ARTICLE
Journal of Cellular Biochemistry 116:551–558 (2015)

Journal of Cellular Biochemistry

A New Role for 5-methoxytryptophol On Bone Cells Function in Vitro

María Satué, Joana Maria Ramis, Maria del Mar Arriero, and Marta Monjo*

Group of Cell Therapy and Tissue Engineering, Research Institute on Health Sciences (IUNICS). University of Balearic Islands, Palma de Mallorca, Spain

ABSTRACT

The present study investigates the direct action of 5-methoxytryptophol (5-MTX) in both MC3T3-E1 and RAW264.7 cells and compares it with melatonin (MEL), another 5-methoxyindol known to play a significant role on bone metabolism. We first screened increasing doses of both 5-MTX and MEL to determine their effect on metabolic activity and viability of preosteoblastic MC3T3-E1 cells. The optimal dose was used to determine its effect on differentiation of MC3T3-E1 cells and preosteoclastic RAW264.7 cells. Finally, we investigated the mechanism of action by adding the melatonin receptor antagonist luzindole (LUZ) and detecting the immunostaining of phospho-ERK. In MC3T3-E1 cells, most of the 5-MTX doses reduced slightly the metabolic activity of osteoblasts compared with the control, while MEL only decreased it for the highest dose (2.5 mM). As regards to cytotoxicity, low doses (0.001–0.1 mM) of both indoles showed a protective effect on osteoblasts, while the highest dose of MEL showed a higher cytotoxicity than the 5-MTX one. After 14 days of cell culture, *Rankl* mRNA levels were decreased, especially for 5-MTX. 5-MTX also induced a higher osteocalcin secretion and mineralization capacity than MEL. In RAW264.7 cells, 5-MTX decreased the number of osteoclast formed and its activity whereas MEL did not affect significantly the number of multinucleated TRAP-positive cells formed and showed a lower activity. Finally, MEL and 5-MTX promoted activation of the ERK1/2 pathway through the phosphorylation of ERK, while LUZ addition suppressed this effect. In conclusion, the present study demonstrates a new role of 5-MTX inhibiting osteoclastogenesis and promoting osteoblast differentiation. J. Cell. Biochem. 116: 551–558, 2015.

KEY WORDS: MELATONIN; 5-METHOXYTRYPTOPHOL; OSTEOBLAST; OSTEOCLAST; BONE REMODELING

Melatonin (MEL) is the main product synthesized from serotonin in the pineal gland. The role of MEL involves numerous processes such as the regulation of circadian rhythms [Redman et al., 1983], reproduction [Reiter et al., 2009], sexual development [Guyomarc′h et al., 2001], immune system [Liebmann et al., 1997] and aging [Karasek and Reiter, 2002]. MEL levels decline with age and its availability to the organism is lower in the elderly than in the youth [Iguchi et al., 1982; Mishima et al., 2001]. Consequently, age-related disorders, such as osteoporosis and deficient implant osseointegration issues, are suspected to be affected by the decrease in MEL secretion. In fact, MEL has been demonstrated to affect bone metabolism through both bone anabolic as well as antiresorptive effects, as reviewed in [Cardinali et al., 2003; López-Martínez et al., 2012; Liu et al., 2013]. Moreover, many authors have suggested potential beneficial effects of MEL on periodontal regeneration or in dental implantology [Carrillo-Vico et al., 2005; Cutando et al., 2007; Gomez-Moreno et al., 2010; Gómez-Florit et al., 2013].

5-methoxytryptophol (5-MTX), another 5-methoxyindole compound synthesized by the pineal gland, is involved in numerous biological actions as recently reviewed in [Ouzir et al., 2013]. Thus, this compound regulates the biological rhythms and the reproductive behavior of several animal species, including humans. In addition, 5-MTX is a matter of high clinical relevance since it has been described as pro- or antigonadotropic hormone according to species [Balemans et al., 1977; Sackman et al., 1977], as a potent supplement in the treatment of cancer [Lissoni et al., 1997; Lissoni et al., 2000], as antioxidant [García et al., 2000; Rodriguez-Naranjo et al., 2012] and as immunomodulatory agent [Lissoni et al., 1996]. However, there is no data to date regarding its role in bone metabolism. 5-MTX is one of the methoxyindoles with potential to scavenge free radicals and with antioxidant activity [García et al., 2000]. Indeed, several indole derivatives related to MEL have been described to play a similar role by preventing oxidative damage in vitro [Poeggeler et al., 1996]. The properties of these indoles as free

Grant sponsor: Ministerio de Ciencia e Innovación del Gobierno de España.
* Correspondence to: Dr. M Monjo. Research Institute on Health Sciences (IUNICS). University of Balearic Islands, PO Box 07122 Palma de Mallorca, Spain. E-mail: marta.monjo@uib.es
Manuscript Received: 2 July 2014; Manuscript Accepted: 24 October 2014
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 30 October 2014
DOI 10.1002/jcb.25005 • © 2014 Wiley Periodicals, Inc.

radical scavengers or antioxidants are probably due to their similar molecular structure, but they show variable effectiveness and potency depending on the presence of additional substituents and changes in their position, which can modify their solubility and affinity.

In our search for new therapeutic agents for bone regeneration, we evaluated 5-MTX as a potential agent for regulating osteoblast and osteoclast cell function. Thus, the aim of the present study was to investigate the action of 5-MTX on bone metabolism, by assessing its direct action in both MC3T3-E1 and RAW264.7 cells. First, increasing concentrations (0.001–2.5 mM) of 5-MTX were used in MC3T3-E1 osteoblasts to investigate their effect on metabolic activity, cell viability and gene expression of markers related to osteoclast activation. Then, the optimum dose of 5-MTX (0.1 mM) was further evaluated in MC3T3-E1 cells for its effect on osteocalcin secretion and mineralization capacity. Finally, RAW264.7 macrophages were used to investigate the effect of 5-MTX on osteoclastogenesis. Given the conclusive effects on bone metabolism of MEL in vitro [Nakade et al., 1999; Roth et al., 1999; Koyama et al., 2002] and in vivo [Roth et al., 1999; Ladizesky et al., 2001; Koyama et al., 2002; Satomura et al., 2007; Uslu et al., 2007; Clafshenkel et al., 2012; Histing et al., 2012; Ramirez-Fernandez et al., 2013], the effects of 5-MTX were compared to those of MEL. Our results clearly indicate for the first time that 5-MTX has a stronger effect in vitro than MEL in terms of stimulating osteoblast differentiation and decreasing osteoclastogenesis.

EXPERIMENTAL PROCEDURES

TREATMENTS

All chemical reagents were of the purest available quality obtainable from commercial sources. 5-MTX and MEL were purchased from Sigma–Aldrich (Madrid, Spain) and dissolved in absolute ethanol (Scharlab, Barcelona, Spain). Final concentration of ethanol (0.9%) was included as vehicle control group in the experiments.

CELL CULTURE OF MC3T3-E1 CELLS

MC3T3-E1 cells (DSMZ, Braunschweig, Germany) were routinely cultured at 37°C in a humidified atmosphere of 5% CO_2, and maintained in the growing medium α-MEM (Pasching, Austria), which contains ascorbic acid (45 μg/ml) and sodium dihydrogen phosphate (140 mg/l) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories) and antibiotics (100 IU penicillin/ml and 100 μg streptomycin/ml).

Cells were seeded in 48-well plates at a density of 3.0×10^4 cells/cm^2. At confluence, cells were treated with different doses (ranging from 0.001 to 2.5 mM) of 5-MTX and MEL. Treatments were added at each medium change every other day.

DETERMINATION OF METABOLIC ACTIVITY AND CYTOTOXICITY

MC3T3-E1 cells were seeded at a density of 6.2×10^3 cells/cm^2 and cultured for 24 h. After cell attachment, culture medium was changed and different doses of MEL and 5-MTX were added. Total metabolic activity was quantified using Presto Blue reagent (Life Technologies, Carlsbad, CA) following manufacturer's protocols and 1 h of incubation time.

The presence of LDH activity in the culture medium after 48 h of incubation was used as an index of cell death. LDH activity was determined spectrophotometrically according to the manufacturer's kit instructions (Cytotoxicity Detection kit, Roche Diagnostics, Mannheim, Germany). Positive control was cell culture medium from cells incubated with Triton X-100 at 1%. Negative control was cell culture medium from cells incubated with the vehicle control.

RNA ISOLATION, RETROTRANSCRIPTION AND REAL-TIME PCR ANALYSIS

Total RNA was isolated from MC3T3-E1 and RAW264.7 cells after 14 and 5 days of cell culture, respectively, using a monophasic solution of phenol and guanidine isothiocyanate (Tripure, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Total RNA was quantified at 260 nm using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). The same amount of total RNA from each sample was reverse transcribed to cDNA using High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA) according to the protocol of the supplier.

Real-time RT-PCR was performed for two reference genes in both cell lines: 18S ribosomal RNA (18S rRNA), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). For MC3T3-E1 cells, two target genes were analyzed: Receptor activator of nuclear factor kappa-B ligand *(Rankl)* and Osteoprogeterin *(Opg)*. For RAW264.7 cells, 3 target genes were analyzed: Tartrate resistant acid phosphatase *(Trap)*, Metalloproteinase-9 *(Mmp-9)* and Cathepsin K *(CtsK)*. The reactions were performed in the Lightcycler 480® (Roche Diagnostics, Germany). Primer sequences used for PCR analysis and reaction conditions have been reported elsewhere [Satué et al., 2013a; Satué et al., 2013b]. Real-time efficiencies were calculated from the given slopes in the LightCycler 480 software (Roche Diagnostics, Mannheim, Germany) using serial dilutions, showing all the investigated transcripts high real-time PCR efficiency rates, and high linearity when different concentrations were used.

All samples were normalized by the geometric mean of the expression levels of *Gapdh* and *18S* and fold changes were related to the control groups using the formula: $\text{ratio} = E_{\text{target}}^{\Delta Cp \text{ target (mean control - sample)}} / E_{\text{reference}}^{\Delta Cp \text{ reference (mean control - sample)}}$, where Cp is the crossing point of the reaction amplification curve as determined by the LightCycler 480 software. Stability of reference genes was calculated using the BestKeeper tool [Pfaffl et al., 2004].

LUMINEX ANALYSIS

Cell culture supernatants were collected at day 14 of cell culture to analyze osteocalcin levels using the multiplex bead immunoassay (Mouse Bone Magnetic Bead Panel Kit, Cat#MBNMAG-41K, Millipore Corporation, Billerica, MA) according to the manufacturer's protocol. Analyte profiling was performed on the Luminex MAGPIX xPONENT 4.2 (Luminex Corporation, Austin, TX).

MINERALIZATION ASSAY

For mineralization assays, MC3T3-E1 cells were allowed to reach confluence and then treated with differentiation medium, consisting on growing medium containing ascorbic acid (50 ug/mL) and beta-glycerolphosphate (10 mM), and 0.1 mM of MEL or 0.1 mM of 5-MTX. After 14 days of cell culture, cells were washed with PBS and

fixed with 100% ethanol for 15 min. Ethanol was removed and cells were stained with Alizarin Red Staining solution (Sigma, St. Louis, MO) for 30 min at room temperature. Stained cells were washed and further photographed.

CELL CULTURE OF RAW 264.7 CELLS

RAW 264.7 cells (ATCC, Manassas, VA) were cultured at 37°C in 5% CO2 atmosphere in Dulbecco modified Eagles medium (DMEM) supplemented with 10% FBS and antibiotics (50 IU penicillin/ml and 50 μg streptomycin/ml).

Cells were seeded in 96-well plates (for TRAP staining) or on dentin slices (for resorption pit assay) at a density of 3.0×10^4 cells/cm^2 and, after an overnight period, the culture medium was replaced with medium containing 4 ng/mL RANKL (R&D Systems, Minneapolis, MN) and different doses of 5-MTX or MEL (1 and 0.1 mM). Treatments were added after changing the medium every 48 h over the course of 5 days.

TRAP STAINING OF RAW 264.7 CELLS

Cultured RAW 264.7 cells were stained for the enzyme tartrate-resistant acid phosphatase (TRAP) using the TRAP-staining kit (Sigma–Aldrich, St. Louis, MO), according to the manufacturer's instructions. TRAP-positive multinucleated (3 or more nuclei) osteoclasts were visualized by light microscopy and photographed.

RESORPTION PIT ASSAY

RAW 264.7 cells seeded on dentine slices (Immunodiagnostic Systems, Boldon, UK) were removed by sonication in 0.1 N NaOH for 2 min, stained in hematoxylin for 40 s and washed in distilled water. The surface of each dentine slice was examined by light microscopy for evidence of lacunar resorption and quantitative analysis of the resorption area was performed with Image J software 1.44p (NIH).

DETECTION OF Phospho-p44/42 MAPK (Erk 1/2)

MC3T3-E1 cells were cultured for 7 days while RAW264.7 cells were cultured for 5 days. Then, they were treated for 2 h with 0.1 mM luzindole and subsequently treated for an additional 2 h with 0.1 mM 5-MTX or 0.1 mM MEL as previously described [Roth et al., 1999]. Cells were fixed for 10 min with 2% formaldehyde in PBS and then permeabilized for 10 min with 0.2% Triton X-100 in PBS. Cells were immunostained with Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody (1:200; Cell Signaling Technology, MA) for 1 h at 37°C followed by 1 h of incubation at room temperature with Cy3 (1:200; Thermo Fisher Scientific, MA). Finally, a drop of Fluoroshield DAPI (Sigma, St. Louis, MO) was added to stain cell nucleous. Images were taken with the confocal microscope (Leica DMI 4000B equipped with Leica TCS SPE laser system).

STATISTICS

All data are presented as mean values ± SEM. A Kolmogorov–Smirnov test was done to assume parametric or non-parametric distributions for the normality tests. Differences between groups were assessed by one-way analysis of variance (ANOVA) test followed by post-hoc pairwise comparisons using Dunnett's test. When ANOVA test was not suitable for data, Mann–Whitney-test or Student *t*-test were run depending on their normal distribution. The SPSS® program (SPSS Inc., Chicago, IL) for Windows, version 17.0 was used. Results were considered statistically significant at P-values ≤ 0.05.

RESULTS

EFFECT OF 5-MTX AND MEL ON METABOLIC ACTIVITY, CYTOTOXICITY AND TOTAL RNA CONTENT IN MC3T3-E1 CELLS

Total metabolic activity was determined after 24 h of 5-MTX and MEL treatment in MC3T3-E1 cells (Fig. 1A). A dose-response effect was observed for both indoles. 5-MTX doses decreased the metabolic activity of osteoblasts whilst only the highest dose of MEL (2.5 mM) reduced the metabolic activity. LDH activity was measured in the culture medium after 48 h of treatment as an index of cytotoxicity (Fig. 1B). Also a dose-response effect was observed with statistical differences in both 5-MTX and MEL treatments. The two highest doses (2.5 and 1 mM) of both compounds produced a higher release of LDH compared with control. In addition, 2.5 mM 5-MTX showed a lower LDH release compared with 2.5 mM MEL. Finally, 0.001 and 0.01 mM 5-MTX and 0.001 mM MEL doses revealed a protective effect for cells. Total RNA content isolated from MC3T3-E1 cells cultured for 14 days was quantified (Fig. 1C). Interestingly and in line with the previous data, a dose-effect was found for both 5-MTX and MEL treatments. Indeed, a lower total RNA content was observed in both 2.5 mM 5-MTX and MEL groups when compared with control (6.23 ± 0.39 μg). Additionally, 1 mM 5-MTX group also showed a higher total RNA content when compared with 1 mM MEL. The visual observations indicated that the highest dose of both 5-MTX and MEL showed lower cell density and different cell morphology in accordance with the metabolic activity, cytotoxicity and total RNA content results (data not shown). Consequently, the 2.5 mM dose was not considered in further analysis.

EFFECT OF 5-MTX AND MEL ON GENE EXPRESSION OF RANKL AND OPG OF MC3T3-E1 CELLS

Real-time RT-PCR was performed to observe the effect of 5-MTX and MEL on markers related to osteoclast activation (Fig. 2). *Rankl* and *Opg* showed a dose-response effect for both 5-MTX and MEL. Interestingly, every dose of 5-MTX and MEL significantly decreased *Rankl* gene expression, except the lowest dose of 0.001 mM MEL. Indeed, the higher the dose of each 5-methoxyindol, the more inhibited gene expression of *Rankl*. In addition, when comparing both indoles, low doses (0.001–0.1 mM) of 5-MTX decreased more *Rankl* gene expression than MEL ones. Regarding the gene expression of *Opg*, only some doses of 5-MTX (0.1–1 mM) decreased its expression. MEL showed similar *Opg* mRNA expression profile than 5-MTX but only 0.1 mM dose showed significant results. In light of the mRNA expression levels results and morphological appearance of cells, the 0.1 mM dose was selected for further analysis.

EFFECT OF 5-MTX AND MEL ON OSTEOCALCIN RELEASE AND MINERALIZATION OF MC3T3-E1 CELLS

Osteocalcin secretion to the culture medium at day 14 of cell culture was determined by Luminex assay. Oc release was higher in 5-MTX

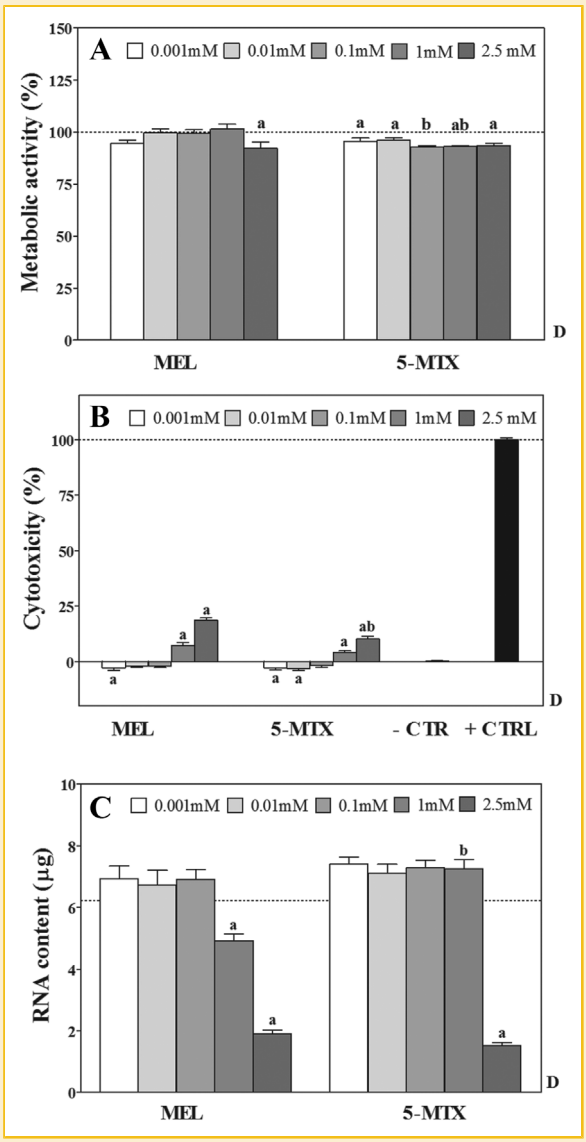

Fig. 1. (A) Metabolic activity was measured in MC3T3-E1 cells after 24 h of treatment using a resazurin based assay (PrestoBlue™ Cell Viability Reagent). 100% metabolic activity control (0.9% ethanol, v/v) was culture medium from control vehicle cells. (B) LDH activity measured in culture medium of MC3T3-E1 cells collected after 48 h of treatment. Positive control (Triton; 100% toxicity) was cell culture medium from cells incubated with Triton X-100 at 1%. Negative control (0.9% ethanol, v/v) was cell culture medium from control vehicle cells. The percentage of LDH activity was calculated using the following equation: Cytotoxicity (%) = (exp.value – negative control)/(positive control – negative control) × 100. (C) Quantification of total RNA content isolated from MC3T3-E1 cells treated with different doses of MEL and 5-MTX for 14 days of cell culture. Values represent the mean ± SEM (N = 6). The dotted line represents the total RNA content of the control group (0.9% ethanol, v/v). The effect of dose was assessed by one-way ANOVA test: D $P \leq 0.05$. Post-hoc analyses were made using Dunnett's test: [a]treatment versus control (0.9% ethanol, v/v). Differences between biomolecules were assessed by Mann–Whitney or by Student's *t* test: [b]$P < 0.05$ for every 5-MTX treatment versus the corresponding MEL dose.

compared with the MEL group, and for both treatments when compared with control (Fig. 3A). Alizarin Red Staining was performed in MC3T3-E1 after 14 days of culture to evaluate the calcium-rich deposits. A higher mineralization was observed in osteoblasts treated with MEL and especially with 5-MTX (Fig. 3B and C).

EFFECT OF 5-MTX AND MEL ON OSTEOCLASTOGENESIS AND RESORPTION ACTIVITY OF RAW 264.7 CELLS

RAW 264.7 cells were treated with 0.1 mM 5-MTX and MEL in the presence of RANKL (Fig. 4 A–C). Treatment with 5-MTX significantly decreased the number of multinucleated (with 3 or more nuclei) TRAP-positive cells (OCL) formed and indeed, smaller OCL and with fewer nuclei than control cells were observed, while no significant differences were found for cells treated with MEL (Fig. 4G). Furthermore, we analyzed the gene expression of several phenotypical and functional markers in order to gain more knowledge about the response of RAW cells to both MEL and 5-MTX. Although the phenotypical marker *Trap* showed similar mRNA levels in 5-MTX and MEL compared to control, the functional markers *Mmp9* and *Ctsk* were significantly decreased in 5-MTX compared with control, while only *Mmp9* was reduced in MEL (Fig. 4I).

We also assessed the effect of the treatments on osteoclast function by evaluating bone resorption on dentin slices (Fig. 4D–F). A significant decrease on bone resorption activity was found for both 5-MTX and MEL treatments (Fig. 4H), despite no differences on TRAP staining were found in cells treated with MEL.

EFFECT OF 5-MTX, MEL AND THE INHIBITOR LUZ ON THE ERK PATHWAY

MC3T3-E1 and RAW264.7 cells were treated with MEL, 5-MTX and also with the inhibitor LUZ, and further immunostained for p-ERK (Fig. 5). A higher expression of p-ERK was observed in cells treated with 5-MTX than MEL, and both treatments were higher than the ethanol control. Furthermore, when LUZ was added, p-ERK expression was significantly decreased in both treatments.

DISCUSSION

In the present investigation, we demonstrate for the first time a new role of 5-MTX inhibiting osteoclastogenesis and promoting osteoblast differentiation. In our attempt to identify new therapeutic agents for hard and soft tissue treatment in the oral cavity and for bone regeneration applications, we compared the effects of 5-MTX and MEL in bone cells. Thus, we tested doses ranged from 0.001 mM up to 2.5 mM for both treatments.

Physiological plasma levels of MEL and 5-MTX [Linsell et al., 1979; Silman et al., 1979] range from 10 to 300 pg/ml for MEL and 2 to 120 pg/ml for 5-MTX. However, we used pharmacological doses based on previous studies using MEL in vitro [Nakade et al., 1999; Roth et al., 1999; Koyama et al., 2002], which have previously shown a positive effect on human osteoblast differentiation [Satomura et al., 2007].

When evaluating metabolic activity and cytotoxicity, while 5-MTX reduced slightly the metabolic activity of osteoblasts in all the doses, MEL only decreased it in the 2.5 mM dose. As regards to

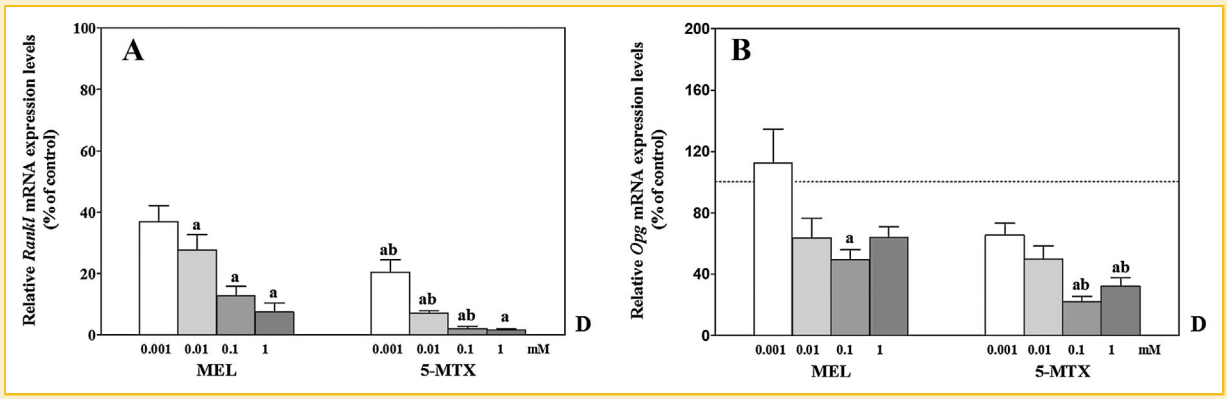

Fig. 2. Effect of MEL and 5-MTX on mRNA expression levels of (A) *Rankl and* (B) *Opg* in MC3T3-E1 cells cultured for 14 days. Data were normalized to reference genes (*Gapdh* and 18 S rRNA), expressed as percentage of control which was set to 100%. Values represent the mean ± SEM (N = 6). The effect of dose was assessed by one-way ANOVA test: D $P \leq 0.05$. Post-hoc analyses were made using Dunnett's test: [a]treatment versus control (0.9% ethanol, v/v). Differences between groups were assessed by Mann–Whitney or by Student's *t* test: [b]$P < 0.05$ for every 5-MTX treatment versus the corresponding MEL dose.

cytotoxicity, only 2.5 mM 5-MTX and MEL were toxic for the cells, as shown by the increased LDH activity, which was related to the lower RNA content and cell density. However, the results revealed a significant decrease in cell death at lower concentrations, although this reduction was no more than 5%. Previously, MEL was found to be non-cytotoxic up to the concentration of 0.5 mM [Park et al., 2011].

Previous reports [Nakade et al., 1999; Roth et al., 1999; Radio et al., 2006; Satomura et al., 2007; Sethi et al., 2010; Zhang et al., 2010; Park et al., 2011; Zhang et al., 2012; Kim and Yoo, 2013] have demonstrated that MEL induces osteoblast differentiation in MC3T3-E1 cells. Indeed, our results showed that MEL increased osteocalcin synthesis as previously reported for these cells [Park et al., 2011]. However, here we show that 5-MTX stimulates osteoblast differentiation in a higher degree than MEL, as clearly indicated by the higher osteocalcin secretion levels to the culture medium. The increased secretion of osteocalcin by 5-methoxyindols could be explained by the fact that osteogenic genes such as osteocalcin

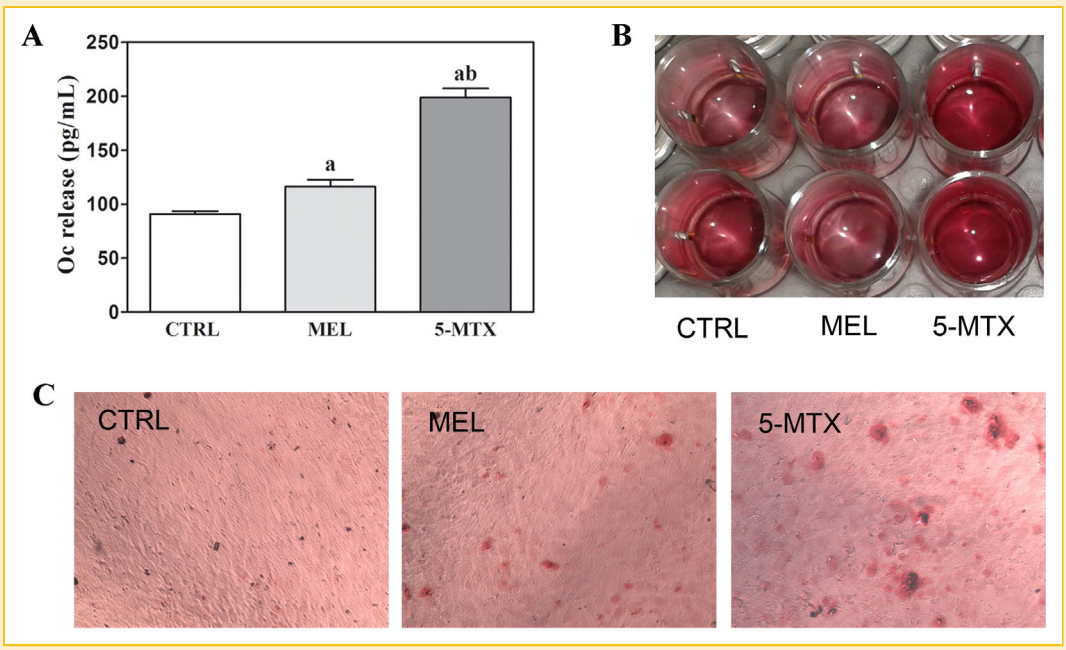

Fig. 3. (A) Osteocalcin released to the culture medium after 14 days of culture and treatments with 0.1 mM of 5-MTX and MEL. Values represent the mean ± SEM (N = 6). Differences between groups were assessed by Student's t-text: [a]$P < 0.05$ treatment versus ethanol control and [b]$P < 0.05$ 5-MTX versus MEL treatment. Plate view (B) and microscopic view (C) of Alizarin Red Staining of MC3T3-E1 cells treated for 14 days with 0.1 mM of 5-MTX and MEL.

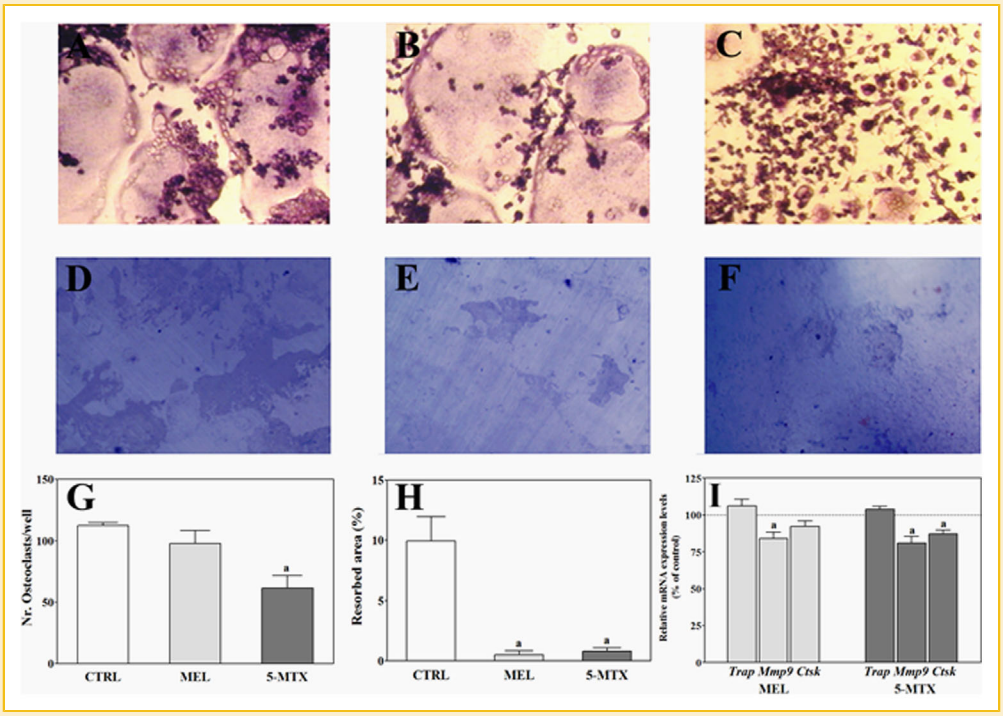

Fig. 4. Effect of MEL and 5-MTX on the generation of multinucleated TRAP-positive cells (OCL) and their effect on bone resorption ability on dentine slices. Trap staining of CTRL (A), MEL (B) and 5-MTX (C) RAW 264.7 cells. Bone resorption ability of CTRL (D), MEL (E) and 5-MTX (F) RAW 264.7 cells evaluated by resorption pit assay on dentine discs. (G) Number of multinucleated TRAP-positive cells with 3 or more nuclei (OCL). (H) Percentage of resorbed area. (I) Effect of MEL and 5-MTX on mRNA expression levels of *Trap*, *Mmp-9* and *Ctsk* in RAW 264.7 cells cultured for 5 days. Data were normalized to reference genes (Gapdh and 18 S rRNA), expressed as percentage of control which was set to 100%. CTRL is RAW 264.7 dosed with 4 ng/mL RANKL for 5 days, MEL is RAW 264.7 cells dosed with 4 ng/mL RANKL and treated with 0.1 mM of MEL and 5-MTX is RAW 264.7 cells dosed with 4 ng/mL RANKL and treated with 0.1 mM of 5-MTX. Values represent the mean ± SEM. Significant differences were assessed by Students *t* test: $^{a}P < 0.05$ treatment versus ethanol control.

contain the putative sequences for binding RZR nuclear orphan receptor, responsible for increasing their transcription and ultimately inducing osteoblast differentiation [Roth et al., 1999]. Interestingly, these results are in agreement with the mineralization images that show a higher amount of calcium deposits in MEL and especially, 5-MTX treated cells, demonstrating that 5-MTX has a greater potential for promoting osteoblast differentiation.

Osteoclastogenesis is regulated indirectly by osteoblasts through the production of RANKL and OPG, a decoy for RANKL. Previous studies support the suppressive action of MEL on the *Rankl* gene expression [Koyama et al., 2002; Histing et al., 2012], here we demonstrate that 5-MTX exerts a higher potential inhibiting *Rankl* mRNA levels than MEL. Although *Opg* gene expression was also decreased in both treatments for some of the doses, the RANKL/OPG ratio was decreased with both treatments, resulting in a lower index of osteoclastogenic stimulus. Decreased *Opg* mRNA levels found for MEL differs from previously reported results, this difference is probably due to the MEL treatment duration; 1 h [Koyama et al., 2002] *versus* 14 days in the present study.

Our results also indicate a higher direct effect of 5-MTX than MEL inhibiting osteoclastogenesis. It has been previously suggested that MEL may act directly on osteoclasts by suppressing their differentiation [Koyama et al., 2002; Suzuki and Hattori, 2002]. The mechanism by which MEL inhibits bone resorption is related to its ability to detoxify the low toxic concentrations of reactive oxygen species (ROS) produced during osteoclastogenesis, which act as an intracellular signal mediator for osteoclast differentiation [Lee et al., 2005]. Thus, RANKL-mediated ROS production serves to regulate RANKL signaling pathways, including JNK and p38 activation that are sensitive to activation by ROS and required for osteoclast differentiation [Lee et al., 2005]. MEL increases the phosphorylative activation of ERK1/2 in order to constrain ROS production [Luchetti et al., 2009]. Furthermore, MEL promotes osteoblastic differentiation of MC3T3-E1 cells though this ERK signaling pathway [Park et al., 2011]. In the same way, we here demonstrated that 5-MTX induces the ERK pathway in both MC3T-E1 and RAW264.7 cells, according to a higher osteoblast differentiation and a lower ROS production respectively. Additionally, in an attempt to prove that the MEL transmembrane receptor was involved in mediating the action of 5-MTX, as demonstrated for MEL [Roth et al., 1999], we demonstrated that exposure to LUZ could attenuate the actions of 5-MTX. Indeed, the ERK signaling cascade was reduced when LUZ was added to MEL and 5-MTX, suggesting that both indoles act through the MEL receptors. All in all, these experiments suggest that 5-MTX mechanism of action occurs through MT1 and MT2 receptors, and it involves the phosphorylation of ERK.

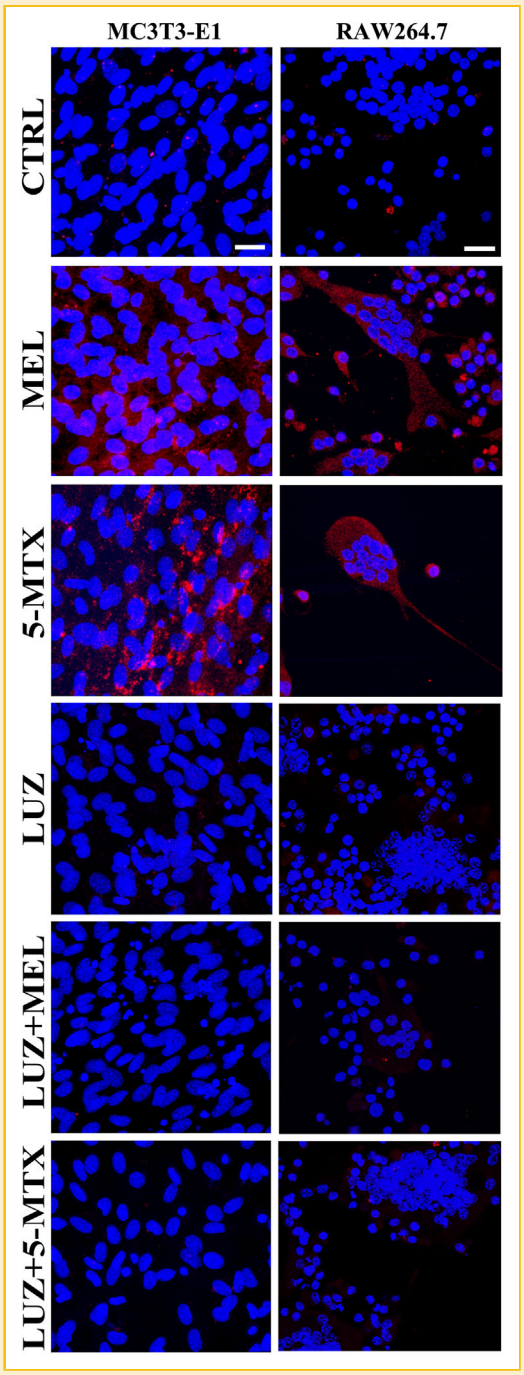

Fig. 5. Effect of MEL, 5-MTX and luzindole (LUZ) on the Erk 1/2 activity in MC3T3-E1 and RAW264.7 cells. Representative images obtained from confocal laser scanning microscope are shown. Cells were stained with anti-phospho-Erk (red) and fluoroshield-DAPI (blue). Bar scale = 25 μm. CTRL are cells treated with ethanol; MEL are cells treated with 0.1 mM of MEL; 5-MTX are cells treated with 0.1 mM of 5-MTX; LUZ are cells treated with 0.1 mM of luzindole; LUZ + MEL are cells trated with 0.1 mM luzindole and 0.1 mM MEL and LUZ + 5-MTX are cells treated with 0.1 mM luzindole and 0.1 mM 5-MTX.

In conclusion, this paper provides for the first time evidences for 5-MTX to promote osteoblast differentiation whilst inhibit osteoclast formation and resorption activity. Further in vivo studies are required to determine its effect in the prevention of bone loss and improvement of bone formation in animal models and its potential for therapeutic applications.

ACKNOWLEDGMENTS

This work was supported by the the Ministerio de Ciencia e Innovación del Gobierno de España (Torres Quevedo contract to JMR, and Ramón y Cajal contract to MM). We thank Dr. P. Villalonga and Dr. S. Fernández de Mattos for kindly providing the Phospho-p44/42 MAPK antibody.

REFERENCES

Balemans MGM, van de Veerdonk FCG, van de Kamer JC. 1977. The influence of 5-methoxytryptophol, a pineal compound, on comb growth, ovarian weight, follicular growth and egg production of juvenile, maturing and adult white leghorn hens (Gallus domesticus L). J Neural Transm 41:37–46.

Cardinali DP, Ladizesky MG, Boggio V, Cutrera RA, Mautalen C. 2003. Melatonin effects on bone: Experimental facts and clinical perspectives. J Pineal Res 34:81–87.

Carrillo-Vico A, Guerrero JM, Lardone PJ, Reiter RJ. 2005. A review of the multiple actions of melatonin on the immune system. Endocrine 27:189–200.

Clafshenkel WP, Rutkowski JL, Palchesko RN, Romeo JD, McGowan KA, Gawalt ES, Witt-Enderby PA. 2012. A novel calcium aluminate-melatonin scaffold enhances bone regeneration within a calvarial defect. J Pineal Res 53:206–218.

Cutando A, Arana C, Gómez-Moreno G, Escames G, López A, Ferrera MJ, Reiter RJ, Acuña-Castroviejo D. 2007. Local application of melatonin into alveolar sockets of beagle dogs reduces tooth removal–induced oxidative stress. J Periodontol 78:576–583.

García JJ, Reiter RJ, Cabrera JJ, Pié J, Mayo JC, Sáinz RM, Tan DX, Qi W, Acuña-Castroviejo D. 2000. 5-Methoxytryptophol preserves hepatic microsomal membrane fluidity during oxidative stress. J Cell Biochem 76:651–657.

Gómez-Florit M, Ramis JM, Monjo M. 2013. Anti-fibrotic and anti-inflammatory properties of melatonin on human gingival fibroblasts in vitro. Biochem Pharmacol 86:1784–1790.

Gomez-Moreno G, Guardia J, Ferrera MJ, Cutando A, Reiter RJ. 2010. Melatonin in diseases of the oral cavity. Oral Dis 16:242–247.

Guyomarc'h C, Lumineau S, Vivien-Roels B, Richard JP, Deregnaucourt S. 2001. Effect of melatonin supplementation on the sexual development in European quail (Coturnix coturnix). Behav Processes 53:121–130.

Histing T, Anton C, Scheuer C, Garcia P, Holstein JH, Klein M, Matthys R, Pohlemann T, Menger MD. 2012. Melatonin impairs fracture healing by suppressing rankl-mediated bone remodeling. J Surg Res 173:83–90.

Iguchi H, Kato K, Ibayashi H. 1982. Age-dependent reduction in serum melatonin concentrations in healthy human subjects. J Clin Endocrinol Metab 55:27–29.

Karasek M, Reiter RJ. 2002. Melatonin and aging. Neuroendocrinol Lett 23:14–16.

Kim CH, Yoo YM. 2013. Fluid shear stress and melatonin in combination activate anabolic proteins in MC3T3-E1 osteoblast cells. J Pineal Res 2013:453–461.

Koyama H, Nakade O, Takada Y, Kaku T, Lau KHW. 2002. Melatonin at pharmacologic doses increases bone mass by suppressing resorption through down-regulation of the RANKL-mediated osteoclast formation and activation. J Bone Miner Res 17:1219–1229.

Ladizesky MG, Cutrera RA, Boggio V, Somoza J, Centrella JM, Mautalen C, Cardinali DP. 2001. Effect of melatonin on bone metabolism in ovariectomized rats. Life Sci 70:557–565.

Lee NK, Choi YG, Baik JY, Han SY, Jeong DW, Bae YS, Kim N, Lee SY. 2005. A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation. Blood 106:852–859.

Liebmann PM, Wölfler A, Felsner P, Hofer D, Schauenstein K. 1997. Melatonin and the immune system. Int Arch Allergy Immunol 112: 203–211.

Linsell C, Mullen PE, Silman RE, Leone RM, Finney M, Carter SJ, Hooper RJ, Smith I, Francis P. 1979. The measurement of the daily fluctuations of 5-methoxytryptophol in human plasma. Prog Brain Res 52:501–505.

Lissoni P, Fumagalli L, Paolorossi F, Rovelli F, Roselli MG, Maestroni GJ. 1997. Anticancer neuroimmunomodulation by pineal hormones other than melatonin: preliminary phase II study of the pineal indole 5-methoxy-tryptophol in association with low-dose IL-2 and melatonin. J Biol Regul Homeost Agents 11:119–122.

Lissoni P, Pittalis S, Rovelli F, Zecchini S, Casati M, Tremolada M, Pelizzoni F. 1996. Immunomodulatory properties of a pineal indole hormone other than melatonin, the 5-methoxytryptophol. J Biol Regul Homeost Agents 10:27–30.

Lissoni P, Rovelli F, Frassineti A, Fumagalli L, Malysheva O, Conti A, Maestroni G. 2000. Oncostatic activity of pineal neuroendocrine treatment with the pineal indoles melatonin and 5-methoxytryptamine in untreatable metastatic cancer patients progressing on melatonin alone. Neuro Endocrinol Lett 21:319–323.

Liu J, Huang F, He HW. 2013. Melatonin effects on hard tissues: Bone and tooth. Int J Mol Sci 14:10063–10074.

López-Martínez F, Olivares Ponce, Guerra PN, Rodríguez M, Martínez Pedraza. 2012. Melatonin: Bone metabolism in oral cavity. Int J Dent 2012:ID 628406.

Luchetti F, Betti M, Canonico B, Arcangeletti M, Ferri P, Galli F, Papa S. 2009. ERK MAPK activation mediates the antiapoptotic signaling of melatonin in UVB-stressed U937 cells. Free Radical Bio Med 46:339–351.

Mishima K, Okawa M, Shimizu T, Hishikawa Y. 2001. Diminished melatonin secretion in the elderly caused by insufficient environmental illumination. J Clin Endocrinol Metab 86:129–134.

Nakade O, Koyama H, Ariji H, Yajima A, Kaku T. 1999. Melatonin stimulates proliferation and type I collagen synthesis in human bone cells in vitro. J Pineal Res 27:106–110.

Ouzir M, Bouhaddou N, Khalki H, Lakhdar-Ghazal N. 2013. Physiological and pharmacological properties of 5-methoxytryptophol. Expert Rev Endocrinol Metab 8:355–364.

Park K-H, Kang JW, Lee E-M, Kim JS, Rhee YH, Kim M, Jeong SJ, Park YG, Hoon K. 2011. Melatonin promotes osteoblastic differentiation through the BMP/ERK/Wnt signaling pathways. J Pineal Res 51:187–194.

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. Biotechnol Lett 26:509–515.

Poeggeler B, Reiter RJ, Hardeland R, Tan DX, Barlow-Walden LR. 1996. Melatonin and structurally-related, endogenous indoles act as potent electron donors and radical scavengers in vitro. Redox Rep 2:179–184.

Radio NM, Doctor JS, Witt-Enderby PA. 2006. Melatonin enhances alkaline phosphatase activity in differentiating human adult mesenchymal stem cells grown in osteogenic medium via MT2 melatonin receptors and the MEK/ERK (1/2) signaling cascade. J Pineal Res 40:332–342.

Ramirez-Fernandez MP, Calvo-Guirado JL, de-Val JE, Delgado-Ruiz RA, Negri B, Pardo-Zamora G, Penarrocha D, Barona C, Granero JM, Alcaraz-Banos M. 2013. Melatonin promotes angiogenesis during repair of bone defects: A radiological and histomorphometric study in rabbit tibiae. Clin Oral Investig 17:147–158.

Redman J, Armstrong S, Ng KT. 1983. Free-running activity rhythms in the rat: Entrainment by melatonin. Science 219:1089–1091.

Reiter RJ, Tan DX, Manchester LC, Paredes SD, Mayo JC, Sainz RM. 2009. Melatonin and reproduction revisited. Biol Reprod 81:445–456.

Rodriguez-Naranjo MI, Moyá ML, Cantos-Villar E, Garcia-Parrilla MC. 2012. Comparative evaluation of the antioxidant activity of melatonin and related indoles. J Food Comp Anal 28:16–22.

Roth JA, Kim BG, Lin WL, Cho MI. 1999. Melatonin promotes osteoblast differentiation and bone formation. J Biol Chem 274:22041–22047.

Sackman JW, Little JC, Rudeen PK, Waring PJ, Reiter RJ. 1977. The effects of pineal indoles given late in the light period on reproductive organs and pituitary prolactin levels in male golden hamsters. Horm Res Paed 8:84–92.

Satomura K, Tobiume S, Tokuyama R, Yamasaki Y, Kudoh K, Maeda E, Nagayama M. 2007. Melatonin at pharmacological doses enhances human osteoblastic differentiation in vitro and promotes mouse cortical bone formation in vivo. J Pineal Res 42:231–239.

Satué M, Arriero MdM, Monjo M, Ramis JM. 2013a. Quercitrin and Taxifolin stimulate osteoblast differentiation in MC3T3-E1 cells and inhibit osteoclastogenesis in RAW 264.7cells. Biochem Pharmacol 86:1476–1486.

Satué M, Petzold C, Cordoba A, Ramis JM, Monjo M. 2013b. UV photoactivation of 7-dehydrocholesterol on titanium implants enhances osteoblast differentiation and decreases Rankl gene expression. Acta Biomater 9:5759–5770.

Sethi S, Radio NM, Kotlarczyk MP, Chen CT, Wei YH, Jockers R, Witt-Enderby PA. 2010. Determination of the minimal melatonin exposure required to induce osteoblast differentiation from human mesenchymal stem cells and these effects on downstream signaling pathways. J Pineal Res 49:222–238.

Silman RE, Hooper RJ, Leone RM, Edwards R, Grudzinskas JG, Gordon YB, Chard T, Savage M, Smith I, Mullen PE. 1979. 5-methoxytryptophol and pituitary function in man. Prog Brain Res 52:507–511.

Suzuki N, Hattori A. 2002. Melatonin suppresses osteoclastic and osteoblastic activities in the scales of goldfish. J Pineal Res 33:253–258.

Uslu S, Uysal A, G O, Yurtseven M, T T, G B. 2007. Constructive effect of exogenous melatonin against osteoporosis after ovariectomy in rats. Anal Quant Cytol Histol 29:317–325.

Zhang L, Su P, Xu C, Chen C, Liang A, Du K, Peng Y, Huang D. 2010. Melatonin inhibits adipogenesis and enhances osteogenesis of human mesenchymal stem cells by suppressing PPARgamma expression and enhancing Runx2 expression. J Pineal Res 49:364–372.

Zhang L, Zhang J, Ling Y, Chen C, Liang A, Peng Y, Chang H, Su P, Huang D. 2012. Sustained release of melatonin from poly (lactic-co-glycolic acid) (PLGA) microspheres to induce osteogenesis of human mesenchymal stem cells in vitro. J Pineal Res 54:24–32.