

## Melatonin Influences the Proliferative and Differentiative Activity of Rat Adipose-Derived Stem Cells

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### Abstract

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**Objective:** This study was performed to determine whether melatonin at physiological concentrations (0.01-10nM) could affect the proliferation and osteogenic differentiation of Rat ADSCs in vitro.

**Materials and Methods:** ADSCs were isolated from the fat of adult rats. After cell expansion in culture media and through three passages, osteogenesis was induced on a monolayer culture with osteogenic medium with or without melatonin at physiological concentrations (0.01-10nM). After 4 weeks cultures were examined for mineralization by Alizarin Red S and von Kossa staining and for alkaline phosphatase (ALP) activity by ALP kit. Cell viability and apoptosis were also assayed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-ulfophenyl)-2H-tetrazolium assay and flowcytometry, respectively. All assays were performed in triplicate.

**Results:** The results indicated that at physiological concentrations, melatonin suppressed proliferation and differentiation of ADSCs. These data indicate that ADSCs exposed to melatonin, had a lower ALP activity in contrast to the cells exposed to the osteogenic medium alone. Similarly, the mineral deposition (calcium level) also decreased. The flow cytometry proved that the cell growth decreased and the apoptotic cells increased.

**Conclusion:** These results suggest that physiological concentration of melatonin has a negative effect on ADSCs osteogenesis.

**Keywords:** Adipose Tissue, Stem Cells, Melatonin, Osteogenic Differentiation

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### Introduction

The repair of bone defects secondary to trauma, osteomyelitis, fracture nonunion, and tumor resection poses a significant problem for many clinicians, particularly plastic, head and neck, and orthopedic surgeons (1).

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

distinct mesenchymal lineages (3).

Although bone marrow provides the most universal source of MSCs and the apparent pluripotent nature of bone marrow stem cells (BMSCs) makes them excellent candidates for tissue engineering, BMSCs 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, 6), and may yield low numbers of MSCs upon processing (7). As

an alternative to BMSCs, adipose tissue is particularly attractive because of its easy accessibility and abundance (8-10). Adipose-derived stem cells (ADSCs) obtained from lipoaspirates have been shown to have the multi-lineage potential to differentiate into adipogenic, chondrogenic, myogenic and osteogenic cells (9, 11).

ADSCs mineralized their extracellular matrix (ECM), and increased the expression of osteocalcin and alkaline phosphatase (ALP) (12). These factors may make ADSCs a viable clinical alternative to BMSCs.

Melatonin has been shown to play a role in many physiological systems including those involved in sleep, gastrointestinal physiology, immune defense, cardiovascular function, detoxification, reproduction, as well as bone physiology (13).

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 (14). 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 (BSP), alkaline phosphatase (ALP), osteopontin, and osteocalcin (15). Several studies using various animal models show that melatonin prevents bone deterioration, including preventing idiopathic scoliosis in adolescents (13,16) and that it stimulates proliferation of normal cells such as human bone cells (13). However, there are no reports of melatonin effects on ADSCs osteogenic differentiation.

In this study, we examined the effects of melatonin at low and high (0.01-10 n M) (17), physiological concentrations on the proliferation, apoptosis and differentiation of the ADSCs derived from adult rat using in vitro culture systems.

## Materials and Methods

### *Isolation of Adipose-Derived Stem Cells*

Three Sprague-Dawley male rats (about 6- to 8-week-old) were killed using Diethylethr. The groin and testicular areas were shaved and

prepared with standard sterile techniques. A 15 mm incision was made along the scrotum and the testes were pulled out, exposing the epididymal fat pad. Epididymal adipose tissue was excised, placed on a sterile glass surface, and finely was minced. The minced tissue after washing in Hanks balanced salt solution was placed in a sterile 50ml 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°C for 1 hr, being shaken every 5 minutes. Then, an equal volume of Dulbecco's Modified Eagle Medium with 10% fetal bovine serum was added to neutralize the collagenase. The tube contents were filtered through a sterile 250 µm nylon mesh to remove undigested debris. The digested tissue was then centrifuged at 250g for 5 minutes, and the mature adipocytes were removed by aspirating the supernatant, leaving a pellet of cells behind. Percent viability was determined by trypan blue dye exclusion. Cell suspension was mixed with 0.04% trypan blue dye, and live and dead cells were scored by counting them on a hemacytometer (2). The viabilities of ADSCs were 98%. The cell pellet was resuspended (500 000 cells/rat) in adipose-derived stem cell medium: DMEM/F12 (Sigma, USA), 10% fetal bovine serum (Gibco, USA), and 100U/ml penicillin, 100µg/ml streptomycin (Sigma, USA). All experimental protocols with animals were pre-approved by research committee of the Tehran University of medical sciences.

### *Cell Culture and Expansion:*

The collected fat-derived stem cells were plated in 75-cm<sup>2</sup> vented tissue culture flasks at a density of 1×10<sup>6</sup> cells per flask in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma, USA). The flasks were maintained in a tissue culture incubator at 37°C, 5% carbon dioxide conditions. The media were replaced the day after the initial stem cell harvest and then every third day. The cells were monitored for confluence on a daily basis.

The cells were subcultured when the flasks reached 80% confluence. The media were

removed and 3 ml trypsin was added to each flask for 5 minutes to allow the cells to deplate. The cell layer was then collected into a 15ml conical tube and was centrifuged at 250g for 10 minutes. The pellet was resuspended with 10 ml control media and the cells were counted. The cells were split into flasks at a density of  $10 \times 10^5$  cells per flask.

ADSCs were cultured and expanded in basal medium, and used for the experiments at passage 3.

### *Osteogenic Differentiation*

At the end of passages, the cells were trypsinized and replated in 25cm<sup>2</sup> tissue culture flasks at  $10 \times 10^5$  cells per flask. Cells were allowed to adhere and grow for 3 days in basal medium, and then the media were replaced with osteogenic media containing: DMEM with 10% fetal bovine serum (Sigma, USA), 0.1µM dexamethasone (Sigma, USA), 10mM β-glycerol phosphate (Sigma, USA), 50µg/ml ascorbic acid-2-phosphate (Sigma, USA) with or without physiological concentrations of melatonin (0.01-10 nM) (Sigma, USA) (2).

### *Treatment Groups*

Three treatment groups, osteogenic medium alone, osteogenic medium with low physiological concentration of melatonin (0.01nM) and osteogenic medium with high physiological concentration of melatonin (10nM), were used throughout this study to analyze melatonin's effect on ADSCs differentiation into osteoblasts. The experiments were conducted for 28 days to determine whether melatonin could modulate osteogenic induced differentiation of ADSCs into osteoblasts.

### *Confirmation of Osteogenic Differentiation*

Confirmation of osteogenesis was done by means of von Kossa and Alizarin Red S staining (to highlight extra cellular matrix calcification), the assessment of alkaline phosphatase activity.

### *Von Kossa Staining*

The cells in flasks (25 cm<sup>2</sup>) were rinsed with phosphate-buffered saline (PBS) (Sigma,

USA) and fixed in 4% Para formaldehyde (Sigma, USA) for 20 min. The cells were incubated in 5% silver nitrate (Gibco, USA) in the dark, and then the flasks were exposed to ultraviolet light for 1 hour. The secretion of calcified extra cellular matrix was observed as black nodules with von Kossa staining (12).

### *Alizarin Red S staining*

The cells in 25cm<sup>2</sup> flasks were washed with PBS and fixed in 10% (v/v) formaldehyde (Sigma, USA) at room temperature for 20 min. The monolayer cells were then washed twice with excess distilled water prior to the addition of ARS 2 % (Sigma, USA) (pH=4.1). The flasks were incubated at room temperature for 20 min while being shaken. After the aspiration of the unincorporated dye, the flasks were washed four times with distilled water while being shaken for 5 min (18). The secretion of calcified extra cellular matrix was observed as reddish areas with Alizarin Red S staining.

### *Quantification of Mineralization*

The analysis of the amount of calcium deposition in osteogenic media was modified from a previous report (18). For the quantification of Alizarin Red S staining, 2 ml 10% (v/v) acetic acid was added to each flask incubated at room temperature for 30 min while being shaken. 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 with a wide-mouth pipette. 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 20,000g for 15 min and 500µl of the supernatant was removed to a new 1.5 ml micro centrifuge tube. Then 200µl of 10% (v/v) ammonium hydroxide was added to neutralize the acid. In some cases, the pH was measured at this point to ensure that it was between 4.1 and 4.5. Aliquots (150µl) of the supernatant were read in triplicate at 405 nm in 96-well format using opaque-walled, transparent-bottomed plates.

### ***ALP Activity***

Cells were lysated by sonication for three cycles, and then protein solutions were centrifuged at 2000g for 15 min at 4°C. The supernatant was kept at -20°C for the analysis of ALP activity and protein content. A total cellular protein analysis was performed on the same samples as the ALP activity measurements. The total protein content of each sample was determined according to Bradford, using bovine serum albumin (BSA) as a standard. The ALP activity was performed using an ALP kit (Ziest Chem., Tehran, Iran) following the manufacturer's instructions. The levels of activity were neutralized with an amount of protein in cell lysate solution (units/mg protein) (19).

### ***Cell Viability Assay***

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Sigma, USA) test measures the mitochondrial (metabolic) activity in the cell culture, which reflects the number of viable cells. In brief, the cultures ( $10 \times 10^5$  was seeded to a 96-well plat) were washed in PBS, and 200µl MTT reagents were added. Following incubation for 3 hrs in the incubator (in 5% CO<sub>2</sub> at 37°C), the absorption of the medium was measured in an ELISA Reader (Anthon 2020) at 440nm (20).

### ***Apoptosis Detection***

DNA fragmentation, as the late feature of apoptosis, was determined by flow cytometry as a percentage of nuclei with hypo diploid DNA content. For DNA content evaluation, the samples were fixed with 70% ethanol at 4°C for at least 30min. They were washed in PBS, resuspended in 400µl citrate buffer (Sigma, USA) and then (PI) (Sigma, USA) stained with 400 µl of a 50 µg/ml propidium iodide PI. The samples were incubated at 37°C for 30 min, and then analyzed. PI red fluorescence was collected on a linear scale: the events in the hypo diploid peak identified the percentage of apoptosis. Sample acquisition was performed by FACScan flow cytometer equipped with Cell Quest software (21)

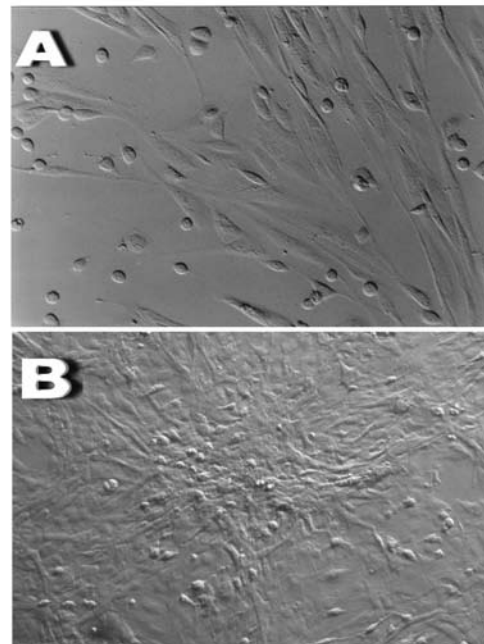
### ***Statistical Analysis***

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

## **Results**

### ***Isolated Rat Adipose-Derived Stem Cells***

Adipose-derived stem cells grown in culture appeared spindle-shaped. Cells cultured in osteogenic media demonstrated a dramatic change in morphology from the day 10 of induction, with the cells changing morphology from an elongated fibroblastic appearance to polygonal shape (Fig 1).



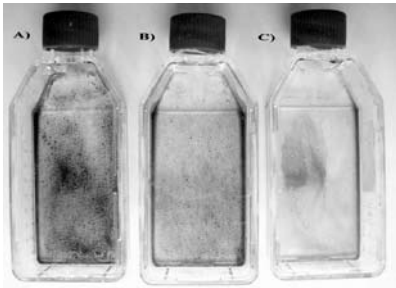
**Fig 1:** Initially adherent Adipocyte stem cells grew as spindle-shaped cells that developed into multi-polar fibroblastoid cells (x200) (A). They gradually reached confluency at 7<sup>th</sup> day(x200) (B). (This Figures has also been printed in full-color at the end issue)

### ***Effects of melatonin on formation of mineralized bone nodules***

The cytological results of ADSCs cultures were convincingly positive, when stained by Alizarin Red S and von Kossa after 28 days of the subculture. Alizarin red S staining was used to investigate mineralized matrix formation by ADSCs. As shown in Fig 2, the intensity of staining increased in cells cultured in osteogenic medium alone when compared with cells exposed to osteogenic

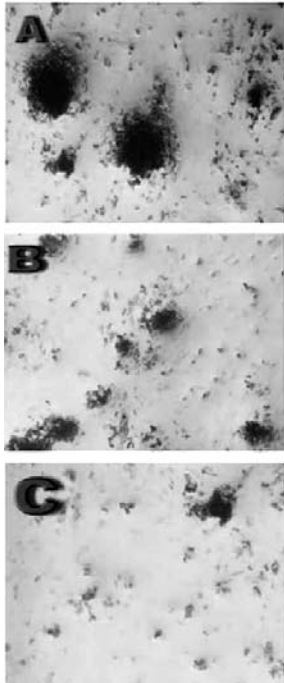
## Melatonin Effect on Rat ADSCs

medium supplemented with melatonin (Fig 2).



**Fig 2:** Alizarin Red S staining after osteogenic differentiation. Staining for mineral deposition was performed for ADSCs after 28 days. The osteogenic medium alone group was the control group. A) Control medium B) in presence of melatonin 10 n M, C) in presence of melatonin 0.01 n M. (This Figures has also been printed in full-color at the end issue)

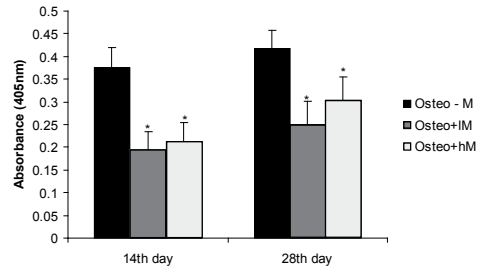
The Von Kossa staining was used to investigate nodule formation by ADSCs. The first mineralized bone nodules formed by ADSCs were observed after 10 days of treatment. The addition of melatonin at physiologic concentrations to the osteogenic media decreased the formation of mineralized nodules compared with osteogenic media alone (Fig3). The experiments were repeated at least three times and showed similar effects.



**Fig 3:** Von Kossa staining after osteogenic differentiation of Adipocyte stem cells. This staining was done only for demonstrating of osteogenic differentiation after 28 days. A) Osteogenic medium alone, B) Osteogenic medium in presence of melatonin 10 n M, C) Osteogenic medium in presence of melatonin 0.01 n M. (x200)

## Quantification of Mineralization

Calcium level quantification was measured in the three groups after 14 and 28 days following differentiation of ADSCs in osteogenic medium with or without of melatonin (Fig 4).



**Fig 4:** Calcium deposition The ADSCs were cultured in osteogenic medium in the absence and presence of physiological concentrations of melatonin (0.01-10 n M) for 28 days in flasks 25cm<sup>2</sup> (10×10<sup>5</sup> cells/flask ) as a monolayer culture. The osteogenic medium alone group was the control group. Calcium depositions were measured as described in Materials and Methods. Values are mean±SD (n=3) (\*p<0.05). AD: Adipose derived stem Cells, Cont: control, IM: melatonin 0.01 n M, hM: melatonin 10 nM.

Calcium level quantification was measured in the control groups as 0.374 ± 0.045 and 0.418 ± 0.086 (p < 0.05) 14 and 28 days after osteogenic induction in ADSCs, respectively.

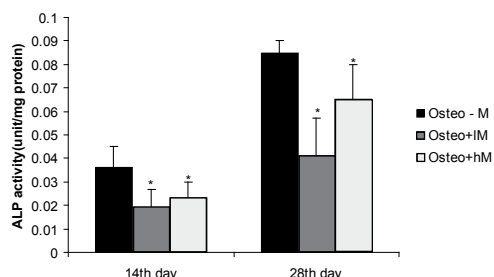
The calcium was more in the control groups. The melatonin containing groups revealed low levels of calcium quantification measured as 0.195 ± 0.039 and 0.025 ± 0.051 (p < 0.05) in IM and 0.211 ± 0.043 and 0.303 ± 0.051 (p < 0.05) in hM after 14 and 28 days, respectively.

## Effects of melatonin on ALP activity

As a marker for ADSCs differentiation into osteoblasts, ALP levels were measured after 14 and 28 days. As shown in Fig 5, ALP activity increased in ADSCs following incubation in osteogenic medium alone when compared with cells exposed to osteogenic medium containing melatonin.

When ADSCs, were exposed to melatonin, in combination with the osteogenic medium, ALP activity significantly decreased beyond that of cells exposed to osteogenic medium alone (control: 14th day 0.036 ± 0.09, 28th days 0.05 ± 0.005 p < 0.05). These data indicate that melatonin suppresses ALP activity in low dose.

But as can be seen, after 28 days ALP activity increased in ADSCs following incubation in osteogenic medium in the presence of melatonin (IM 0.019±0.008, 0.041±0.0016; hM 0.023±0.007, 0.065±0.0015 14th & 28th days respectively p<0.05). A concentration-dependent effect of melatonin on ALP activity was also observed in cell populations.



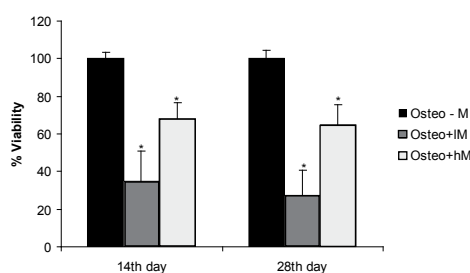
**Fig 5:** ALP activity in Adipocyte stem cells on osteogenic differentiation at day 14 and 28. The cells were plated at  $10 \times 10^5$  cells/flask and cultured in osteogenic medium with or without physiologic concentration of melatonin. The osteogenic medium alone group was the control group. Values are means± S.D. (n=3) (\*p<0.05). ADSCs: Adipose derived stem Cells, Cont: control, IM: melatonin 0.01 nM, hM: melatonin 10 nM.

**Effects of melatonin on viability of ADSCs**

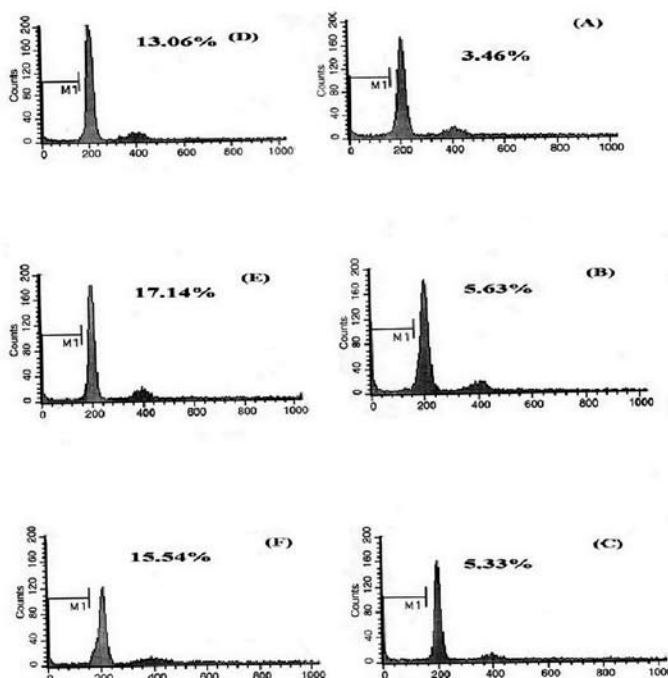
As shown in (Fig 6) the physiological concentrations of melatonin (0.01-10 n M) affect the cell viability of ADSCs at 14 and

28 days after the melatonin treatment as assessed by MTT assay.

The data indicated that viable cells among the control groups (14th day: 100±3.46%; 28th day 100±4.33%, p<0.05) were higher than the cells in melatonin containing groups. As can be seen, when ADSCs were exposed to melatonin, cell viability increased beyond that of cells exposed to osteogenic medium alone (IM. 34.7±16.1, 27.3±13.51; hM 68±8.83, 65±10.39 14th & 28th days respectively p<0.05).



**Fig 6:** Effect of physiologic concentration melatonin on the viability of Adipose derived stem cells. The cells were plated at  $10 \times 10^5$  cells/well and cultured in osteogenic medium with or without physiologic concentration of melatonin for 28 days. Cell viability was measured by MTT assay at 14<sup>th</sup>, 28<sup>th</sup> day. The osteogenic medium alone group was the control group. Values are means± S.D. (n=3) (\*p<0.05). Cont: control, IM: melatonin 0.01 n M, hM: melatonin 10 nM.



**Fig 7:** Flow cytometric analysis of DNA content for detection of apoptosis after 24 and 72 hr of melatonin treatment. The cells were plated at  $10 \times 10^5$  cells/flask and cultured in osteogenic medium with or without of melatonin. A, D after 24, 72 hr treatment with osteogenic medium alone, respectively. B, E: after 24, 72 hr treatment with osteogenic medium in presence of melatonin 0.01nM, respectively. C, F: after 24, 72 hr treatment with osteogenic medium in presence of melatonin 10 nM, respectively.

### *Effects of melatonin on apoptosis of ADSCs*

To quantitate accurately the incidence of apoptotic cells in defined ADSCs population, we used flow cytometry to detect content of DNA in cells labeled by propidium iodide. As shown in Figure 7, the incidence of apoptotic cells is increased in ADSCs following incubation in osteogenic medium supplemented with melatonin when compared with cells exposed to osteogenic medium alone. Melatonin treatment induced a cell growth down regulation detectable after 24 and 72 hrs of incubation and increased with the treatment time among ADSCs (Fig 7).

### **Discussion**

Our results clearly show that melatonin modulates the proliferative and differentiative potentials of ADSCs in a concentration- and exposure-timing-dependent manner.

One of the most interesting functions of physiological concentrations of melatonin in the present study was that ADSCs which had been propagated in melatonin-containing media exhibited a decrease in the proliferation and osteogenic differentiation. When ADSCs were exposed to melatonin, We observed that ALP activity and mineral deposition decreased. This can be due to the reduction in the number of cells, which was demonstrated by the results of MTT assay and flow cytometry. With regard to the results of MTT and ALP activity assays it should be mentioned that induction of osteogenic medium on stem cells differentiation is a gradual process which takes four weeks, that means, there are lots of osteoblasts at the end of 4<sup>th</sup> week so ALP activity is higher than 2<sup>nd</sup> week (Fig 5) although the whole number of cells is decreased (Fig 6). In the present study, it was demonstrated that in melatonin-containing media, the progression of the apoptotic type of ADSCs is not prevented; rather, it was exaggerated by melatonin. The induction of apoptosis by melatonin without the addition of other drugs 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 (14). Anti-proliferative effects of melatonin have been demonstrated in vivo and in vitro in a number of cancer cells (22, 23) and normal cells (17, 24). In contrast, pharmacological concentration melatonin

(10mM, 100mM) stimulates proliferation of normal cells such as human bone cells (25). The fact that melatonin may affect cell growth in biphasic manner was first reported by Slominski and Pruski in experiments with cultured rat melanoma cells (26). In that study, low concentrations of melatonin suppressed human melanoma cell growth, while higher concentrations stimulated such growth. These findings led Roth et al. to explore the effect of melatonin on cell growth in rat pheochromocytoma cells (PC12 cells) (27). Similar to the above findings with human melanoma cells, the melatonin induced a biphasic dose response for cell growth in the PC12 cell model. More specifically, at low concentrations, melatonin suppressed PC12 cells growth, while at a higher concentration; it prevented cell death (27). Thus, melatonin differentially suppressed proliferation in cell lines of different behavior.

As mentioned before one of the most interesting actions of melatonin in the present study was that the ADSCs, which had been propagated in melatonin-containing medium, exhibited a decrease in the proliferation and osteogenic differentiation even when incubated without melatonin. These results suggest that melatonin exhibited the aftereffect on the function of the ADSCs. This effect of physiological concentrations of melatonin is also reported in its protective actions against PC12 cell death (27). Although we could not explain why melatonin influences the fate of the ADSCs in physiological concentrations, it is possible that melatonin might not affect the cellular components regulating the cell cycle and differentiation process via cellular membrane receptors (17) or could be related to the cell-line specific pattern of melatonin cellular receptors and cytosolic binding protein expression (27).

### **Conclusion**

It is premature to offer firm conclusions about such findings except to indicate that biphasic apoptotic responses reliably occur. However, the fact that these observations are quite recent suggests that they will stimulate more exploration in this important low-dose research area.

### **Acknowledgments**

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