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The Role of Melatonin Preconditioning on Survival of Bone Marrow-Derived Mesenchymal Stem Cells in Differentiation to Osteoblasts

Ali Rafat [1], Maryam Bahmanzadeh [2], Sara Soleimani Asl [1], Amaneh Mohammadi Roushandeh [3] and Zoleikha Golipoor [4, *]

[1]Department of Anatomical Sciences, School of Medicine, Hamadan University of Medical Sciences, Hamadan, IR Iran
[2]Endometrium and Endometriosis Research Center, Hamadan University of Medical Sciences, Hamadan, Iran
[3]Medical Biotechnology Research Center, Paramedicine Faculty, Guilan University of Medical Sciences, Rasht, Iran
[4]Cellular and Molecular Research Center, Faculty of Medicine, Guilan University of Medical Sciences, Rasht, Iran

[*]*Corresponding author*: Department of Anatomical Sciences, School of Medicine, Guilan University of Medical Science, Rasht, Iran. Tel: +981333690884, +98-9122239929, Fax: +981333690036, E-mail: masoomeh_golipoor@yahoo.com

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Abstract

Background: Preconditioning has become an increasingly promising strategy and applied in cell therapy and shows multiple therapeutic benefits in several diseases. Some bioactive agents, such as hormones, are utilized to a develop cell therapy outcome. Melatonin is secreted from the pineal gland and is famous for its antioxidant capability and cytoprotection potential. Evidence suggest that melatonin may play an important role in the regulation of mesenchymal stem cells (MSCs) differentiation to osteoblasts.
Objectives: The aim of this study was to evaluate the influence of melatonin preconditioning on the differentiation of bone marrow derived mesenchymal stem cells (BM-MSCs) to osteoblasts.
Methods: This experimental study was performed at molecular genetic and embryology laboratories of Hamadan University of Medical Sciences, Hamadan, Iran, from October 2015 to April 2017. The bone marrow stem cells (BMSCs) were obtained, cultured, expanded, and preconditioned with 5 μM melatonin and analyzed for their multi-potency and immunophenotypic potential at passage five. There were three groups composed of the control group BMSCs + osteogenic medium group, and melatonin-bone marrow stem cells (MT-BMSCs) group. The BMSCs and MT-BMSCs were cultivated in an osteogenic medium. After three weeks of cell survival, osteogenic capability and apoptosis were assessed in three groups.
Results: Bone marrow-derived mesenchymal stem cells expressed CD44 and CD90, yet not CD45 and showed differentiation to adipocytes and osteoblast. The BMSCs were preconditioned with MT expressed metallothionein 1 (MT1) and metallothionein 2 (MT2) ($P < 0.05$). Group three showed lower expression of *Bax* gene yet higher expression of *Bcl2* ($P < 0.05$). Group three had a high ability of osteogenic differentiation capability and reduced apoptosis.
Conclusions: The current study detected that melatonin pretreatment promotes MSC survival, reduce apoptosis, and has a positive effect on osteogenic factors in vitro. The preconditioning strategy may represent a safe approach to improve the beneficial effects of stem cell therapy in bone regenerative medicine.

Keywords: Antioxidant, Bone Marrow, Mesenchymal Stem Cell, Melatonin Osteoblast, Osteogenesis, Preconditioning

1. Background

Stem cells have a high potential for therapeutic properties in the curing of a vast variety of diseases (1, 2). Mesenchymal stem cells are pluripotent non–hematopoitic stromal cells that can be harvested from many tissues, such as bone marrow, umbilical cord, umbilical blood, Wharton jelly, synovial membrane and fluid, and also perodental ligament (3). The BM-MSCs simply produces single cell-derived colonies that can be highly propagated and differentiated to mesenchymal originated tissues, including bone, cartilage, and skeletal muscle (4, 5). Osteogenic BMSCs preparations have been used as new cell-based therapies to repair damaged skeletal tissues (6, 7). However, the cells viability and differentiation diminish dramatically after transplantation in vivo and their therapeutic efficiency is weakened (8). Of note, the transplanted cells in ischemic or injured tissues reside in harsh environments. The efficacy of direct application of MSC is limited because the transplanted cells do not survive efficiently within the in-

jured organ and more than 80% to 90% of grafted cells die within 72 hours after injection (9). Different factors occur in the early death of grafted cells, including free radicals, hypoxia, food deprivation, thermal stress, and inflammation (10-12). Therefore, BMSCs need to protect themselves from potentially detrimental effects of thermal shock, food shortage, oxidative stress, and ischemia (13). Therefore, establishing strategies to improve cells survival and homing before BMSC-based therapies in clinical trials is the necessity. Preconditioning has been an increasingly promising strategy and applied in cell therapy and represents multiple therapeutic benefits after injury in different tissues (14, 15). Pharmacological preconditioning utilizes some bioactive agents, including hormones, cytokines vitamins, and proteins to develop cell therapy outcome (16, 17). Melatonin (N-acetyl-5-methoxytryptamine) is secreted from the pineal gland and is known for its antioxidant property and cytoprotection potential (18). It has been declared that melatonin increases MSCs survival and motility. Emerging pieces of evidence suggest that melatonin may act as a critical regulator of precursor cell commitment and differentiation. Furthermore, MSC differentiation is finely controlled by the action of mechanical and molecular signals that originate from the extracellular environment. Melatonin is considered as a potent free radical scavenger and inducer of cellular antioxidants in different cell types and provides crucial protection mechanisms for MSC after their grafting into damaged tissues (19). Another evidence suggests that melatonin has a critical role in the regulation of MSC differentiation to osteoblasts (20). Bone growth was also promoted by melatonin through improving osteoblastic differentiation, which is shown by increase in expression of alkaline phosphatases (ALP), osteocalcin (OC), osteopontin, bone sialoprotein (BSP). The apoptosis and survival of preconditioned cells with melatonin have not been reported in differentiation to osteoblasts.

2. Objectives

Therefore, the current study was designed to determine how melatonin affects differentiation rate of MSCs and its effects on apoptosis and survival of differentiated osteoblasts.

3. Methods

The present research was an experimental study and was supported financially by the research deputy of Hamadan University of Medical Sciences.

3.1. Bone Marrow-Derived Mesenchymal Stem Cells Isolation

Six-week old male Wistar rats were sacrificed using diethyl ether and their tibia and femur bones were collected under sterile conditions. Each bone was flushed with α-minimum essential medium eagle (α-MEM) (Sigma, USA) containing 1% PenStrep (Gibco, Germany) to harvest its bone marrow. The cells were centrifuged at 1500 rpm for five minutes and dispersed in α-MEM with 10% fetal bovine serum (FBS) (Sigma, USA), containing 100 U/mL penicillin and 100 μg/mL streptomycin (21).

3.1.1. Cell Culture and Expansion

The harvested cells were cultivated in T75 tissue culture flasks at a concentration of 10×10^5 cells per flask. The flasks were incubated at 37°C and 5% CO_2 and the medium was changed every three days. Cell subculture was performed using trypsin/ethylenediamine tetraacetic acid (EDTA) (Sigma, USA) when the flasks reached 90% confluency.

3.1.2. Flow Cytometric Analysis

BMSCs from three to five passages were harvested by trypsinization and then stained using antibodies CD44 (ab157107, Abcam), CD90-FITC (ab226, Abcam), CD45 (ab10558, Abcam), and secondary antibodies (Goat Anti-Rabbit FITC, ad97050, Abcam and Donkey Anti-Rabbit PE, 12-4739-81, Ebioscience) for 20 minutes. Afterward, they were rinsed twice in PBS and analyzed using a Attune™ NxT Flow Cytometer from Thermo Fisher Scientific.

3.1.3. Bone Marrow-Derived Mesenchymal Stem Cells Multi-Lineage Differentiation Potential

Passage two MSCs were disseminated at a density of 5×10^3 cells/cm^2 in 96-well plates with α-MEM with 10% fetal bovine serum containing 1% PenStrep. After 24 hours, the medium was replaced by osteogenic and adipogenic differentiation media. Osteogenic differentiation medium composed of α-MEM, 10% fetal bovine serum, 50 mg/mL L-ascorbic acid (A92902, Sigma), and 1% PenStrep, 10 nM dexamethasone (D4902, Sigma), 2 mM L-glutamine (G3126, Sigma), and 10 mM b-glycerophosphate (G6501, Sigma). Adipogenic differentiation medium composed of 10 nM dexamethasone (D4902, Sigma), 200 mg/mL indomethacin (I7378, Sigma), 0.5 mM IBMX (I5879, Sigma), and 5 mg/mL insulin (I1507, Sigma). Every three days, the differentiation media were changed. Osteogenic deposits and adipocytes cells were found after 21 days using alizarin red (Alizarin-Red Staining Solution, TMS-008-C, Millipor) and oil red O (oil red O Kit, ab150678 , Abcam) staining, respectively.

3.2. Pretreatment of Mesenchymal Stem Cells with Melatonin

To detect the preconditioning effect of melatonin on BMSCs survival and differentiation, the cells were subjected to 24 hours of treatment with 5 μM melatonin (M5250, Sigma, USA). Then, melatonin-pretreated BMSCs (MT-BMSCs) were washed three times with PBS (Sigma, USA) for complete discard of the hormone (18).

3.3. Osteogenic Potential of MT-BMMSCs

After passage five, BMSCs and MT-BMMSCs were cultivated at 10^6 cells/cm^2 concentration and were incubated in an osteogenic medium, including 0.05 mM ascorbate, 1 μM dexamethasone, and 10 mM b-glycerophosphate for 21 days (22).

3.3.1. Quantification of Alizarin Red Protocol

Amount of calcium deposition in osteocytes was detected, according to Gregory et al.'s protocol. Briefly, 2 mL of 10% (v/v) acetic acid was added to the flask. After 30 minutes, the monolayer was separated from the plate using a cell scraper and all solutions were transferred to a 15-mL tube. After vortexing for 30 seconds, the slurry was covered with 1.25 mL mineral oil (Sigma, USA), heated to 85°C for 10 minutes, and placed on ice for five minutes. The slurry was then centrifuged at 20 000 $\times$g for 15 minutes and 500 μL of the supernatant was discarded to a new 1.5-mL tube. Afterward, 200 μL of 10% (v/v) ammonium hydroxide was added to neutralize the acid. Next, 150 μL of the supernatant was read at 405 nm in 96-well format using opaque-walled, transparent-bottomed plates and their OD was recorded for statistical analysis (23).

3.4. Cell Viability Assay

3-(4, 5-Dimethylthiazol- 2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (M2128, Sigma, USA) measures the mitochondrial metabolic activity in cell culture, which shows the number of viable cells. Briefly, 10×10^5 cells were cultured in a 96-well plate. After 24 hours, the cells were washed with PBS and 50 μL of MTT reagent was added. Following three hours of incubation at 37°C and 5% CO_2, the plate was read with the ELISA Reader at 630 nm and the groups OD was recorded (Microplate Reader, Rayto, RT-2100C, Germany).

3.5. Real Time-Polymerase Chain Reaction

Four weeks after osteogenic induction, total RNA was extracted from the cells using RNA extraction solution (RNX™, CinnaGen, Iran) and stored at -70°C for future analysis. The cDNA synthesis was carried out from 5 μg total RNA, using the Fermentas kit (Fermentas, Canada), according to the manufacturer's instructions. The primers of the genes detected in the present study are listed in Table 1. Overall 25 μL of PCR product contained components including 0.2 pM of each primer, 0.3 mM dNTP, 1.5 mM $MgCl_2$, 1U taq DNA polymerase, and 1$\times$PCR buffer (Fermentas, Canada). The PCR reactions were conducted in a programmable thermocycler (Bio-rad, USA) with the following temperature profile: 94°C for five minutes, 35 cycles at 94°C for 45 seconds, 55°C for 45 seconds, 72°C for 45 seconds, and a final extension at 74°C for 10 minutes. Furthermore, 10 μg of the PCR product was ran on a 1.5% agarose gel. The density of the PCR bands was quantified using Image J software.

3.6. Apoptosis Detection

The cells in the control, BMSCs, and MT-BMMSCs groups were seeded to a 96-well plate. The apoptosis detection was performed by in situ cell detection kit (11684817910, Roche, Canada), based on the manufacturer's manual. Briefly, the cells were rinsed with PBS and fixed in 4% paraformaldehyde. Then, endogenous peroxidase activity was blocked by methanol and H_2O_2 and permeabilized with TritonX-100 (T9284, Sigma). The TUNEL reaction solution was added to the cells and incubated for one hour at 37°C followed by 30 minutes of incubation in Converter-POD. Finally, the reaction was developed using 3, 3′-diaminobenzidine (DAB) and the apoptotic cells were observed under a light microscope. The brown cells were considered as apoptotic cells.

3.7. Statistical Analysis

The findings are presented as mean $\pm$ SD. The data were analyzed by one-way analysis of variance (ANOVA) followed by the post hoc test. All experiments were performed in triplicates and $P < 0.05$ was considered significant.

4. Results

4.1. Rat BMSCs Characterization

Rat BM-MSCs appeared as a monolayer of large, fibroblast-like flattened cells (Figure 1A) and showed osteogenic (Figure 1B) and adipogenic (Figure 1C) phenotype when cultured in differentiation media. Flow cytometry findings of rat passages three to five BMSCs confirmed the presence of CD44 and CD90 and was negative for CD45 (Figure 1D).

4.2. Melatonin Improved Cell Viability

The cells underwent a 24-hour preconditioning with melatonin followed by incubation with osteogenic medium (OS) for 21 days. Based on MTT findings, the rate of viability in MT-BMSCs was significantly higher than BMSCs without melatonin ($P < 0.05$) (Table 2). As shown in Table 2, the rate of viability was more than 50% in MT-BMSCs compared to 25% in BMSCs.

Table 1. Primers and Expected Length of Products: Sense, Antisense

Primer	Sequence	Length, bp
MT1	S: 5´-CGGACAGCAAACCCAAACTG-3´	152
	A: 5´AACTAGCCACGAAGAGCCAC-3´	
MT2	S: 5´-TGACCTGTTACTGAATGTTGCC-3´	199
	A: 5´GAACTGCGATTTCTGGGTTAC-3´	
Bax	S: 5´-AACAACATGGAGCTGCAGAGG-3´	304
	A: 5´-GAAGTTGCCGTCTGCAAACAT-3´	
Bcl-2	S: 5´-TGACTTCTCTCGTCGCTACC-3´	116
	A: 5´-CACAATCCTCCCCCAGTTCA-3´	
Osteocalcin	S: 5´-AGGACCCTCTCTCTGCTAC-3´	138
	A: 5-AACGGTGGTGCCATAGATGC-3´	

Abbreviations: A, Antisense; MT1, Melatonin receptor 1; MT2, Melatonin receptor 2; S, Sense.

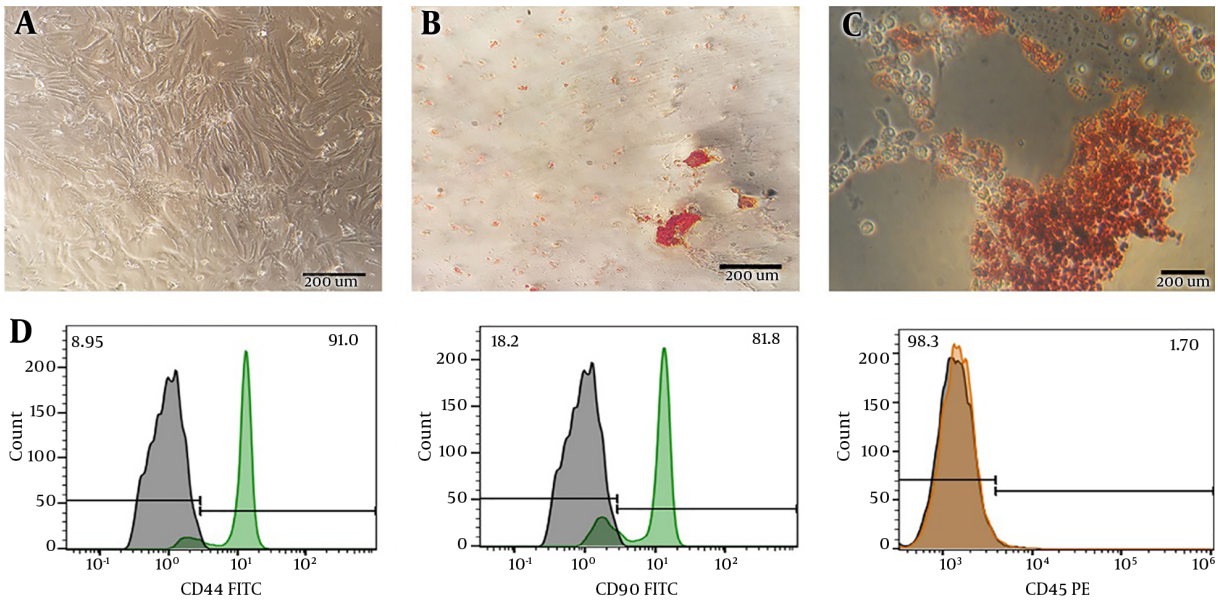

Figure 1. Undifferentiated bone marrow stem cells (BMSCs) (A) under phase contrast microscopy, displayed a flattened fibroblast-like morphology. Alizarin red staining of mineralized bone tissue and oil red O positive intracellular lipid droplets indicates that BMSCs could differentiate to osteoblasts (B) and adipocytes (C), respectively. Flow cytometric analysis of BMSC showing that they expressed CD44, CD90 and did not express CD45 (D).

4.3. Expression of Pro/Anti Apoptotic, MT1 and MT2 Receptors, and Osteocalcin Genes in Differentiated BMSCs and MT-BMSCs

The current results indicated that melatonin decreased the pro-apoptotic gene expression (*Bax*) and upregulated anti-apoptotic (*Bcl2*) gene expression that was detected by RT-PCR ($P < 0.05$) (Figure 2A and Table 3). Also, the presence of the melatonin receptors (MT1 and MT2 gene transcripts) was detected. It was shown that after melatonin preconditioning, its two receptors MT1 and MT2 were upregulated significantly in the MT-BMSCs group compared with BMSCs ($P < 0.05$) (Figure 2B and Table 3).

The presence of the osteoblast cell marker gene transcript was confirmed by RT-PCR. The findings showed that MT-BMSCs expressed a high level of osteocalcin compared to BMSCs ($P < 0.05$) (Figure 2C and Table 3).

4.4. Osteogenic Differentiation Capability of Melatonin

Quantification analysis of alizarin red staining with one-way analysis of variance (ANOVA) demonstrated that

Table 2. Results of Post Hoc Analysis After 21 Days[a]

Variables	Groups		
	Control	BMSC	MT-BMSC
Optical absorption[b]	0.621 ± 0.042	0.207 ± 0.043*	0.047 ± 0.038**
Number of mineralized nodules[c]	55.85 ± 8.42	462.1 ± 11.31*	2184 ± 36.56**
Number of apoptotic cells[d]	94.6 ± 7.461	529.8 ± 25.240*	93.8 ± 16.360**

Abbreviations: BMSC, bone marrow stem cells; MT-BMSC, melatonin-bone marrow stem cells.
[a] Data are presented as mean ± SD.
[b] Optical Absorption (nm): *, P < 0.001 compare to Control. **, P < 0.05 compared to BMSC.
[c] Number of mineralized nodules: The Data shows the quantification of osteogenesis by analysis of alizarin red concentration. Note the significant increase in osteogenesis in MT-BMSC group compared to the control and BMCs *, P<0.005 compare to the control. **, P < 0.01 compared to BMCS.
[d] Number of apoptotic cells: The number of apoptotic cells were counted and presented as percentage in different groups *, P<0.001, compare to the control. **, P < 0.001 compared to BMCS.

Table 3. After 24 Hours, the Optical Density of Each Band Calculated by the Image J software Results of Post Hoc Analysis[a]

Density of Bands on an Agar Gel	Groups	
	BMSC	MT-BMSC
Bax	14.2113 ± 0.2643	8.027 ± 0.2945[b]
Bcl2	7.7628 ± 0.76	15.029 ± 0.83[b]
MT1	12.107 ± 0.6554	31.341 ± 0.6239[b]
MT2	6.349 ± 0.734	32.558 ± 0.8517[b]

Abbreviations: MT1, Melatonin receptor 1; MT2, Melatonin receptor 2; BMSC, bone marrow stem cells ; MT-BMSC, melatonin-bone marrow stem cells.
[a] Data are presented as mean ± SD of Bax, Bcl2, MT1, MT2 density (pixels).
[b] P < 0.05, compared to BMSC.

the cells exposed to melatonin-induced higher osteogenic differentiation and represented more mineralized nodules (P < 0.01) (Figure 3 and Table 4).

4.5. Melatonin Attenuated Apoptosis in the Bone Marrow Stem Cells

In situ cell death detection was performed to determine whether melatonin might decrease apoptosis in MSCs. The results showed that in the BMSC group, many positively stained apoptotic cells with round shape and brown nucleus were observed (Figure 4A-4C); while, the number of apoptotic cells decreased significantly in MT-BMSCs compared with the BMCs group (P < 0.001) (Table 2).

5. Discussion

The present study attempted to investigate protective and osteogenic differentiation capability effects of melatonin preconditioning on BM-MSCs. The results showed that melatonin might regulate MSCs behavior and prepares the cells more efficiently for stem cell therapy. Indeed, this study found that melatonin protects MSCs from apoptosis. These effects were associated with increased BMSCs survival after differentiation to osteoblasts based on osteocalcin gene expression of differentiated BMSCs and MT-BMSCs and severity of alizarin red. Taken together, the present study revealed that preconditioning of BM-MSCs with melatonin could improve their therapeutic outcomes.

Furthermore, MT could exert its effect via receptor-mediated or through receptor-independent mechanisms on BM-MSCs (2). This research found that there was higher level of MT1 and MT2 receptors expression in MT-BMSCs rather than BMSCs. It has been documented that MT proteins have protective potential through their receptor-mediated mechanisms and result in improvement of MSCs survival (3, 24) and reduce apoptosis (3). It was declared that pretreatment of melatonin has cytoprotective potential against H_2O_2 toxicity and increases MSCs viability through increase in antioxidants capacity and decline in apoptosis and secretion of inflammatory cytokines (25). Han et al. showed that melatonin could increase the therapeutic efficiency of MSCs through activation of antioxidants induction pathways, such as silent information regulator 1 (SIRT1) and increase the expression of anti-apoptotic genes (26). Also, Zhang et al. showed that using constant concentration of melatonin for a time course supports osteogenic differentiation in hMSCs in vitro and could be considered seriously as a growth stimulator in re-

Table 4. Results of post hoc analysis after 21 days from the optical density of each band that was calculated by the image J software[a]

Density of Bands on an Agar Gel	Groups	
	BMSC	MT-BMSC
Osteocalcin	13.483 ± 0.5393	28.59 ± 0.3279[b]

Abbreviations: BMSC, bone marrow stem cells; MT-BMSC, melatonin-bone marrow stem cells.
[a] Data are presented as mean ± SD of osteocalcin density (pixels).
[b] $P < 0.05$, compared to BMSC.

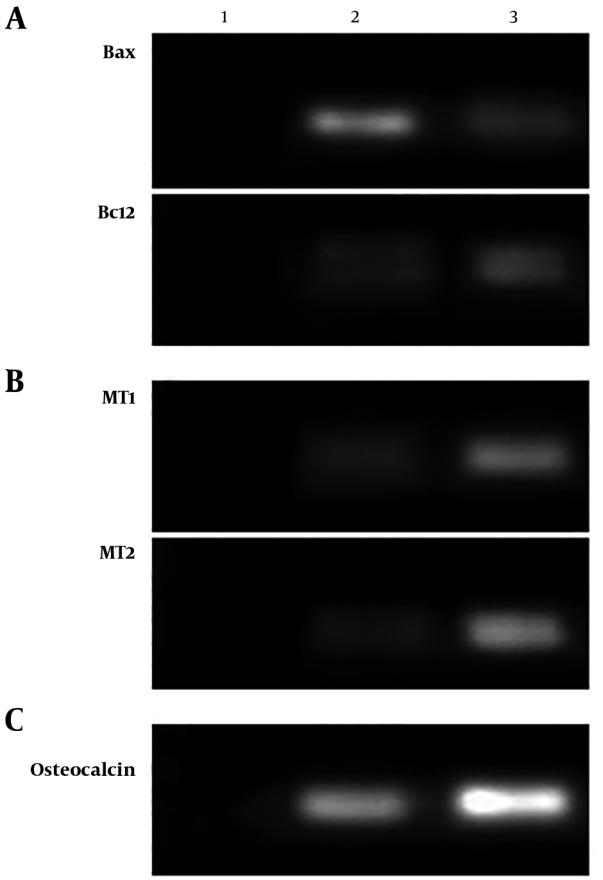

Figure 2. Analysis of *Bax* and *Bcl-2* after 24 hours of preconditioning of melatonin by RT-PCR. It is shown that melatonin decreased pro-apoptotic gene (*Bax*) expression and upregulated anti- apoptotic (*Bcl2*) (A). Gene expression of melatonin receptor (MT1, MT2) mRNA expression after 24 hours (B) presents osteocalcin expression level extracted from bone marrow stem cells (BMSCs) and melatonin-bone marrow stem cells (MT-BMSC) differentiated osteoblast (C). Negative control of RT-PCR (H2o) (Lane 1), BMSC (Lane 2), and MT-BMSC (Lane 3).

generative medicine clinics for bone healing (27). There is a body of studies in the field of mesenchymal stem cell signaling that supports the positive effect of melatonin in the process of osteogenesis differentiation. It is suggested that MT regulates transcription factors, such as Runx2 that control osteogenesis (28). In addition, mesenchymal stem cell signaling studies have also described the role of the Wnt signal in differentiation to osteoblasts (29, 30). It seems that melatonin strongly enhances the release of osteogenic factors, which is shown as the concentration of alizarin red and expression of osteocalcin gene in the present study. A body of studies confirmed the capacity of melatonin to promote bone growth through development in osteoblast cells differentiation and functional competence (27, 29). It has been reported that melatonin could regulate the osteogenic differentiation of MSCs (20). Some aspects of the cell signaling demonstrated the routes of MSC differentiation in osteoblast cell lineages, also providing suggestions on the possible effect of melatonin and its osteogenesis potential (27). Melatonin preconditioning could decline the number of apoptotic cells and enhance BMSC viability. There are some previous studies that have supported the current findings regarding apoptosis and cell viability (3, 31, 32). Although there are promising messages using melatonin to increase cell therapy outcomes, yet, considerations, including the dose and time of melatonin application and also the sources of MSCs and different diseases, should be noted.

5.1. Weak Points and Limitations

Using human mesenchymal stem cells would be more efficient and the results will be more applicable. Therefore, using human-derived MSCs in the current study was better that Rat-BMMSCs. Of note, the period of cell differentiation for each experimental group was long. In addition, there was a budget limitation.

5.2. Strong Point

Bone regeneration is a highly important topic in regenerative medicine. This study reports that melatonin preconditioning could activate the pathway that improves osteogenesis in BMSC. On the other hand, it increases cell viability and decreases apoptosis. The researchers hope that these findings are promising and a preliminary study for clinical application in bone regenerative medicine.

5.3. Conclusions

The current study suggested that melatonin pretreatment promotes BMSCs survival reduces apoptosis and has

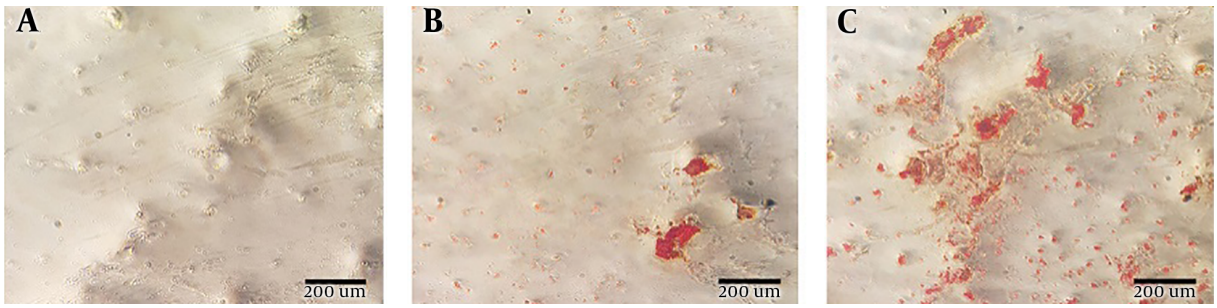

Figure 3. Alizarin red staining for mineral deposition after osteogenic differentiation. Control (bone marrow stem cells (BMSC) without any pretreatment and any differentiation) (A), BMSC (B), melatonin-bone marrow stem cells (MT-BMSC) (C), after 21 days (C).

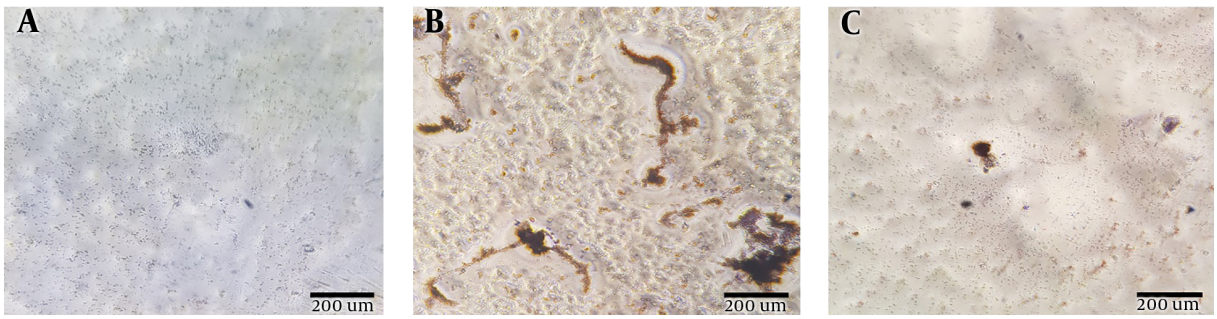

Figure 4. Apoptosis induction was detected using the TUNNEL assay kit after 21 days of differentiation period into osteoblast cells and observed under a light microscope. Control group (A), bone marrow stem cells (BMSC) (B), melatonin-bone marrow stem cells (MT-BMSC) (C).

positive effects on osteogenic factors in vitro. The preconditioning strategy in stem cells may represent an efficient approach to improve the beneficial effects of stem cell therapy in bone regenerative medicine.

Footnote

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