM and the flasks were incubated with a medium replacement of 2 times a week until became confluent. At this stage, cultures were trypsinized and subcultured at 5×10^5 cells/cm^2. Cell passages were repeated up to subculture 3 when sufficient cell were available to continue the experiments.

Differentiation

To evaluate in vitro differentiation potential, passaged-3 cells were cultivated under osteogenic, chondrogenic and adipogenic conditions at the end of which the culture explored for the differentiation using specific staining as well as RT-PCR analysis.

Osteogenesis: Confluent passaged-3 cells in 6-well culture plates were used to induce bone differentiation. Osteogenic medium was consisted of DMEM supplemented with 50 mg/ml ascorbic 2-phosphate (Sigma, USA), 10 nM dexamethasone (Sigma, USA) and 10 mM ß-glycerole phosphate (Sigma, USA). The cultures were incubated at 37 C° and 5% CO2 for 21 days with the medium replacement of three times a week. Alizarin red staining was used to detect whether or not the mineralized matrix was formed in the cultures. For staining, the cultures were first fixed by methanol for 10 minutes, then subjected to alizarin red solution for 2 minutes, washed by distilled water and observed with light microscope.

Chondrogenesis: To induce the cartilage differentiation, micro mass culture system was used. For this purpose, 2.5×10^5 passaged-3 cells were pelleted under 300 g for 5 minute and cultured in a chondrogenic DMEM medium supplemented with 10 ng/ml TGF- ß (transforming growth factor-ß; Sigma, USA), 10 ng/ml BMP-6 (bone morphogenetic protein-6; Sigma, USA), 50 mg/ml insulin/ transferin/selenium+ premix (Sigma, USA), 1.25 mg BSA (bovine serum albumin; Sigma, USA) and 1% FBS (Gibco, UK). The chondrogenic culture was maintained at 37 °C, 5% CO2 for 21 days with a medium replacement of three times a week. To examine cartilage differentiation, the pellets were subjected to the following: fixing in 10% formalin; dehydrating in ascending concentrations of ethanol; clearing in xylene; embedding in paraffin wax and sectioning at 5µm. The sections were stained in toluidine blue for 30 second at room temperature and viewed by light microscope.

Adipogenesis: Adipogenic DMEM medium containing 100 nM dexamethasone (Sigma, USA) and 50 mg/ml indomethasine (Sigma, USA) was added on the confluent passaged-3 cells in 6-well culture plate. The cultures were incubated at 37 °C and 5% CO2 for 21 days during which the medium was changed 3 times a week. At the end of culture period, the cells were fixed with 4% formalin at room temperature, washed with 70% ethanol, stained by oil red solution in 99% isopropanol for 15 minute and observed by light microscope.

RT-PCR analysis

This was performed to assess expression of a set of chondrocyte, osteocyte and adipocyte related genes in differentiated cultures of MSCs. Total RNA was isolated using the RNX^{TM}(-Plus) Isolation of RNA(RN7713C; CinnaGen Inc., Tehran, Iran). Before RT, a sample of the isolated

RNA was treated, with 1U/µl of RNase-free DNaseI (EN0521; Fermentas,Opelstrasse 9,Germany) per 1 mg of RNA in order to eliminate residual DNA in the presence of 40 U/µl of ribonuclease inhibitor (E00311; Fermentasm, Germany) and 1×reaction buffer with MgCl2 for 30 min at 37°C. To inactivate the DNaseI, 1 µl of 25mM EDTA was added and incubated at 65°C for 10 min. Standard RT reactions were performed with 2 µg total RNA using Random hexamer as a primer designed for equine genes (see below) and a RevertAid™ First Strand cDNA Synthesis Kit (K1622; Fermentas, Germany) according to the manufacturer's instructions. For every reaction set, one RNA sample was prepared without RevertAid™**M-MuLV** Reverse Transcriptase (RT⁻ reaction) to provide a negative control in the subsequent PCR. To minimize variation in the RT reaction, all RNA samples from a single experimental setup were reverse transcribed simultaneously. Reaction mixtures for PCR included 2 µl cDNA, 1×PCR buffer(AMS™; CinnaGen Co., Tehran, Iran), 200 µM dNTPs, 0.5 µM of each antisense and sense primer, and 1U Taq DNA polymerase.

Table 1: The similarity percent of amplified sequences from ovine differentiated cultures using equine primers to sequences of other species

Gene Name	Other Speices	Similarity Percent	Other Species	Similarity Percent
Aggrecan	Other speices	96%	Equus caballus	89%
Collagen II	Bos taurus	87%	Equus caballus	85%
Peroxisome proliferator-activated receptor alpha (PPARA)	Bos taurus	95%	Equus caballus	91%
Peroxisome proliferator-activated receptor gamma (PPARG)	Bos taurus	99%	Equus caballus	93%
lipoprotein lipase	Bos taurus	96%	Equus caballus	90%
Collagen I	Bos taurus	97%	Equus caballus	97%

Because of the lack of specific gene sequence for sheep osteocyte, chondrocyte and adipocyte (except for GAPDH and Osteocalcin), we designed the primers for equine bone, cartilage and adipose-specific genes and used them in order to amplify the genes in sheep differentiated cultures. The bands of amplified fragments were recovered from the gel by QIAquick® Gel Extraction kit and then sequenced by Chromas Software version 2.31. BLAST results of these sequences, show that there are many similarities between them and sequences related to genes of other species close to sheep. Therefore, it confirmed that the equine specific primers were able to

amplify our interest fragments in sheep genome. Sequenced gene fragments were registered in NCBI site and they were assigned the accession numbers. The similarity percent of these sequences to sequences of other species and also primers sequences are indicated in Table 1 and 2 respectively.

Table 2: Primers used in RT-PCR analysis; * indicates the genes that were not found in gene banks.

Accession number	Gene name	Primer sequence	A.T
FJ200438*	Aggrecan	F: 5' ttg gac ttt ggc aga ata cc 3' R: 5'ctt cca cca atg tcg tat cc 3'	59
FJ200439*	Collagen II	F: 5' gcg gag act act gga ttg R: 5' ttt ctt gtc ctt gct ctt gc 3'	58
FJ200440*	peroxisome proliferator-activated receptor alpha (PPAR a)	F: 5' agaacaaggaagcggaagtc 3' R: 5' atcccgtctttgttcatcac 3'	60
FJ200441*	peroxisome proliferator-activated receptor gamma(PPAR g)	F: 5' aagagcagagcaaagaggtg3' R: 5' gggcttcacattcaacaaac 3'	60
FJ200442*	Collagen1	F: 5' ccc aga aca tca cct acc ac 3' R: 5' gga ggg agt tta cag gaa gc 3'	58
FJ200435*	LPL	F: 5' tct ctt ggg ata cag cct tg 3' R: 5' atg ccc tac tgg ttt ctg 3'	59
XM001504101.1	Collagen type X	F: 5' act gag cga tac caa aca cc 3' R: 5'ggt cca ttt agt cct ctc tcc 3'	62
DQ152956.1	GAPDH	F: 5' gga gaa acc tgc caa gta tg 3' R: 5' tga gtg tcg ctg ttg aa gtc 3'	60
NM_001040009.1	Osteocalcin	F: 5'agc gag gtg gtg aag aga c 3' R:5'gct cat cac agt cag ggt tg 3'	61
XM_001917620.1	Collagen type III	F: 5' ttg atg gtg cta ctt tga a 3' R: 5' aac atc ctc ctt caa cag c 3'	54

Sheep MSC culture requirements

Since MSCs are present in a low quantity in bone marrow samples, culture expansion of them is a necessary step prior to their applications. To recognize culture requirements of these cells, in this

study, FBS concentrations in medium and initiating cell seeding density were optimized for maximum proliferation of the cells.

Initiating cell seeding density: Passaged-3 cells were plated at densities of 100, 500, 1000, 2000 and 5000 cells/cm^2 in 6-well culture plates with a DMEM medium supplemented with 10% FBS. A week after culture initiation, the cells were trypsinized, lifted and counted with hemocytometer. Fold increase were then calculated for each cultures which, in this term, the cultures with different initiating cell densities were statistically compared.

Serum concentration: MSCs expansion is strongly dependent on FBS presence in medium. To determine optimal serum concentration, passaged-3 cells were plated at density of 100 cells /cm^2 in 6-well culture plates with the DMEM medium supplemented with FBS concentrations of 5%, 10%, 15% and 20%. One week after culture initiation, the cells were lifted and counted with hemocytometer. Fold increase in cell number was determined for each culture group and in this regard, the FBS concentrations were statistically compared.

Population doubling time (PDT)

After optimal cell seeding density and FBS concentrations was determined, the cells were cultivated under these conditions and their PDT was calculated. For this purpose, passaged-3 cells were plated at 100 cells/cm^2 in a DMEM supplemented with 15% FBS and incubated for a period of 10 days when the culture terminated, the cell were lifted and counted with hemocytometer. Using the following equation, PDT was determined. In this equation N is the initiating cell number, N0 the harvesting cell number and C.T stands for culture time.

$$\mathbf{(PDT)} = \frac{C.T}{Log\frac{N}{N_0} \times 3.31}$$

Colonogenic assays: MSCs are known to be colonogenic cells since they were first isolated and introduced. Colonogenic potential of the cells is indeed indicative of their expansion capacity in vitro. In this study, passaged-3 MSCs from sheep were plated at 100 cells/ 10 cm Petri dish and allowed to generate cell colonies for 10 days when the cultures were observed under phase contrast invert microscope in order to count the colonies number.

Statistical analysis: Repeated Measure ANOVA with one within subject effect was used to analyze the data. Pairwise comparison of the data of FBS concentrations and initial cell seeding density was performed using LSD post hoc. Furthermore, Mauchly method was used to test the sphericity of the data. All values stated as statistical means ± standard deviations. The used software was the SPSS version 13. A P value of <0.05 was considered to be statistically significant.

Results

Cell culture

At early days, the primary cultures were consisted mainly of fibroblastic cells and a few small clear cells (Fig. 1A). Two weeks after culture initiation, all available surfaces of the culture dish covered with a monolayer in which the bundles of fibroblastic cells extended in different directions (Fig. 1B). During subcultures, the cells maintained their fibroblastic morphology and approach confluency in 10 days.

Differentiation

Osteogenesis: At osteogenic culture, nodule-like aggregations were appeared. Upon alizarin red staining, these nodules stained red indicating that they were mineralized during the induction period (Fig. 2A). RT-PCR analysis were indicated that the mRNA of bone specific proteins including osteocalcin, and collagen I was largely produced in the culture (Fig. 2B).

Chondrogenesis: The sections from micomass cultures for cartilage differentiation were stained methachromatically purple with toluidine blue staining (Fig. 2C). RT-PCR analysis revealed that the mRNA of collagen II, X and aggrecan macromolecules were largely produced in either differentiated cells (Fig. 2D).

Adipogenic cultures: about 10 days after induction initiation, the first lipid droplets were appeared inside of the cells and increased in number as the time progressed. These droplets were positively stained red with oil red staining for adipocyte detection (Fig. 2E). RT-PCR analysis were further confirmed the expression of adipocyte marker genes including PPAR-alpha, PPAR-gamma2 and LPL in the cultures (Fig. 2F).

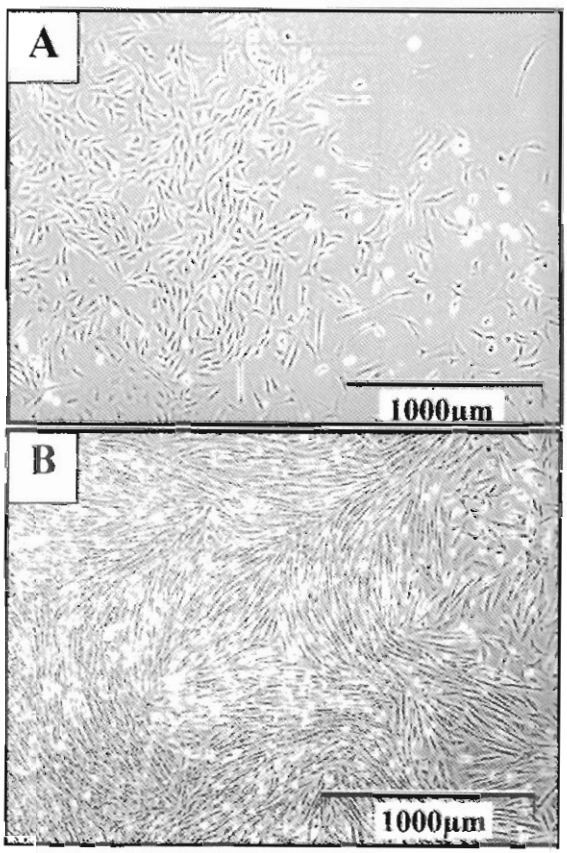

Figure 1: Ovine Marrow cell culture. A) The cells at primary cultures, day 5 after culture initiation B) primary culture at confluency, phase contrast invert microscopic images.

Culture requirements

Initiating cell seeding density: According to our data, significantly more fold increase were observed at cultures with seeding density of 100 cell/cm^2 compared with the cultures initiated at 500, 1000, 2000 and 5000 cells/cm^2 (Fig.3 up) ($P<0.05$).

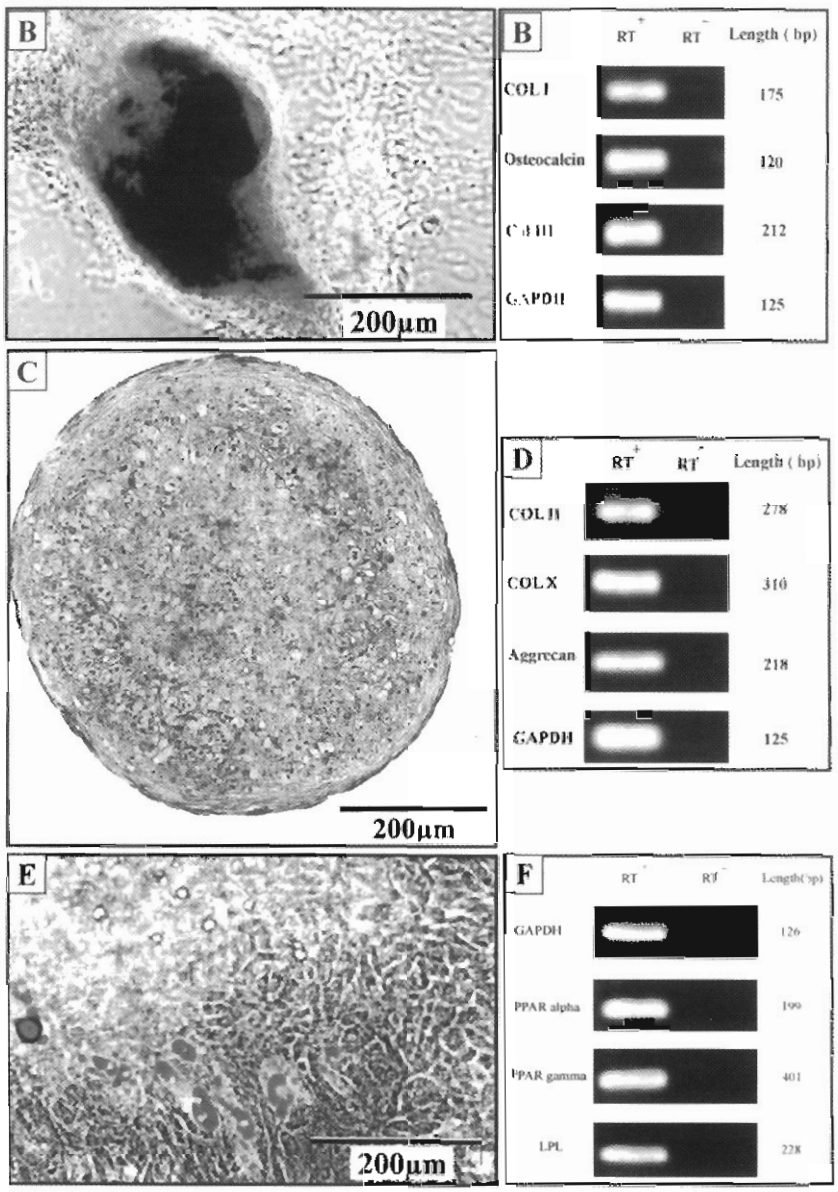

Figure 2: Ovine MSCs differentiation potential: A: The cells were able to differentiate into bone. This was evident in alizarin red staining (A) and RT-PCR analysis (B). More over, ovine cells had the capacity to differentiate into chondroblast and deposit cartilage matrix that was methachromatically stained purple with toluidine blue (C). At differentiated cultures cartilage specific genes were expressed. Ovine MSCs isolated in this study, were also able to differentite into adipocytes when cultivated at adipogenic culture. Lipid droplet in differentiated cells were stained red by oil red staining (E). According to the RT-PCR analysis, adipocyte-specific genes were expressed at adipogenic cultures. RT+ denotes the sample with Reverse Transcriptase Enzyme and RT- the sample without Reverse Transcriptase Enzyme.

FBS concentrations: Statistical comparison indicated that more fold increase occurred at the cultures supplemented with 15% FBS than 5%, 10% and 20% FBS concentrations (Fig.3 down).

Population doubling time (PDT)

Population of passaged-3 sheep MSCs appeared to have a rather high expansion rate so that their number was doubled in 24.94 hours when plated at 100 cells/cm^2 in a medium supplemented with 15% FBS..

Colonogenic ability

According to our results, sheep MSCs appeared to produce an average of 50.2 colonies per 100 cells that were plated in 10 cm culture dishes.

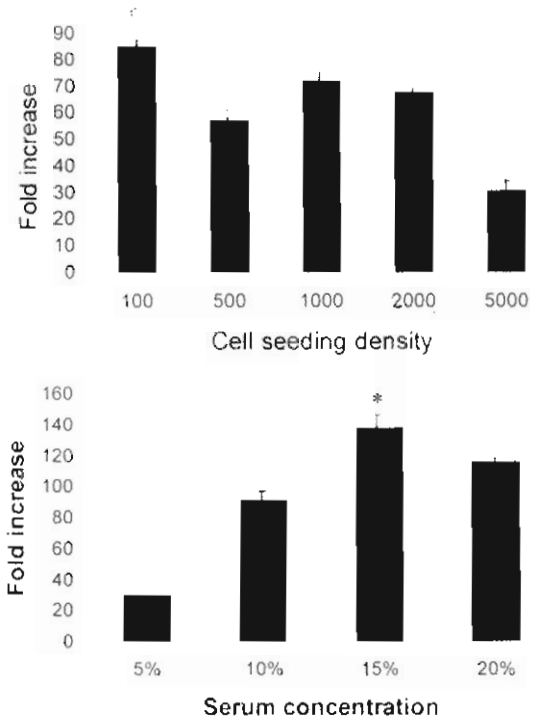

Figure 3: Ovine MSCs culture requirements. Up: The cells possessed significantly more fold increase when initiated at 100 cells/ cm^2 ; *: P<0.05. Down: They also exhibited maximum proliferation at the presence of 15% FBS in culture medium.*: P<0.05

Discussion

In this study, fibroblastic cell monolayer developed at ovine marrow cell culture were collected and examined in terms of their tripotent differentiation potential that is proposed as a criterion indicating MSCs identity of the cells in question.[25] Our findings indicated that the cells are able to undergo differentiation into three skeletal cell lineages as bone, cartilage and adipose cells hence could be considered as MSCs that are described elsewhere.[2-6] In previous investigations, the cells isolated from ovine marrow have directly been transplanted into tissue defect as MSCs without confirmation of their MSC nature[22-23] as it was done in this study. Furthermore, investigations considering in vitro differentiations of MSCs would also be of great importance especially with respect to their applications in cell based-treatment of tissue defects. In cell therapy strategies; it is believed that transplanted cells should be of differentiated lineages in order to avoid their unwanted differentiation into the cells other than those native to defect site. Therefore, in vitro differentiations of the cells into interest cells could be a necessary step prior their transplantation. In the present investigation, we also determined culture requirement of the isolated cells which is so far not yet to be reported.

Large animals in stem cell investigations are of great importance since in contrast to rodents they are genetically more closely related to humans. In the other words, the scaling up and preclinical development of therapeutic procedures, and particularly those involving cell or gene therapies, require an intermediate step between rodent or in vitro assays and clinical trials, both for safety and for the investigation of efficacy. Results from large animal models have been much more predictive with regard to human clinical trials than have results from in vitro assays or rodent models.[26,27] Furthermore one limitation associated with mice as a model is their limited life

expectancies especially the inbred or immunodeficient strains used for human xenografts that preclude long-term follow-up. Given the significance of large animal model in stem cell investigation, in this study MSCs from sheep bone marrow were isolated and characterized. This has not been done before.

According to the previous investigations, MSCs occur at low frequencies in bone marrow samples.[28] Therefore, any experimental work with MSCs requires in vitro expansion of the cells which is largely dependent on the presence of FBS in culture medium. On the other hand, MSCs developed in a medium containing FBS would be immunogenic and may transfer bovine pathogens upon transplantation.[29] In spite of extensive attempt that has been made to substitute the bovine serum, less success has been achieved.[30,31] In this study, therefore, ovine MSCs were cultivated in different concentrations of FBS to determine the least concentration with the highest mitogenic effect, which this was achieved by 15% FBS in the medium.

The other factor affecting the rate of MSCs proliferation would be the cell seeding density at culture initiation.[32] Based on our findings, the best seeding density in which the more fold increase could occur was determined as 100 cells /cm^2. This data can be used to optimize the culture condition for maximum proliferation of the cells. This is of utmost importance especially at cell therapy strategies where the rapid expansion of cells would be desired.

In spite of the considerable attempts that have been made to define the antigenic profile of human MSCs, no definitive single marker has so far been introduced. In this regard, several markers including CD133, LNGFR (low affinity nerve growth factor receptor) and STRO-1 have been proposed as markers of human MSCs.[32-34] However, the identification of MSCs, especially those from animal source, among the other cells and hence their isolation would be a difficult task owing to the lack of distinct specific marker. To overcome this problem, MSCs committee of the international society for cell therapy has proposed that MSCs from animal source should possess two characteristic criteria: a) They must be adherent on plastic surfaces of culture dish. b) They must be able to produce bone, cartilage and adipose cell lineages.[25] Since the isolated cells of the present study were easily differentiated into bone, cartilage and adipose cells and also they were plastic adherent cell, we convinced that they were the MSCs described elsewhere.

Taken together, ovine marrow contains a population of fibroblastic cells that are able to differentiate into bone, cartilage and adipose cells hence being mesenchymal stem cell in nature. These cells exhibited extensive proliferation when being provided with a medium supplemented with 15% FBS. Furthermore, the culture of these cells exhibit extensive expansion when being initiated at the density of 100 cells / cm^2. In general, the cells possessed rapid expansion rate as it was well evident in their population doubling time (PDN). Using the data introduced in this study, it could be possible to prepare ovine MSCs in sufficient numbers for tissue regeneration application. Moreover, in this study we determine the sequence of a number of ovine related genes and registered them in NCBI site.

References

1. Friedenstein, AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* 1970;3:393-403.

2. Digirolamo CM, Stokes D, Colter D, et al. Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br J Haematol* 1999;107:275–281.
3. Javazon EH, Colter DC, Schwarz EJ, et al. Rat marrow stromal cells are more sensitive to plating density and expand more rapidly from single-cell-derived colonies than human marrow stromal cells. *Stem Cells* 2001;19:219–225.
4. Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* 1970; 3:393–403.
5. Rickard DJ, Sullivan TA, Shenker BJ, et al. Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2. *Dev Biol* 1994; 161:218–228.
6. Awad HA, Butler DL, Boivin GP, et al. Autologous mesenchymal stem cell-mediated repair of tendon. *Tissue Eng* 1999;5:267–277.
7. Croft AP, Przyborsk SA. Mesenchymal stem cells from bone marrow stroma: Basic Biology and Potential for cell therapy. Curr Anesthesia & critcare 2004;5:865-875.
8. Barry FP, Murphy JM. Mesenchymal stem cells: clinical application and biological characterization. *Int Biochem cell Biol* 2004;36:568-84.
9. Romanov YA, Svintsitskaya VA, Smirnov VN. Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. *Stem Cells* 2003; 21:105-110.
10. Baksh D, Song L, Tuan RS. Adult mesenchymal stem cells: Characterization, differentiation and application in cell therapy. *Mol Med 2004*;8:301-136.
11. Horwitz EM, Gordon PL, Koo WK, et al. Isolated allogenic bone marrow- derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. *Proc Natl Acad Sci* USA 2002;99:8932-8937.
12. Koe ON, Gerson SL, Cooper BW, et al. Rapid hematopoietic recovery after confusion of autologous - blood stem cells in advanced breast cancer patients receiving high dose chemotherapy. *J Clin Oncol* 2000;18:307-316.
13. Petite H, Viateau V, Bensaid W, et al. Tissue engineered bone regeneration. *Nat Biotechnol* 2000;18:959-963.
14. Quarto R, Mastrogiacomo M, Cancedda R, et al. Repair of large bone defect with the use of autogenic bone marrow stromal cell. *N Engl J Med* 2001;344:385-386.
15. Grinnemo KH, Mansson A, Dellgren G, et al. Xenoreactivity and engrafment of human mesenchymal stem cells transplantation into infarcted rat myocardium. *J Thorac Cardiovasc Surg* 2004;127:1293-1300.
16. Barry FP. Mesenchymal stem cell therapy in joint disease. Novartis Found Symp 2003; 249:86-96.
17. Jafarian M, Eslaminejad MB, Khojasteh A, et al. Marrow-derived mesenchymal stem cells-directed bone regeneration in the dog mandible: A comparison between Biphasic calcium phosphate and Natural bone mineral. *Oral Surg Oral Med Oral Pathol Oral Radiol Endodont* 2008;105:14-24.
18. Eslaminejed MB, Mirzadeh H, Mohamadi Y, et al. Bone differentiation of the marrow-derived mesenchymal stem cells using tricalcium phosphate/alginate/gelatin scaffolds. *J Tissue Eng Reg Med* 2007;6:417-424.

19. Eslaminejad MB, Jafarian M, Khojasteh A, et al. Enhancing ectopic bone formation in canine masseter muscle by loading mesenchymal stem cells onto natural bovine bone minerals. *Iran J Vet Surg* 2007: 4:25-35.
20. Khojasteh A, Eslaminejad MB, Nazarian H. Mesenchymal stem cells enhance bone regeneration in rat calvarial critical size defect more than platelete rich plasma. *Oral Surg Oral Med Oral Pathol Oral Radiol Endodont* 2008;106: 356-362.
21. Eslaminejad MB, Jafarian M, Khojasteh A, et al. In vivo bone formation by canine mesenchymal stem cells loaded onto HA/TCP scaffolds: Qualitative and quantitative analysis. *Yakhteh Med J* 2008,3:205-212.
22. Chen J, Wang C, Lu S, et al. In vivo chondrogenesis of adult bone-marrow-derived Autologous mesenchymal stem cells. *Cell Tissue Res* 2005;319:429-438.
23. Frosch KH, Drengk A., Krause P, et al. Stem cell-coated titanium implants for the partial joint resurfacing of the knee. *Biomaterials* 2006;27:2542-2549.
24. Rhodes NP, Srivastava JK. Heterogeneity in proliferative potential of ovine mesenchymal stem cell colonies. *J Sci Mater med* 2004;15:397-402.
25. Dominci M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The international societry for cellular therapy position statement. *Cytotherapy* 2006;4:315-317.
26. Donahue RF, Dunbar CE. An update on the use of non-human primate models for preclinical testing of gene therapy approaches targeting hemotopoiesis cells. *Hum Gene Ther* 2001;12:607-617.
27. Horn PA, Thmasson BM, Wood BL, et al. Distinct hematopoietic stem/progenitor cell populations are responsible for repopulating NOD/SCID mice compared with nonhuman primates. *Blood* 2003;102:4329-4335.
28. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;284:143-147.
29. Spees JL, Gregory CA, Singh H, et al. Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. *Mol Ther* 2004;95: 747-756.
30. Kobayashi T, Watanabe H, Yanagawa T, et al. Motility and growth of human bone-marrow mesenchymal stem cells during ex vivo expansion in autologous serum. *J Bone Joint Surgery* 2005;87:1426-1428.
31. Kusnetsov SA, Mankani MH, Robey PG. Effect of serum on human marrow stromal cells: Ex vivo expansion and in vivo bone formation. *Transplantation* 2000;70:1780-1787.
32. Bartmann C, Rohde E, Schallmoser K, et al. Two steps to functional mesenchymal stromal cells for clinical application. *Transfusion* 2007; 47:1426-1435.
33. Kuçi S, Wessels JT, Bühring HJ, et al. Identification of a novel class of human adherent CD34- stem cells that give rise to SCID-repopulating cells. *Blood* 2003;3:869-876.
34. Quirici N, Soligo D, Bossolasco P, et al. Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. *Exp Hematol* 2002;30:783-791.
35. Gronthose S, Simmons PJ. The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-derived condisions in vitro. *Blood* 1995;85: 929-940.

چکیده

پتانسیل تمایز و نیاز های کشت سلول های بنیادی مزانشیمی جدا شده از مغز استخوان گوسفند جهت استفاده در بازسازی بافتی

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[1] مرکز تحقیقات علوم سلولی جهاد دانشگاهی، پژوهشکده رویان، تهران، ایران.
[2] مرکز نگهداری و پرورش حیوانات پژوهشکده رویان، کرج، ایران.

هدف- جداسازی، تکثیر و تمایز سلو لهای بنیادی مزانشیمی از مغز استخوان گوسفند و تعیین نیاز های کشت آنها برای حداکثر رشد

طرح مطالعه- مطالعه تجربی

حیوانات- ۵ گوسفند با نژاد شال.

روش کار= در این مطالعه، سلول های مغز استخوان گوسفند ها کشت گردید و با انجام سه پاساژ متوالی تکثیر گردید. سلول های حاصل در شرایط تمایز به استخوان، غضروف و چربی قرار گرفت تا ماهیت بنیادی مزانشیمی آنها مشخص سود. همچنین، ما نیاز های کشت این سلول ها را از نظر غلظت سرم گاوی و تراکم آغاز گر کشت سلولی تعیین کردیم. در این مطالعه زمان لازم برای دو برابر شدگی جمعیت سلولی نیز محاسبه گردید.

نتایج= بر اساس مشاهدات ما، سلول های بنیادی مزانسیمی گوسفندی از لحاظ ظاهر به شکل فیبروبلاسی بودند. این سلول ها توانستند براحتی به سه رده استخوان، غضروف و چربی تمایز یابند. این مسئله در بررسی RT-PCR و رنگ آمیزی اختصاصی برای سلول ها کاملا مشخص بود. طبق یافته های ما، سلول های بنیادی مزانشیمی گوسفندی زمانیکه با تراکم ۱۰۰ سلول در سانتیمتر مربع و در حضور ۱۵٪ سرم گاوی کشت شدند، حداکثر تکثیر را نشان دادند. همچنین یافته های این تحقیق نشان داد که سلول های حاصل زمان دو برابر شدگی جمعیتی نسبتا کوتاهی دارند (۲۴/۹۴±۲/۶۷ ساعت).

نتیجه گیری و کاربرد بالینی- سلول های فیبروبلاستی جدا شده از مغز استخوان گوسفند قادرند بطور وسیعی تکثیر شوند و همچنین توانایی تمایز به رده های اسکلتی را دارا هستند، بنابراین جهت اهداف سلول درمانی مناسب هستند.

کلید واژگان- مغز استخوان گوسفند، سلول بنبادی مزانشیمی، استخوان، غضروف، چربی، سرم جنینی گاو، تراکم سلولی