

In vitro osteogenesis of rat adipose-derived stem cells: comparison with bone marrow stem cells

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Abstract

Introduction: Adipose-derived stem cells (ADSCs) have been shown to differentiate into osteoblasts, adipocytes or myoblasts. However, it is not certain that ADSCs are equal to bone marrow stem cells (BMSCs) in their osteogenic differentiation potential. The purpose of this study was to answer the question.

Material and methods: Mesenchymal stem cells (MSCs) were isolated from bone marrow and fat of adult rats. After cell expansion in culture media and three passages, osteogenesis was induced on a monolayer culture with osteogenic medium containing dexamethasone, b-glycerophosphate and ascorbate. After 4 weeks, expression of the osteocalcin gene was analyzed by RT-PCR, alkaline phosphatase (ALP) activity assayed, and Alizarin Red S and Von Kossa staining were done. Cell viability and apoptosis were also assayed by MTT and flow cytometry, respectively.

Results: In the test of osteogenesis, the osteoblastic differentiation of ADSCs as demonstrated by ALP activity was less than that of the BMSCs. The amount of matrix mineralization shown by Alizarin Red S and Von Kossa staining also showed statistical differences between the two MSCs. The incidence of apoptotic cells among ADSCs was higher than BMSCs. The flow cytometry proves that cell growth reduction is due to a decrease of the cells entering the S phase of the cell cycle. The data demonstrated by MTT assay indicated that viable cells among ADSCs were lower than BMSCs in control groups.

Conclusions: The results of our study suggest that ADSCs may have an inferior potential for osteogenesis compared with BMSCs.

Key words: osteogenesis, bone marrow stem cells, adipose-derived stem cells, rat.

Introduction

Various methods have been proposed for the repair of bone defects in the field of plastic and orthopaedic surgery. A previous approach to this problem focused on the development of various artificial materials that might be used instead of autogenous bone [1]. Current studies, however, rely mainly on the use of fresh autograft bone, various bone graft substitutes [2] and tissue-engineering techniques that incorporate appropriate implants seeded with cells having osteogenic potential [3]. Mesenchymal stem cells have recently received widespread attention because of their potential use in tissue-engineering applications [4]. Mesenchymal stem cells (MSCs), also known as marrow stromal cells or mesenchymal progenitor cells, are defined as self-renewable, multipotent progenitor cells with the capacity to differentiate into several distinct mesenchymal lineages [5]. To date, MSCs of various adult

vertebrate species have been demonstrated to differentiate into lineage-specific cells that form the bone, cartilage, fat, tendon, and muscle tissue [6-8]. Bone marrow derived mesenchymal stem cells have been shown to be multipotential in that they differentiate in culture [8] or after implantation *in vivo* into osteoblasts [9]. Although bone marrow provides the most universal source of MSCs, other tissues such as periosteum [10], muscle [11], synovial membrane [12] and adipose tissue also appear to possess MSCs [13]. Adipose tissue is particularly attractive because of its easy accessibility and abundance [14-16]. Adipose tissue-derived mesenchymal stem cells (ADSCs) obtained from lipospirates have been shown to have the multi-lineage potential to differentiate into adipogenic, chondrogenic, myogenic and osteogenic cells [13, 14]. Adipose-derived stem cells mineralized their extracellular matrix (ECM), and increased the expression of osteocalcin and alkaline phosphatase (ALP) [16]. However, it is not well established whether ADSCs have the same potential for osteogenesis and chondrogenesis as bone marrow-derived mesenchymal stem cells (BMSCs). Thus, the purpose of this study is to investigate whether ADSCs are equal to BMSCs with regard to their osteogenesis potential.

Material and methods

Isolation and culture of mesenchymal stem cells

About 6- to 8-week-old male Wistar rats of the albino strain were killed using diethyl ether and the bones were collected under sterile conditions, then all the bones were cut at both ends. The bone marrow from each bone was collected by flushing the bone with Dulbecco's Modified Eagle's Medium (Sigma) containing 1000 U/ml Penicillin G. The cells after filtering were centrifuged at 3,000 rpm for 5 min. The purified cells were finally dispersed in DMEM with 15% fetal bovine serum (Sigma) containing 100 U/ml penicillin, 100 µg/ml streptomycin [17].

Primary adipose-derived stem cells were harvested from the scrotal fat pad of the same age rats. Epididymal adipose tissue was excised, placed on a sterile glass surface, and finely minced. The minced tissue was placed in a 50 ml conical tube (Greiner, Germany) containing 0.05% tissue culture grade collagenase type 1 (Sigma) and 5% bovine serum albumin (Sigma). The tube was incubated at 37°C for 1 h, being shaken every 5 min. The tube contents, after filtering through a sterile 250 µm nylon mesh, were centrifuged at 250 g for 5 min. The cell pellet was resuspended in adipose-derived stem cell medium: DMEM/F12 (Sigma), 10% fetal bovine serum (Gibco), and 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma). Cell count was determined with a haemocytometer [18].

Cell culture and expansion

The isolated stem cells were plated in T75 tissue culture flasks, containing appropriate stem cell medium at a density of 10×10^5 cells per flask. The flasks were maintained in a tissue culture incubator at 37°C and 5% carbon dioxide. The medium was replaced every third day afterwards. Cell viability was confirmed by continued cell division and the cells were subcultured using 3 ml of trypsin/EDTA (Sigma) when the flasks reached 90% confluence.

Osteogenic differentiation

Both types of cells were used to assess melatonin's effect on osteogenic differentiation. Cells (passage 3) were then seeded at an initial density of 10×10^5 cells per flask (25 cm²) in osteogenic medium (OS) containing 0.05 mM ascorbate, 1 µM dexamethasone and 10 mM β-glycerophosphate for 4 weeks [18].

Confirmation of osteogenic differentiation

Confirmation of osteogenesis was confirmed by means of Von Kossa and Alizarin Red S staining (highlights extracellular matrix calcification) and assessment of alkaline phosphatase activity and expression of osteocalcin gene.

Von Kossa Staining

Cells in flasks (25 cm²) were rinsed with phosphate-buffered saline and were fixed in 4% paraformaldehyde for 20 min. The cells were incubated in 5% silver nitrate in the dark, then the flasks were exposed to ultraviolet light for 1 h. Secretion of calcified extracellular matrix was observed as black nodules with von Kossa staining [1].

Alizarin Red S Staining

Cells in flasks (25 cm²) were washed with phosphate-buffered saline (PBS) and fixed in 10% (v/v) formaldehyde (Sigma-Aldrich). After 15 min ARS 2% (pH = 4.1) was added to each flask. The flasks were incubated at room temperature for 20 min. Then the flasks were washed four times with dH₂O while being shaken for 5 min [19].

Quantification of mineralization

The analysis of the amount of calcium deposition in osteogenic media was modified from a previous report [19]. In brief, 2 ml 10% (v/v) acetic acid was added to each flask. After 30 min the monolayer was scraped off the plate with a cell scraper and transferred with 10% (v/v) acetic acid to a 15 ml microcentrifuge tube. After vortexing for 30 s, the slurry was overlaid with 1.25 ml mineral oil (Sigma-Aldrich), heated to exactly 85°C for 10 min, and transferred to ice for 5 min. The slurry

was then centrifuged at $20,000 \times g$ for 15 min and 500 μl of the supernatant was removed to a new 1.5 ml microcentrifuge tube. Then, 200 μl of 10% (v/v) ammonium hydroxide was added to neutralize the acid. Aliquots (150 μl) of the supernatant were read in triplicate at 405 nm in 96-well format using opaque-walled, transparent-bottomed plates.

ALP activity

The cells were lysed by sonication for three cycles, and then protein solutions were centrifuged at $2000 \times g$ for 15 min at 4°C . The total protein content of each sample was determined according to Bradford [20]. ALP was performed using an ALP kit (Ziest Chem, Tehran, Iran) and following the manufacturer's instructions. The levels of activity were neutralized with an amount of protein in cell lysate solution (units/mg protein).

RNA extraction and reverse transcription-polymerase chain reaction analysis of gene expression

After extraction of total RNA, reverse transcriptase polymerase chain reaction assays were performed as described [21].

Flow cytometry

DNA fragmentation, as a late feature of apoptosis, was evaluated using a flow cytometer. PI staining was performed as previously described [22]. Sample acquisition was performed by a FACScan flow cytometer equipped with Cell Quest software.

Cell viability assay

The MTT (Sigma) test measures the mitochondrial (metabolic) activity in the cell culture, which reflects the number of viable cells [23]. In brief, the cultures (5×10^4 were seeded to a 96-well plate) were washed in PBS, and 200 μl of MTT reagent were added. Following incubation for 3 h

in the incubator (in 5% CO_2 at 37°C) the absorption of the medium was measured in an ELISA Reader (Anthos 2020) at 440 nm.

Statistical analysis

The results are listed as the mean \pm SD. The statistical difference was analyzed by one-way ANOVA followed by Dennett's test. The p value < 0.05 was considered to be significant. All assays were performed in triplicate.

Results

Cultivation and passaging of human mesenchymal stem cell culture

No adherent cells were removed from the dish during medium changes and the subsequent passaging. Typically 80-90% of confluence was reached by day 10 in BMSC cultures and by day 5 for ADSC cultures. Cells at third passage were used for the experiments.

Rat bone marrow stem cells and adipose-derived stem cells grown in culture appeared spindle-shaped. Cells cultured in osteogenic media demonstrated a dramatic change in cell morphology from day 5 induction, with the cells changing morphology from an elongated fibroblastic appearance to polygonal, more cuboidal shape (Figure 1).

The cells of the two cell populations were cultured for 28 days in osteogenic media. After this period, samples were taken for analyses.

Quantitative estimation of ALP and mineralization

As a marker for BMSCs and ADSCs differentiation into osteoblasts, ALP levels were measured in both groups. In our study, ALP activity was measured in BMSCs and ADSCs in osteogenic medium after 14 and 28 days (Figure 2). The data showed that BMSCs underwent a much higher increase in ALP activity compared with ADSCs.

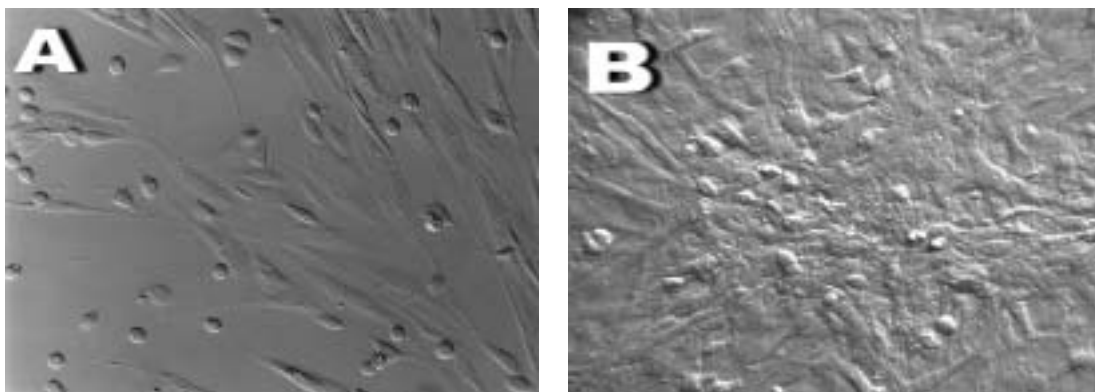


Figure 1. Initially adherent mesenchymal cells grew as spindle-shaped cells (A) that developed into multi-polar fibroblastoid cells (B). They gradually reached confluency at about 7 days for ADSCs (adipose-derived stem cells) and 10 days for BMSCs (bone marrow stem cells) (300 \times)

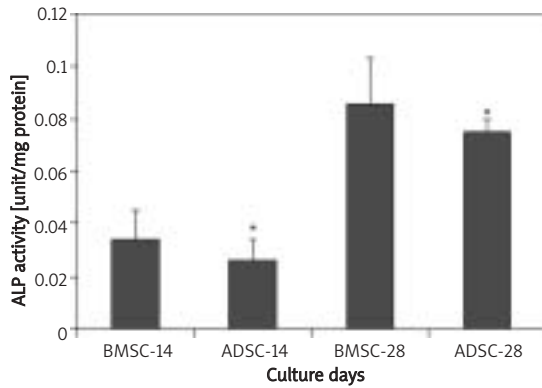


Figure 2. ALP activity in MSCs on osteogenic differentiation at day 14 and 28. The cells were plated at 10^6 cells/flask and cultured in osteogenic medium for 28 days. Values are means \pm SD ($n = 3$)
ADSCs – adipose-derived stem cells, BMSCs – bone marrow stem cells

The cells were stained positively for extracellular mineralization after 2 and 4 weeks of culture in osteogenic media, as confirmed by Von Kossa and Alizarin Red S staining (Figure 3). Calcium level

quantification was measured in both groups after 2 and 4 weeks after osteogenic induction in ADSCs and BMSCs (Figure 4).

The calcium measurement indicated more calcium for the BMSCs. The ADSC groups revealed a low level.

Osteocalcin gene expression

To determine gene expression of osteocalcin mRNA in both MSCs, RT-PCR was carried out with primer specific for osteocalcin. Figure 5 shows the results. The presence of the osteocalcin mRNA band indicated osteogenesis.

Apoptosis and cell viability

In individual experiments, the incidence of apoptotic cells among ADSCs was higher than BMSCs (Figure 6). The flow cytometry proves that cell growth reduction is due to a decrease of cells

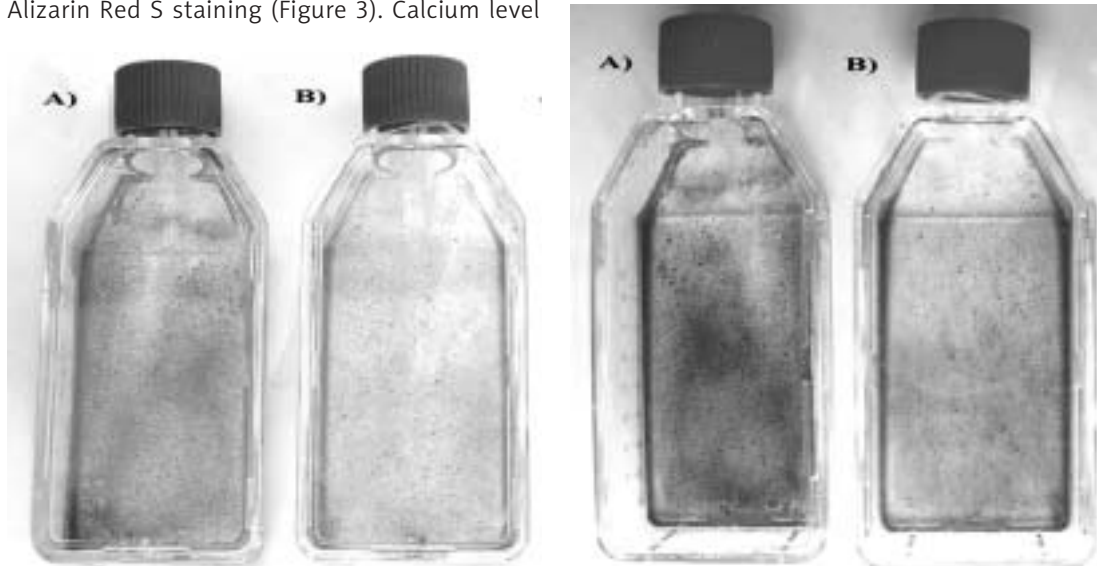


Figure 3a. Alizarin Red S staining after osteogenic differentiation. Staining for mineral deposition was performed for ADSCs and BMSCs after 14 (left) and 28 (right) days. A) BMSCs, B) ADSCs
BMSCs – bone marrow stem cells, ADSCs – adipose-derived stem cells

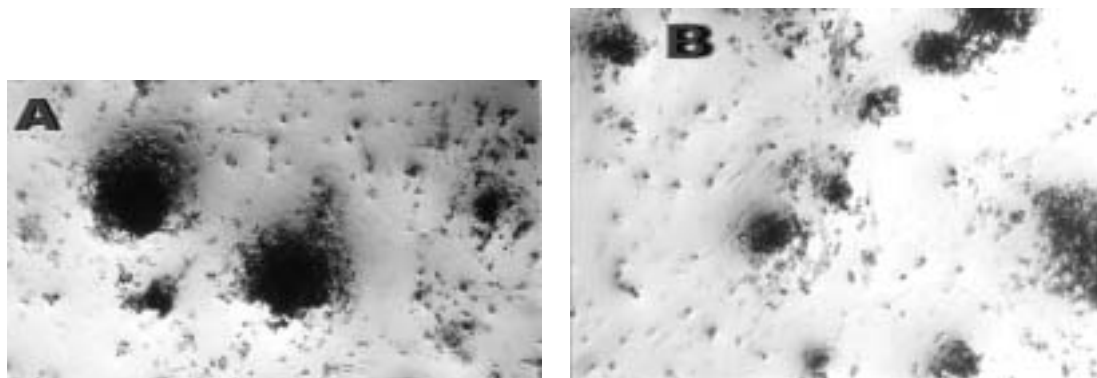


Figure 3b. von Kossa staining after osteogenic differentiation. Staining for mineral deposition was performed for BMSCs (A) and ADSCs (B). This staining was done only for demonstration of osteogenic differentiation (400 \times)
BMSCs – bone marrow stem cells, ADSCs – adipose-derived stem cells

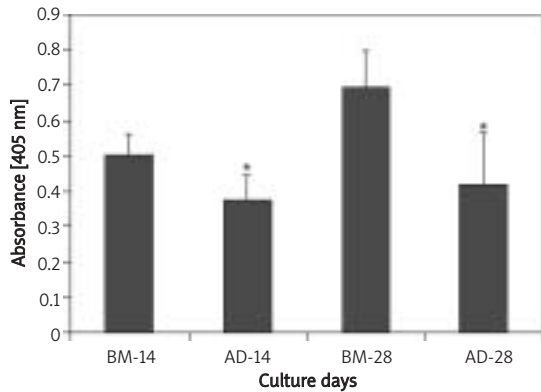


Figure 4. Calcium deposition. The BMSCs and ADSCs were cultured in osteogenic medium for 4 weeks in flasks (25 cm²) as a monolayer culture. Calcium depositions were measured as described in Material and methods. Values are mean ± SD
AD – adipose-derived stem cells, BM – bone marrow stem cells

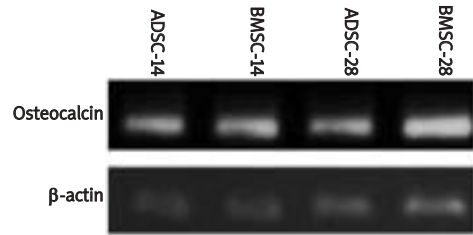


Figure 5. Differentiation of ADSCs and BMSCs into osteoblasts. MSCs were incubated in osteogenic differentiation medium for 4 weeks as indicated. RNA samples were isolated at the indicated time, thereby also controls of undifferentiated MSCs, fully differentiated osteoblasts (28 days under osteogenic conditions) were investigated. RT-PCR was performed for osteogenic marker gene osteocalcin

Discussion

In the present study, we confirm that both BMSCs and ADSCs have the potential to differentiate into an osteogenic lineage. However, BMSCs have a greater osteogenesis potential as evidenced by greater matrix production when compared with ADSCs.

entering the S phase of the cell cycle. These data were demonstrated by MTT assay (Figure 7). These data indicated that viable cells among ADSCs were lower than BMSCs in the control groups.

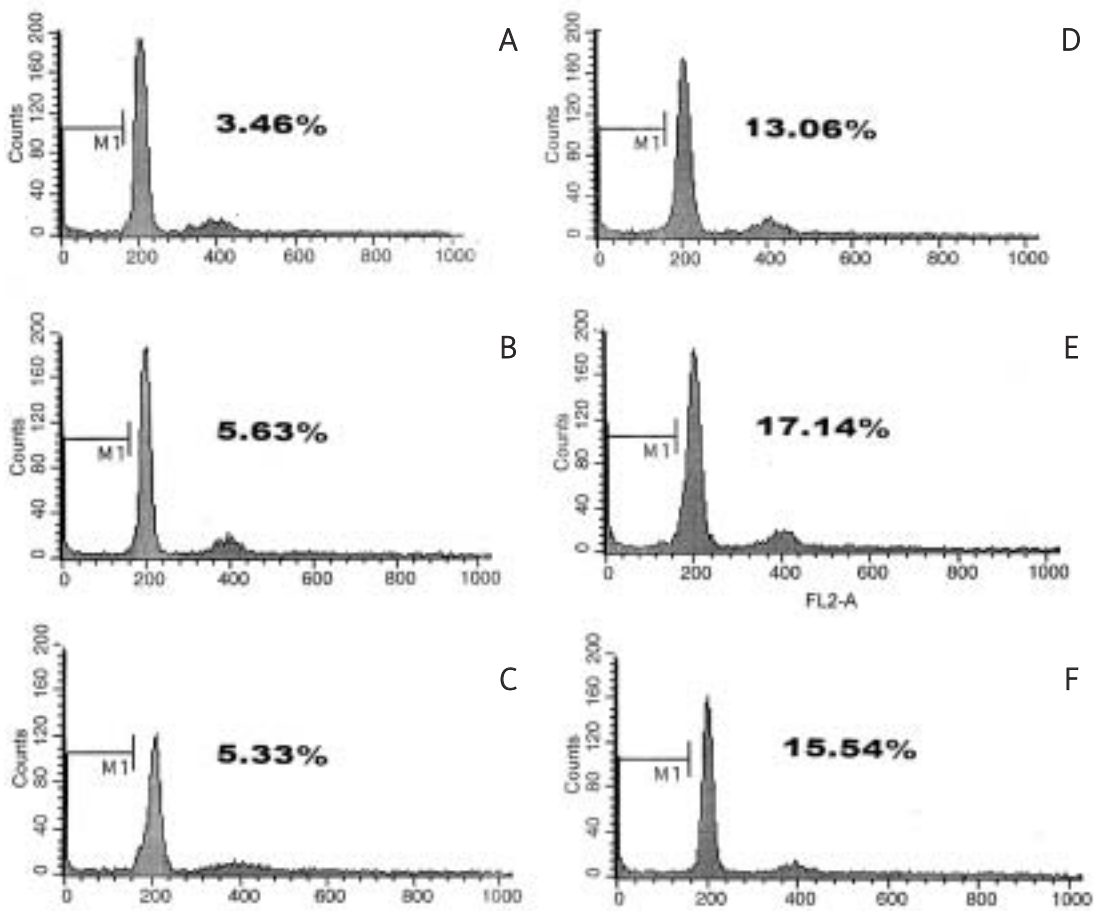


Figure 6. Flow cytometric analysis of DNA content for detection of apoptosis after 72 h of osteogenic treatment. The cells were plated at 10⁶ cells/flask and cultured in osteogenic medium for 72 h
A, B, C – bone marrow stem cells, D, E, F – adipose-derived stem cells, os – osteogenic medium

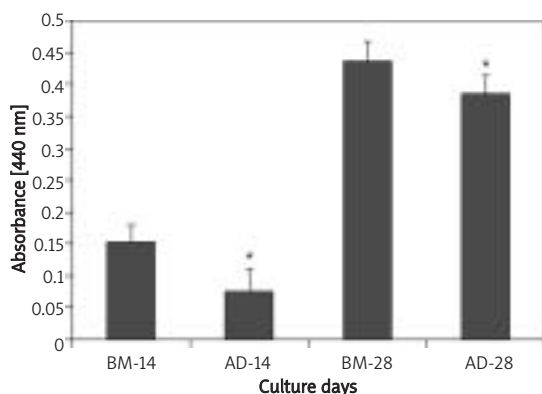


Figure 7. Analysis of the effect of osteogenic stimulation on BMSCs and ADSCs. The cells were plated at 5×10^4 cells/well and cultured in osteogenic medium for 28 days. In a separate experiment, cell viability at day 14, 28 was quantified using the reagent MTT. Values are means \pm SD ($n = 3$)
ADSCs – adipose-derived stem cells, BMSCs – bone marrow stem cells

Over recent years, stem cells have generated great interest given their potential therapeutic use. Recent reports have provided clear evidence that multipotent adult stem cells exist in many more organs and tissues than previously expected. Mesenchymal cells capable of differentiation into a variety of specific cell phenotypes have been isolated from tissues such as bone marrow, muscle, fat, periosteum and synovial membrane both from rodents and humans [8, 12, 13, 24, 25].

Although bone marrow provides a universal source of MSCs, adipose tissue also possesses abundant and easily accessible MSCs. Recent advances in cosmetic surgery add to its advantage with a huge amount of available fatty tissue. It may have a further advantage when the morbidity associated with large volume bone marrow harvests is taken into consideration [26, 27].

Regarding comparison between BMSCs and ADSCs there are similar findings [28-30] but the overall results of the present study do not corroborate the results of the previous studies that suggested equal or comparable capacity of ADSCs for osteogenesis [13, 31, 32]. There are several possible reasons for such a difference. The reason suggested by Gun *et al.* [28] was that ADSC isolates may represent a fairly heterogeneous population of cell types with only a small number of progenitor cells capable of osteogenic differentiation. This would be consistent with the results of some studies which indicated differences in cell surface antigen expression between cellular preparations of adipose-derived and bone marrow-derived stromal cells [33-35]. Another explanation is that ADSCs may represent distinctly different cell populations that are at different stages of lineage-specific commitment from BMSCs [36].

Generally, studies have shown that BMSCs and ADSCs are not a homogeneous population of multilineage progenitors; rather, they are made up of a heterogeneous population of pluripotent stem cells and tripotent, bipotent, and unipotent progenitors [13, 37, 38].

Therefore, the differences between BMSCs and ADSCs observed here may not be due to the inherent difference between multipotent BMSCs and multipotent ADSCs. Rather, it could be due to the fact that BMSC cultures may be dominated by osteogenic and chondrogenic progenitors, whereas ADSCs have mainly adipogenic progenitors [38].

There are limitations of this study that preclude definite conclusions. First, we had different rates for BMSCs and ADSCs. Second, the gene expression profiles were not thoroughly investigated using a quantitative PCR technique. This will be pursued in the following study.

In conclusion, the results of this study show that ADSCs differ from BMSCs in their osteogenic potential. When equal amounts of bioactive factors are given, ADSCs have inferior capacity to differentiate into bone, suggesting the limited utility of ADSCs as a source of cells needed for tissue engineering of bone. As a further step forward, a search for the culture conditions that would induce successful osteogenesis from ADSCs is warranted.

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