

Melatonin promotes osteoblast differentiation of bone marrow mesenchymal stem cells in aged rats

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Abstract. – OBJECTIVE: The current study was to explore the effect of melatonin on osteoporosis and relevant mechanisms.

MATERIALS AND METHODS: We performed micro-CT to detect bone microstructure and ELISA to detect the contents of osteocalcin (OCN) and bone alkaline phosphatase (BAP) in serum. Double fluorescence labeling of calcein and tetracycline and toluidine blue staining were used to determine morphological indexes of bone tissues. Alizarin red staining and Oil Red O staining were performed to recognize bone cells and adipocytes. RT-PCR was performed to determine the expression of osteoblast differentiation related genes.

RESULTS: In the current study, data from micro-CT indicated that melatonin significantly increased the bone mass density (BMD), bone volume/tissue volume (BV/TV), trabecular number (Tb.N) and trabecular thickness (Tb.Th), and decreased the Structure Model Index (SMI) and trabecular Separation/Spacing (Tb.Sp) in elderly rats. Melatonin reduced calcium and phosphorus losses in urine and increased BAP and OCN levels in serum in elderly rats and increased bone formation rate (BFR) and bone mineralization rate (MAR) in elderly rats. Melatonin increased the number of osteoblasts in bone marrow and reduced the number of adipocytes in elderly rats. Melatonin also promoted the expression of osteogenic differentiation genes and suppressed the expression of adipogenic differentiation genes.

CONCLUSIONS: WE suggest that melatonin could alleviate osteoporosis in aged rats' models probably by promoting osteoblast differentiation.

Key Words:

Melatonin, Osteoporosis, Micro-CT, Osteoblast differentiation, Adipogenic differentiation.

Introduction

Osteoporosis (OP) is a type of progressive bone disease characterized by low bone mass, bone microstructure damage, increased bone fragility and easy fracture^{1,2}. Bone tissue is a balanced internal environment. There are osteoblasts and osteoclasts in the marrow cavity. The osteoblast is differentiated into the osteocytes, secreting bone matrix and improving bone quality, and the osteoclast in the marrow cavity absorbs bone matrix, acidize bone and hydrolyze digestion, thus destroying bone matrix and bone formation³. The maintenance of bone tissue depends on the continuity and circulation of bone remodeling. The essence of osteoporosis is the imbalance between bone formation and bone resorption, leading to the loss of bone mineral. Moreover, the loss of lots of bone mineral increases the fracture risk⁴⁻⁶. The expenses of medical care for osteoporosis-related fracture pose a huge financial burden for the individuals, families, and society. With the situation of the aging population being increasingly severe, osteoporosis has become a global problem which seriously threatens human's health.

Melatonin is a type of neuroendocrine hormone which was mainly secreted by the pineal body. It has a variety of physiological effects, such as the regulation of biological rhythm, improvement of sleep quality, increase of body immunity and anti-oxidative stress, anti-tumor and anti-aging, etc.⁷. In addition to the pineal body, other tissues, such as the retina, thymus, spleen, ovary, testis, gastrointestinal tract, bone marrow tissues, etc., can secrete melatonin. The

melatonin level in bone marrow is 2 times of that in plasma at night⁸. With the increase of age, the peak concentration of melatonin at night is gradually decreased, and the circadian rhythm in the elderly population has tendency to be mild or even disappear. Melatonin level in plasma at night and duration of melatonin secretion in postmenopausal females are lower than those in perimenopausal females. However, there is no statistically significant difference in the time point of peak concentration of melatonin between the both⁹. OP development speed is closely related to the age. It is reported in the study that for OP patients, the more damaged of the bone substance, the lower the melatonin level in plasma¹⁰. It is found in the animal experiments that the artificial light or β -adrenergic receptor blocker inhibits the secretion of melatonin in rats, and decreases the serum calcium levels. However, the serum calcium level is recovered after supplementing the exogenous melatonin^{11,12}, which suggesting that melatonin has the effect of increasing serum calcium level under the physiological state. The cancellous bone mass is decreased and Pyr and D-Pyr, the bone resorption markers, are increased at 6 months after the excision of pineal body in sheep¹³.

Mesenchymal stem cell (MSC) is a type of pluripotent stem cell that can differentiate into many different types of cells, including osteoblasts, chondrocytes and adipocytes¹⁴. The abnormal differentiation ratio of mesenchymal stem cells is an important reason for the occurrence of senile osteoporosis. Bone marrow mesenchymal stem cells (BMSCs) are mainly differentiated into osteoblasts and adipocytes. And the normal differentiation ratio plays an important role in maintaining the bone tissue reconstruction and bone metabolism. A large number of clinical data show that with the increase of age, the differentiation capacity of bone marrow mesenchymal stem cells (BMSCs) into osteoblast is inhibited. The differentiation ratio into adipocytes is increased, and the size and number of bone marrow adipocytes are also increased linearly¹⁵. Data obtained from rats at different ages shows that compared with 3-week-old rats. The number of BMSCs colony forming units (CFU-f) is decreased by 57% in 56-week-old rats; the size is decreased by 52%. The alkaline phosphatase (ALP) activity is decreased by 25%¹⁶, which suggesting that the decreased osteogenic differentiation capacity of BMSCs is the typical age-related change. Therefore, clari-

fying the bi-directional differentiation factors of BMSCs and promoting the differentiation into osteoblasts have become hotspots in the field of prevention and treatment of osteoporosis.

Roth et al¹⁷ detected and found in the experimental study that MC3T3-E1 precursor osteoblast cell line was differentiated and mineralized under the action of 50 nmol/L melatonin. Satomura et al¹⁸ confirmed that the expression level of melatonin 1A receptor in human osteoblasts was decreased with age. Fjelldal et al¹⁹ found that in the study on the long-term influence of excision of pineal body in Atlantic salmon, the mineral content in vertebral body, hardness, elasticity and plastic deformation capacity of vertebral body were decreased significantly, which suggesting that melatonin is related to the growth and development of vertebral body bone. At present, the study on the improvement of osteoporosis via melatonin has not clarified whether melatonin can alleviate the aged-related osteoporosis through regulating the differentiation direction of mesenchymal stem cells. Therefore, this experimental work whether melatonin could improve the aged-related osteoporosis and related mechanisms with the elderly rats as the model.

Materials and Methods

Animal Grouping

Sprague-Dawley (SD) male rats (Taconic, Hudson, NY, USA) with 20 mon age were taken as the experimental subjects and randomly divided into the vehicle group (solvent group) and the melatonin treatment (Haoran Biotech Co., Ltd., Shanghai, China) group (melatonin, 50 mg/kg/day) with 8 rats in each group. Rats received the intraperitoneal injection of saline (control group) or melatonin (experimental group) for 12 weeks. The general conditions were observed and the weight was recorded every week. This study was approved by the Ethical Committee of Nanjing Medical University.

Sample Collection

Double fluorescence labeling of calcein and tetracycline. Rats received the subcutaneous injection of tetracycline (25 mg/kg) at 14 d and 13 d before they were executed. And the subcutaneous injection of calcein (5 mg/kg) at 4 d and 3 d before they were executed. Then the bone tissues in rats received the double fluorescence labeling.

Collection and treatment of urine sample. Before rats were executed, they were placed in metabolic cage, and fasting but fed with water. The 24 h urine was collected and centrifuged for 5 min, and the supernatant was taken and stored at -80°C in a dark place.

Collection and treatment of blood sample. The animals were executed after urine was collected. Then the blood was drawn from abdominal aorta and centrifuged for 10 min; the serum was collected and stored at -80°C in a dark place.

Collection and treatment of bone tissue sample. The bilateral femurs and tibias of eight rats in each group were taken. We divided each femur and tibia into several parts for following analysis. 16 pieces' femurs of each rat were stored at -80°C for MicroCT (Bruker, Billerica, MA, USA) and biomechanical analysis. 16 pieces' tibias of each rat were fixed using 4% paraformaldehyde, and received the paraffin-embedded sections, followed by hematoxylin-eosin (HE) staining (ThermoFisher, Waltham, MA, USA) and toluidine blue staining; 16 pieces' tibias of each rat were fixed using 80% alcohol for hard tissue sections.

Micro-CT Analysis

The rat femur samples were unfrozen at room temperature, and the bone microstructure was analyzed via Micro-CT scan. Scan parameters were set as: current of 400 μA , voltage of 60 kV, 360° rotational scan, angle gain of 2° , scanning time for each sample of about 20-30 min. The whole femoral image was scanned to reconstruct the stereo image with the resolution of 12 μm . The region of interest (ROI) selected was located in the metaphysis of the femur, and at least 100 layers were scanned. MicroView v.2.1 software (Beijing, China) was used to integrate these planar images into 3D images.

Detection of Biochemical Indexes of Bone Metabolism

The automatic biochemical analyzer was used to detect the levels of Ca^{2+} and P^{3+} in serum and urine, and creatinine (CREA) level in urine. The enzyme-linked immunosorbent assay (ELISA) method was used to detect the contents of osteocalcin (OCN) and bone alkaline phosphatase (BAP) in serum: 50 μl primary antibody solution was added to 96-well

plate and placed at room temperature for 1 h. Then, the antibody was discarded, and buffer was added. The 96-well plate was tapped on filter paper to remove the residual liquid. 50 μl conjugate was added to each well, and the samples were added after 15 min of incubation at room temperature for 2 h. Then, the sample was discarded. 200 μl substrate solution was added to each well for incubation at room temperature for 30 min in the dark. Finally, 100 μl stop solution was added to each well and vibrated gently. The concentration at 450 nm wavelength was measured.

Determination of Morphological Indexes of Bone Tissues

Materials and Treatment of Undecalcified Section. The tibia samples fixed by 80% ethyl alcohol were dehydrated using ethyl alcohol layer by layer, and embedded with methyl methacrylate, dibutyl phthalate and catalyst. The embedded samples were sliced using hard tissue microtome along the sagittal plane of tibia for the double fluorescence labeling and observation.

Double fluorescence labeling of calcein and tetracycline and analysis. The bone mineral apposition rate (MAR, $\mu\text{m}/\text{d}$) and bone formation rate (BFR, $\mu\text{m}/\text{d}$) were measured and calculated by automatic image analysis system.

Material and treatment of decalcified section. Tibial samples were immersed in 10% ethylene diamine tetraacetic acid (EDTA) (Haoran Biotech Co., Ltd., Shanghai, China) decalcifying solution for decalcification until the needle can pierce through the bone tissue.

HE Staining. The procedures included the paraffin embedding, slice, dehydration, dewaxing and staining, sealing and counting.

Toluidine Blue Staining. The proximal tibia of rats was taken and fixed with 4% paraformaldehyde, embedded in paraffin, and sliced, dewaxed and dehydrated, followed by toluidine blue staining for 30 min; and the tibia was soaked in glacial acetic acid for rapid differentiation until the cellular morphology could be clearly observed; then it was washed with distilled water, quickly dried, followed by transparency via xylene and neutral resin sealing.

Induced Differentiation, Culture and Staining of Mesenchymal Stem Cells

The acquisition and culture of MSCs. SD rats aged 3 weeks were executed. Bilateral femurs and tibias were cut from epiphysis and rinsed with α -minimum essential medium (α -MEM) (Gibco, ThermoFisher, Waltham, MS, USA) to get bone medullary cells suspension. Bone medulla cells suspension was centrifuged and abandoned super-fluid. mesenchymal stem cells (MSCs) were cultured with α -MEM supplemented with 10% FBS (ThermoFisher, Waltham, MS, USA) and penicillin as well as streptomycin.

Alizarin Red Staining (Beijing Dakewei Biotech Co., Ltd., Beijing, China). Mesenchymal stem cells cultured were divided into the osteogenic induction culture group, osteogenic induction + melatonin group; after 21 days of osteogenic induction, 1 g alizarin red powder was added into 100 ml 0.1 M Tris-HCL to prepare the staining solution; after the cells were fixed by 70% alcohol, they were stained by 40 mM alizarin red at room temperature for 10 min, and the staining condition was observed under microscope;

Oil red O staining (Beijing Dakewei Biotech Co., Ltd., Beijing, China). Cells were divided into the adipogenic induction culture group, adipogenic induction culture + melatonin group. After 17 days of adipogenic differentiation and culture, 0.5 g oil red O powder and 100 ml isopropyl alcohol were prepared into the 0.5% saturated oil red O mother solution. The mother solution was heated in boiling water bath for 10 min, and then cooled naturally at room temperature. A certain amount of supernatant was taken and diluted into the working solution, and then the solution was left to stand for 5-10 min and filtered twice. 4% paraformaldehyde was used to fix the cells after induction for 45-60 min, and isopropyl alcohol was used for dehydration; oil red O working solution was added and incubated at room temperature in a dark place for 30 min; the excess dye liquor was washed off, and the hematoxylin was used for re-staining, followed by observation after drying.

RT-PCR

1 mL RNAiso Plus (TaKaRa, Dalian, Liaoning, China) was added into the well-processed

cells which were later blown and then placed on the ice for 5 min. Then, cells were blown again, and transferred to a 1.5 mL Eppendorf tube, in which 200 μ L chloroform was added. Then the tube was vibrated for 30 s and the layered solution was then centrifuged at 12000 g for 10 min at 4°C. Thereafter, the supernatant was transferred into a new Eppendorf tube for mixing with the 0.5 mL pre-cooled isopropanol that had been added. Then the solution was centrifuged at 12000 g for 1000 min at 4°C. The supernatant was discarded, and 0.5 mL 70% ethanol that was prepared by the anhydrous ethanol and diethyl pyrocarbonate (DEPC) water was added to the solution followed by 12000 g centrifugation at 4°C for 5 min. The supernatant was abandoned again and the rest of solution was dried in the air. RNA was resolved using an appropriate volume of DEPC water and the concentration and purification of RNA were assayed using micronuclei acid quantitative apparatus (ThermoFisher, Waltham, MS, USA). Thereafter, reverse transcription was carried out following the protocols of reverse transcription kit (ThermoFisher, Waltham, MS, USA), and samples were added and amplified according to the instructions of the amplification kit (ThermoFisher, Waltham, MS, USA).

Statistical Analysis

All experiment results were statistically analyzed using SPSS 17.0. All experiment results were expressed by mean $\pm$ standard deviation. The *t*-test was performed in the intergroup comparison, and each result was analyzed for at least 3 times. $p < 0.05$ suggested that the difference was statistically significant.

Results

Melatonin Improved the Bone Trabecular Microstructure of Elderly Rats

Micro-CT was used for the analysis of bone morphology. Compared with solvent group, melatonin could significantly increase BMD, BV/TV, Tb.N and Tb.Th in elderly rats, and could significantly decrease SMI and Tb.Sp in elderly rats (Figure 1 and Figure 2). These results suggested that melatonin can significantly increase the bone mass in elderly rats with osteoporosis and improve the femoral trabecular microstructure.

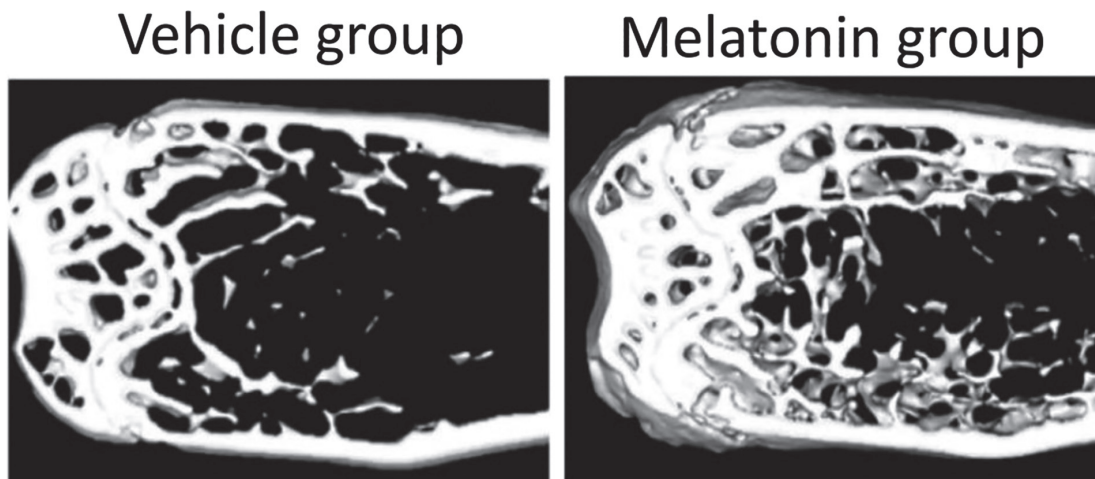


Figure 1. Melatonin increases bone mass and ameliorates osteoporosis in old rats (n=8/group). Representative three-dimensional reconstructive images from micro-CT of trabecular bone from the distal femur metaphyses of rats (aged 20 months) treated with vehicle or melatonin (50 mg/kg/day) for 12 weeks by intraperitoneal injection.

Melatonin Reduced Calcium and Phosphorus Losses in Urine and Increased BAP and OCN Levels in Serum in Elderly Rats

Serum calcium, serum phosphorus, urinary calcium, urinary phosphorus, serum bone-spe-

cific alkaline phosphatase (BAP) and serum osteocalcin (OCN) are important indicators reflecting the steady equilibrium of bone. Serum calcium, serum phosphorus, serum OCN and serum BAP are indicators of bone formation. The bone formation is increased if their

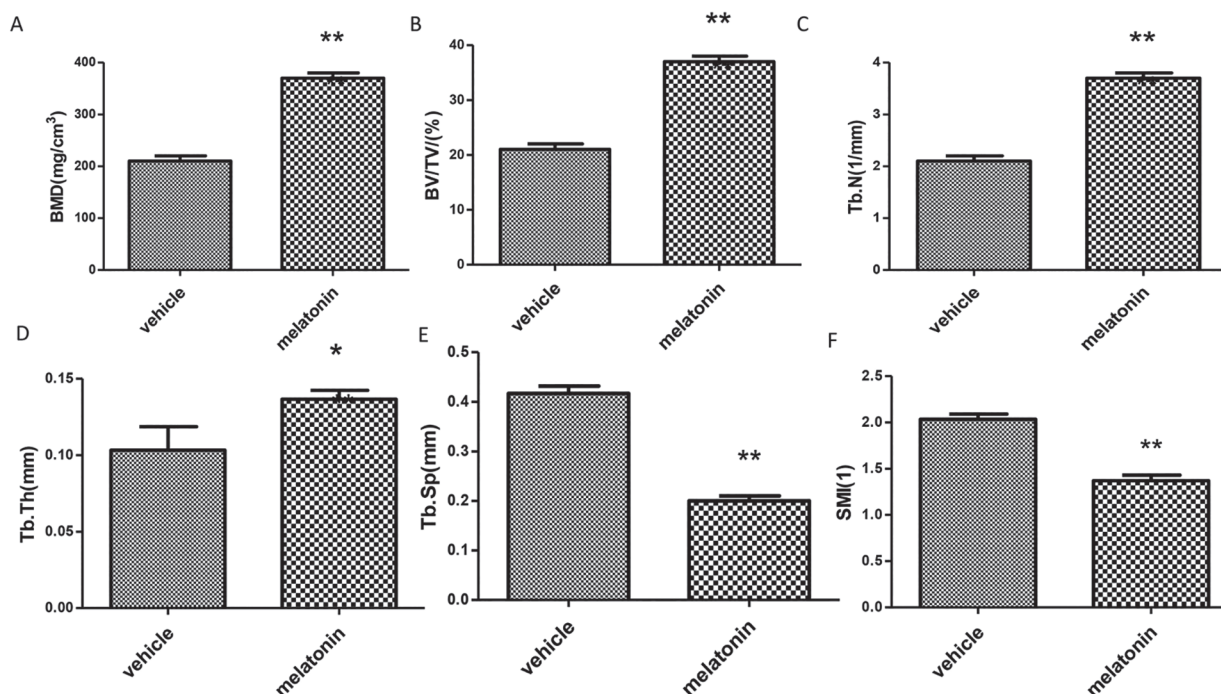


Figure 2. Micro-CT analysis of distal femur metaphyses from the groups of rats indicated above (n=8/group). BMD, bone mineral density; BV/TV, bone volume/total volume; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; SMI, structure model index. Bars represent mean and SD. Results are mean of three independent experiments. *, $p < 0.05$, **, $p < 0.01$ vs. Vehicle rats by one-way ANOVA followed by a Student-Newman-Keuls t test.

concentration increases. The urinary Ca/Cr and P/Cr are indicators of bone resorption. The bone resorption is enhanced if the ratio of Ca/Cr to P/Cr increases. Integra 400 plus fully automatic biochemical analyzer (ROCHE COBAS, West Sussex, England) was used to detect the Ca^{2+} and P^{3+} in serum and Ca^{2+} and P^{3+} in urine. ELISA method was used to detect BAP and OCN in serum. Compared with solvent group, melatonin could significantly increase the concentration of Ca^{2+} in serum in melatonin treatment group (Figure 3A). However, melatonin had no significant effect on P^{3+} concentration in serum (Figure 3B); melatonin significantly decreased the ratio of Ca/Cr to P/Cr in urine (Figure 3C and 3D), and significantly increased the levels of BAP and OCN in serum (Figure 3E and 3F). The above results indicated that the melatonin can significantly reduce the calcium and phosphorus loss in urine in elderly rats and increase levels of BAP and OCN in serum, suggesting that melatonin can inhibit osteoclast activity and promote osteoblast activity.

Melatonin Increased Bone Formation Rate (BFR) and Bone Mineralization Rate (MAR) in Elderly Rats

The double fluorescence labeling method of calcein and tetracycline was adopted. The bone mineral deposition rate was analyzed to investigate the bone formation activity. The study results showed that compared with solvent group, MAR and BFR in elderly rats could be significantly improved in melatonin treatment group, suggesting that melatonin plays a role in promoting bone formation (Figure 4).

Melatonin Increased the Number of Osteoblasts in Bone Marrow and Reduced the Number of Adipocytes in Elderly Rats

It can be observed that the number of adipocytes in melatonin-treated elderly rats was significantly decreased. And the osteoblasts was significantly increased (Figure 5), suggesting that melatonin can inhibit the formation of adipocytes and promote the formation of osteoblasts with the function of promoting bone formation.

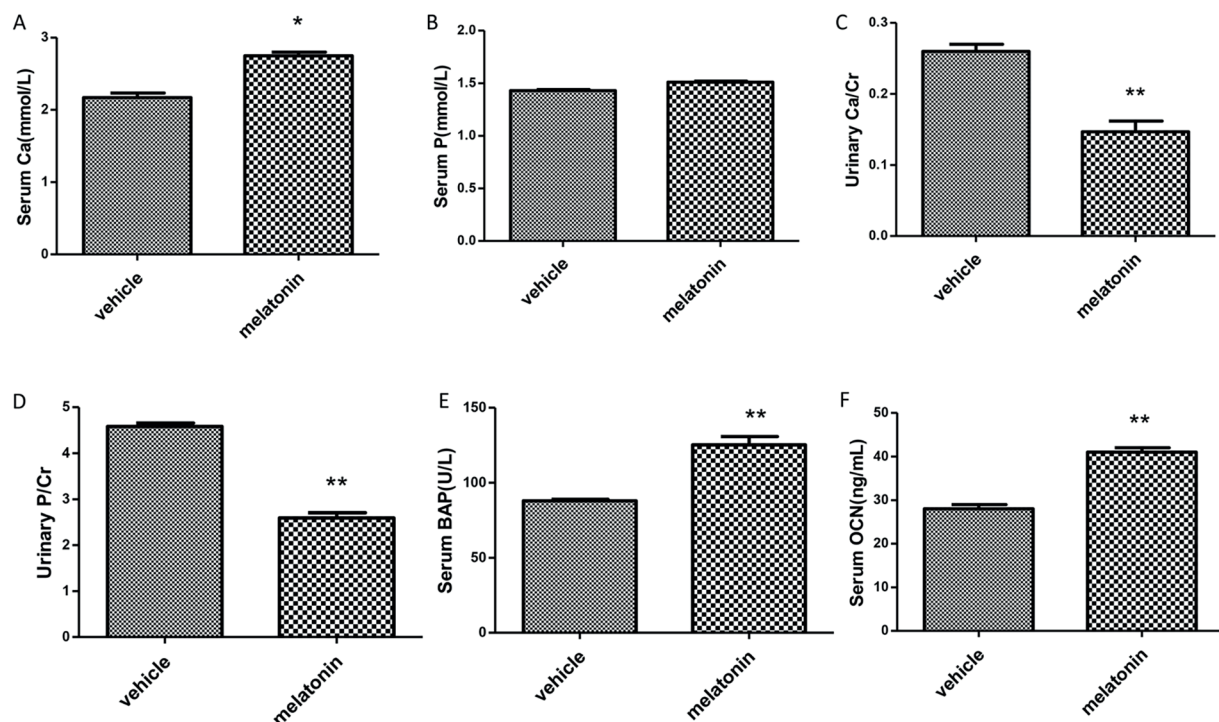


Figure 3. Melatonin increases bone formation. Bone formation markers: osteocalcin (OCN), UrinaryCa/Cr, Urinary P/Cr, Serum bone alkaline phosphatase (BAP) and Serum osteocalcin (OCN) were measured by ELISA systems in serum and urinary from the groups of rats indicated above (n=8/group). Bars represent mean and SD. Results are mean of three independent experiments. **, $p < 0.01$ vs. Vehicle rats by one-way ANOVA followed by a Student-Newman-Keuls t test.

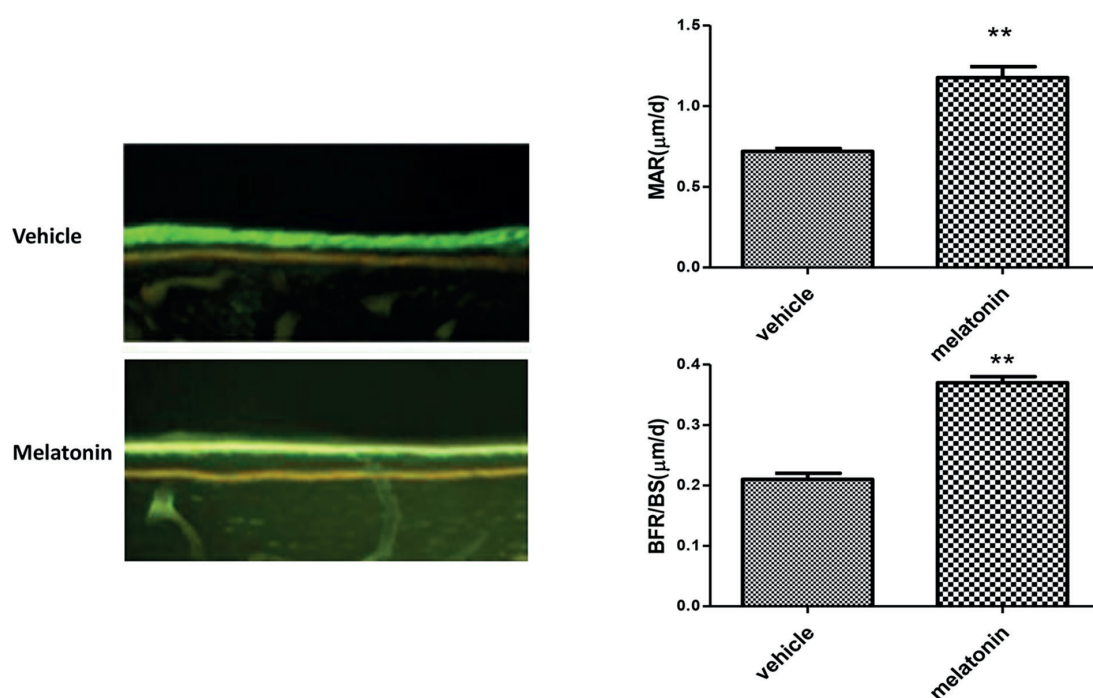


Figure 4. Melatonin increases bone formation. Representative images showing new bone formation examined by calcein and tetracycline labeling of the femur trabecular bone sections of rats treated with vehicle or melatonin (50 mg/kg/day) for 12 weeks, Scale bars: 10 μ m. Analysis of dynamic bone histomorphometric parameters at femur trabecular bone sections (n=8/group). MAR, mineral apposition rate; BFR/BS, bone formation rates/bone surface area. Bars represent mean and SD. Results are mean of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. Vehicle rats by one-way ANOVA followed by a Student-Newman-Keuls t test.

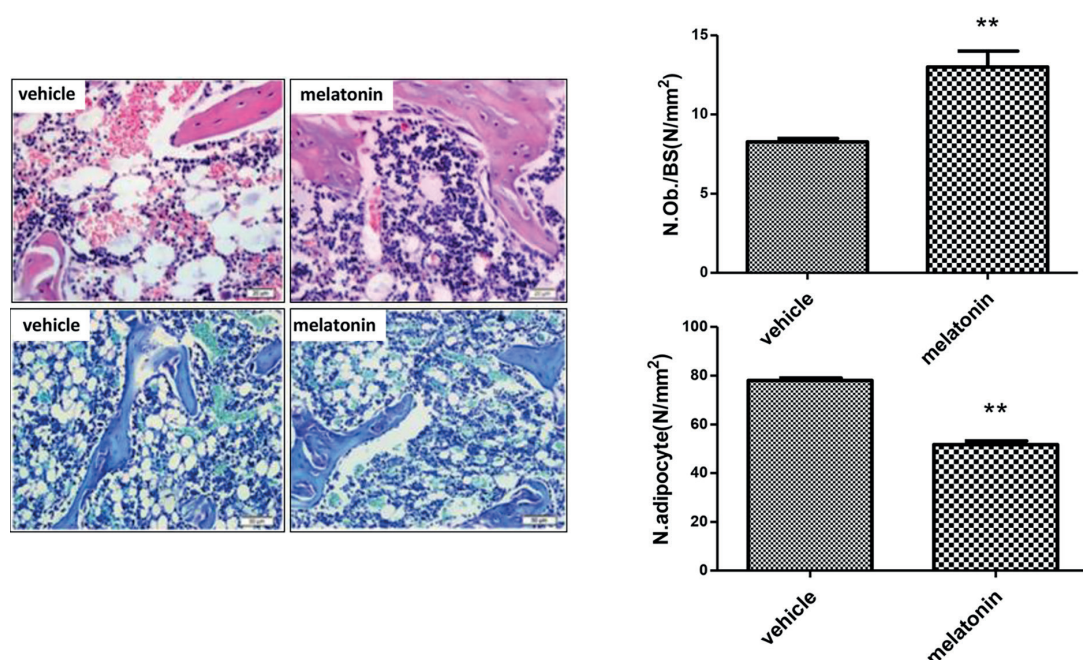


Figure 5. Melatonin increases osteogenesis and inhibits adipogenesis. Tibial sections from rats were stained with hematoxylin and eosin to detect osteoblasts and stained with toluidine blue to visualize adipocytes, the numbers of osteoblasts (N. Ob) and the numbers of adipocytes (N. Adipocyte) per millimeter of trabecular bone surface (BS) were counted (n=8/group). Scale bar: 100 μ m. Bars represent mean and SD. Results are mean of three independent experiments. **, $p < 0.01$ vs. Vehicle rats by one-way ANOVA followed by a Student-Newman-Keuls t test.

Melatonin Promoted Osteogenic Differentiation and Inhibited Adipogenic Differentiation

In the experiment, bones were divided into the osteogenic induction group, osteogenic induction + melatonin group, adipogenic induction group and adipogenic induction + melatonin group, and cultured for 21 days, followed by staining and observation. The result of alizarin red staining showed that the number of mineralized nodules in osteogenic induction group was less than that in osteogenic induction + melatonin group. The difference was statistically significant, suggesting that melatonin promotes the formation of mineralized nodules and promotes osteogenic differentiation of BMSCs. The result of oil red O staining showed that the number of lipid droplets in adipogenic induction group was more than that in adipogenic induction + melatonin group. The difference was statistically

significant, suggesting that melatonin inhibits the formation of lipid droplets in BMSCs and inhibits the differentiation of BMSCs towards adipocytes (Figure 6).

Melatonin Promoted the Expression of Osteogenic Differentiation Genes and Suppressed the Expression of Adipogenic Differentiation Genes

The grouping in experiment was the same as above. PCR was used to detect the expression of Runx-2 and Oc, the marker genes of osteogenic differentiation, and Ppar γ and C/ebp α , the marker genes of adipogenic differentiation. Osteogenic induction detection showed that the expression levels of Runx-2 and Oc, the marker genes of osteogenic differentiation, in melatonin group were higher than those in induction group. The differences were statistically significant, suggesting that melatonin can promote the

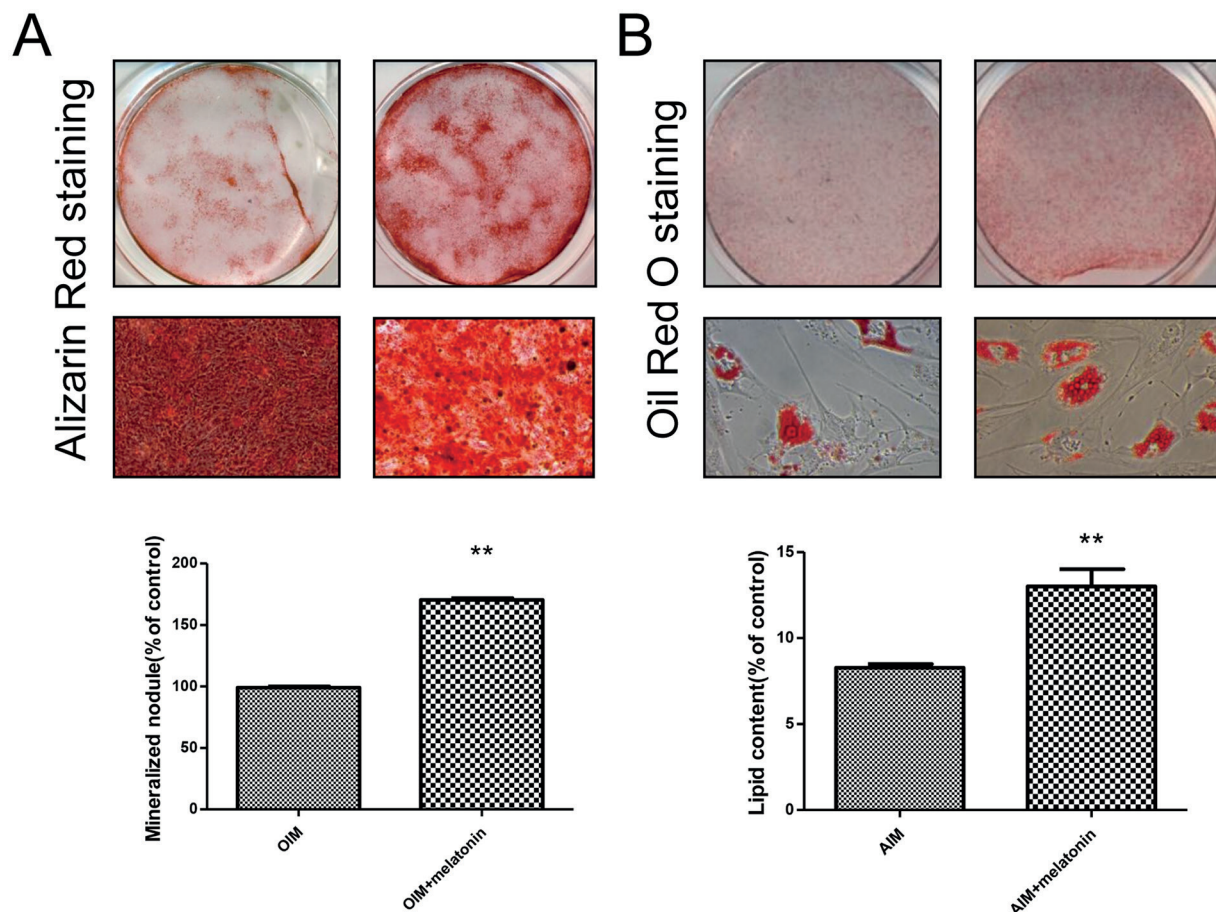


Figure 6. Melatonin promotes osteogenesis and inhibits adipogenesis. Mineralized matrix were visualized in cells by Alizarin Red staining after treatment with melatonin. Lipid content accumulation in cells was visualized in cells by Oil Red O staining after treatment with melatonin. Bars represent mean and SD. Results are mean of three independent experiments. **, $p < 0.01$ vs. vehicle group and by one-way ANOVA followed by a Student-Newman-Keuls t test.

expression of osteogenic differentiation gene and promote the osteogenic differentiation of cells. Adipogenic induction detection showed that the expression levels of PPAR γ and C/EBP α , the marker genes of adipogenic differentiation, in melatonin group were lower than those in control group, and the differences were statistically significant, suggesting that melatonin can inhibit the expression of adipogenic differentiation gene and the adipogenic differentiation of cells (Figure 7).

Discussion

Primary osteoporosis (OP) is a type of systemic bone disease characterized by decreased bone mass, thinner bone trabecula, fracture, and less number, eventually increasing the bone fragility and increased risk of fracture². The number of osteoporosis (OP) patients is also increased year by year. Estrogen and calcitonin are used to treat the bone reabsorption caused by osteoclasts and avoid further bone destruction. However, there are many pathogenic factors of OP patients, the application of estrogen or calcitonin alone cannot completely eliminate the pathogenesis, therefore the effects of these therapies are limited.

Melatonin is a kind of neuroendocrine hormone mainly secreted by pineal body, which has a variety of physiological effects, such as the regulation of biological rhythm, improvement of sleep quality, increase of body immunity, anti-oxidative stress, anti-tumor and anti-aging, etc^{7,20}. Human osteoblasts express melatonin-1 receptor (MT1), and the expression level is decreased with age. Melatonin has a wide range of

effects, and its mechanisms of action are not the same²¹. Melatonin can play a role via the combination with cell-membrane receptor (MT1), melatonin-2 receptor (MT2), and melatonin-3 receptor (MT3) or nuclear receptor (ROR/RZR). Melatonin can pass the cell membrane and act on the subcellular organelle due to its lipophilicity. And it can also be combined with cytoplasmic proteins, such as protein kinase C, calmodulin and calreticulin. Bones maintain the mineralization balance and complete structure through continuous reconstruction. The mutual regulation between osteoblasts and osteoclasts is the basis for the balance between bone formation and bone resorption. Bony remodeling is regulated by various hormones and cytokines, including melatonin²². Like melatonin, bone cells also have circadian rhythm, and the bone tissue growth rate is the highest when the level of melatonin is the highest.

In this study, the elderly SD rat model was adopted. Micro-CT technique showed that the melatonin can significantly increase the bone mass of elderly rats with osteoporosis and improve the femoral trabecular microstructure. The analysis of serology and bone metabolism-related ion level in urine in elderly rats showed that melatonin can significantly decrease the calcium and phosphorus loss in urine and increase BAP and OCN levels in serum. Also, we adopted the double fluorescence labeling method of calcein and tetracycline, and investigated the bone formation activity via analyzing the bone mineral deposition rate. The results showed that compared with solvent group, MAR and BFR in elderly rats can be significantly improved in melatonin treatment group, suggesting that melatonin has a function

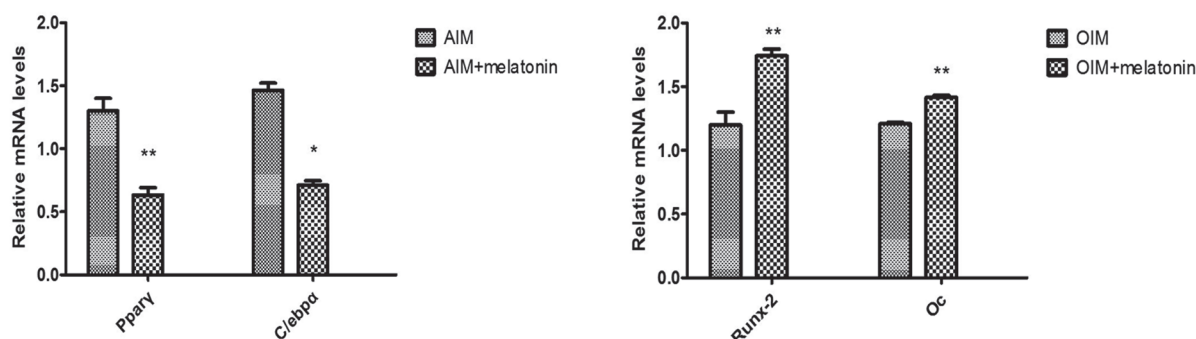


Figure 7. Melatonin promotes the expression of osteogenic genes and inhibits the expression of adipogenic genes. The expression of osteogenesis genes were increased after treatment with melatonin, the expression of adipogenic genes were decreased after treatment with melatonin. Bars represent mean and SD. Results are mean of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. vehicle group and by one-way ANOVA followed by a Student-Newman-Keuls t test

of promoting bone formation. We studied the osteogenic mechanism of melatonin and found that mechanism can promote the osteogenic differentiation of bone marrow mesenchymal stem cells. This effect can be achieved by up-regulating osteogenesis-related genes.

With the gradual increase of aging population, the number of osteoporosis patients is increased year by year, resulting in increasingly serious harm. Although there are many drugs for the prevention and treatment of bone mass loss at present, there are adverse reactions and safety issues. Melatonin levels decrease with the increase of age, and it decreases significantly in perimenopausal period. The recovery of peak melatonin level at night can promote the bone health of perimenopausal females. Melatonin can increase bone mass and induce new bone formation via a variety of ways, which may be a new method for the treatment of osteoporosis.

Conclusions

Our study indicated that melatonin could alleviate osteoporosis in aged rats' models probably by promoting osteoblast differentiation.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

- 1) WU J, SHANG DP, YANG S, FU DP, LING HY, HOU SS, LU JM. Association between the vitamin D receptor gene polymorphism and osteoporosis. *Biomed Rep* 2016; 5: 233-236.
- 2) ZHANG Y, WANG N, MA J, CHEN XC, LI Z, ZHAO W. Expression profile analysis of new candidate genes for the therapy of primary osteoporosis. *Eur Rev Med Pharmacol Sci* 2016; 20: 433-440.
- 3) FONSECA H, MOREIRA-GONCALVES D, CORIOLOANO HJ, DUARTE JA. Bone quality: the determinants of bone strength and fragility. *Sports Med* 2014; 44: 37-53.
- 4) COOK DJ, GUYATT GH, ADACHI JD, CLIFTON J, GRIFFITH LE, EPSTEIN RS, JUNIPER EF. Quality of life issues in women with vertebral fractures due to osteoporosis. *Arthritis Rheum* 1993; 36: 750-756.
- 5) GOLD DT. The clinical impact of vertebral fractures: quality of life in women with osteoporosis. *Bone* 1996; 18: 185S-189S.
- 6) KADO DM, DUONG T, STONE KL, ENSRUD KE, NEVITT MC, GREENDALE GA, CUMMINGS SR. Incident vertebral fractures and mortality in older women: a prospective study. *Osteoporos Int* 2003; 14: 589-594.
- 7) WITT-ENDERBY PA, RADIO NM, DOCTOR JS, DAVIS VL. Therapeutic treatments potentially mediated by melatonin receptors: potential clinical uses in the prevention of osteoporosis, cancer and as an adjuvant therapy. *J Pineal Res* 2006; 41: 297-305.
- 8) CUTANDO A, ANEIROS-FERNANDEZ J, LOPEZ-VALVERDE A, ARIAS-SANTIAGO S, ANEIROS-CACHAZA J, REITER RJ. A new perspective in oral health: potential importance and actions of melatonin receptors MT1, MT2, MT3, and RZR/ROR in the oral cavity. *Arch Oral Biol* 2011; 56: 944-950.
- 9) TOFFOL E, KALLEINEN N, HAUKKA J, VAKKURI O, PARTONEN T, POLO-KANTOLA P. Melatonin in perimenopausal and postmenopausal women: associations with mood, sleep, climacteric symptoms, and quality of life. *Menopause* 2014; 21: 493-500.
- 10) CARDINALI DP, LADIZESKY MG, BOGGIO V, CUTRERA RA, MAUTALEN C. Melatonin effects on bone: experimental facts and clinical perspectives. *J Pineal Res* 2003; 34: 81-87.
- 11) HAKANSON DO, BERGSTROM WH. Phototherapy-induced hypocalcemia in newborn rats: prevention by melatonin. *Science* 1981; 214: 807-809.
- 12) HAKANSON DO, PENNY R, BERGSTROM WH. Calcemic responses to photic and pharmacologic manipulation of serum melatonin. *Pediatr Res* 1987; 22: 414-416.
- 13) EGERMANN M, GERHARDT C, BARTH A, MAESTRONI GJ, SCHNEIDER E, ALINI M. Pinealectomy affects bone mineral density and structure--an experimental study in sheep. *BMC Musculoskelet Disord* 2011; 12: 271.
- 14) YANG L, GE D, CAO X, GE Y, CHEN H, WANG W, ZHANG H. MiR-214 Attenuates Osteogenic Differentiation of Mesenchymal Stem Cells via Targeting FGFR1. *Cell Physiol Biochem* 2016; 38: 809-820.
- 15) DUQUE G. Bone and fat connection in aging bone. *Curr Opin Rheumatol* 2008; 20: 429-434.
- 16) STOLZING A, SCUTT A. Age-related impairment of mesenchymal progenitor cell function. *Aging Cell* 2006; 5: 213-224.
- 17) ROTH JA, KIM BG, LIN WL, CHO MI. Melatonin promotes osteoblast differentiation and bone formation. *J Biol Chem* 1999; 274: 22041-22047.
- 18) SATOMURA K, TOBIUME S, TOKUYAMA R, YAMASAKI Y, KUDOH K, MAEDA E, NAGAYAMA M. Melatonin at pharmacological doses enhances human osteoblastic differentiation in vitro and promotes mouse cortical bone formation in vivo. *J Pineal Res* 2007; 42: 231-239.
- 19) FJELLDAL PG, GROTMOL S, KRYVI H, GJERDET NR, TARANGER GL, HANSEN T, PORTER MJ, TOTLAND GK. Pinealectomy induces malformation of the spine and

- reduces the mechanical strength of the vertebrae in Atlantic salmon, *Salmo salar*. *J Pineal Res* 2004; 36: 132-139.
- 20) COLOMBO J, MACIEL JM, FERREIRA LC, DA SR, ZUCCARI DA. Effects of melatonin on HIF-1alpha and VEGF expression and on the invasive properties of hepatocarcinoma cells. *Oncol Lett* 2016; 12: 231-237.
- 21) LIU J, HUANG F, HE HW. Melatonin effects on hard tissues: bone and tooth. *Int J Mol Sci* 2013; 14: 10063-10074.
- 22) HARDELAND R, MADRID JA, TAN DX, REITER RJ. Melatonin, the circadian multioscillator system and health: the need for detailed analyses of peripheral melatonin signaling. *J Pineal Res* 2012; 52: 139-166.