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Effects of melatonin on adult human mesenchymal stem cells in osteoblastic differentiation. An experimental in vitro study

TO CITE THIS ARTICLE
Calvo-Guirado JL, Pérez-Albacete C, Pérez Sánchez C, Boquete-Castro A, Maté–Sánchez De Val JE, Delgado Peña JE, Ramírez Fernández MP, Garcés M, Meseguer-Olmo L, Gómez Moreno G. Effects of melatonin on adult human mesenchymal stem cells in osteoblastic differentiation. An experimental in vitro study. J Osseointegr 2015;7(2):23-32.

KEYWORDS Bone regeneration; Melatonin; Osteoblast differentiation; Stem cells.

ABSTRACT

Aim The purpose of this study was to determine if different melatonin concentrations enhances human adult mesenchymal stem cells (MSCs) differentiation into osteoblasts, in comparison with MSCs cultured with dexamethasone (DEX).

Material and Methods MSCs were treated with different melatonin concentrations. Specifically: Group I: untreated MSCs; Group II: MSCs exposed to physiological doses of melatonin of 0.01 µM; Group III: MSCs exposed to 50 µM melatonin; Group IV: MSCs exposed to 100 µM melatonin; Group V: MSCs exposed to 150 µM melatonin; Group VI: MSCs exposed to 100 µM of DEX. Cell viability, adhesion, growth, differentiation and activity were evaluated at different time points (3, 7, 14, 21 and 28 days) using the following assays MTT, SEM, Flowcytometry, ALP activity, Alizarin Red staining and RT-PCR.

Results Melatonin stimulated the viability and alizarin red activity of MSCs in a dose-dependent manner. Melatonin 50 µM significantly increased ALP activity, especially after 21 and 28 days of culture. A significant decrease in the expression of membrane markers CD75, CD105 and CD90 was recorded over time, in the presence of melatonin; therefore, the MSCs were early differentiated into osteoblasts regardless of the melatonin concentration.

Conclusion These results demonstrated that melatonin directly accelerated the differentiation of human stem cells into osteoblasts and also suggested that melatonin could be applied as a pharmaceutical agent to promote bone regeneration.

INTRODUCTION

Melatonin, or N-acetyl 5-methoxytryptamine, is a hormone synthesized and secreted mainly in the pineal gland (1). Melatonin synthesis does not only take place in the pineal gland, but also in other areas such as the eyes, lymphocytes, gut, bone marrow, skin and gonads, where it acts either as a paracrine or an autocrine agent (2). Melatonin is released in response to darkness; conversely, it is inhibited by light (3). Because of its diverse activity, melatonin is not a hormone in the strictest sense. Melatonin has significant bone protecting properties (4). It is involved in osteogenesis and osteolysis through a variety of mechanisms. The effects of melatonin on bone tissue have been attributed to its action on osteoblasts, that induces either their differentiation or proliferation (5-8) or inhibits osteoclast activity (9, 10). It is also known that the age-related decrease in melatonin is related to an increased risk of osteoporosis (7-11). The results obtained in the review by Cardinali et al. (2003) (12) indicate that melatonin may positively influence age-associated bone loss in a number of ways. One is its direct effect on bone by acting on osteoclasts and perhaps on osteoblasts and turning the calcium balance positive. In the case of osteoclasts, melatonin activity could be associated with its potent antioxidant properties.

Ladizesky et al. (13) found that melatonin inhibited in vitro the increased calcium uptake in bone samples

of rats treated with pharmacologic amounts of methylprednisolone. Numerous studies (14-19) have documented melatonin's importance as a mediator in bone formation. Indeed, melatonin acts as a promoter of bone formation in vivo by enhancing both the proliferation and differentiation of osteogenic cells (20). In micromolar concentrations, melatonin stimulates the synthesis of type-I collagen fibers in human osteoblasts in vitro (21). In addition, Roth et al. (6) found that it increased gene expression of bone sialoprotein, as well as other proteins and bone markers including alkaline phosphatase (ALP), osteopontin and osteocalcin in preosteoblasts, reducing the osteoblast differentiation period from 21 to 12 days.

The action of melatonin on bone tissue is of interest as it may be possible to apply melatonin as a biomimetic agent during endosseous dental implant surgery (12). There have been some reports that demonstrate that melatonin in bone defects has an effect of augmenting cortical bone width and length.

A study by Calvo-Guirado et al. (2009) found that topical applications of melatonin accelerate bone formation associated with implants in a canine model; four weeks after implant insertion, melatonin in addition to collagenized porcine bone increased osteointegration and reduced crestal bone resorption significantly (16).

Furthermore, a latter study published by Calvo-Guirado et al. (2010) using metaphyseal defects in a rabbit model corroborated the results of the former, reporting that melatonin regenerated the width and length of cortical bone around implants more quickly than around control implants without the addition of melatonin (22).

Moreover, animal experiments have observed that melatonin works synergistically together with growth hormone, remodeling the socket and making it heal with new bone formation in the first weeks after tooth extraction. This indicates that melatonin maintains capillary homeostasis under normal conditions, as the tissue observed was made up of numerous endothelial sprouts and capillary blood vessels with abundant mesenchymal cells of irregular morphology. In the control group, it was observed that angiogenesis was enhanced time-dependently during the natural healing process, whereas melatonin accelerated the healing process, pointing to its strong angiogenic potential (19, 23).

The goal of this study was to observe different quantities of melatonin exposure over different time periods in order to define the ideal dose of melatonin exposure required to differentiate undifferentiated MSCs into osteoblasts. These MSCs were used as a model to analyze the continuing effect of different doses of melatonin on the capacity of differentiation to osteoblasts, comparing MSCs with dexamethasone (DEX), used as a control.

MATERIALS AND METHODS

Isolation and culture of bone marrow MSCs

Stem cells (MSCs) were isolated from bone marrow obtained by percutaneous direct aspiration from the iliac crest of three male human volunteers (50 mL/patient) in good physical condition ranging from 27 to 35 years old, who were undergoing elective surgery for slipped disc and a vascular necrosis of the femoral head as result of posterior dislocation. All the volunteers signed an informed consent. The study was approved by the ethics and clinical trials committee of the Virgen de la Arrixaca University Hospital, Murcia (Spain), where the study was performed. For the isolation, the aspirated material was transferred to a sterile tube containing sodium heparin (20 U mL^{-1} of aspirated material).

Mononuclear cell fraction was obtained from buffy coat cells by Ficoll density gradient-based separation and cell washing using the closed automated SEPAX™ System (Biosafe, Eysines, Switzerland). After estimating the number and viability of cells with a vital dye (tripan blue staining) using a Neubauer chamber, the mononuclear cells were plated out in 75 cm^2 culture flasks (Sarstedt, Nümbrecht, Germany) with 10 mL of basal culture growth medium (GM) and incubated at 37°C, in 5% CO2 and 95% humidity atmosphere to attach undisturbed for seven days. The growth media (GM) used was Minimum Essential Medium (MEM) (Sigma-Aldrich, St Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and penicillin/streptomycin (100 U/mL^{-1} and 100 µg/mL^{-1} respectively) (Sigma-Aldrich). The culture medium was renewed twice a week, removing the non-adherent hematopoietic cells and selecting the MSCs by means of their proven capacity for attaching to the plastic of the flasks.

When the cellular cultures became near-confluent (80-85%) (passage 0, P0), they were subcultured in a 1:3 ratio, treating the cells with trypsin-EDTA (0.25%/0.25%) in phosphate-buffered saline (PBS, pH 7.4) for five minutes. To obtain a homogenous sample and reduce inter-individual variability among donors, as well as the number of donors needed, all the cells from the first subculture (passage 1, P1) were regrouped and cultured in 150 cm^2 flasks, growing a sufficient number of cells for use in the study (passage 3, P3).

Phenotypic characterization of bone marrow-derived MSCs. Flow cytometry

In order to confirm the identity of isolated cells, the adherent ones, considered as MSCs, were tested for CD73, CD90 and CD105 marker expression and the non-expression of the hematopoietic markers CD34 and CD45, following the criteria of the International Society of Cellular Therapy (ISCT). For flow cytometry, specific mouse monoclonal antibodies were used at a concentration of 2.5 mg/mL: CD34 PE/clone 581, CD45 FITC/clone HI30, CD73 PE/clone AD2, CD90

APC/clone 5E10 and CD105 FITC/clone 266 (Becton Dickinson, Madrid, Spain). As isotype controls anti-mouse immunoglobulin IgG1 marked with fluorescein isothiocyanate (FITC), IgG1 K marked with phycoerythrin (PE) and IgG1 k marked with allophycocyanin (APC) (Becton Dickinson) were used. The samples were acquired by the flow cytometer FACSort (Becton Dickinson) and analyzed using the CellQuest program, calculating the geometric mean of the distribution of fluorescence for each fluorochrome. These values determined mean fluorescence for each surface antigen and were performed for each of the specimen's different passages of culture, fixing the FACSort cytometer acquisition parameters.

Study design

To conduct the study, six groups were established: Group I: control group of untreated MSCs; Group II: MSCs exposed to physiological doses of melatonin of 0.01 µM; Group III: MSCs exposed to 50 µM melatonin; Group IV: MSCs exposed to 100 µM melatonin; Group V: MSCs exposed to 150 µM melatonin; Group VI: MSCs exposed to 100 µM of dexamethasone (DEX). The cultivation conditions were the same for the six groups as described previously and the application to MSCs of the different doses of melatonin and DEX was continuous throughout the study. All tests were performed at least in triplicate and repeated 8 times.

MTT Assay

To assess whether melatonin affected MSCs proliferation, cellular viability was assessed using 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue (MTT; Sigma-Aldrich Química, Madrid, Spain) assay after 3, 7, 14 and 21-days of exposure. The MTT assay is dependent on cellular reduction of MTT by the mitochondrial dehydrogenase in living cells producing a formazan product that represents the number of living cells. The amount of formazan product produced from the MTT assay was solubilized using 100 µL Dimethylsulfoxide (DMSO) and spectrophotometrically analyzed at 690 nm.

Contrast phase optical microscopy observation

The isolated MSCs attached to plastic were observed with a Nikon, Eclipse TE2000 U phase contrast optical microscope (Nikon Instruments, Amsterdam, Holland), recording the evolution of cultures, changes to their morphological features, adhesion properties on plastic and growth under the effect of different doses of melatonin and DEX. Observations were made at 3, 7, 14 and 21 days.

Scanning Electron Microscopy (SEM)

SEM was used to evaluate the morphology, cell adhesion and growth of MSCs cultured with different doses of melatonin. Cells at a density of 3.0 x 10^3 cells/cm^2 were seeded onto two-well chamber slides (4.2 cm^2) and cultured for different periods (3, 7, 14, and 21 days). Afterwards, the cultured cells were rinsed with PBS and fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2-7.4) for one hour at 4°C. Subsequently, they were rinsed and postfixed with osmium tetroxide for one hour before being dehydrated through a graded ethanol series (30, 50, 70, 90 vol.%), with final dehydration in absolute alcohol. After this, samples were dried by the critical point method in an acetone and CO_2 bath in a CPDO2 Balzers Union chamber (Balzers, Liechtenstein) and then sputter coated with gold (200 Å thickness) in a Bio-Rad Polaron Division vaporizer (Labexchange, Burladingen, Germany). The samples were observed with a Jeol T-6100 (Croissy-sur-Seine, France) scanning electron microscope with an acceleration of 15 to 20 kV.

Energy Dispersive Microanalysis

To determine the chemical elements of which the nodules (observed by SEM) were composed, in cultures exposed to the action of melatonin (0.01, 50, 100 and 150 µM) and DEX, samples were processed as described for MEB and carbon coated. For spectroscopic microanalysis an INCA SEM-EDS (Oxford Instruments, Bristol, UK) was used.

Flow Cytometry

Flow cytometry was performed in order to determine the phenotypic changes in the expression of MSCs surface markers before and after exposure to different concentrations of melatonin and DEX. The criteria of the International Society of Cellular Therapy were applied: the presence of clusters of differentiation (CD) – CD73, CD90 and CD105 – and the absence of hematopoietic markers CD34 and CD45. The analysis were performed at 3, 7, 14 and 21 days of culture.

Alkaline Phosphatase Activity (ALP)

ALP activity was determined by staining using a commercially available quantitative assay kit (Quantitative Alkaline Phosphatase ES Characterization kit SCR 066, Millipore, Billerica, MA, USA). Cells at a density of 3.0 x 103 cells/cm^2 were seeded on 12-well plates with different treatments. The commercial assay kit was carried out at 14, 21 and 28 days, following the manufacturer's instructions. The results were spectrophotometrically analyzed at 405 nm.

Alizarin Red Staining

The presence of calcium deposition (nodules of mineralization) in cultures was evaluated qualitatively by selective binding of Alizarin Red Solution (ARS) (Osteogenesis Assay Kit, Millipore, Billerica, MA, USA) to calcium salts following the manufacturer's protocol. Briefly, after culturing the cells with different melatonin concentrations and DEX at a density of 3.0 x 103 cells/cm^2 on 48-well plates, after 21 and 28 days of culture

Gene name	Gen symbol	Amplicon lenght (bp)	ID Test
Osteocalcine	BGLAP	90 bp	Hs_BGLAP_1_SG
Octamer Binding Protein 4	OCT-4	77 bp	Hs_POU5F1_1_SG
Colagen type I	COL1A1	118 bp	Hs_COL1A1_1_SG
Glyceraldehyde-3-phosphate dehydrogenase	GADPH	95bp	Hs_GADPHH_1_SG

TABLE 1 The primer sequences employed in real time polymerase chain reaction (RT-PCR).

samples were rinsed three times in DPBS, fixed with 8% paraformaldehyde at room temperature for 15 minutes, and then stained for 30 min. After fully washing with distilled water, the stained areas were visualized under an optical microscope.

Real Time Polymerase Chain Reaction (RT-PCR)

Cells were seeded at 3.0 x 103 cells/cm² in 75 cm² flasks and cultured in the presence of different melatonin concentrations (0.01, 50, 100 and 150 µM) and also with the osteoinducer DEX (100 µM) for 3, 7, 14, 21 and 28 days. Total RNA was isolated using the RNeasy™ mini kit (Qiagen Ibérica SL, Madrid, Spain) following the manufacturer's instructions.

Briefly, the cells were lysed directly in the flask with lysis buffer and the help of a cell scraper; the lysate was homogenized by vortexing and then 1:1 volumes of ethanol were added before transfer to an RNeasy spin column. Ribonucleic acid (RNA) was eluted in 30 µL of RNase-free water and stored at -80°C; 1 µg RNA from each sample was reverse transcribed into complementary DNA (cDNA) using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Madrid, Spain) following the manufacturer's recommendations. Gene-specific primers were obtained from Qiagen's GeneGlobe Web Portal. Gene expression was quantified using real time reverse transcription-polymerase chain reaction (RT-PCR) analysis using the SYBR™ green master mix kit (Takara Bio INC. Shiga, Japan) in the Rotor-Gene™ Q (Qiagen Ibérica SL, Madrid, Spain). Rotor-Gene run conditions were as follow: activation at 95°C for 3 min, 40 amplification cycles at 95°C for 10 s and at 60°C for 34 s. Melting curve analysis was performed to ensure that all transcripts under investigation were represented by a single peak, indicating specificity (melting ramp from 70 to 95°C). Data analysis was carried out using Rotor-Gene software (Qiagen, Ibérica SL, Madrid, Spain). Each sample was run in triplicate. Gene expression levels were normalized to the expression of the housekeeping gene GAPDH. A comparative CT method was used to quantify the samples. Comparison of values was performed utilizing the formula: $2^{-\Delta C}{}_{T}$, to obtain a relative quantification. The CT value describes the difference between the CT value of the target gene and the CT value of the corresponding endogenous reference gene GAPDH. Expression of osteogenic marker genes including Col-I, OCT-4, BGLAP (osteocalcin) and GAPDH was examined (Table 1).

Statistical Analysis

Differences in outcome parameters were analyzed using the ANOVA. All pairwise multiple comparison procedures were performed applying the Bonferroni correction. For mean values, Welch and Brown-Forsythe tests were applied. P values of <0.05 were considered significant.

RESULTS

Isolation and Culture of Bone Marrow MSCs

During the first three days of culture, abundant blood cells could be observed as well as non-adherent cells with a round shape, and slightly elongated cells attached to the plastic. After seven days, the culture medium was renewed, removing the non-adherent hematopoietic cells and selecting just the attached MSCs to the plastic of the flasks. Once the cells were confluent, they were subcultured.

Phenotypic Characterization of Bone Marrow-Derived MSCs

The identity of isolated cells from primary culture was confirmed by flow cytometry. Cells expressed membrane markers CD75, CD105 and CD90 and did not express CD34 or CD45.

MTT Assay

Melatonin did not inhibit MSCs viability; cells treated with melatonin showed higher absorbance levels at 28

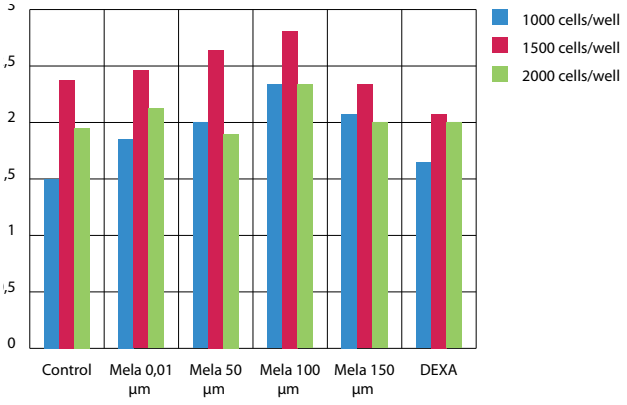

FIG. 1 MTT assay of cell viability. Levels of absorbance under the different dosages of melatonin and dexamethasone in different seeding densities after 28 days of culture.

days when compared to the control and DEX groups (Fig. 1).

Contrast Phase Optical Microscopy Observations

At 7 days, the control cells formed aggregates or colonies consisting of 25-40 cells; they generally showed elongated and polygonal shapes. After 28 days, cells showed a fibroblast-like morphology and formed a cohesive layer, covering the whole surface of the flask. When cultured cells were subjected to the action of melatonin, morphology behavior varied according to the dose and the duration of its action. The most remarkable findings were observed with doses of 0.01, 50 and 100 µM after 7 days of treatment, where a faster proliferation of cells treated with melatonin than with DEX and control cells was observed. At 28 days all cultures, regardless of the dose, reached confluence without significant morphological differences. The morphological appearance of MSCs did not vary between treatments and only small deposits of refractive material were noted in the cultures with DEX.

Scanning Electron Microscopy

After 3 days, cells of control group (absence of melatonin) showed a rounded shape and were clustered to form colonies. At 7 days, MSCs had a flattened and elongated appearance with the presence of cytoplasmic prolongations. At day 14, when the cell culture had reached confluence, MSCs adopted a spindle-shaped and polygonal morphology with widespread cytoplasmic extensions (filopodia). Finally, between days 21 and 28, it became difficult to individually recognize the cells as they covered the whole surface. At days 3 and 7, the MSCs undergoing treatment with melatonin had a similar appearance to the control samples. Although the number of cells and therefore the degree of confluence was greater than in the control group. Morphological variations between groups subjected to the action of different doses of melatonin were not observed. Only after 28 days, when they reached confluence, MSCs changed their morphology, becoming more flattened and spindle-shaped with a tendency to align in the same direction. At this time point, the nodules of mineralization isolated from cultures treated with melatonin (50 µM, 100 µM) and DEX were notable (Fig. 2).

Energy Dispersive Microanalysis

The spectrum showed birefringence nodules highlighting a peak that corresponded to phosphorus and a lower intensity for calcium, while the adjacent peaks correspond to spectral artifacts caused by different elements. This spectrum proved the presence of a calcium-like material indicating mineral apatite bone tissue fraction (Fig. 3).

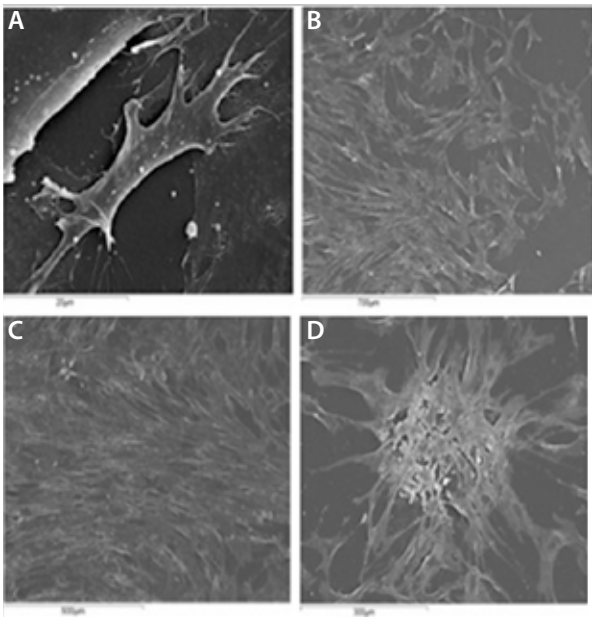

FIG. 2 Scanning electron microscope images of (A) MSCs cultured with melatonin at 3 days; (B) MSCs cultured with melatonin at 7 days; (C) MSCs cultured with melatonin at 14 days; (D) Nodules of mineralization isolated from cultures treated with melatonin at 21 days. Magnification A and D 200X, B and C 100X.

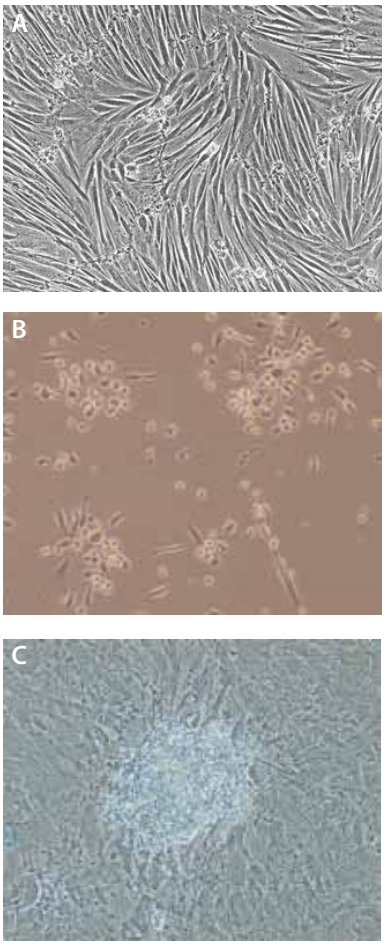

FIG. 3 Energy Dispersive Microanalysis. (A) Scanning electron microscope image of nodules of mineralization (magnification 200X); (B) The spectrum of the nodules of mineralization found at 21 days in groups III, IV and VI proved the presence of a calcium-like material indicating mineral apatite bone tissue fraction; (C) Percentages of calcium, phosphorous, and silicon found in the nodules of mineralization.

	Control	Mela 100 µm	Mela 150 µm	Mela 0.01 µm	DEX 0.01 µm	Control	Mela 150 µm	Mela 100 µm	Mela 0.01 µm	Mela 150 µm	Mela 0.01 µm	DEX 100 µm	DEX 100 µm	Mela 0.01 µm	DEX 100 µm
	Mela 50 µm	Mela 50 µm	Mela 50 µm	Mela 50 µm	Mela 50 µm	Mela 100 µm	Mela 100 µm	Mela 100 µm	Mela 100 µm	Control	Control	Control	Mela 150 µm	Mela 150 µm	Mela 0.01 µm
15 days	-0,447	-0,730	0,000	-0,447	-	-1,342	-0,674	-1,069	-0,447	0,000	-1,342	-	-	-1,604	-
	(0,655)	(0,465)	(1,000)	(0,655)	-	(0,180)	(0,500)	(0,285)	(0,655)	(1,000)	(0,180)	-	-	(0,109)	-
21 days	-0,943	0,000	-1,064	-1,782**	-2,023*	-1,095	-0,943	-1,604	0,000	-1,609	-1,159	-2,023*	-1,604	-0,535	-1,214
	(0,345)	(1,000)	(0,109)	(0,075)	(0,043)	(0,273)	(0,345)	(0,109)	(1,000)	(0,285)	(0,249)	(0,043)	(0,109)	(0,593)	(0,225)
28 days	-1,214	-1,363	-1,572	-2,203**	-2,001**	-0,944	-1,124	-1,424	-1,214	-0,674	-0,674	-0,135	-1,782**	-0,675	-1,782**
	(0,225)	(0,173)	(0,116)	(0,075)	(0,028)	(0,345)	(0,225)	(0,120)	(0,225)	(0,500)	(0,500)	(0,893)	(0,075)	(0,500)	(0,075)
Significant values in brackets. (*) Signiftcance at 5%. (**) Significance at 10%. Mela: melatonin.															

TABLE 2 Wilcoxon test of the results obtained with the ALP activity assay. At 15 days there was no significant results. At 21 and 28 days the group of melatonin 50 µM showed significant results when compared to the groups of melatonin 0.01µM and DEX 100 µM.

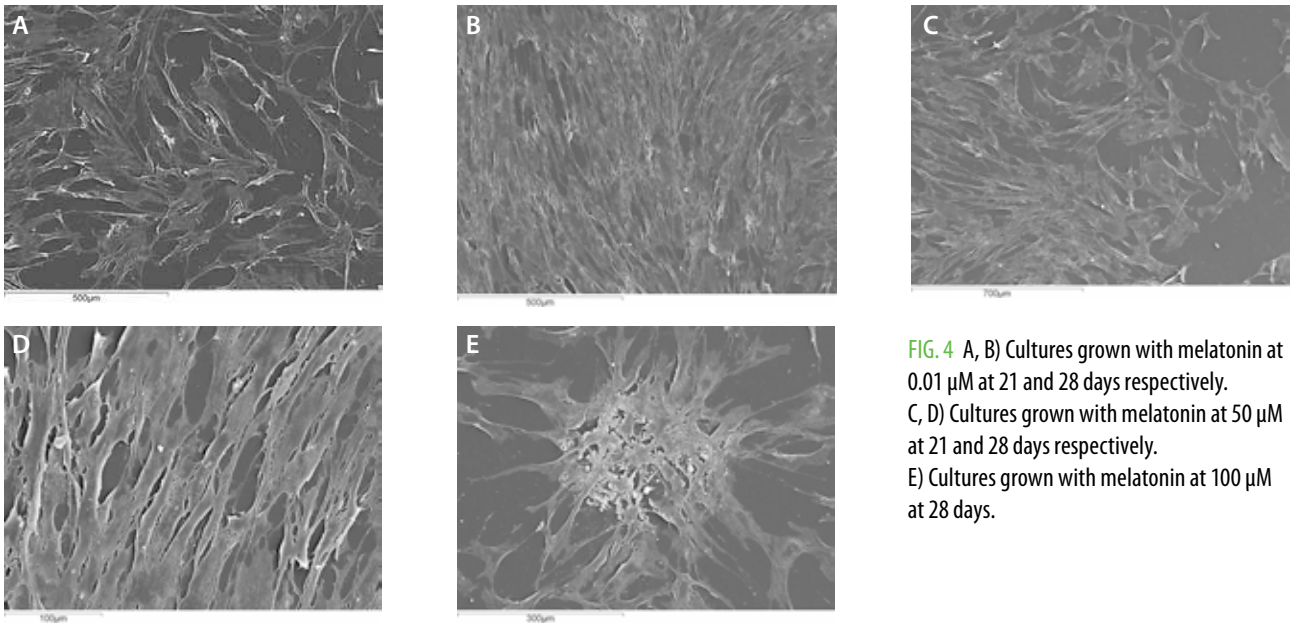

FIG. 4 A, B) Cultures grown with melatonin at 0.01 µM at 21 and 28 days respectively.
C, D) Cultures grown with melatonin at 50 µM at 21 and 28 days respectively.
E) Cultures grown with melatonin at 100 µM at 28 days.

Alkaline Phosphatase Activity

An increase of specific ALP activity (ALP activity related to cell number) at all examined time points of culture was found. Specifically, ALP activity was significantly higher in presence of 50 µM melatonin when compared to the other groups, especially after 21 and 28-days of culture (Table 2).

Alizarin Red Staining

All cultures treated with melatonin (0.01 µM, 50 µM, 100 µM and 150 µM) and DEX (100 µM) were alizarin red-positive at 21 and 28 days.

Flow Cytometry

Melatonin, at a physiological concentration of 0.01 µM, induced a significant ($p<0.05$) decrease in the expression of CD105 ($p=0.001$), CD73 ($p=0.0005$) and CD90 ($p=0.0005$) at 7, 14 and 21 days of continuous treatment in comparison with cells treated for 3 days. Treatment of MSCs with 50 µM of melatonin produced a significant ($p<0.05$) decrease in the expression of CD105 ($p=0.001$), CD73 ($p=0.001$) and CD90 ($p=0.002$) at 7, 14 and 21 days of continuous treatment in comparison with 3 days. Treatment with 100 µM of melatonin produced statistically significant ($p<0.05$) differences in expression of CD105 ($p=0.002$), CD73 ($p=0.009$), and CD90 ($p=0.002$) at 7, 14 and 21 days of continuous treatment in comparison with 3 days. Treatment with 150 µM of melatonin levels produced very similar results to the dose of 50 µM.

Finally, the treatment of MSCs with DEX did not produce modifications ($p<0.05$) in the expression of the markers CD105, CD73 and CD90 at 7, 14 and 21 days of continuous treatment with respect to 3 days. However, after 21 days of treatment, a greater decrease of CD90 expression was detected, that was statistically significant ($p<0.05$) in comparison with the CD105 and CD73 (Fig. 5).

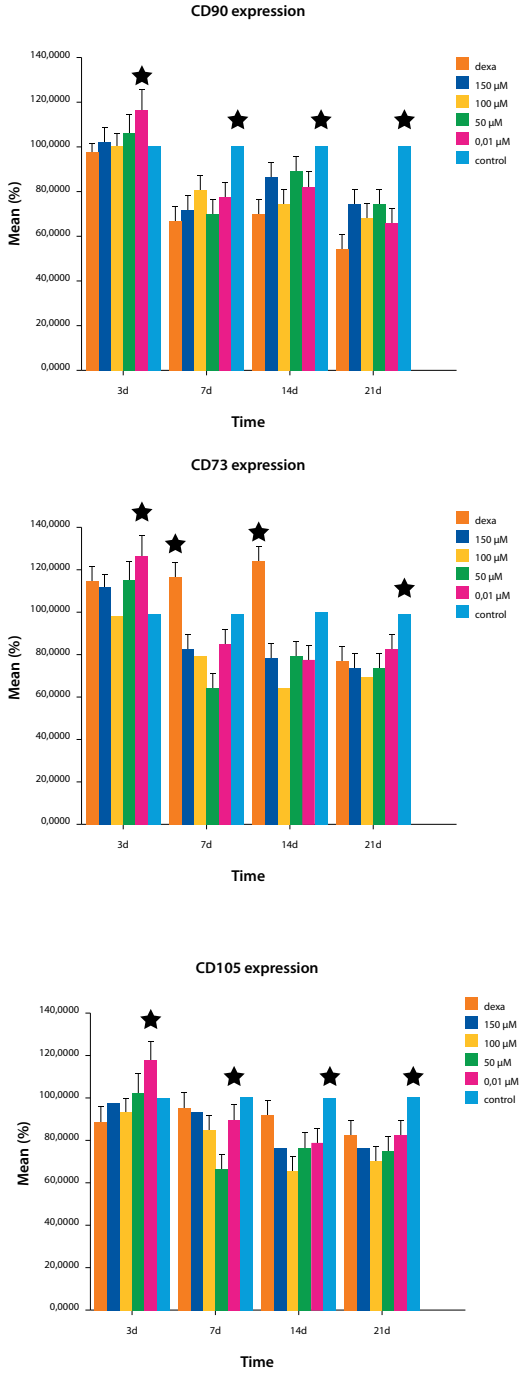

FIG. 5 Flow citometry was made to determine the phenotypic changes in the expression of MSCs surface markers before and after exposure to different concentrations of melatonin and DEX. (A) Evaluation of CD90 expression; (B) Evaluation of CD73 expression; (C) Evaluation of CD105 expression.

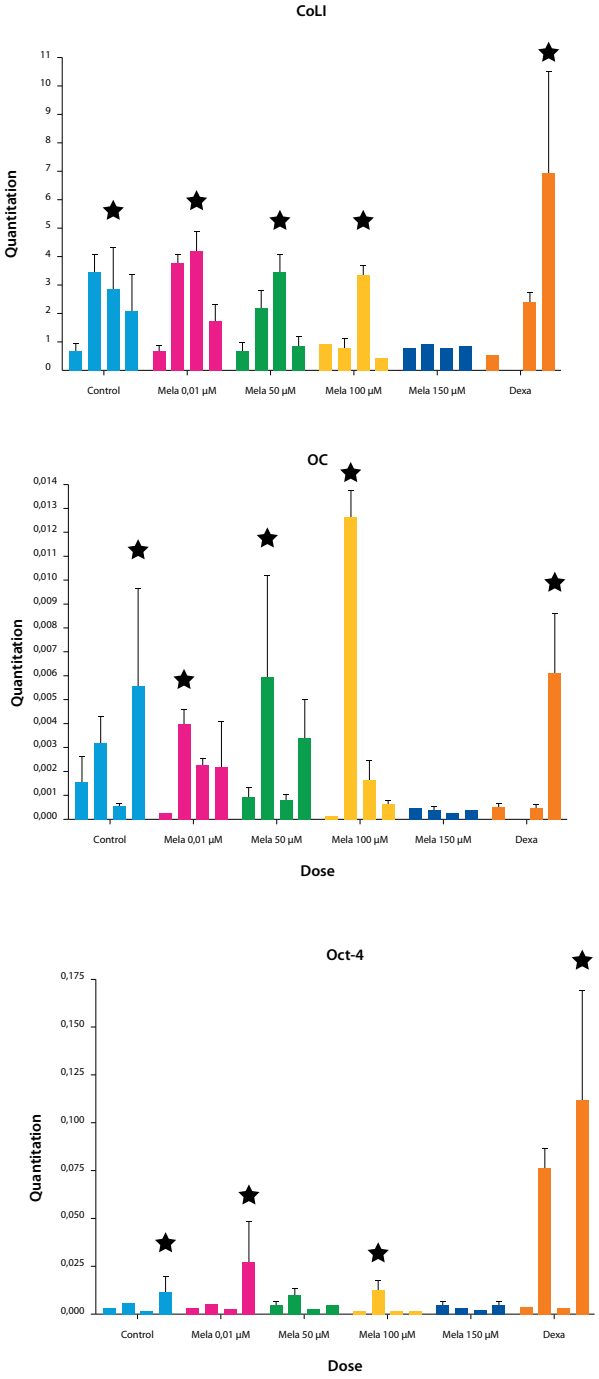

FIG. 6 Results obtained with RT-PCR. (A) Expression of Col I gen; (B) Expression of OC gen; (C) Expression of Oct-4 gen.

Real-time PCR

Collagen type I expression analyzed for all doses and all study intervals was significantly higher ($p \leq 0.05$) between 14 and 21 days, particularly in the cultures exposed to doses of 100 µM and 50 µM. When osteocalcin expression was analyzed, significant variations were found in all cases of cultures exposed to the dose of 100 µM of melatonin between 7 and 14 days, diminishing during the third week. This also occurred with the 0.01 µM dose between the 3-days time point and the first week and the second week, respectively. For the rest of the doses, no significant variations were observed between study times. Oct-4 expression was low and without significant variations for all doses and at all study points (Fig. 6).

DISCUSSION

Melatonin can either inhibit or stimulate proliferation depending on the type of cells and on the dose of melatonin used. Zang et al. showed that melatonin, both at physiological (0.01 µM, 1 nM) and pharmacological concentrations (100 µM) did not stimulate cell proliferation (24). The authors postulated that the effect of melatonin on cell division might depend on the specific cell type. As in a study by Sethi et al. (2010), MSCs exposed to melatonin (50 nM) for 2, 10 and 21 days in the presence of osteogenic medium (OM) did not show significant differences in proliferation in comparison with control cells (25). In the present study, melatonin at concentrations of 0.01 µM, 50 µM, 100 µM and 150 µM promoted cell viability at 28 days, achieving higher levels than the control group and the group treated with 100 µM DEX; this growth pattern was produced for all seeded cell concentrations (1000, 1500 and 2000 cells/well), however, differences were not statistically significant.

Melatonin has the capacity to directly promote osteoblast maturation (26). This was demonstrated for the first time with preosteoblastic clonal calvarial cells (MC3T3 cells) originating in new-born mice and in rat osteoblastic osteosarcoma cells (ROS cells), whereby determined concentrations of melatonin increased the gene expression of bone marker proteins such as BSP, ALP, osteopontin, collagen type I and osteocalcin. More specifically, in MC3T3 cells, concentrations of melatonin as low as 10 nM (0.01 µM) were sufficient to stimulate transcription of the BSP gene in presence of β-glycerophosphate and ascorbic acid (6). Melatonin in growth medium had less effect on osteopontin and collagen gene expression when compared with control cultures in osteogenic medium. Furthermore, ALP activity was significantly greater after the sixth day in culture for the cells treated with melatonin (10 µM or more) compared with a control group. ALP is the most widely recognized biochemical marker in osteoblast activity and so the activity of this enzyme is used to measure osteoblast differentiation and its activity increases as cellular differentiation progresses (27). In the present study, this enzyme's activity reached a maximum on the twelfth day in culture in the group treated with melatonin (50 µM or more) and decreased thereafter until day 15, while in the control group it continued to increase until day 15. This finding supported the ones of earlier studies with regard to the increase in ALP activity with the maturation of osteoblasts followed by a decrease. These results suggested that melatonin stimulated human osteoblast differentiation. Similar results were reported in normal human mandibular bone cells (HOB-M cells) and in human osteoblast cell lines (SU-HFO cells), for which micromolar concentrations of melatonin (50 µM, 100 µM) significantly increased collagen type I production; however, neither ALP activity nor osteocalcin secretion increased (5).

The in vitro action of melatonin on bone tissue may be different using animal mother cells and the results cannot be extrapolated to humans reliably. For this reason, it is very important, having carried out preliminary trials using animals, to proceed to human studies. In this sense, the double-blind study of Kotlarcyk et al. (2012) (28) evaluated the effects of nightly melatonin supplementation on bone health and quality of life in 18 perimenopausal women. Authors concluded that melatonin supplementation improved physical symptoms associated with perimenopause, and may improve bone remodeling thus preventing bone loss. This conclusions demonstrated that melatonin induces osteoblast activity and reduces osteoclast activity. Nevertheless, only Radio et al. (2006), Sethi et al. (2010) and Zang et al. (2010) used MSCs for studying the effects of melatonin on osteoblast differentiation (24, 25,29). There again, it must be stressed that results derived from studies of preosteoblastic cells or osteoblasts should not be compared with results obtained in studies using MSCs, as the former investigated whether melatonin stimulates or accelerates osteoblastic processes, while the latter investigated whether melatonin was able to induce MSCs differentiation into osteoblasts (MSCs can also differentiate into chondrocytes and adipocytes). In Radio et al. (2006) study, melatonin (50 nM=0.05 uM) was not seen to produce an increase ALP activity in MSCs cultivated in normal growth medium, but this did occur when the cells were cultivated in OM, which contains ascorbate, dexamethasone and β-glycerophosphate (29). The present study found the contrary as the group cultivated with melatonin 50 µM produced the greatest increases in ALP activity. The group treated with 100 µM also showed increases in ALP production. This difference in results could be interpreted as a difference in the concentrations used in the two studies. Radio et al. (2006) used melatonin at 50 nM, a concentration lower than the physiological dose (0.01 µM). It may be that such a low concentration was insufficient to produce any effect on MSCs differentiation to osteoblasts (29).

Zhang et al. (2010) found that ALP activity of cells treated with melatonin and cultivated in OM increased significantly depending on concentration (0.01 µM, 1 nM, 100 µM) (24). They concluded that melatonin produced osteoblastic differentiation but the melatonin was added to OM, which has been shown to induce MSCs differentiation to osteoblast lineage cells. The same study suggested that melatonin increases ALP activity directly and by itself (24). Although, since OM were always added, the data might be misleading as any increase in ALP production observed could be due to the dexamethasone content in OM or to a synergic action of this in combination with melatonin. On the other hand, the results of the present study did not show a decrease in ALP activity over time; indeed, it was after

day 21 when statistically significant results were found in the group treated with 50 µM melatonin. It should be noted that the group treated with 100 µM DEX showed low levels of ALP production, especially at day 28. Concentrations of 50 µM and 100 µM melatonin produced high levels of collagen type I and osteocalcin gene expression at 14 days, while at the same time, levels of OCT-4 gene expression were negligible. These data are in agreement with the levels of ALP production, as the group cultivated at 50 µM concentration induced greater ALP production, which suggested that melatonin at 50 µM may stimulate the stem cells to differentiate into osteoblasts. The study by Sethi et al. (2010) demonstrated that under continuous exposure to melatonin in OM for 21 days, MSCs increased their differentiation into osteoblasts, marked by increases in ALP activity and mineralization, in comparison with a control group (OM alone) (25). A significant increase in ALP activity was observed when melatonin was added to the medium for 2 days towards the end of the 21 days of OM culture, compared to cells cultivated in OM without melatonin for 21 days. However, the effect on ALP activity was not significant for cells treated with melatonin for 5, 10 or 14 days compared with the control (OM) (25). These results were similar to those described by Roth et al. (1999), who suggested that MC3T3 cells (preosteoblastic cells) must first differentiate before they will be receptive to melatonin (6). As before, it was not possible to compare data derived from the Sethi et al. (2010) with the present one as the cell culture conditions were not the same, since the melatonin was added to OM (25).

Alizarin red staining has been in use for many years as a quick and convenient method for visualizing the presence of calcium and apatite crystals (30). Sheti et al. (2010) observed that MSCs cultured with 50 nM concentrations of melatonin produced calcium deposits after 21 days (25). Satomura et al. (2007), in a study of osteoblasts, found that melatonin concentrations of 100 µM and 200 µM stimulated a statistically significant formation of mineralized matrix at 18 days (31). Results of the present study support these early results, obtaining calcium deposit production in cultures supplemented with melatonin (0.01 µM, 50 µM, 100 µM and 150 µM) at 21 days. At 28 days, melatonin-dosed cultures, with the exception of the 0.01 µM concentration, reached a very intense alizarin red staining, which confirms melatonin's capacity for promoting or facilitating MSCs differentiation to osteoblastic cells. Furthermore, when spectroscopic microanalysis of birefringent nodules was performed for cultures with 100 µM melatonin and DEX, these were composed of calcic material similar to the mineral fraction of bone tissue. The results of a study by Cui et al. (32) suggest that melatonin's action may depend on the cell type, the functional state of the cell or other factors such as drug concentration and melatonin concentration, which may be crucial.

To our best knowledge, no other study analyzed the action of melatonin on MSCs in osteoblastic differentiation, by evaluating the loss of membrane marker expression. Flow microscope evaluation is important as it indicated that as membrane marker CD105, CD90 and CD73 expression decreased, the cells started their differentiation process. The results obtained in the present study point to 50 µM melatonin as the concentration that produces the greatest overall loss of membrane marker expression, although concentrations of 100 µM and 150 µM produced similar expression levels as 50 µM. The present study is unique in demonstrating that melatonin produces a decrease in CD105, CD90 and CD73 marker expression, with this progressive loss of expression interpreted as a stage of MSCs differentiation to osteoblasts.

CONCLUSIONS

Our results support the possibility that melatonin plays an important role in the differentiation of MSCs into osteoblasts, especially melatonin at a concentration of 50 µM, when membrane marker CD105, CD90 and CD73 expression were seen to decrease, levels of ALP production to increase, production of calcium-like deposits were facilitated and collagen type I and osteocalcin gene expression increased, while OCT-4 gene expression diminished. Moreover, melatonin at any concentration did not inhibit cell viability. Calcium deposits were observed in cultures with melatonin at 100 µM.

On the basis of these results, it may be asserted that melatonin behaves as a cytocompatible and bioactive material able to induce osteoblastic differentiation of MSCs by itself and an effective substrate promoter of bone tissue regeneration suitable for bone tissue bioengineering.

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