

Osteogenic Differentiation of Rat Mesenchymal Stem Cells from Adipose Tissue in Comparison with Bone Marrow Mesenchymal Stem Cells: Melatonin As a Differentiation Factor

Arash Zaminy¹, Iraj Ragerdi Kashani^{*1}, Mohammad Barbarestani¹, Azim Hedayatpour¹, Reza Mahmoudi² and Ahmadreza Farzaneh Nejad³

¹Dept. of Anatomy, School of Medicine, Medical Sciences/University of Tehran, Tehran; ²Dept. of Anatomy, School of Medicine, Yasouj University of Medical Sciences, Yasouj, ³School of Allied Medical Sciences, Medical Sciences/University of Tehran, Tehran, Iran

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ABSTRACT

Background: Adipose-derived stem cells (ADSC) could be an appealing alternative to bone marrow stem cells (BMSC) for engineering cell-based osteoinductive grafts. Meanwhile, prior studies have demonstrated that melatonin can stimulate osteogenic differentiation. Therefore, we assayed and compared the melatonin effect on osteogenic differentiation of BMSC with that of ADSC. **Methods:** Mesenchymal stem cells (MSC) were isolated from the bone marrow and fat of adult rats. Both cell types were cultured in osteogenic medium in the absence and presence of melatonin at physiological concentrations (20-200 pg/ml). After 4 weeks, the expression of osteocalcin gene was analyzed by reverse transcription-PCR, alkaline phosphatase (ALP) activity was assayed and alizarin red S and von Kossa staining were done. Cell viability and apoptosis were also assayed by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole (MTT) and flow cytometry, respectively. **Results:** The osteoblastic differentiation of ADSC as demonstrated by ALP activity was less than that of BMSC. The amount of matrix mineralization has shown by alizarin red S and von Kossa staining also showed statistical differences between the two MSC. The incidence of apoptotic cells was higher among ADSC than BMSC. The flow cytometry proves that cell growth reduction is due to a decrease in the number of the cells entering the S phase of the cell cycle. MTT assay indicated that viable cells were fewer among ADSC than BMSC in control groups. **Conclusion:** The results of the study suggest that BMSC have greater osteogenic potential than ADSC and that melatonin promotes osteogenic differentiation to BMSC, but has a negative effect on ADSC osteogenic differentiation. *Iran. Biomed. J. 12 (3): 133-141, 2008*

Keywords: Mesenchymal stem cells (MSC), Bone marrow, Adipose tissue, Osteogenic, Melatonin

INTRODUCTION

Mesenchymal stem cells (MSC) have recently received widespread attention because of their potential use in tissue-engineering applications [1]. MSC are defined as self-renewable, multipotent progenitor cells with the capacity to differentiate into several distinct mesenchymal lineages [2].

Although bone marrow provides the most universal source of MSC [3] and the apparent pluripotent nature of bone marrow stem cells (BMSC) makes them excellent candidates for tissue

engineering, BMSC have been reported to require selective sera lots and growth factor supplements for culture expansion [4]. Moreover, traditional bone marrow procurements, particularly in volumes larger than a few milliliters may be painful, frequently requiring general or spinal anesthesia [5], and may yield low numbers of MSC upon processing [6]. As an alternative to BMSC, in many studies, a population of cells isolated from other adipose tissue appears to possess MSC [4]. Adipose-derived stem cells (ADSC) obtained from lipoaspirates have been shown to have the multi-lineage potential to differentiate into adipogenic, chondrogenic,

*Corresponding Author; Tel. (+98-21) 6443 2348; Fax: (+98-21) 6641 9072; E-mail: ragerdi@sina.tums.ac.ir

myogenic and osteogenic cells [3, 7]. Adipose tissue is particularly attractive because of its easy accessibility and abundance [8].

ADSC mineralized their extracellular matrix (ECM), and increased the expression of osteocalcin and alkaline phosphatase (ALP) [9]. These factors may make ADSC a viable clinical alternative to BMSC. However, it is not well established whether ADSC have the same potential for osteogenesis as BMSC.

On the other hand, melatonin has been shown to play a role in many physiological systems including those involved in sleep, gastrointestinal physiology, immune defense, cardiovascular function, renal function, detoxification, reproduction and retina, as well as bone physiology [10].

Melatonin influences cell proliferation, and the effect of stimulation or suppression of cell division appears to depend on its concentration and the cell type examined [11]. Melatonin's ability to directly promote osteoblast maturation was first demonstrated in preosteoblast and rat osteoblast-like osteosarcoma cells where low concentrations of melatonin increased the mRNA levels of several genes expressed in osteoblasts including bone sialoprotein, ALP, osteopontin, and osteocalcin [12]. Several studies using various animal models show that melatonin prevents bone deterioration; including preventing idiopathic scoliosis in adolescents [13] and that it stimulates proliferation and differentiation of normal cells such as human bone cells [10]. The effects of melatonin on BMSC and ADSC are unknown. Thus, the purpose of this study is to investigate whether ADSC are equal to BMSC in regard to their osteogenesis potential and examination of melatonin's effect at physiological concentrations (20-200 pg/ml) on the proliferation, apoptosis and differentiation of the BMSC and ADSC derived from adult rat using in vitro culture systems.

MATERIALS 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 of Eagle's Medium (DMEM) (Sigma, USA) containing 1000 u/ml Penicillin G. After filtering, the cells were

centrifuged at $1100 \times g$ for 5 min. The purified cells were finally dispersed in DMEM with 15% fetal bovine serum (Sigma, USA) containing 100 U/ml penicillin and 100 $\mu g/ml$ streptomycin [14]. Primary ADSC were harvested from 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, USA) and 5% bovine serum albumin (Sigma, USA). The tube was incubated at $37^{\circ}C$ for 1 h and shaken every 5 minutes. After filtering through a sterile 250- μm nylon mesh, the tube contents were centrifuged at $250 \times g$ for 5 minutes. The cell pellet was resuspended in ADSC medium: DMEM/F12 (Sigma, USA), 10% fetal bovine serum (Gibco, USA), 100 U/ml penicillin and 100 $\mu g/ml$ streptomycin (Sigma, USA). Cell count was determined with a hemacytometer [15].

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 $37^{\circ}C$ 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, USA) 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 cm^2) in osteogenic medium (OS) containing 0.05mM ascorbate, 1 μM dexamethasone and 10mM b-glycerophosphate for 4 weeks [16].

Treatment groups. For each cell type, three treatment groups [OS + M-, OS + M + (20 pg/ml and 200 pg/ml)] were used throughout this study to analyze melatonin's effect on bone marrow and ADSC differentiation into osteoblasts. The groups (OS + M-, OS + M+) were exposed to OS+ in the absence (OS + M-) or presence (OS + M+) of melatonin to promote osteoblasts differentiation. These studies were conducted for 28 days to determine if melatonin could modulate osteogenic induced differentiation of MSC into osteoblasts.

Confirmation of osteogenic differentiation.

Confirmation of osteogenesis was done by means of von Kossa and alizarin red S staining (to highlight ECM calcification), the assessment of ALP activity, and the expression of osteocalcin gene.

von Kossa staining. The cells in flasks (25 cm²) were rinsed with PBS and fixed in 4% paraformaldehyde for 1 h. The cells were incubated in 5% silver nitrate in the dark for 30 minutes, and then the flasks were exposed to ultraviolet light for 1 h. The secretion of calcified ECM was observed as black nodules with von Kossa staining [9].

Alizarin red S staining. The cells in flasks (25 cm²) were washed with PBS and fixed in 10% (v/v) formaldehyde (Sigma, USA). After 15 min, ARS 2% (pH 4.1) was added to each flask. The flasks were incubated at room temperature for 20 min and then they were washed four times with dH₂O shaken for 5 min [16].

Quantification of mineralization. The analysis of the amount of calcium deposition in OS was modified from a previous report [16]. In brief, 2 ml of 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 micro centrifuge tube. After vortexing for 30 s, the slurry was overlaid with 1.25 ml mineral oil (Sigma, USA), heated to exactly 85°C for 10 min, and transferred to ice for 5 min. The slurry was then centrifuged at 20,000 ×g for 15 min and 500 µl of the supernatant was removed to a new 1.5-ml micro centrifuge 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 lysated by sonication for three cycles, and then protein solutions were centrifuged at 2000 ×g at 4°C for 15 min. The total protein content of each sample was determined according to Halvorsen [9]. ALP was performed using an ALP kit (Ziest Chem, Iran) 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 (RT)-PCR analysis of gene expression. After extraction

of total RNA, RT-PCR, assays were performed as described [12].

Flow cytometry. DNA fragmentation, as the late feature of apoptosis, was evaluated using flow cytometer. Propidium iodide staining was performed as previously described [13]. Sample acquisition was performed by FACScan flow cytometer (Becton Dickinson, USA) equipped with Cell Quest software.

Cell viability assay. The 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma, USA) test measures the mitochondrial (metabolic) activity in the cell culture, which reflects the number of viable cells. In brief, the cultures (10 × 10⁵), seeded to a 96-well plate, were washed in PBS, and 500 µl of culture medium containing 50 µl MTT reagents was added. Following incubation in the incubator at 37°C for 1 h (in 5% CO₂), the absorption of the medium was measured in an ELISA Reader (Anthos 2020) at 440 nm [17].

Statistical analysis. The results are listed as the mean ±S.D. The statistical difference was analyzed by one-way ANOVA followed by Dennett's test. *P*<0.05 was considered to be significant. All assays were performed in triplicate.

RESULTS

Establishment of primary cultures. In this experiment, and according to many experimental studies [7, 8, 12], the cells obtained from the bone marrow and adipose tissue in toto, without the selection of any particular population, constitute an optimal source of MSC which can be differentiated towards an osteoblastic phenotype. The mesenchymal stem-cell nature of the cells derived from the bone marrow of adult rats is demonstrated by their capacity of extensive proliferation and of differentiation towards several different lineages. Because of their proliferative and differentiating abilities, they are referred to as MSC in this study.

Quantitative estimation of ALP and mineralization. The data showed that BMSC underwent a much higher increase in ALP activity compared with ADSC in control groups (Fig. 1). Additionally, as shown in Figure 2, ALP activity increased in BMSC following 28 days of incubation

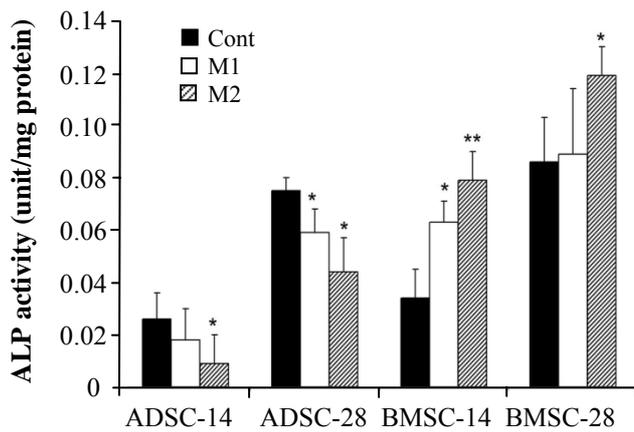
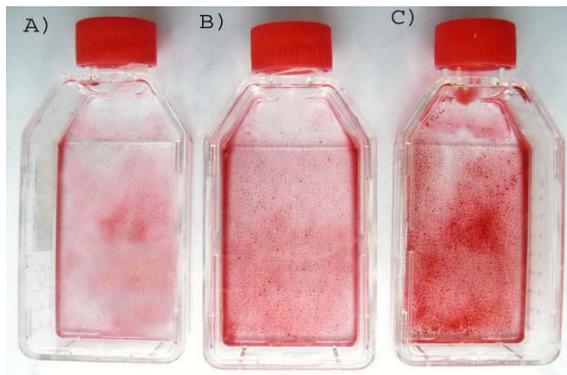


Fig. 1. Effect of melatonin on ALP activity in MSC on osteogenic differentiation at day 14 and 28. The cells were plated at 10^6 cells/flask and cultured in osteogenic medium for 28 days. Values are means \pm S.E.M (* $P<0.05$ and ** $P<0.001$). ADSC, adipose derived stem cells; BM, bone marrow stem cells; Cont, Control; M1, melatonin (20 pg/ml) and M2, melatonin (200 pg/ml).

in OS in the presence of melatonin beyond that of cells exposed to OS alone. But, as it can be seen, ALP activity decreased in ADSC following incubation in OS in the presence of melatonin. These data indicate when ADSC were exposed to melatonin, ALP activity decreased in contrast to the cells exposed to OS alone (Fig. 1).

Confirmation of osteogenic potential. The cells were stained positively for extracellular mineralization after 4 weeks of culture in OS, as confirmed by alizarin red S staining (Fig. 2a) and von Kossa (Fig. 2b). Calcium level quantification was measured in both control groups after 2 and 4 weeks following osteogenic induction in ADSC and BMSC (Fig. 3). Calcium measurement indicated more calcium for BMSC groups on days 14 and 28 than that for ADSC groups.

(a)



(b)

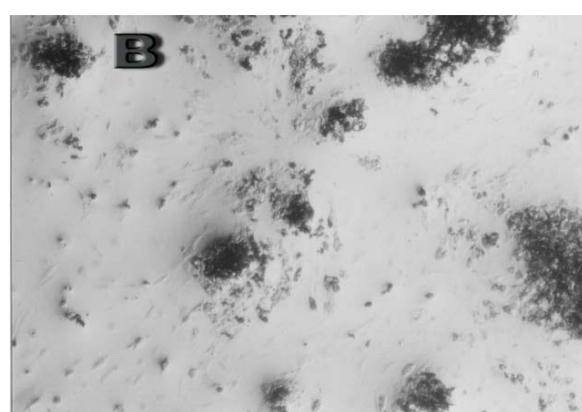
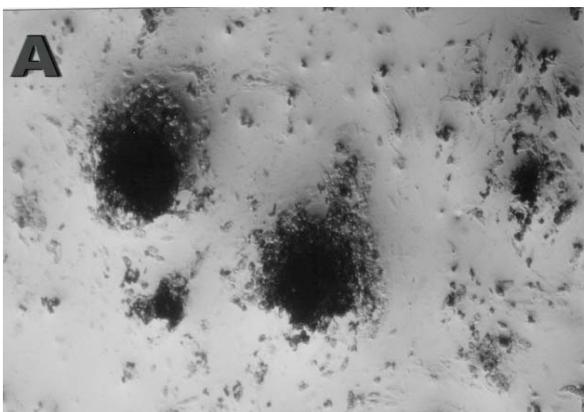


Fig. 2. Staining after osteogenic differentiation. (a) Alizarin red staining for mineral deposition was performed for BMSC (Left) and ADSC (Right) after 28 days. (A) Control medium (B) in presence of melatonin (20 pg/ml) and (C) in presence of melatonin (200 pg/ml); (b) von Kossa staining for mineral deposition was performed for BMSC (A) and ADSC (B). BMSC, bone marrow stem cells, ADSC, adipose derived stem cells.

Osteocalcin gene expression. To determine gene expression of osteocalcin mRNA in both MSC, RT-PCR was carried out with primer specific for osteocalcin (Fig. 4).

Apoptosis and cell viability. Melatonin treatment induced a detectable cell growth down-regulation after 72 h of incubation and increased with the treatment time among ADSC (Fig. 5). The flow cytometry proves that cell growth reduction is due to a decrease in the cells entering the S phase of the cell cycle. In individual experiments, the incidence of apoptotic cells among ADSC was higher in melatonin-treated groups than in the control group and BMSC, whereas the apoptosis incidence of BMSC reduced after melatonin treatment (Fig. 5). As shown in Figure 6, when BMSC were exposed to melatonin, cell viability increased beyond that of the cells exposed to OS alone, whereas the cell viability incidence of ADSC decreased in contrast to the control group and BMSC.

DISCUSSION

In the present study, we confirm that both BMSC and ADSC have the potential to differentiate into osteogenic lineage. However, BMSC have a greater osteogenesis potential as evidenced by greater matrix production. Additionally, in the

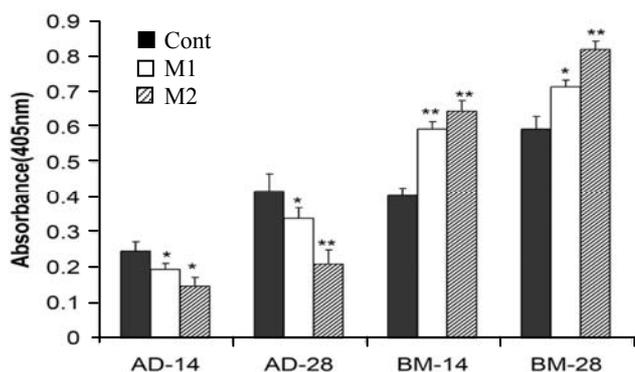


Fig. 3. Calcium deposition. The BMSC and ADSC were cultured in osteogenic medium in the absence and presence of melatonin for 4 weeks in flasks (25 cm²) as a monolayer culture. Calcium depositions were measured as described in Materials and Methods. Values are mean \pm S.E.M (* P <0.05 and ** P <0.001). AD, adipose derived stem cells; BM, bone marrow stem cells; Cont, control; M1, melatonin (20 pg/ml) and M2, melatonin (200 pg/ml).

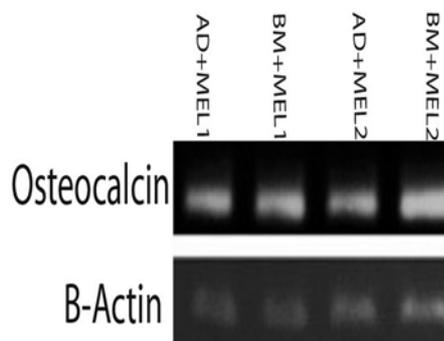


Fig. 4. ADSC and BMSC were induced to differentiate into osteoblast by incubation in osteogenic differentiation medium containing physiological dose of elatonin. RNA was isolated from the cells at the indicated time points (h) and expression of osteocalcin and β -actin was measured by RT-PCR. AD, adipose derived stem cells; BM, bone marrow stem cells; MEL1, melatonin (20 pg/ml) and MEL2, melatonin (200 pg/ml).

present study, we observed an increase of ALP activity and extracellular mineralization in the presence of melatonin in BMSC grown in OS, but when ADSC were exposed to melatonin, ALP activity and mineral deposition decreased.

When BMSC and ADSC are compared, the findings are similar [3, 17, 18] but the overall results of the present study do not corroborate the results of the previous studies, in which equal or comparable capacity of ADSC for osteogenesis has been suggested [8, 19, 20]. There are several possible reasons for such a difference. One reason explained by Gun *et al.* [3] might be that ADSC isolates may represent a fairly heterogeneous population of cell types with only a small number of progenitor cells capable of osteogenic differentiation.

However, this would be consistent with the results of some studies that indicate differences in cell surface antigen expression between cellular preparations of adipose-derived and bone marrow-derived stromal cells [21-23]. Another explanation is that ADSC may represent distinctly different cell populations that are at different stages of lineage-specific commitment from BMSC [20]. Generally, studies have shown that BMSC and ADSC are not a homogeneous population of multilineage progenitors; rather, they are made up of heterogeneous population of pluripotent stem cells and tripotent, bipotent, and unipotent progenitors [8, 24, 25].

Therefore, the differences between BMSC and ADSC observed in this study may not be due to the inherent difference between multipotent BMSC and multipotent ADSC; rather, it could be due to the fact

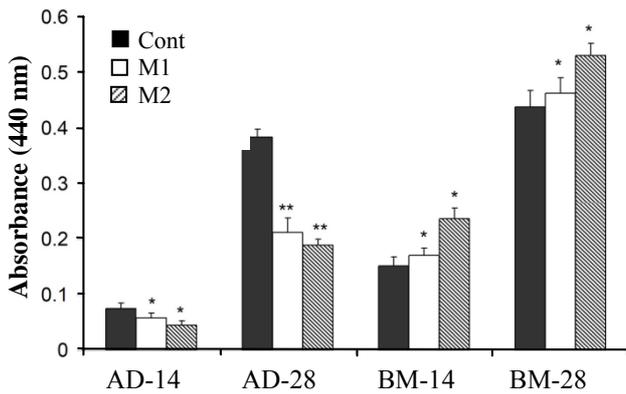


Fig. 6. Analyses of the effect of osteogenic stimulation in the absence and presence of melatonin on BMSC and ADSC. The cells were plated at 5×10^4 cells/well and cultured in osteogenic medium for 28 days. In a separate experiment, cell viability at day 14 and 28 was quantified using the reagent MTT. Values are means \pm S.E.M (* $P < 0.05$ and ** $P < 0.001$). AD, adipose derived stem cells; BM, bone marrow stem cells; Cont, control; M1, melatonin (20 pg/ml) and M2, melatonin (200 pg/ml).

that BMSC culture may be dominated by osteogenic and progenitors, whereas ADSC mainly have adipogenic progenitors [25].

Also, the results of current study show that melatonin enhanced osteogenic differentiation on BMSC but has negative effect on ADSC.

Melatonin's ability to directly promote osteoblast maturation was first demonstrated in preosteoblast and rat osteoblast-like osteosarcoma cells through melatonin transmembrane receptors [26]. In a study, the effect of melatonin on mesenchymal stem cells was examined by Radio and co-workers [27]. The results of that study suggest that melatonin increases ALP activity in differentiating human adult mesenchymal stem cells via MT2 melatonin receptors.

The signaling mechanisms underlying how melatonin enhances osteoblast cells formation are less well understood. In general, the promotion of osteoblast differentiation has been shown by numerous laboratories to be mediated via the mitogen activated protein kinase (MEK) cascade [28-30]; however, the effect of p38, MEK/extracellular signal-regulated kinases (ERK) 1/2 has remained unclear [31]. Delagrangé *et al.* [30]

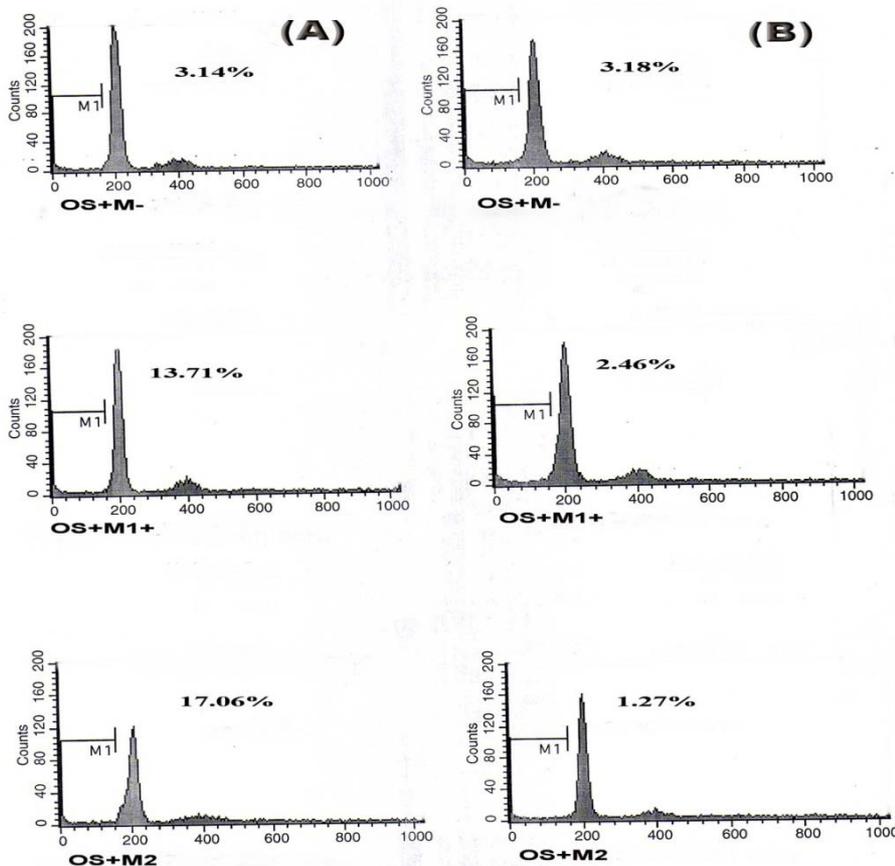


Fig. 5. Flow cytometric analysis of DNA content for detection of apoptosis after 72 h of melatonin treatment. The cells were plated at 10^6 cells/flask and cultured in osteogenic medium in presence of melatonin for 72 h. Column A, adipose derived stem cells and column B, bone marrow stem cells. OS, osteogenic medium; M1, melatonin (20 pg/ml); M2, melatonin (200 pg/ml).

revealed that inhibition of p38 MAP-K does not block ascorbic acid induced mesenchymal cell differentiation and Radio *et al.* [27] also showed the inhibition of ALP activity in the presence of a MEK 1/2 inhibitor, PD98059. So, melatonin induced differentiation of BMSC is not due to p38 MAP-K and is probably through MEK and ERK [27, 32]. It can be due to the reduction in the number of cells, which was demonstrated by the results of MTT assay and flow cytometry. The study demonstrated that in melatonin-containing media, the progression of the apoptotic type of ADSC was not prevented, but rather exaggerated by melatonin.

The induction of apoptosis by melatonin is not a common effect of this indole in normal cells. Melatonin influences cell proliferation and differentiation, and the effect of stimulation or suppression of cell division appears to depend on its concentration and the cell type examined [11, 28]. Anti-proliferative effects of melatonin have been demonstrated *in vivo* and *in vitro* in a number of cancer cells [33, 34]. In contrast, it stimulates proliferation of normal cells such as human bone cells [35]. That melatonin may affect cell growth in biphasic manner was first reported by Slominski and Pruski [36] in experiments with cultured rat melanoma cells. In that study, low concentrations of melatonin suppressed human melanoma cell growth, while higher concentrations stimulated such growth.

Similar to the above findings with human melanoma cells, the melatonin at low concentrations, suppressed PC12 cells growth, while at a higher concentration; it prevented cell death [35]. Melatonin's behavior was complex, affecting cell cycle presumably by different mechanisms according to dose [27]. The intensity of the different responses to melatonin could be related to the cell-line specific pattern of melatonin cellular receptors and cytosolic binding protein expression [37].

In conclusion, the results of this study show that ADSC differ from BMSC in their osteogenic potentials. When an equal amount of bioactive factors is given, the ADSC have an inferior capacity to differentiate into bone, suggesting the limited utility of ADSC as a source of cells needed for tissue engineering of the bone. As a step forward, a search for the culture conditions that would induce a successful osteogenesis from ADSC is warranted. It is premature to offer firm conclusions about such findings except to indicate that biphasic apoptotic responses reliably occur.

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